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DEVELOPMENTAL REGULATION OF THE MONOAMINE FEAR CIRCUITRY

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Abstract

The central thesis of these studies was to examine the regulatory mechanisms that govern the development of neurotransmitter systems of the mammalian monoamine fear circuitry. We first examined the central orchestrator of the monoamine fear circuitry, the extended amygdala, comprised of the amygdala proper, as well as its rostral extension along the bed nucleus of the stria terminalis to the shell of the nucleus accumbens. We identified a series of GABAergic gating structures along the entire rostro-caudal axis of the extended amygdala that are dopamine D1 receptor rich, but have variable dopamine-like terminal input. In addition, we identified a complimentary distribution of the μ-opioid receptor 1, which also receives variable opioid peptide terminal input. We proposed a model in which the regions lacking direct terminal input maintain a tonic inhibition of amygdaloid fear outputs, whereas the regions receiving direct dopaminergic input disinhibit amygdaloid behavioural outputs, enhancing fear response.

Following on from these brain circuitry studies we turned our attention to the critical developmental regulators of the monoamine fear circuitry. We first examined how a Parkinson's disease mutation in the transcription factor NURR1, might lead to impairment in dopamine development that may also affect fear circuitry. We also investigated, *in vivo* and *in vitro*, two transcriptional regulators that control development of the serotonin system (Pet-1 and Hes1) and how they impact serotonergic modulation of receptor expression. *In vitro* we showed Pet-1 and Hes1 directly enhance or repress, respectively, the expression of serotonin-1A receptors. Using mouse models lacking Pet-1, which specifically promotes serotonergic differentiation, we found unexpected compensatory alterations in the regulation of pre- and post-synaptic serotonin-1A

receptors. In mice lacking Hes1, which restricts neuronal differentiation, we observed striking up-regulation receptor expression.

Taken together, these studies provide (1) an anatomical template for understanding how emotionally relevant stimuli, including the subjective experience of stress, are processed by the extended amygdala, the core of the monoamine fear circuitry and (2) a molecular explanation for how this processing machinery may be predisposed to certain emotional or behavioural responses via early developmental regulatory events.

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List of Abbreviations

5-HT	**5-Hydroxytryptamine**
5-HTT	5-Hydroxytryptamine Transporter
6-OHDA	6-Hydroxydopamine
8-OH-DPAT	8-hydroxy-2-(di-η-propylamino)tetralin
AADC	**Aromatic L-Amino Acid Decarboxylase**
AC	Adenylate Cyclase
Acb	Nucleus Accumbens Core and Shell divisions
AcbC	
AcbSh	
ACTH	Adrenocorticotropic Hormone
ADHD	Attention Deficit Hyperactive Disorder
AF	Activation Function-1 and -2
AMPA	(±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid
ATP	Adenosine Triphosphate
BDNF	**Brain-Derived Neurotrophic Factor**
bHLH	Basic Helix-Loop-Helix
BLA	Basolateral Complex of the Amygdala Basolateral (BL), Basomedian (BM), Lateral (La) Divisions
BST	Bed Nucleus of the Stria Terminalis Medial and Lateral Divisions
BSTM	
BSTL	
CCK	**Cholecystokinin/Gastrin**
Ce	Central Amygdaloid Nucleus Central (CeC), Lateral (CeL) and Medial (CeM) Divisions
CEA	Central Extended Amygdala
Co	Cortical Amygdaloid Nucleus
COMT	Catechol-O-Methyltransferase
CNS	Central Nervous System
CPu	Caudate and Putamen
CRE	cAMP response element
CREB	cAMP response element binding protein
D2S	**Dopamine D2 Receptor, short isoform**
D2L	Dopamine D2 Receptor, long isoform
DA	Dopamine
DARPP-32	Dopamine and cyclic AMP Regulated Phosphoprotein, 32 kDa
DAT	Dopamine Transporter
DBH	Dopamine β Hydroxylase
DEAF-1	Drosophila Deformed Epidermal Autoregulatory Factor 1
DNMT	DNA Methyltransferase
DOPA	3,4-dihydrophenylalanine, a.k.a. L-DOPA

DOR	δ Opioid Receptor
DRE	Dual Repressor Element (See TRE, FRE)
DRN	Dorsal Raphe Nucleus
DSL	Delta/Serrate/Jagged/LAG-2
ECF	**Extracellular Fluid**
EEG	Electroencephalogram
EN	Engrailed 1/2
EMSA	Electrophoretic Mobility Shift Assay
ENK	Enkephalin, Leu- and Met-
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Related Kinase (See MAPK)
ETS	E26 Transformation-Specific
FA	**Field Areas**
FEV	Fifth Ewing Variant
FGF	Fibroblast Growth Factors
FITC	Fluorescein Isothiocyante
FRE	5' Repressor Element (See TRE, DRE)
Freud	Five' Repressor Element Under Dual Repression Binding Protein-1/-2
GABA	**γ-Aminobutyric Acid**
GAD	Glutamic Acid Decarboxylase
GDP	Guanosine Diphosphate
GIRK	G Protein Regulated Inwardly Rectifying K^+
GPCR	G Protein Coupled Receptor
GR	Glucocorticoid Receptor
GRK	GPCR Serine/Threonine Kinases
Gro	Groucho
GTP	Guanosine Triphosphate
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
Hes	Hairy/Enhancer of Split 1-7
HLH	Helix-Loop-Helix
HPA	Hypothalamic-Pituitary-Adrenal Axis
ICM	**Intercalated Cell Masses** Main (Im) and Paracapsular (Ip) Islands
Im	
Ip	
Intr	Initiator Motif
IP_3	Inositol Triphosphate
IPAC	Interstitial Nucleus of the Posterior Limb of the Anterior Commissure Lateral and Medial divisions
IPACL	
IPACM	
IR	Immunoreactivity

JNK	**Jun N-Terminal Kinase**
KOR	**κ Opioid Receptor**
LBD	**Ligand Binding Domain**
LC	Locus Coeruleus
L-DOPA	See DOPA
Lmx1b	LIM Homeobox Transcription Factor 1 β
LSS	Lateral Stripe of the Striatum
LTP	Long Term Potentiation
MAO	**Monoamine Oxidase**
MAPK	p42/p44 Mitogen Activated Protein Kinase (a.k.a. ERK)
MAZ	Myc-Associated Zinc Finger Protein
MDD	Major Depressive Disorder
Me	Medial Amygdaloid Nucleus
MEA	Medial Extended Amygdala
MEK1	MAPK/ERK kinase
mGluR5	Metabotropic Glutamate Receptor 5
MHO	Midbrain-Hindbrain Organizing Center
MOR	μ Opioid Receptor
MPEP	2-Methyl-6(phenylethenyl) Pyridine
MRN	Median Raphe Nucleus
NA/A	Noradrenaline/Adrenaline (See NE/E)
NBRE	NGFI-B Response Element
NCID	Notch Intracellular Domain
NE/E	Norepinephrine/Epinephrine (NA/A)
NF-κB	Nuclear Factor kappa B
NMDA	N-Methyl-D-Aspartic Acid
NO	Nitric Oxide
NOR1	Neuron-Derived Orphan Receptor 1 (a.k.a. NR4A3)
NOT	Nuclear Receptor of T Cells (a.k.a. human NURR1)
NRSF	Neuron-Restrictive Silencer Factor (a.k.a. REST)
Nur77	Nuclear Receptor 77 (a.k.a. NGF-1B, NR4A1)
NURR1	Nuclear Receptor Related 1 (a.k.a. NR4A2, NOT, RNR-1)
OCD	Obsessive Compulsive Disorder
OT	Optic Tract
PBS	Phosphate Buffered Saline
ρCPA	Para-Chlorophenylalanine
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PEA3	Polyomavirus Enhancer Activator 3
PET	Positron Emission Tomography
Pet-1	Pheochromocytoma 12 *Ets* Factor

$PI(4,5)P_2$	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	cGMP-Dependent Protein Kinase
PLCβ	Phospholipase C Beta
PP-1	Phosphoprotein-1
PP-2A	Phosphoprotein-2A
PP-2B	Phosphoprotein-2B
PTX	Pertussis Toxin
RE-1	Repressor Element 1
REST	Repressor Element 1 Silencing Transcription Factor (a.k.a. NRSF)
RGS	Regulator of G Protein Signalling
RNR-1	Regenerating Liver Nuclear Receptor 1 (a.k.a. rat NURR1)
SAND	Sp100, AIRE-1, NucP41/75, DEAF-1
Shh	Sonic Hedgehog
SN	Substantia Nigra Pars Compacta and Pars Reticulata
SNc	
SNr	
SNP	Single Nucleotide Polymorphism
SSRI	Selective Serotonin Reuptake Inhibitor
TAF	TATA Associated Factors
TBP	TATA Binding Protein
TCA	Tricyclic Antidepressants
TH	Tyrosine Hydroxylase
TLE	Transducin-Like Enhancer of Split
TPH	Tryptophan Hydroxylase
TRE	3' Repressor Element (See FRE, DRE)
VDCC	**Voltage-Dependent Calcium Channels**
VIP	Vasoactive Intestinal Peptide
VGAT	Vesicular GABA Transporter
VMAT2	Vesicular Monoamine Transporter 2
VT	Volume Transmission
VTA	Ventral Tegmental Area
VZ	Ventricular Zone
WT	**Wiring Transmission**

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Thesis Format

This thesis is presented in manuscript format. There are seven chapters including a general introduction (Chapter I), a short discussion of the entire work (Chapter VII) and 5 papers in manuscript format. Chapters II-IV are published and available through PubMED. Chapters V and VI have been submitted for publication. Between manuscripts, a short linking statement will appear in order to maintain the flow of the overall body of work.

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CHAPTER I: Introduction

1: Development of the Central Nervous System

The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory. Santiago Ramón y Cajal (1852-1934)

1 A: Central Nervous System Development

The central nervous system (CNS) is the largest part of the nervous system and includes the brain and the spinal cord (Nolte, 1993). Embryonic stem cells give rise *in vivo* to ectoderm, which forms the nervous system and outer part of the integumentary system; the mesoderm, from which bones, heart, muscles, and the circulatory, urinary, reproductive and gastrointestinal organ systems are derived; and the endoderm, which gives rise to the gastrointestinal and respiratory tracts, as well as the endocrine glands (Binetruy et al., 2007).

1 A(i): Development of the Cerebral Cortex

During embryonic development, the neural plate is formed from a longitudinal band of thickened ectoderm that folds inward, eventually fusing along the neural groove and forming the neural tube, whose cavity forms the cerebrospinal fluid (CSF)-filled ventricular system (Nolte, 1993). The neuroepithelial cells lining the neural tube begin expressing makers of radial glia around the time of neural tube closure. These radial glia proliferate along the ventricular zone (VZ) surface, giving rise to intermediate progenitor cells which in turn migrate along the long radial processes that extend towards the pial surface and form the subventricular zone (Heins et al., 2002; Ever and Gaiano, 2005; Noctor et al., 2007). The radial glia and intermediate progenitors both give rise to the

cortical neurons of the cortical plate, and once cortical neurogenesis is complete, radial glia also give rise to astrocytes, leaving only a single layer of VZ cells postnatally.

1 A(ii): Cerebral Vesicles

Along the neural tube, even before the closure of the rostral and caudal neuropores, three distinct bulges form, marking the primary cerebral vesicles (Encha-Razavi and Sonigo, 2003). These vesicles represent the developing prosencephalon, mesencephalon and rhombencephalon. As brain development continues, secondary vesicles become distinguishable, with the prosencephalon giving rise to the telencephalon (cerebral hemispheres, including limbic system, cortex, basal ganglia and olfactory bulbs) and diencephalon (thalamus, hypothalamus), and the rhombencephalon subdividing into the metencephalon (pons and cerebellum) and myelencephalon (medulla) (Nolte, 1993).

1 A(iii): Specification of Neuronal Subtypes

Along the rostrocaudal and dorsoventral axes of the neural tube, progenitor cells produce distinct and diverse populations of neurons and glia (Bertrand et al., 2002). The development of these different neural lineages is regulated by organizing centers which divide the developing brain into a virtual Cartesian space of compartments (Hynes and Rosenthal, 1999; Baek et al., 2006) that express a variety of signalling molecules. In different combinations, these factors induce the specification of distinct neural cell types (Crossley and Martin, 1995; Lumsden and Krumlauf, 1996; Hynes and Rosenthal, 1999; Roussa and Krieglstein, 2004). Some of the best characterized signalling pathways are Sonic hedgehog (Shh), Wnt, fibroblast growth factor (FGF), retinoic acid, transforming growth factor-beta family members and Notch, all of which induce transcriptional or signalling cascades important for stem cell maintenance, proliferation and differentiation

(Yoon et al., 2004; Steventon et al., 2005; Basch and Bronner-Fraser, 2006; Katoh and Katoh, 2007).

One signalling cascade that has been extensively studied for its role in influencing cell differentiation processes throughout development and into adult life is the Notch pathway (Figure I-1) (Dang et al., 2006). Notch is a single-pass transmembrane receptor and has four human homologues (Notch1-4) (Bianchi et al., 2005). It is important in the fate determination of neurons and glia (Wang and Barres, 2000; Mason et al., 2005), neurite outgrowth and neuronal maturation (Sestan et al., 1999; Redmond et al., 2000). Both Notch and its ligands, members of the Delta/Serrate/Jagged/LAG-2 (DSL) family of proteins, are single-pass transmembrane receptors (Yoon and Gaiano, 2005). Notch is activated by an interaction between the extracellular portions of Notch and its DSL ligands on neighbouring cells (Figure I-1, step (1)). This signalling between adjacent cells represents a form of lateral-inhibition that results in the cleavage of the Notch intracellular domain (NICD) (Bertrand et al., 2002). The NICD is then able to translocate to the nucleus and regulate gene expression by interacting with the CBF1/RBP-J/Lag-1 (CSL) DNA-binding protein complex, converting this repressor-complex into an activator complex (Bianchi et al., 2005; Yoon and Gaiano, 2005). The NICD/CSL activator complex then activates transcription of *Drosophila Hairy/Enhancer of split (Hes)* homologues *Hes1* and *Hes5* (Figure I-1, step (2)), inhibitors of neurogenesis that are downstream effectors of Notch (Kageyama et al., 2005). Hes1 and Hes5 are inhibitory basic helix-loop-helix (bHLH) transcription factors and are highly expressed by neural stem cells. Another related bHLH, Hes3, is also localized to neural stem cells, although Hes3 is not thought to be regulated by Notch signalling since it is downregulated at E8.5 in the mouse, prior to expression of Notch (Hatakeyama et al., 2004). A C-terminal

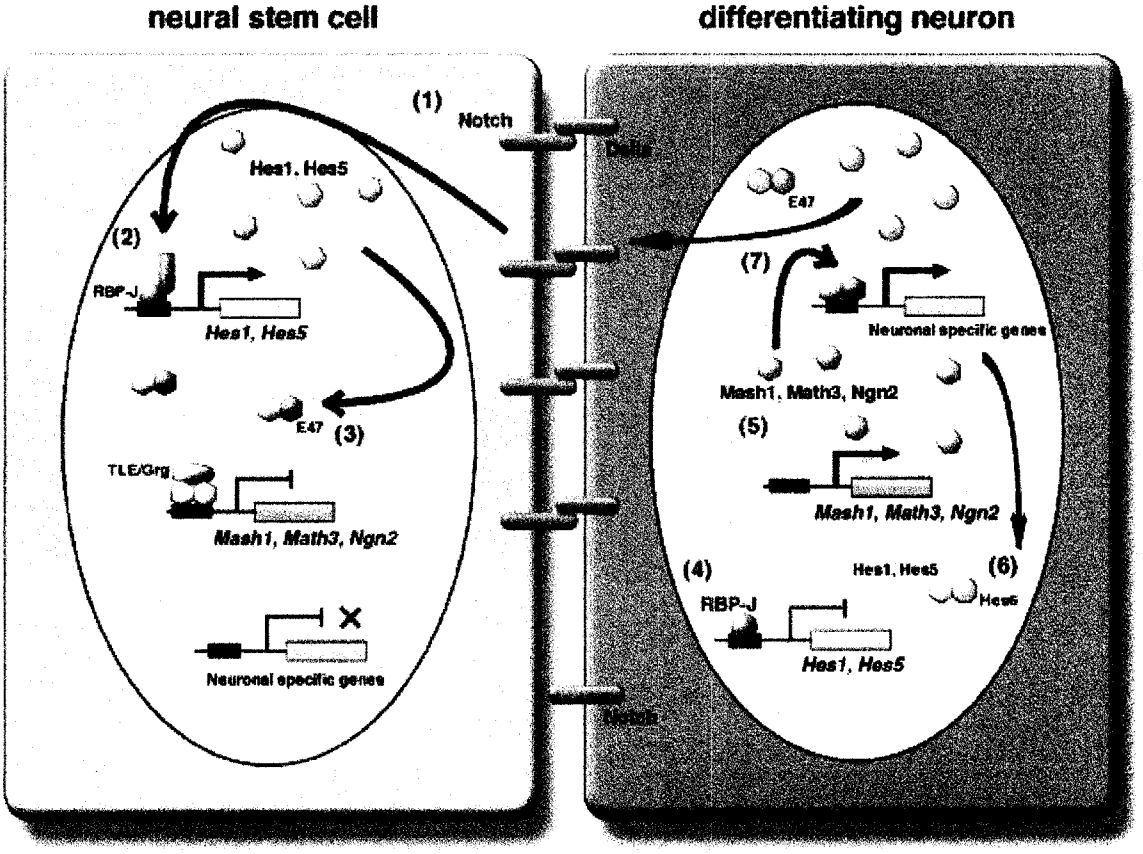


Figure I-1: Notch-Signalling Pathway.

(1) In a neural stem cell, Notch is activated by its ligand, Delta on a neighbouring cell. (2) Upon activation, the NICD translocates into the nucleus and forms a complex with RBP-J. This complex induces Hes1 and Hes5 expression. (3) Hes1 and Hes5 inhibit the activator-type bHLH factors by sequestering E47. In addition, Hes1 and Hes5 repress transcription of the activator-type bHLH genes by binding to their promoters and recruiting the co-repressor TLE/Grg. (4) During neuronal differentiation, Notch is not activated, and RBP-J represses Hes1 and Hes5 expression. (5) The activator-type bHLH genes are expressed and (6) induce expression of Hes6, which inhibits Hes1 function and reinforces the neurogenic process. (7) The activator-type bHLH factors also induce neuron specific genes (Kageyama et al., 2005). Copyright © 2005 Elsevier Inc.

WRPW motif of Hes1 and Hes5 mediates interaction with co-repressors of the Groucho (Gro)/TLE family (Jennings et al., 2006), together binding and transcriptionally repressing pro-neural bHLHs such as *Mash1, Math1, Math3* and *Ngn2* (Bertrand et al., 2002; Kageyama et al., 2005; Baek et al., 2006). Hes1 can also inhibit activator-type bHLHs by sequestering the co-activator E47 (Figure I-1, step (3)) (Sasai et al., 1992). In the absence of activated NICD, the CSL-mediated repression of the inhibitory bHLHs is maintained, leaving pro-neural bHLHs to interact with E47 and drive transcription of a number of neuron-specific genes, including Hes6 (Figure I-1, steps (4-7)). Hes6 is able to inhibit Hes-mediated repression of pro-neural genes by a number of mechanisms (reviewed later, Figure I-17), further promoting neurogenesis (Bae et al., 2000)

1 B: The Fear Circuitry of the Central Nervous System

James Papez first described the limbic system as the anatomical mechanism of emotion in 1937 (Papez, 1995), which includes afferent and efferent connections with the hippocampal formation, now commonly referred to as the circuit of Papez.

> *Is emotion a magic product, or is it a physiologic process which depends on an anatomic mechanism?* James W. Papez, (1883-1958)

The hippocampal formation includes several distinct structures, with information flowing from the dentate gyrus, to the hippocampus proper (*Cornu Ammonis* fields CA1-CA3), to the subiculum (Nolte, 1993). The major afferent connections of the hippocampal formation come from the amygdala, and the cingulate, temporal, orbital and olfactory cortices via the entorhinal cortex (see Figure I-2 for overview of structures). Additional connections from the hypothalamus and septal nuclei reach the hippocampus via the fornix. The hippocampal formation in turn shares a number of reciprocal connections with the entorhinal and other cortical regions, and its major output pathway is via

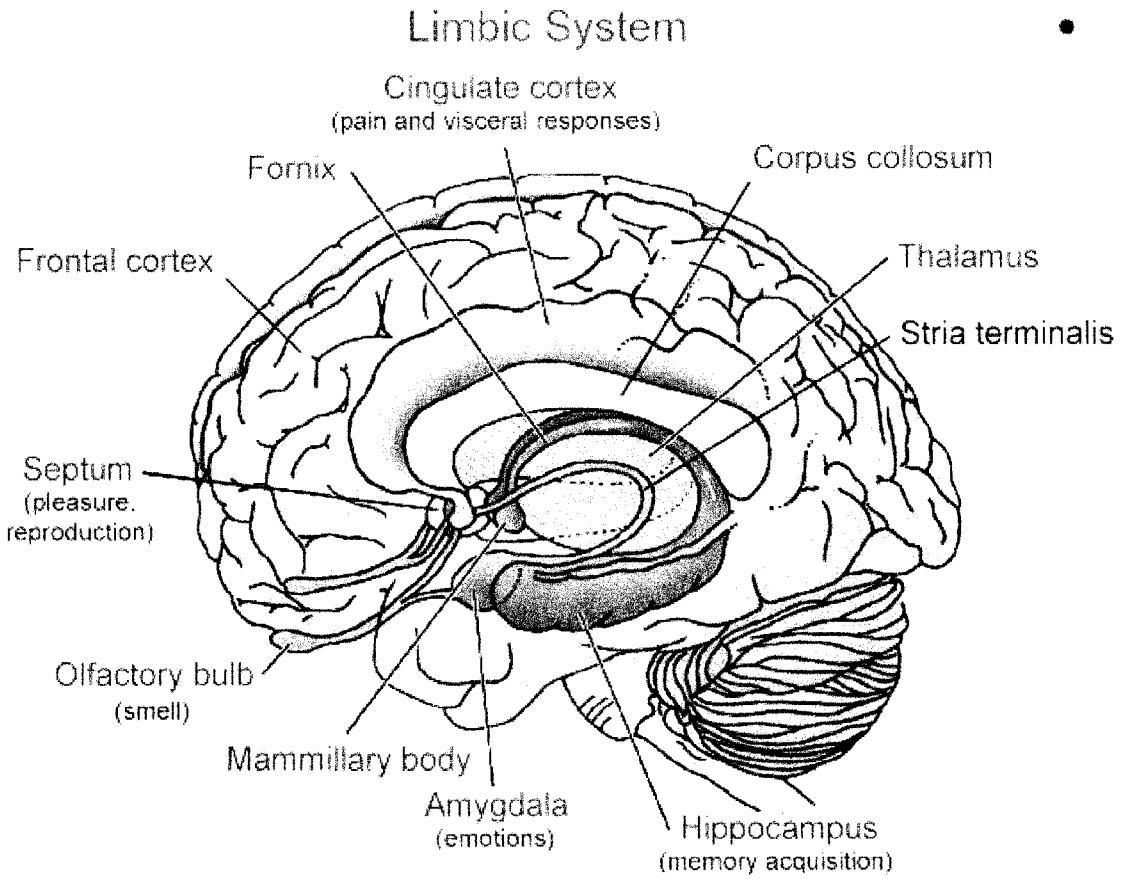


Figure I-2: Overview of the Major Limbic System Pathways.

Cartoon representation of the limbic system with the basal ganglia removed. Important structures for the purposes of this review include cortical regions such as the cingulate and frontal cortices, olfactory structures, hippocampus, amygdala, thalamic relay nuclei, hypothalamic nuclei such as the mammillary bodies. Copyright © University of Colorado at Boulder.

The fornix, through which it reaches thalamic nuclei, hypothalamic nuclei such as the mammillary body and other forebrain structures such as the septal area, orbital cortex and anterior cingulate cortex. In addition, the hippocampus projects directly to the amygdala. While Papez's mapping of emotional 'anatomy' to a discrete circuit centered on the hippocampal formation provides important insights into the functional anatomy of fear and anxiety, more recent research has shed light on the vastness and shear complexity of the fear circuitry.

1 B(i): The Limbic System and Beyond

The boundaries of the fear circuitry are somewhat ambiguously defined. Not only is the functional anatomy of the fear circuitry still being elucidated, even the known structures, such as the amygdala, have been redefined over the last 20 years in a struggle to reconcile the structural boundaries with function and connectivity. Of primary focus in the understanding of fear and emotion is the amygdala, or amygdalar complex (Swanson and Petrovich, 1998; Pare et al., 2002; Schafe et al., 2005). The amygdala plays a role in consolidation of emotionally laden memories, conditioned and unconditioned fear responses, appetitive conditioning, and has been linked to many psychiatric disorders including schizophrenia, obsessive compulsive disorder, anxiety disorder, major depression and addiction (Alheid and Heimer, 1988; Davis, 1992; Heimer, 2000; Ghashghaei and Barbas, 2002; Koob, 2003b, a; Pare et al., 2004; Pelletier and Pare, 2004; Eggers, 2007).

The amygdala represent the legacy of Karl Friedrich Burdach's discovery of the "amygdalar nucleus" between 1819 and 1826 and its subdivision into a series of defined nuclei a century later by J.B. Johnston (Swanson and Petrovich, 1998): the basolateral complex [BLA, with Lateral (La), basolateral (BL) and basomedian (BM) subdivisions];

corticomedial nuclei [central (Ce), cortical (Co) and medial (Me)]; and the anterior group (Ulfig et al., 2003). It receives all modalities of sensory input, including olfactory bulb projections onto the Co and periamygdaloid complex, a region that processes olfactory information; taste and visceral afferents from the nucleus of the solitary tract via the parabrachial nucleus and thalamus; and projections from sensory association cortices such as vision (inferior temporal lobe), audition (superior and anterior temporal cortex) and somatosensation (insula) onto the BLA (Price, 2003). With the exception of the thalamic input to these amygdaloid nuclei, most connections are bi-directional. The Ce and BLA also project to the frontal cortex and striatum, including the point of fusion of the putamen and the head of the caudate, the nucleus accumbens (Acb), raising the possibility that the amygdala influences forebrain motor mechanisms (Kita and Kitai, 1990; Shinonaga et al., 1992; Stevenson and Gratton, 2003). Of the amygdaloid output nuclei, the Ce is most relevant for regulating emotion. The Ce projects extensively to the hypothalamus, midbrain, brainstem and forebrain. A summary of the major direct connections of the Ce and the subsequence physiological and behavioural implications as they related to fear and anxiety responses can be seen in Table I-1. This fear/anxiety response involves the connection with the hypothalamic-pituitary-adrenal (HPA) stress axis, activation of autonomic and somatic responses and hypoalgesia (Ninan, 1999; Heisler et al., 2007).

Through its widespread interconnections, the amygdala compiles sensory information (Pitkanen et al., 1997; Price, 2003) and influences striatal and accumbal stress responses (Shinonaga et al., 1992; Stevenson and Gratton, 2003) and the entire stress axis (Ninan, 1999). The amygdala also receives substantial input from brainstem nuclei, including projections from serotonergic raphe nuclei, especially the dorsal raphe

Table I-1: **Regulation of Fear/Anxiety Responses by the Central Amygdaloid Nucleus**

Target of Projection		Effect		Test or Sign of Fear/Anxiety
Lateral hypothalamus	→	Sympathetic Activation	→	Tachycardia, Galvanic skin response, paleness, pupil dilation, blood pressure elevation
Dorsal motor nucleus of vagus, nucleus ambiguous	→	Parasympathetic activation	→	Ulcers, urination, defecation, bradycardia
Parabrachial nucleus	→	Dyspnea/ hyperventilation	→	Panting, respiratory distress
Ventral tegmental area, locus coeruleus, dorsolateral tegmental nucleus	→	Activation of dopamine, norepinephrine and acetylcholine	→	Behavioural and EEG arousal, increased vigilance
Nucleus of reticularis pontis caudalis	→	Increased reflexes	→	Increased startle
Midbrain central grey, trigeminal, facial motor nucleus	→	Cessation of behaviour, mouth open, jaw movements	→	Freezing, facial expressions of fear, conditioned emotional response, social interaction
Paraventricular nucleus	→	ACTH release	→	HPA axis activation

Direct efferent connections of the central amygdaloid nucleus involved in the fear-stimulated fear/anxiety response. EEG – electroencephalogram, ACTH – adrenocorticotropic hormone. Modified from (Davis, 1992).

nucleus (DRN) (Peyron et al., 1998; Bauman and Amaral, 2005; Eggers, 2007); from dopaminergic cells of the substantia nigra (SN) and ventral tegmental area (VTA) (Oades and Halliday, 1987; Roder and Ciriello, 1993); and from noradrenergic cells of the locus coeruleus (LC) and medullary cell groups (Emson et al., 1979). The main connections of the amygdala are summarized in Figure I-3.

1 B(ii): The Intercalated Cell Masses

Interposed between the BLA and the corticomedial complex, are a series of interconnected γ-aminobutyric acid (GABA)ergic intercalated cell masses (ICM) that receive excitatory glutamatergic afferents from the BLA and send feedforward inhibitory projections to the Ce and Me (McDonald and Augustine, 1993; Pare and Smith, 1993a; Royer et al., 1999). Using a variety of electrophysiological techniques, Royer and colleagues have demonstrated that the ICM effectively gate the impulse traffic between the amygdala input (BLA) and output (Ce, Me) nuclei, and thereby modulate the influence of the BLA on fear responses (Royer et al., 2000b, a). In addition, there is a lateromedial correlation between ICMs and the regions of the Ce (or other ICMs) that they inhibit, adding a spatiotemporal layer to their impulse gating ability (Royer et al., 1999). The ICM is an important locus of synaptic plasticity critical to fear conditioning. Studies examining the effects of intra-amygdala administration of N-methyl-D-aspartic acid (NMDA) agonists (facilitate fear extinction) and NMDA antagonists (block extinction learning) have suggested that the ICM might be important targets for treating clinical fear (Davis, 2002). Further to this, the low- and high-frequency stimulation (respectively) of BLA afferents onto the ICM results in NMDA-dependent long term depression or long term potentiation (LTP), the cellular mechanisms underlying learning

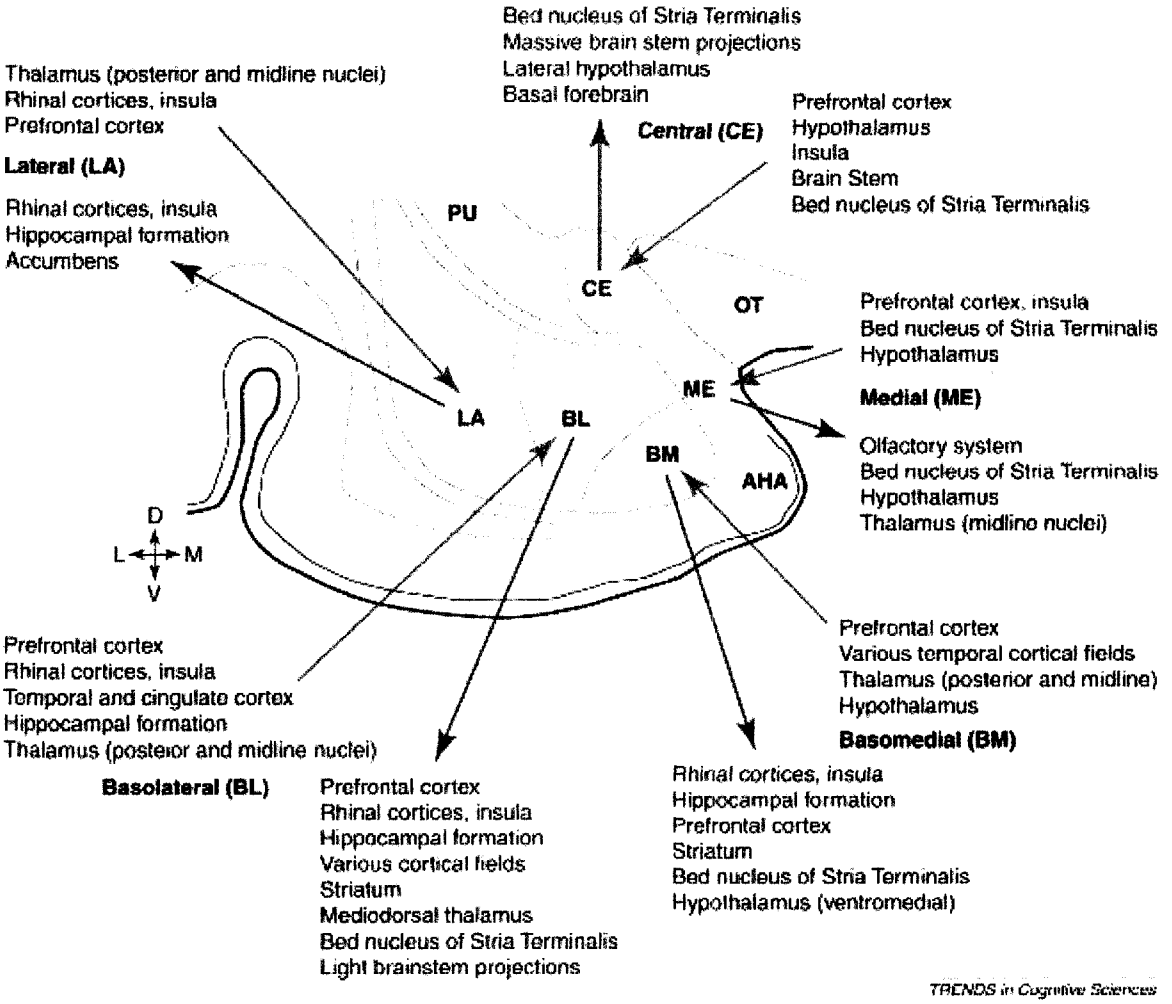


Figure I-3: The Main Connections of the Amygdala.

The amygdala receives information about all sensory modalities from the thalamus and cerebral cortex, most of which is relayed to the BLA and are then conveyed to the corticomedial group via a network of glutamatergic intra-amygdala connections. The amygdala then sends descending projections to the hypothalamus and brainstem as well as ascending cortical projections. Inputs are represented by green arrows, outputs by purple arrows. Note that for simplicity, inputs from and projections to neuromodulatory systems of the brainstem and basal forebrain have been omitted. AHA, amygdalohippocampal area; OT, optic tract; PU, putamen. D, dorsal; V, ventral; L, lateral; M, medial. (Pare et al., 2002) Copyright © 2002 Elsevier Science Ltd.

and memory (Royer and Pare, 2002). The ICM also show a slowly deinactivating K^+ current that enables them to maintain a heightened level of excitability for a prolonged period of time, and thus to exert a lasting influence on behaviour (Royer et al., 2000a; Pare et al., 2003). The ICM, therefore, represent key structures in the influence of emotional behaviour, consolidation of fear-related memory and potential targets of drugs that modulate affective phenotypes.

1 B(iii): Defining the 'Extended Amygdala'

In Johnston's 1923 characterization of the various nuclei of the amygdala, he noted a continuity of the Me and Ce with the bed nucleus of the stria terminalis (BST) (Johnston, 1923; McDonald, 2003). A host of tract-tracing, electrophysiological and immunohistochemical evidence has corroborated this observation, and the BST represents part of the rostral extension of the amygdala proper, or "extended amygdala" (See Figure I-4) (Alheid and Heimer, 1988; McDonald, 2003). The extended amygdala has medial and central divisions (MEA and CEA, respectively), which are functionally distinct subdivisions associated, in the case of the MEA, with the Me, medial BST (BSTM) and medial sublenticular extended amygdala; and in the case of the CEA, with the Ce, lateral BST (BSTL), central sublenticular extended amygdala, the medial division of the interstitial nucleus of the posterior limb of the anterior commissure (IPACM) and caudomedial transition area of the nucleus accumbens (Acb) (Alheid et al., 1995). The efferent and afferent connections of the CEA and MEA are also distinct, with the CEA receiving input from the BLA, thalamus, and insular and frontal cortices (Shammah-Lagnado et al., 2000; Koob, 2003b). The CEA projects to the lateral hypothalamus and brainstem regions and is of primary interest in the study of the amygdala's role in fear and anxiety (summarized in Figure I-4). By contrast, the MEA projects to the medial

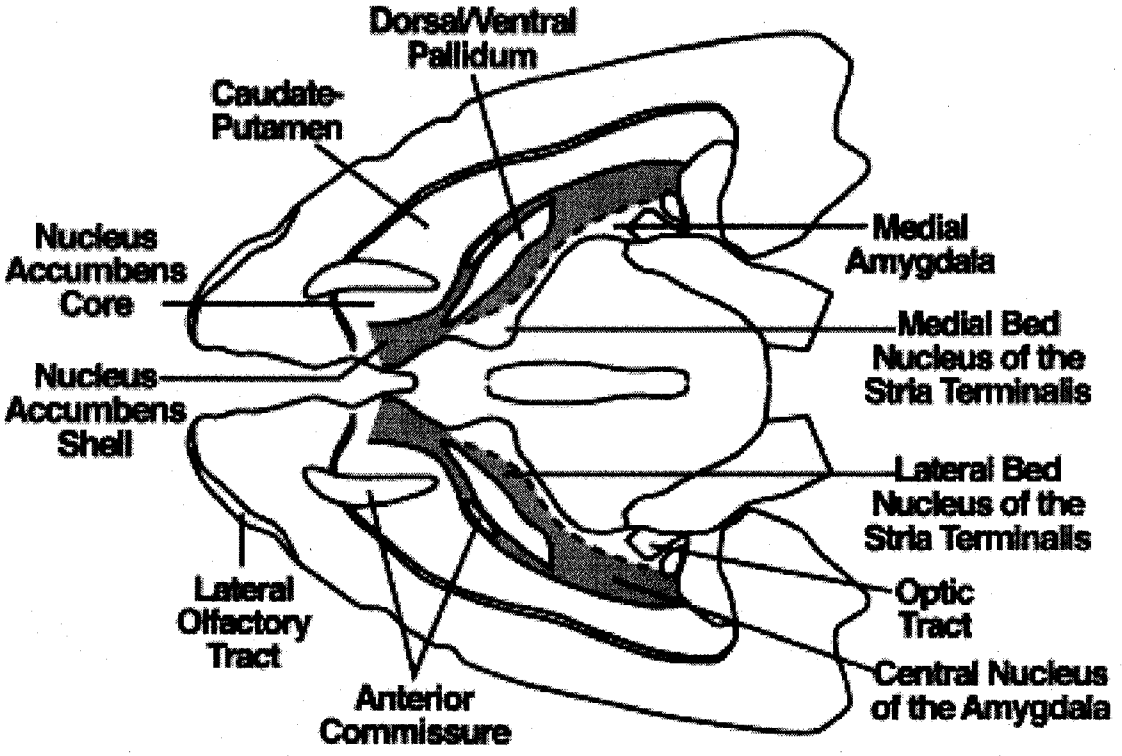


Figure I-4: Summary of the Central Extended Amygdala

Horizontal schematic diagram illustrating the central division of the extended amygdala (highlighted in grey). The central extended amygdala includes a corridor of neurons connecting the nucleus accumbens shell region, the lateral division of the bed nucleus of the stria terminalis, which wraps around the internal capsule, and the central amygdaloid nucleus. Not shown in this diagram is the interstitial nucleus of the posterior limb of the anterior commissure which would appear dorsal to the anterior commissure. (Koob, 2003a). Copyright © Research Society on Alcoholism.

hypothalamus and striatum, and receives largely olfactory input, as well as afferents from the BM (Alheid et al., 1995; Koob, 2003b). Importantly, the MEA does play a role in appetitive behaviour and sexual motivation (Cooke et al., 2001; Cooke et al., 2002).

The interstitial nucleus of the posterior limb of the anterior commissure (IPAC) lies below the posterior limb of the anterior commissure (ac) at the junction of the striatopallidal system and the BST (**Figure I-4**). It has immunohistological characteristics and interconnectivity representative of both the extended amygdala and the striatum (Shammah-Lagnado et al., 1999, 2001). The lateral division (IPACL) is more striatal-like, whereas the IPACM is part of the CEA (Alheid et al., 1995; Veinante and Freund-Mercier, 1997; Shammah-Lagnado et al., 1999; Rosin, 2000). The lateral and medial distinctions of the IPAC are also evident from their respective efferent and afferent connections and immunohistochemistry (Shammah-Lagnado et al., 2001). The IPACM, for example, shares largely reciprocal connections with the Ce and CEA, while the IPACL is interconnected with the pallidal complex, substantia nigra and retrorubral field (Heimer et al., 1997; Bourgeais et al., 2001; Shammah-Lagnado et al., 2001). This has lead to the further subclassifcation of the IPAC into the extended amygdala medial division and the striatal-like lateral division.

The dual striatal/amygdaloid nature of the IPAC is reflected in other extended amygdala structures, such as the nucleus accumbens (Nauta et al., 1978; Maurice et al., 1998). The nucleus accumbens core (AcbC) is interconnected primarily with the striatum, and has a similar immunohistochemical signature. The nucleus accumbens shell (AcbSh), on the other hand, projects to the hypothalamus, amygdala and midbrain (Nauta et al., 1978), and is histochemically similar to the CEA (Zaborszky et al., 1985). In addition, several dopamine D1 receptor rich 'islands' have been identified in the AcbSh that were

associated with very low TH and dopamine D2 receptor immunoreactivity (Jansson et al., 1999). The existence of receptor rich substructures that are relatively devoid of the corresponding neurotransmitter terminal input throughout the CEA is a major focus of this research project and similar substructures have been identified in the ICM and IPACM (Fuxe et al., 2003a; Jacobsen et al., 2006). The functional implications of these patches within the CEA are not yet clear. In this thesis we attempt to address this question, and examine how these patches might be involved in the modulation of amygdala function by a number of important fear circuitry pathways.

1 C: Neurotransmitters of the Fear Circuitry

GABA is the primary inhibitory neurotransmitter of the brain and GABAergic interneurons and projection neurons are present throughout the limbic system (Barnard et al., 1998; Bowery and Enna, 2000; Millan, 2003). Glutamate, the primary excitatory brain neurotransmitter, also has an established role in modulating anxiety pathways through its ionotropic and metabotropic receptors, both classes of which are distributed throughout the limbic system (Davis and Myers, 2002; Walker and Davis, 2002; Millan, 2003; Shekhar et al., 2005). Communication between the primary input (BLA) and output structures (Ce) of the amygdala is achieved by a combination of stimulatory glutamatergic projections from the BLA to the Ce and feed-forward inhibition by the GABAergic ICM (Royer et al., 2000a). The glutamatergic projections from the BLA reach the Ce both directly and via the ICM, which contain inhibitory GABAergic interneurons as well as sending feed-forward GABAergic inhibitory projections to the Ce (Pare et al., 2004). This is analogous to the organization of the striatum, in which glutamatergic projections onto the input nuclei (caudate and putamen) target GABAergic interneurons and projection neurons that gate output (i.e. from the globus pallidus interna

and substantia nigra pars reticulata) (Nolte, 1993). In addition, the striatum also has a series of intermediate nuclei that relay information between the input and output structures (the globus pallidus externa, subthalamic nucleus and substantia nigra pars compacta) and can be modulated by monoamine innervation (i.e. dopamine) (Davis and Myers, 2002; Avshalumov et al., 2003). While the hierarchical organization of the striatum has been extensively studied, we are only beginning to understand how a similar architecture might be involved in modulating amygdala function. In this thesis, we will focus on modulation of the basic intra-amygdala circuitry by neurotransmitter systems involved in regulating emotion.

Perhaps most relevant to the function and dysfunction of the fear circuitry are the monoamines: dopamine (DA), norepinephrine/epinephrine (NE/E, also called noradrenaline/adrenaline – NA/A) and 5-hydroxytryptamine (serotonin, 5-HT) (Millan, 2003) which are derivatives of the amino acids tyrosine (DA and NE/E) and tryptophan (5-HT), respectively (Figure I-5). While the focus of this literature review is on the DA and 5-HT systems, the contribution of NE to the neurobiology of anxiety cannot be overlooked. NE projections from the brainstem noradrenergic nuclei, especially the LC (Aston-Jones et al., 1991; Tanaka et al., 2000), innervate many regions of the limbic system, including the amygdala, hippocampus and hypothalamus, in addition to substantial cortical projections. Prolonged activation of this pathway by anxiogenic or stressful stimuli is a marked feature of the anxiety response (Ressler and Nemeroff, 2000; Morilak et al., 2005). Neuropeptides and their receptors have likewise been studied for their role in fear and anxiety. Cholecystokinin (CCK) and its receptors CCK1 and CCK2 are distributed in a variety of limbic structures and play an important role in anxiety through interactions with a number of other neurotransmitter systems including DA, 5-HT

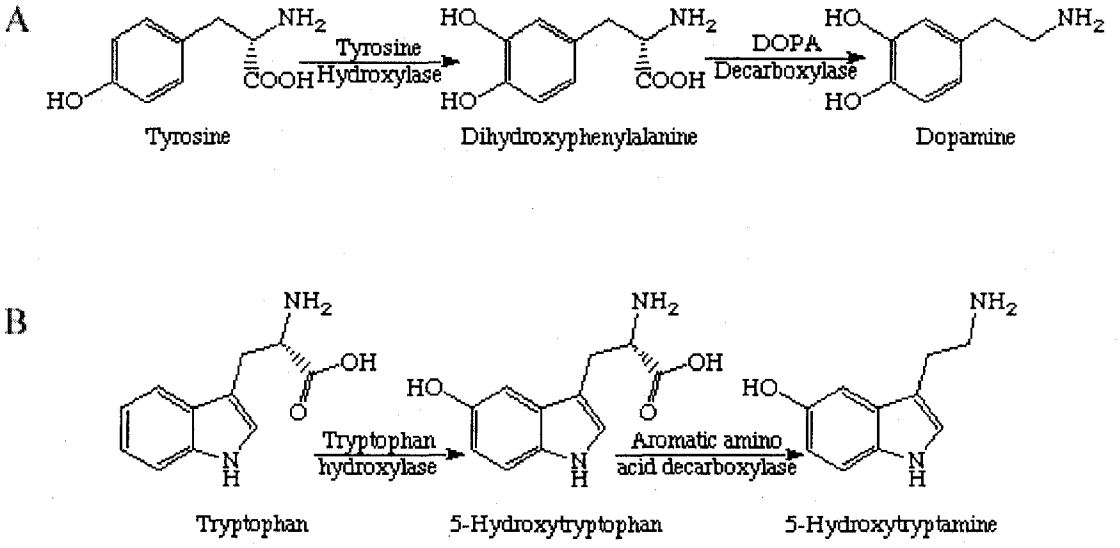


Figure I-5: Synthesis of Dopamine and Serotonin

(A) Dopamine Synthesis: the first step in the synthesis of dopamine is catalyzed by the rate-limiting enzyme, tyrosine hydroxylase, which converts the amino acid tyrosine into 3,4-dihydrophenylalanine (DOPA). DOPA is then converted to dopamine by DOPA decarboxylase [a.k.a. aromatic L-amino acid decarboxylase (AADC)]. In adrenergic cells, dopamine can be further converted into noradrenaline by dopamine β hydroxylase (DBH). (B) Serotonin synthesis: Similar to dopamine, serotonin is synthesized from an amino acid (tryptophan) by the rate limiting enzyme, tryptophan hydroxylase, which converts serotonin to 5-hydroxytryptophan. This is then converted to serotonin (5-hydroxytryptamine) by AADC.

and GABA (Gilles et al., 1983; Agnati et al., 1985; van Megen et al., 1996; Fratucci De Gobbi et al., 2001; de la Mora et al., 2007). Peptides involved in the HPA axis also have a clear role in the stress response. These include corticotrophin releasing factor (CRF), adrenocorticotropic hormone (ACTH), vasopressin, oxytocin, neuropeptide Y, substance P, galanin, somatostatin and neurotensin (Mathe, 1999; Chrousos, 2000; Tsigos and Chrousos, 2002; Barrera et al., 2006; Ebner and Singewald, 2006; Sajdyk et al., 2006; Bao et al., 2007). For the purposes of this thesis, we will focus on a final group of peptide neurotransmitters, the opioid peptides, which are expressed throughout the corticolimbic system and have contrasting and often complimentary roles in the control of anxiety, fear and aggression (Mansour et al., 1988; Adler et al., 1990; Sugita and North, 1993; Mansour et al., 1995a; Drolet et al., 2001; Tordjman et al., 2003; Wilson et al., 2003).

1 C(i): The Opioid Peptide Neurotransmitters

The primary opioid receptor subtypes are μ, δ, and κ, so named for the chemicals first identified to bind to them (for example, m - morphine and k - ketocyclazocine) (Corbett et al., 2006). The μ-, δ-, and κ- opioid receptors (MOR, DOR and KOR, respectively) are G-protein coupled receptors that inhibit adenylyl cyclase as do other Gi/Go-coupled receptors (Childers, 1991), increase conductance of inwardly rectifying potassium channels (North et al., 1987), inhibit voltage-activated calcium channels (Millan, 2003) and increase inositol (1,4,5) trisphosphate formation (Smart et al., 1994) upon agonist stimulation. Many opioid peptides have been identified, although only those with known precursor peptides and affinities for the known opioid receptors will be discussed here. [Met]- and [Leu]-enkephalin (Met-ENK and Leu-ENK) are derived from pro-enkephalin and have the highest affinity for DOR; β-endorphin is derived from pro-opiomelanocortin

which also gives rise to ACTH and has the highest affinity for MOR and DOR; and dynorphin is derived from prodynorphin and binds KOR most strongly, although dynorphin also has a high affinity for MOR and DOR (Corbett et al., 2006). The three opioid receptors are expressed in different and sometimes overlapping brain regions, with MOR having the most extensive and dense distribution, and having the unique property of being localized to discrete 'patches' in several regions, whereas DOR and KOR are localized throughout the brain in a more diffuse pattern (Mansour et al., 1987; Daunais et al., 2001). All three receptors localize throughout the monoamine fear circuitry, with all three being expressed in the nucleus accumbens, hypothalamus, amygdala and extended amygdala structures, such as the BST (Mansour et al., 1994; Mansour et al., 1995b; Mansour et al., 1996; Sim and Childers, 1997); however, the only MOR localizes to the GABAergic ICM of the amygdala (Wilson et al., 2002).

As even the most well-mannered Neoliths knew, opioids are famous for their antinociceptive properties, with the cultivation of opium poppies for anaesthesia dating back, literally, to the Stone Age (Corbett et al., 2006). Opioid peptide activation of MOR in the amygdala, especially the BLA, produces antinociception, an effect that is also activated by exposure to stressful or fear-inducing stimuli (Pavlovic et al., 1996; Tershner and Helmstetter, 2000). On the other hand, stimulation of DORs in the amygdala does not produce an antinociceptive response. In keeping with this, mice lacking DOR display no alterations in their spontaneous nociceptive threshold, in contrast with mice lacking either MOR or KOR, which both have defined roles in the analgesic response (Matthes et al., 1998; Simonin et al., 1998; Filliol et al., 2000). There is, however, evidence suggesting that DORs in the periaqueductal gray are involved in the full expression of morphine and β-endorphin-induced analgesia in the amygdala (Pavlovic et al., 1996). Opioid peptide

stimulation of opioid receptors in the amygdala and nucleus accumbens have an involvement in the rewarding and anxiolytic properties of ethanol and cocaine (Heyser et al., 1999; Yuferov et al., 1999; Cowen and Lawrence, 2001; Wilson et al., 2003), the emotional component of feeding behaviour (Glass et al., 1999), reducing the affective/distressing component of pain (Drolet et al., 2001; Nandigama and Borszcz, 2003) and regulating a number of aspects of the stress response (Drolet et al., 2001).

1 C(ii): Serotonin

Serotonin was first isolated and named by Maurice M. Rapport in 1948 (Rapport et al., 1948). It is one of the earliest neurotransmitters expressed during brain development and was identified in the cell bodies of brainstem raphe-nuclei (B1-9) by Dahlström and Fuxe in 1964 (Dahlstrom and Fuxe, 1964). There is a caudal (B1-5) raphe group that projects to the brainstem and spinal cord, and a rostral (B6-9) raphe group that projects throughout the brain (see Figure I-6a) (Gaspar et al., 2003). Specification of 5-HT neurons is tightly linked to the specification of DA neurons. The intersection of Shh production in the notochord, FGF4 in the primitive streak and FGF8 in the midbrain-hindbrain organizing center (MHO) forms a three dimensional Cartesian space that directs development of 5-HT neurons caudal to the MHO (See Figure I-6b), and induces the genetic cascade which follows (Ye et al., 1998; Hynes and Rosenthal, 1999; Gaspar et al., 2003). Downstream of Shh is the homeodomain gene *Nkx2.2* which interprets Shh signals and is involved in selecting neuronal identity (Briscoe et al., 1999). *Nkx2.2* and the related *Nkx6.1* induce downstream factors in this pathway, including *Gata-2* and *Gata-3*, which are zinc-finger proteins involved in the specification of the rostal and caudal raphe groups, respectively Craven, 2004 #682; van Doorninck, 1999 #646}. *Lmx1b* (LIM homeobox transcription factor 1 β), is also involved in the commitment and maintenance of 5-HT neurons that is

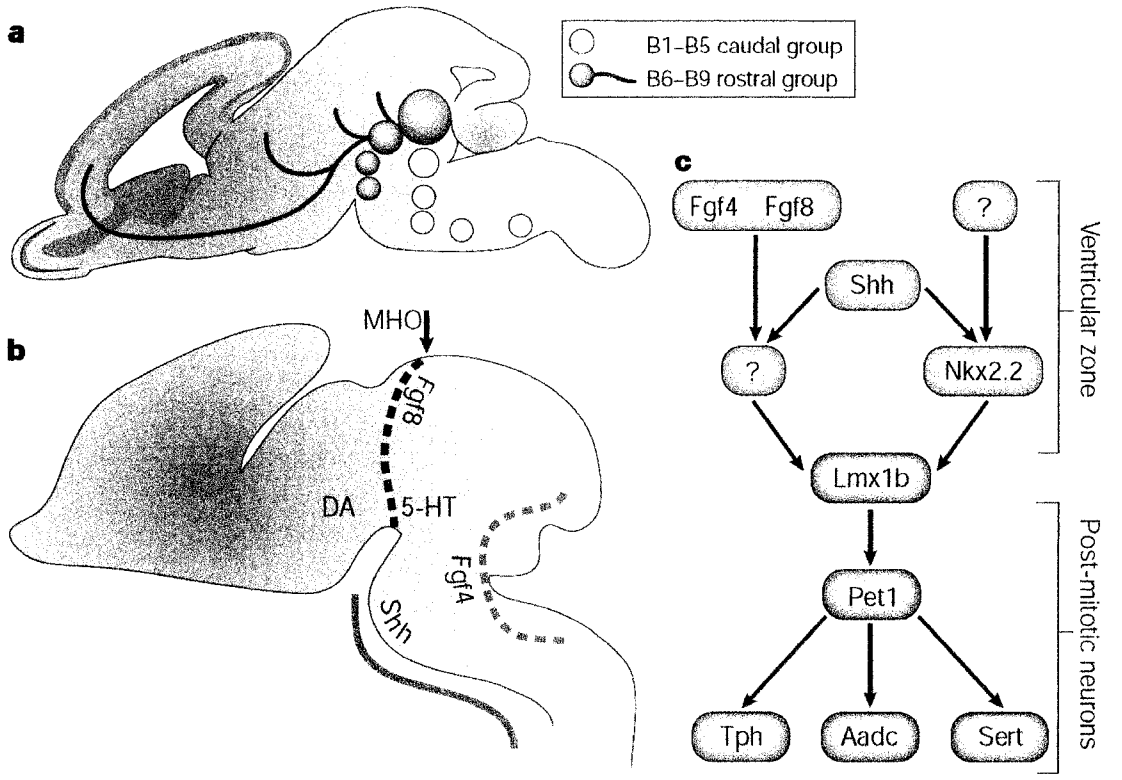


Figure I-6: Brainstem Serotonin Neurons and their Specification

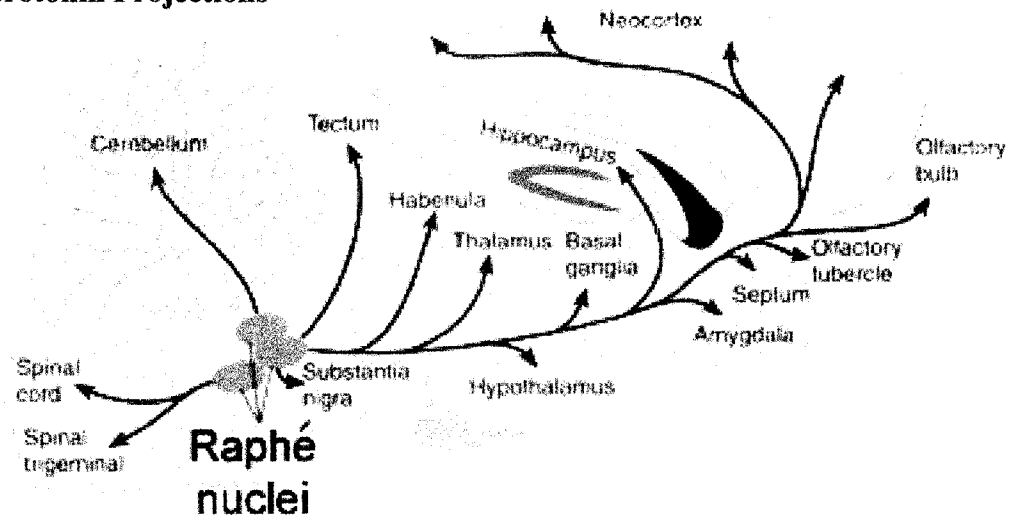
(a) The serotonergic raphe cell groups are divided into the caudal (B1-5, yellow) and rostral (B6-9, red) groups which project to the brainstem and spinal cord, and diencephalon and telencephalon, respectively. (b) Specification of 5-HT precursor neurons in the neural tube is determined by a number of secreted factors, including FGF8 in the MHO and Shh in the ventral midline of the notochord. Shh and FGF8 drive DA neuron specification rostral to the MHO, and expression of FGF4 along the primitive streak caudal to the MHO allows these signals to drive 5-HT neuron specification. (c) FGF4, FGF8 and Shh are necessary for initial specification, and Shh induces *Nkx2.2*. Downstream of *Nkx2.2, Lmx1b* and *Pet-1* are also essential for normal 5-HT phenotype. Other factors, are likely to be involved in this transcriptional cascade, and are represented by a question mark (Gaspar et al., 2003). Copyright © 2007 Nature Publishing Group.

lost in either *Nkx2.2* or *Gata-3* null mice (Cheng et al., 2003; Ding et al., 2003). *Lmx1b* null mice fail to express the ETS transcription factor *Pet-1* [(pheochromocytoma 12 *Ets* (E26 transformation-specific) factor, a.k.a. Fifth Ewing Variant, FEV), which is essential for the differentiation of 5-HT neurons, as well as the expression of a number of serotonergic genes, such as tryptophan hydroxylase (*TPH*), *AADC* and the serotonin transporter (*5-HTT*) (Hendricks et al., 2003, Cheng, 2003 #397). See Figure I-6c for a schematic of the pathway driving 5-HT development. Through conserved Pet-1 binding elements in several serotonergic genes, Pet-1 is thought to act as a transcriptional regulator in addition to having a role in directing differentiation and specification of 5-HT neuron fate (Hendricks et al., 1999). Disruption of any of the transcriptional regulators involved in the specification of 5-HT neurons results in substantial deficits in 5-HT neuron differentiation, migration and signalling; however, interestingly, few of the gene disruptions studied to date result in a complete deficit in serotonergic development. *Nkx2.2* null mice still synthesize 5-HT in the dorsal raphe (Briscoe et al., 1999; Cheng et al., 2003), *Gata-2* and *Gata-3* are only required for specification of the rostral and caudal 5-HT groups (respectively) (van Doorninck et al., 1999; Craven et al., 2004) and *Pet-1* null mice retain 20-30% of their 5-HT neurons (Hendricks et al., 2003). *Lmx1b* null mice express *Nkx2.2* and *Gata-3*, but this knockout results in gross defects in both DA and 5-HT neuron development, as well as limb and kidney development, making it hard to examine its specific role in regulating 5-HT development (Chen et al., 1998; Smidt et al., 2000; Ding et al., 2003). Recently, however, a targeted deletion of *Lmx1b* specifically in the raphe nuclei using the *Pet-1* enhancer revealed that *Lmx1b* is required for maintenance and differentiation of 5-HT neurons (Zhao et al., 2006). Another transcriptional regulator that appears to lay upstream of *Lmx1b*, *Pet-1* and *Gata3*, along

with *Nkx2.2*, is the bHLH gene *Ascl/Mash1*, which is also critical for the differentiation of NE neurons (Pattyn et al., 2004). Other regulators of the commitment, differentiation and development of serotonergic phenotype are almost certain to be discovered.

The role of 5-HT in fear, anxiety and other emotionally relevant behaviours is very well established (Ninan, 1999; Ressler and Nemeroff, 2000; Millan, 2003). Alterations in 5-HT signalling are thought to lie at the root of a number of affective disorders, and SSRIs, as well as other 5-HT-specific drugs, provide therapeutic benefits for patients with depression by increasing 5-HT neurotransmission (Charney et al., 1990a; Ninan, 1999; Ressler and Nemeroff, 2000). Responses to contextual fear conditioning can also be reduced by lesioning of specific raphe nuclei or local stimulation of the inhibitory presynaptic 5-HT1A autoreceptors (Silva et al., 2002). Furthermore, reduced 5-HTT expression, which results in an increase in 5-HT transmission, is associated with increased fear and anxiety behaviours (Hariri et al., 2002; Schmitt et al., 2007). 5-HT raphe neurons project strongly to the amygdala and extended amygdala, including the Ce, the BLA and the gating GABAergic ICM of the amygdala proper (Ma et al., 1991; Bauman and Amaral, 2005; Eggers, 2007). 5-HT neurons also project to extended amygdala structures such as the BST (Emson et al., ; Freedman and Shi, 2001) and serotonergic raphe projections to the limbic system have been implicated in the acquisition of contextual fear (Silva et al., 2002), as well as other cortical-hippocampal-amygdala pathways such as anxiety and aggression (Ressler and Nemeroff, 2000). In this way, critical serotonergic input into the fear circuitry provides an important mechanism for modulating, or possibly directing, affective behaviours, and in the case of serotonergic signalling disruptions, affective disorders. See Figure I-7 for an overview of the 5-HT projections in the CNS.

Serotonin Projections



Dopamine Projections

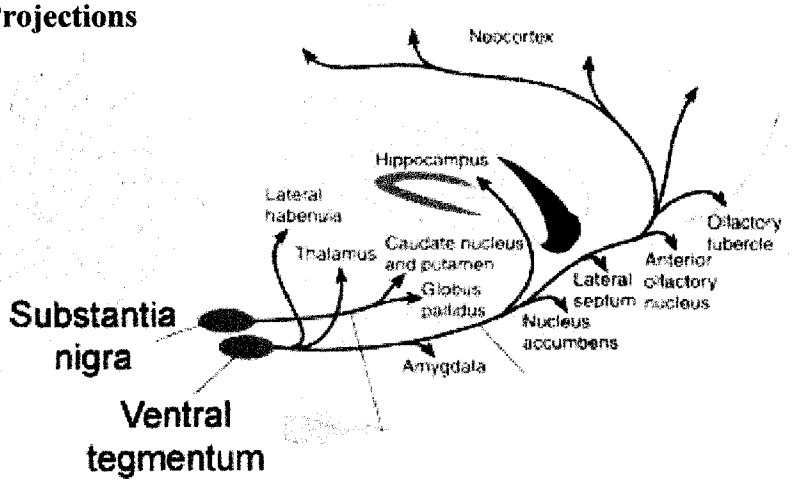


Figure I-7: Summary of Dopamine and Serotonin Projections in the Rodent Brain

Sagittal representation of 5-HT (top) and DA (bottom) pathways in the rodent. 5-HT neurons originate in the raphe nuclei and send both descending and ascending projections throughout the CNS, including to the SN where they mediate dopaminergic signalling. DA neurons originating in the SN and VTA send widespread ascending projections throughout the brain. Copyright © University of Colorado at Boulder.

1 C(iii): Dopamine

Like serotonin, specification of dopamine neurons relies on the overlapping signalling of organizing centers that secrete Shh and FGF8 (Figure I-6), although, unlike 5-HT, DA neuron development does not rely on these signals being preceded by FGF4 (Hynes and Rosenthal, 1999; Roussa and Krieglstein, 2004; Smidt and Burbach, 2007). Catecholamines DA and NE/E are synthesized from the amino acid tyrosine, with the first, rate-limiting step being catalyzed by TH. Dahlström and Fuxe were the first to illustrate that striatal and limbic DA projections originate from mesencephalic DA neurons (Dahlstrom and Fuxe, 1964; Fuxe, 1965b). DA cell bodies are located primarily in the ventral midbrain (denoted A8-10) and diencephalon (A11-15), with two additional smaller populations in the telencephalon (A17 and A18). A1-7 represent NE neuron populations, including the LC (A6) (Hynes and Rosenthal, 1999). Two important midbrain DA neuron populations are the substantia nigra (SN, A9) and ventral tegmental area (VTA, A10), which project to multiple brain regions including the striatum, limbic system, and cortex (Paxinos, 1997; Smidt and Burbach, 2007). The loss of DA neurons from the SN (nigrostriatal pathway) is associated with Parkinson's disease, while DA VTA projections to the Acb, amygdala, extended amygdala and hippocampus (mesolimbic pathway), as well as prefrontal, cingulate and perirhinal cortices (mesocortical pathway) are involved in schizophrenia, addiction and other emotion-based behaviours such as motivation and reward (Chinta and Andersen, 2005).

Midbrain DA neurons can be detected in rodents by about embryonic day E11.5 (Roussa and Krieglstein, 2004). Following induction by FGF8 and Shh, a genetic cascade of transcription factors is involved in the differentiation, phenotype development and maintenance of DA neurons. The specific molecular mechanisms regulating these

processes have not been fully elucidated; however, many of the key players have been identified, including *Engrailed (En 1* and *2)*, *Pax2* and *5*, *Wnt1*, and the more recently identified *Lmx1b* (Smidt et al., 2000; Asbreuk et al., 2002). Many transcription factors implicated in development of the 5-HT system are also required for the differentiation (*Nkx6.1*), development (*Gata3*) and survival (*Lmx1b*) of DA neurons (Smidt et al., 2000; Hong et al., 2006b). For example, the zinc finger protein *Gata3,* as well as the orphan nuclear receptor *NURR1* (Nur-related factor 1), transcriptionally regulate the expression of *TH* (Sakurada et al., 1999; Kim et al., 2003; Hong et al., 2006b). *NURR1* may also be important for the survival of DA neurons, along with *Pitx3* (Nunes et al., 2003; Chinta and Andersen, 2005), and the latter is thought to interact with *Lmx1b* to cooperatively regulate differentiation of midbrain DA neurons (Asbreuk et al., 2002). In support of this, Lmx1b-null mice fail to induce *Pitx3*, but not *TH* or *NURR1*, and these neurons, which are initially formed and express markers of early differentiation, are lost during embryogenesis (Smidt et al., 2000).

The dopamine innervation of the amygdala and extended amygdala has clear implications for the regulation of mood and emotion. Dopaminergic input to the BLA has been shown to be critical for the neuronal plasticity that underlies affective conditioning (Bissiere et al., 2003; Rosenkranz and Grace, 2003), and activation of dopamine D1 receptors in the paracapsular ICM is involved in the increased affective behaviour (i.e. removal of cortical inhibition) during the stress response (Marowsky et al., 2005). Dopaminergic afferents to the entire CEA, including the AcbSh, BST and IPAC (Freedman and Cassell, 1994; Beltramino et al., 1996; Cheung et al., 1998; Freedman and Shi, 2001; Hasue and Shammah-Lagnado, 2002) further support a role for DA in controlling the affective responses to stress and other environmental stimuli.

1 D: Distance Communication: What Light Through Yonder Window Breaks!

For more than a century it has been clear that the idea that the CNS is hardwired in a continuous set of interneuronal connections is inadequate for explaining its global mode of operations. A great deal of evidence suggests a mode of communication other than the well characterized synaptic transmission must exist in order to explain how the CNS functions as a global network, rather than as a series of relatively independent neuronal elements (Vizi and Labos, 1991; Agnati et al., 1995; Fuxe et al., 2007). In order to begin trying to explain this non-synaptic transmission, Nicholson described diffusible ion currents in the extracellular fluid (ECF) as an important mode of communication in the "neuron microenvironment" (Nicholson and Phillips, 1979, 1981). Vizi also characterized several modes of non-synaptic inter-neuronal communication, focussing largely on the cross-talk between adjacent axon termini (Vizi, 1982; Vizi and Labos, 1991). The non-synaptic or "parasynaptic" (Schmitt, 1984) communication was later coined "volume transmission" (VT) by Agnati and Fuxe in 1986 (Agnati et al., 1986; Agnati et al., 1995) in order to better reflect the broad nature of inter-neuronal, as well as neuron-glia and glia-glia communication. The hard-wired communication occurring through a well defined connecting structure such as a synapse, gap junction or membrane juxtaposition was coined "wiring transmission" (WT). See Figure I-8 for a summary of WT versus VT. The combined actions of the fast, one-to-one, hardwired WT and slower, pervasive, one-to-many, diffusion-based VT, provide a foundation for understanding how the CNS functions as a complex cellular network regulating the global neural environment (Agnati and Fuxe, 2000; Bloom, 2000). Understanding the broad scale of VT is also important in neuropsychopharmacology in the sense that the pharmacokinetics and pharmacological actions of psychoactive drugs are subject to the same constraints as endogenous VT

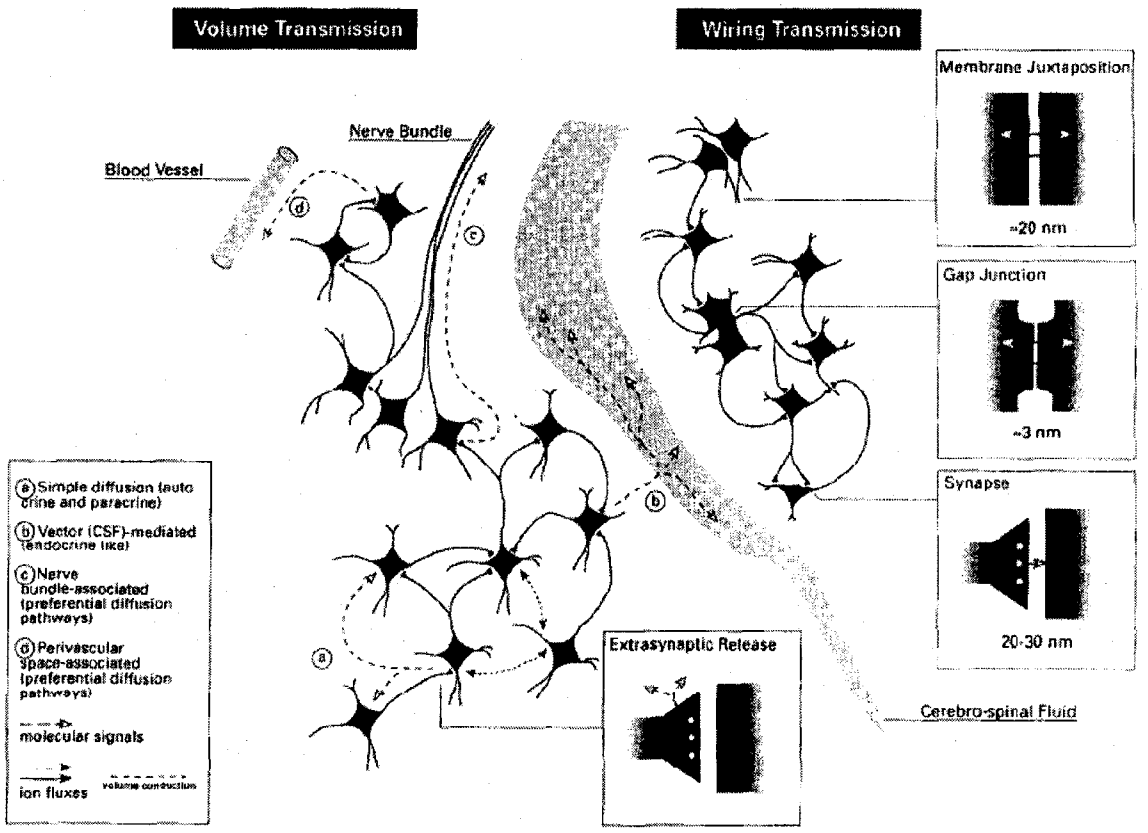


Figure I-8: Volume Transmission vs. Wiring Transmission in the Brain

Wiring transmission, in the form of membrane juxtapositions, gap junctions and synapses, enables a fast, one-to-one form of intercellular communication. In contrast, volume transmission occurs through the local extracellular environment (autocrine and paracrine (a)), CSF (b), in small channels located outside the axons along nerve bundles (c), or perivascular VT along blood vessels (d). In all cases, VT is a one-to-many form of communication that is slower, but more widespread than WT, reaching target cells by diffusing over short (parasynaptic) or long (paracrine) distance (Agnati et al., 1995).

signals (Zoli et al., 1999). That is to say that they must diffuse through the ECF intact and biologically active, reach their appropriate targets and effect a cellular response.

1 D(i): Volume Transmission in the Fear Circuitry

A number of factors are important in determining whether WT or VT occurs for a given neurotransmitter network. The rate, amount and site of neurotransmitter release, rate of diffusion intact in the ECF, reuptake by specific neurotransmitter transporters, recycling, and number and type of locally and distally situated receptors all influence the mode of neurotransmission (Hensler, 2006). Evidence exists of VT for virtually every system involved in modulating the fear circuitry, including 5-HT (Bunin and Wightman, 1998; Jansson et al., 2001a; Hensler, 2006), DA (Fuxe et al., 1988a; Jansson et al., 1999; Rice, 2000; Freedman and Shi, 2001) and the Opioid peptides (Agnati et al., 1986; MacMillan et al., 1998; Drake et al., 2002; Fuxe et al., 2005). The majority of 5-HT varicosities in the cortex and hippocampus of the rat do not appear to associate with synaptic junctions, suggesting that extrasynaptic transmission plays a major role in these regions (Descarries and Mechawar, 2000). Studies using carbon fibre microelectrodes to monitor the easily oxidatable 5-HT (as with DA) have further demonstrated that, in the substantia nigra pars reticulata and the DRN, which has very sparse identifiable synaptic junctions, 5-HT is able to diffuse away from the release site in concentrations sufficient to activate distant 5-HT receptors (Bunin and Wightman, 1998). In these regions, lower clearance rates than other regions, such as of DA in the striatum, enable 5-HT to diffuse through the ECF for a prolonged period of time (Bunin et al., 1998). Also compelling is the impulse- and calcium-dependent, tetrodotoxin-sensitive release of 5-HT from vesicles stored both in the cell body and terminal regions of 5-HT neurons (O'Connor and Kruk, 1991b, a). The localization of 5-HT receptors in extrasynaptic regions, further supports the importance of

VT for 5-HT neurotransmission. The 5-HT1A and 5-HT1B receptors, for example, functions as a somatodendritic autoreceptors, regulating the firing of 5-HT raphe neurons, and are predominantly localized extrasynaptically on cell bodies and dendrites (Gothert et al., 1987; Hjorth and Tao, 1991; Hjorth et al., 1995; Riad et al., 2000; Riad et al., 2001). 5-HT VT to other neurotransmitter receptors has also been suggested, including 5-HT2A receptors in the forebrain (Jansson et al., 2001a), which, along with 5-HT1A and 5-HT1B receptors, is highly implicated the modulation of emotion, and all three receptors are prime targets for drugs modulating affective disorders (Hensler, 2006). These results are in stark contrast to the evidence for neurotransmitter systems such as GABA and glutamate, which appear to communicate exclusively by WT (Bunin and Wightman, 1998).

The DA system, too, has been the subject of much VT research. DA is stored in vesicles and released from both the dendrites and terminals of DA neurons (Pickel et al., 2002). Extrasynaptic release sites have been identified for DA in a number of brain regions (Descarries et al., 1996), and extrasynaptic dopamine transporters (DAT) and dopamine receptors localized to dendritic and axonal plasma membranes are common features of dopamine projections to the striatum and cortex, respectively (Smiley et al., 1994; Nirenberg et al., 1996). A series of revealing studies involving the unilateral 6-hydroxydopamine (6-OHDA) lesioning of rat striatal DA neurons have revealed that DA on the unlesioned side can diffuse to the contralateral, DA-depleted side, and partially restore DA transmission through supersensitive DA receptors (Bjelke et al., 1994). This so-called 'biochemical reinnervation' occurs in the absence of normal synaptic junctions, and has also been observed after grafting of catecholamine-rich tissue into the DA-lesioned striatum, which results in a substantial restoration of DA function even though

no synaptic contacts or outgrowth occurs (Stromberg et al., 2000; Zigmond, 2000). DA neurons, as with 5-HT neurons, also contain somatodendritic autoreceptors. The dopamine D2 receptor has both short (D2S) and long (D2L) isoforms, alternate splice variants that differ by 29 amino acids in the third intracellular loop (Grandy et al., 1989; Civelli et al., 1993; Xu et al., 2002b), with the D2S receptor being localized primarily in presynaptic sites and functioning as an autoreceptor (Usiello et al., 2000). Voltammetric microelectrode and microdialysis studies have further shown that DA can diffuse in the ECF (Kehr et al., 2000), and that VT occurs over short distances in regions where there is an abundance of uptake mechanisms, such as the striatum, and over much longer distances (on the order of 100 μm) in the retina (Rice, 2000). In terms of understanding the global implications of DA VT in the brain, a great deal of work has been done towards understanding the relationship between receptor- and transmitter-distribution in different brain regions. Receptor-transmitter mismatches provide important clues for understanding the spatiotemporal activation of receptors in response to different stimuli. In the ventral striatum, for example, patches rich in dopamine D1 receptor were identified in the AcbSh, that were relatively devoid of dopamine D2 receptors, DAT or TH terminal input (Jansson et al., 1999). DA signalling in target cells is thought to be largely modulated by the 32 KDa potent protein phosphatase inhibitor, dopamine and adenosine 3', 5'-monophosphate-regulated phosphoprotein (DARPP-32) (Greengard et al., 1998; Greengard et al., 1999; Greengard, 2001). DARPP-32 is enriched in all regions that receive DA terminal input, and many extrasynaptically located dopamine receptors do not correlate with DARPP-32 localization, suggesting that DARPP-32 is important primarily for DA WT (Fienberg et al., 1998). The patch-like distribution of the D1 receptor is a consistent feature of numerous systems throughout the extended amygdala, forming a

large basis of this thesis (Jansson et al., 1999; Jansson et al., 2001b; Fuxe et al., 2003a; Jacobsen et al., 2006).

Neuropeptides in the brain have also been widely examined as VT signals. They are primarily stored in large dense core vesicles localized to non-synaptic or parasynaptic sites, and do not have a reuptake mechanism analogous to the monoamines and other classical neurotransmitters, leaving them to diffuse into the ECF until they are cleaved by extracellular proteases and neuropeptidases (Agnati et al., 1995). β-endorphin, an opioid peptide, is able to diffuse, intact, over long distances through the ECF (MacMillan et al., 1998; Höistad et al., 2005). Short range VT of enkephalin, which, like β-endorphin also binds the μ-opioid receptor is also evident in the dentate gyrus (Drake et al., 2002). Neuropeptides commonly coexist with other neurotransmitters, a phenomenon which may be explained by the ability of the former to diffuse over long distances and reach multiple targets. The opioid peptide dynorphin is co-stored with glutamate in mossy fibres (Weisskopf et al., 1993) and NE can be co-released with enkephalin and neuropeptides Y (Ceccatelli et al., 1989; Torres et al., 1992; Morris and Pavia, 2004) glutamate with substance P (De Biasi and Rustioni, 1988) and DA with CCK in mesolimbic neurons (Hokfelt et al., 1980; Hamilton et al., 2000). Mechanisms of co-transmission have also been proposed for the classical neurotransmitters (Trudeau and Gutierrez, 2007), which, together with the concepts of VT and WT, provide insight into the global system of connections able to deliver a hugely varied, sustained and spatiotemporally organized response to a given stimulus.

1 D(ii): Serotonin as a Developmental Signal

Serotonin has long been thought to act as a developmental signal during embryogenesis, mediating a number of histogenic and developmental processes. Combined with the early appearance of 5-HT in the developing brain and widespread projections that coincide spatiotemporally with the expression of 5-HT receptors in target regions (Whitaker-Azmitia et al., 1987; Lauder, 1990; Whitaker-Azmitia et al., 1996; Lauder et al., 2000), a dual role for 5-HT has emerged during embryogenesis, as is the case for other neurotransmitters such as GABA, prior to its role as a classical neurotransmitter: (i) as an autoregulator of serotonergic raphe neuron development and (ii) as a modulator of target neuron differentiation and receptor expression, as well as the development of other neurotransmitter systems such as the dopamine system through the early serotonergic afferents to the substantia nigra (Whitaker-Azmitia et al., 1996). See Figure I-7 for a summary of serotonergic projections throughout the brain.

Maternal administration of ρ-chlorophenylalanine (ρCPA), a competitive tryptophan hydroxylase inhibitor that depletes 5-HT, or 5-methoxytryptamine, a methylated derivative of 5-HT that is a general 5-HT1/2 receptor agonist, delays the differentiation of target cells (Lauder, 1990), reduces 5-HT1 (including 5-HT1A) receptor expression (Lauder et al., 2000), and alters [^{3}H]5-HT binding sites throughout the brainstem and forebrain (Whitaker-Azmitia et al., 1987), axonal pathfinding and neurite outgrowth (Bonnin et al., 2007 and Shemer, 1991 #737) and animal behaviour (Shemer et al., 1988). Other studies have shown that ρCPA treatment (E12-17) alters the maturation, dendritic arborisation and complexity of target regions such as the somatosensory cortex (Vitalis et al., 2007) and disrupts 5-HT axon growth in snails (Baker et al., 1993), a

phenomenon that has also been observed *Drosophila* mutants that do not synthesize 5-HT (Budnik et al., 1989). Lesioning of 5-HT projections to the hippocampus using the 5-HT neurotoxin 5,7-dihydroxytryptamine results in increased levels of 5-HT1A protein in the hippocampus (Patel et al., 1996). By contrast, the short allele of a variable number tandem repeat polymorphism in the 5-HTT gene (5-HTTLPR short), which reduces 5-HTT expression, has been associated with reduced 5-HT1A receptor density in humans (David et al., 2005). Likewise, 5-HTT knockout mice, in which the synaptic 5-HT levels are persistently increased (Bengel et al., 1998; Schmitt et al., 2007), 5-HT1A receptor protein and mRNA levels were significantly decreased in the dorsal raphe (Fabre et al., 2000; Li et al., 2000). Additionally, acute treatment with the 5-HT1A antagonist WAY100635 was shown to upregulate 5-HT1A receptors in cortex and hippocampus (Abbas et al., 2007).

5-HT is further thought to modulate neuronal coupling, both of chemical synapses and gap junctions (Rorig and Sutor, 1996), neuronal excitability (Hayashi et al., 1997; Beique et al., 2004), plasticity (Whitaker-Azmitia et al., 1996), and proliferation of adult neural stem cells in the hippocampus (Schmitt et al., 2007). 5-HT is additionally able to regulate the spatiotemporal expression of other neurotransmitter phenotypes, such as GABA, via 5-HT1A and 5-HT1B receptors (Allain et al., 2005). By tightly modulating the biochemical and morphological development of both serotonergic raphe neurons and their widespread CNS targets, 5-HT plays a critical role in determining the functionality of the entire fear circuitry. As a result, disruptions of the 5-HT signalling during development are inextricably linked to the affective and psychiatric disorders of the fear circuitry.

1 D(iii) Dopamine as a Developmental Signal

Although not as well characterized as 5-HT, there is evidence to suggest a developmental role for DA, both in the anatomy and resultant behaviours associated with the fear circuitry. Especially compelling is that dopamine D1 and D2 receptors are overexpressed in the immature brain, with receptors, synapses and cell number undergoing pruning through development (Whitaker-Azmitia, 1991; Andersen et al., 1997). In fact, both the dopamine D2 and 5-HT1A receptors are expressed transiently in regions through development where they are not seen in the adult, as is the case in the frontal cortex (D2) and cerebellum (5-HT1A). These over- or transiently-expressed receptors may represent programmable receptors whose maintenance is dependent on the level of stimulation or, alternatively, may actually direct development by producing second messengers, both of which have been shown for the dopamine system. Disruption of dopaminergic terminal ingrowth to post-synaptic D2 receptors by neonatal 6-OHDA lesioning (Broaddus and Bennett, 1990) or D2-blocking neuroleptic drugs (Rosengarten and Friedhoff, 1979) reduces D2 receptor binding long into adulthood. In D2 -/- mice, the number of TH-positive neurons was substantially reduced, and stimulation of D2 receptors in wild type mice with the specific agonist Quinpirole increased the number of TH-containing neurons (Kim et al., 2006). Furthermore, enrichment of D1 receptors in growth cones is thought to decrease growth cone motility via cAMP-dependent mechanisms, and begin the differentiation into a mature nerve terminal. The role of DA as a developmental signal in the retina has also been extensively studied (Reis et al., 2007). This important function of DA during development means, however, that subtle pharmacological alterations can result in permanent changes in the mature brain.

2: G-Protein Coupled Receptors

With over 1000 family members, G-Protein Coupled Receptors (GPCRs) constitute the largest and possibly most diverse group of protein families (Fredriksson et al., 2003; Kroeze et al., 2003). GPCRs are 7-transmembrane-spanning receptors that can be classified in an estimated 6 different categories, the largest of which is rhodopsin/β2-adrenergic receptor family (type A) which includes 701 members according to a 2003 review by Fredriksson and collaborators (Fredriksson et al., 2003). Receptors related to the glucagon receptor family (family B) and to the metabotropic neurotransmitter receptors (family C) are smaller and have not been as extensively studied (Gether, 2000). Rhodopsin, the only GPCR for which a crystal structure has been elucidated owing to its natural abundance in metazoan photoreceptors, serves as the structural template for other GPCRs (Palczewski et al., 2000; Stenkamp et al., 2002). In fact, the lab of Brian Kobilka has recently determined the crystal structure of the β2-adrenoreceptor, which may provide more relevant structural information for certain GPCR subtypes (*In Press,* Presented at the International GPCR Conference, 2007). Not only do GPCRs comprise 1% of the human genome (Miller et al., 2004), but they are the targets of more than 40% of all approved prescription drugs (Eglen et al., 2007; Maudsley et al., 2007). They play important roles in regulating a wide range of physiological processes (Kohout and Lefkowitz, 2003) and respond to a host of stimuli, including light and odorants; peptides such as endorphins and enkephalins; lipids; amino acids like glutamate; and biogenic amines such as serotonin and dopamine (Kroeze et al., 2003). GPCRs, aptly named, transmit extracellular signals to a variety of intracellular signalling pathways by coupling to heteromeric G proteins (αβγ) (Kohout and Lefkowitz, 2003). There are at least 18

different Gα proteins, 5 types of Gβ and 11 types of Gγ (Hermans, 2003; Wong, 2003), enabling a vast array of intracellular signalling pathways.

2 A: Overview of G-Protein Coupled Receptor Signalling

The classical paradigm of GPCR signalling involves five principal features which are summarized in Figure I-9. For review see (Gether, 2000; Fredriksson et al., 2003; Kohout and Lefkowitz, 2003; Kroeze et al., 2003; Krasel et al., 2004; Milligan, 2004; Armbruster and Roth, 2005; Ferguson et al., 2005): **(1)** Ligand binding induces a conformational change that allows it to couple to, and thereby activate, the Gαβγ heterotrimer (Gether, 2000; Ayoub et al., 2002). Within the Gα subunit, guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP), enabling dissociation of the Gα and Gβγ subunits. The intrinsic GTPase activity of Gα is facilitated by the RGS (Regulator of G Protein Signalling) proteins (Armbruster and Roth, 2005). **(2)** The activated Gα and Gβγ subunits are then free to activate or inhibit a number of effector enzymes and ion channels (Albert and Robillard, 2002; Krasel et al., 2004; Maudsley et al., 2007). Gαi/o, for example, inhibit adenylyl cyclase (AC) which catalyzes the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Gαs, on the other hand stimulates AC driven cAMP production. The principal target of cAMP is protein kinase A (PKA), which can phosphorylate a number of intracellular targets, including transcription factors such as the cAMP response element binding protein (CREB) which can bind CRE elements in many different gene targets. Another important pathway is the Gαq and Gβγ dependent activation of phospholipase Cβ (PLCβ). PLCβ cleaves the membrane associated phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to the cytosol-soluble Inositol 1,4,5-trisphosphate (IP_3), which releases intracellular calcium (Ca^{2+})

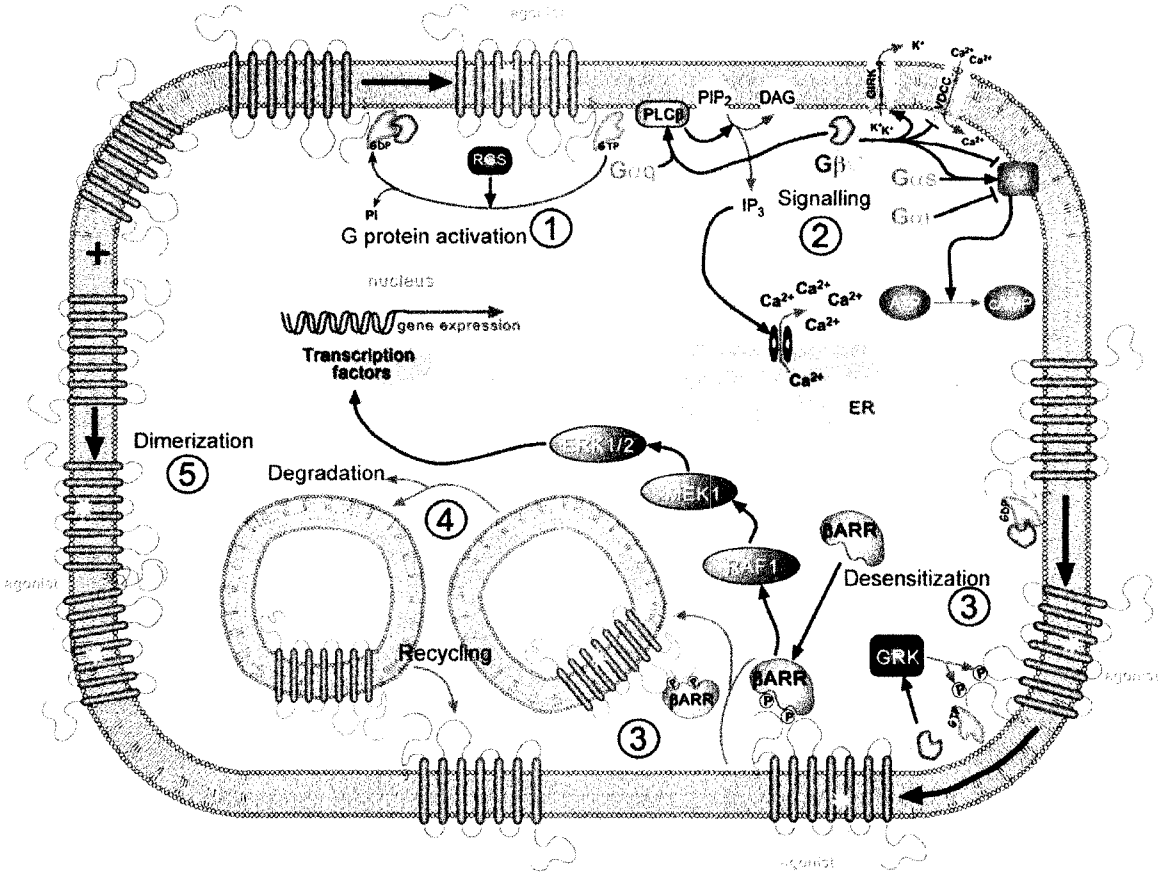


Figure I-9: G-Protein Coupled Receptor Signalling

G-Protein Coupled Receptor Signalling involves 5 main features: (1) G protein activation, (2) Intracellular signalling pathways, (3) Receptor Desensitization, (4) Internalization and Recycling and (5) GPCR oligomerization. Modified from (Armbruster and Roth, 2005).

stores in the endoplasmic reticulum (ER), and diacylglycerol. Diacylglycerol remains membrane-associated and activates the Ca^{2+}-dependent protein kinase C (PKC) which also has numerous protein targets. The Gβγ subunit is further thought to activate G protein-regulated inwardly rectifying K+ (GIRK) channels and inhibit voltage-dependent Ca^{2+} channels (VDCC). Gαi also inhibits the VDCC, while Gαs stimulates it. In addition to these pathways, there are numerous other second messenger pathways downstream of GPCRs. The p42/p44 mitogen activated protein kinase [MAPK, a.k.a. ERK 1/2 (extracellular signal-related kinase)], Jun N-ternimal kinase (JNK), p38 MAPK, big mitogen-activated kinase 1 (BMK1 or ERK5) and nuclear factor-kappa B (NF-κB) pathways have all been linked to G protein signalling. Several G protein independent pathways also exist (Maudsley et al., 2007). **(3)** In addition to initiating any number of intracellular signalling cascades, ligand binding also initiates the process of receptor desensitization (Ferguson, 2001; Kohout and Lefkowitz, 2003; Krasel et al., 2004). This is facilitated by the GPCR serine/threonine kinases (GRKs), which bind and phosphorylate the agonist-associated conformation of the GPCR, and the β-arrestins (βARR), which bind with high affinity the phosphorylated form of the GPCR and prevents further G protein coupling. PKA, PKC and c-Src can also phosphorylate the GPCRs. This feedback mechanism, which can reduce receptor activity by as much as 80%, prevents the potentially harmful effects of acute or chronic receptor overstimulation. β-arrestin is also able to couple GPCRs to G protein independent pathways such as MAPK and JNK. **(4)** β-arrestin then targets the GPCR to clathrin-coated pits, in addition to binding clathrin via association with a larger adaptor complex, and the GPCR is internalized via clathrin-coated vesicles where it is either recycled to the

cell membrane or degraded in lysosomes (Ferguson, 2001; Kohout and Lefkowitz, 2003; Krasel et al., 2004; Eglen et al., 2007). The region of many GPCRs that interacts with arrestins also mediates interactions with other proteins, such as spinophilin, a ubiquitous F-actin and protein phosphatase 1 (PP1) associated protein that localizes to dendritic spines (Allen et al., 1997; Hsieh-Wilson et al., 1999; Feng et al., 2000). By competing with GRKs for association with the ligand-stabilized GPCR-Gβγ complex, spinophilin is able to block the actions of arrestin, a reversible process that represents yet another level of finely-tuned regulation of GPCR signalling (Figure I-10). Importantly, the processes of GPCR desensitization and internalization can also occur via arrestin-independent pathways, as is the case with the 5-HT2A receptor (Bhatnagar et al., 2001; Gray et al., 2003; Bhatnagar et al., 2004). **(5)** Finally, while not, strictly speaking, one of the 'steps' of GPCR signalling, the ability of GPCRs to oligomerize, and the purported functional importance of homo- and heterodimers, is of great interest. Fluorescence and bioluminescence resonance energy transfer and a host of other molecular tools have illustrated *in vivo* GPCR dimerization in a number of systems, and such interactions may even be a requirement for certain GPCRs (Franco et al., 2000; Ayoub et al., 2002; Ramsay et al., 2002; Sacchetti et al., 2002; Milligan, 2004). In fact, GPCR heterodimers, in some cases, even display distinct signalling properties to those of either receptor subtype alone. This has been observed, for example, with DOR and KOR (Ramsay et al., 2002), GABAB R1 and R2 (Jones et al., 1998), adenosine A2A and dopamine D2 receptors (Franco et al., 2000; Canals et al., 2003), adenosine A1 and dopamine D1 receptors (Gines et al., 2000), 5-HT1B and 5-HT1D receptors (Xie et al., 1999), dopamine D2 and D3 receptors (Scarselli et al., 2001) and, although controversial, is

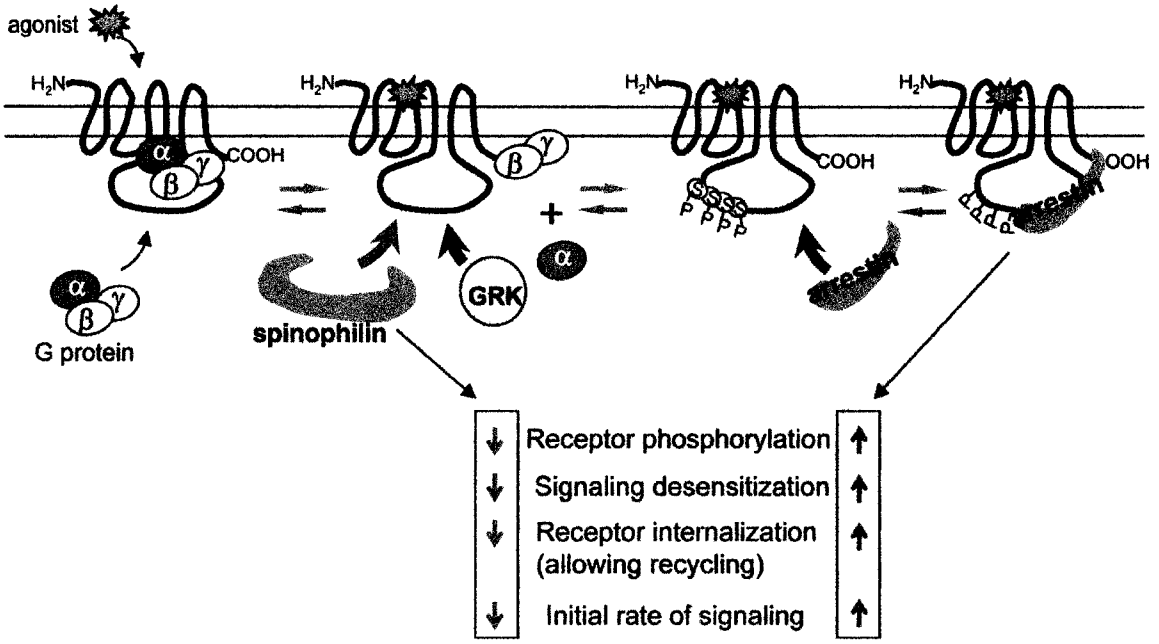


Figure I-10: Model of GPCR interactions with G proteins, arrestin, and spinophilin, and their functional consequences.

Agonist activation of GPCR enhances or stabilizes interactions with G proteins. GRK interacts with the GPCR-Gβγ complex and mediates GPCR phosphorylation. Spinophilin competes for GRK association with the GPCR-Gβγ complex. Activation of the GPCR enhances spinophilin interaction and treatment with Pertussis toxin, which uncouples the GPCR from G proteins, or co-expression of the GRK C terminus blocks this interaction. The result of these reciprocal interactions is the finely-tuned regulation of GPCR signalling. Model based on the α2-adrenergic receptor. Adapted from (Wang et al., 2004).

proposed for the dopamine D1 and D2 receptors as well (Scibilia et al., 1992; Yung et al., 1995; Aizman et al., 2000).

2 B: GPCRs of the Monoamine Fear Circuitry

Central to our understanding of the regulation of human mood and emotion are the ascending pathways of the monoaminergic system, which include 5-HT, DA and NE. The serotonin system is believed to play a critical role in the regulation of many aspects of mood and emotion, including anger (Millan, 2003), aggression (Hendricks et al., 2003; de Boer and Koolhaas, 2005), arousal (Abrams et al., 2005), anxiety (Albert and Lemonde, 2004) and fear (Gross et al., 2000). Alterations in serotonergic signalling, likewise have been associated with major depressive disorder (MDD), obsessive-compulsive disorder (OCD), migraine, irritable bowel syndrome, bipolar disorder, alcoholism and anxiety disorders (Barnes and Sharp, 1999; Ninan, 1999; Koob, 2003a; Lemonde et al., 2003; Overstreet et al., 2003; Albert and Lemonde, 2004; Freitag et al., 2006; Lowry et al., 2007). Indeed, several of the 15 known mammalian 5-HT receptors, most notably the 5-HT1A receptor, are of clinical relevance in the treatment of anxiety and depression. Likewise, dopamine, which is involved in the regulation of locomotor activity, emotion, reward reinforcement, food intake and emotional behaviour has been associated with a number of CNS disorders, including Parkinson's disease (PD), schizophrenia, addiction, Attention-Deficit Hyperactivity Disorder (ADHD) and anxiety (Missale et al., 1998; Vallone et al., 2000; Millan, 2003). Curiously, the DA system has received relatively little attention in the study of affective disorders compared to 5-HT or NE, despite the co-morbidity of "dopaminergic diseases" such as PD and schizophrenia with anxiety and depression, well characterized stress-responsiveness of the mesolimbic and mesocortical pathways, receptor distribution throughout the fear circuitry and anxiolytic properties of

stimulating dopaminergic pathways in the amygdala (McGrath et al., 1999; Tsigos and Chrousos, 2002; Millan, 2003; de la Mora et al., 2005; Jacobsen et al., 2006). As with 5-HT, NE projects to virtually all regions of the fear circuitry (Westlund and Coulter, 1980; Lindvall et al., 1984). Tricyclic antidepressants (TCAs), which block NE reuptake, are commonly used in the treatment of depression, and elevated NE or activation of NE pathways is associated with fear and anxiety (Ninan, 1999; Tanaka et al., 2000). Benzodiazepines, such as Valium, are also used in the treatment of anxiety (Ninan, 1999; Tanaka et al., 2000; Millan, 2003). These target ionotropic GABAA receptors, especially those on the NE cell bodies of the LC, which are believed to produce their anxiolytic effects in part by reducing NE transmission. The interactions between these neurotransmitter systems are critical components of the stress response and the control of mood and emotion. The current review will focus primarily on two receptors critical to the development and treatment of psychiatric disorders: the 5-HT1A receptor and the dopamine D2 receptor.

2 B(i): Serotonin Receptors: focus on 5-HT1A

There are seven distinct subfamilies of 5-HT receptor (5-HT1A/B/D/E/F, 5-HT2A/B/C, 5-HT3A/B, 5-HT4, 5-HT5A/B, 5-HT6 and 5-HT7) (Hoyer and Martin, 1997; Raymond et al., 1999; Millan, 2003; Albert and Lemonde, 2004). The G-protein coupling, agonist and antagonist affinities, intracellular signalling paradigms and distribution of each receptor subtype is quite distinct, underscoring the broad range and versatility of the serotonergic regulation of the brain. The 5-HT1A receptor, which functions both as a presynaptic autoreceptor and a post-synaptic receptor (Hjorth et al., 1995), is a Gi/o coupled receptor, that decreases neuron firing by inhibiting adenylyl cyclase (↓ cAMP levels), opening

GIRK channels and inhibiting VDCC channels (Albert et al., 1990; Barnes and Sharp, 1999; Raymond et al., 1999; Kroeze et al., 2003; Albert and Lemonde, 2004). The presence of somatodendritic autoreceptors on 5-HT neurons provides a sensitive mechanism of self-regulation, whereby 5-HT, possibly released from axon collaterals, reaches the 5-HT1A autoreceptors and decreases neuron firing rate (Sotelo et al., 1990; Hjorth et al., 1995). It is this autoregulatory mechanism that is thought to account for the delay in efficacy of selective serotonin reuptake inhibitors (SSRIs), which block the 5-HTT and increase 5-HT transmission (Bonhomme and Esposito, 1998). The antidepressant effects of SSRIs, delivered acutely, are mitigated by the autoreceptor inhibition of firing and subsequent decrease in 5-HT release; however, with chronic treatment, homologous desensitization of 5-HT1A autoreceptors (see Figure I-9) disinhibits the serotonergic neuron and restores firing frequency, thereby increasing serotonergic neurotransmission and producing clinical effects (Figure I-11) (Albert and Lemonde, 2004).

The 5-HT1A receptor distribution in the brain has been mapped extensively by autoradiography, PET studies and immunocytochemistry (Pazos and Palacios, 1985; Khawaja et al., 1995; Pike et al., 1995; Hoyer and Martin, 1997; Barnes and Sharp, 1999; Bonnin et al., 2006). 5-HT1A receptors are highly expressed in the limbic system, including the hippocampus, lateral septum and amygdala, as well as the brainstem raphe nuclei and cortex (especially limbic-associated entorhinal and cingulate cortex). Much lower levels are found in the striatum, and 5-HT1A is virtually undetectable in the adult cerebellum.

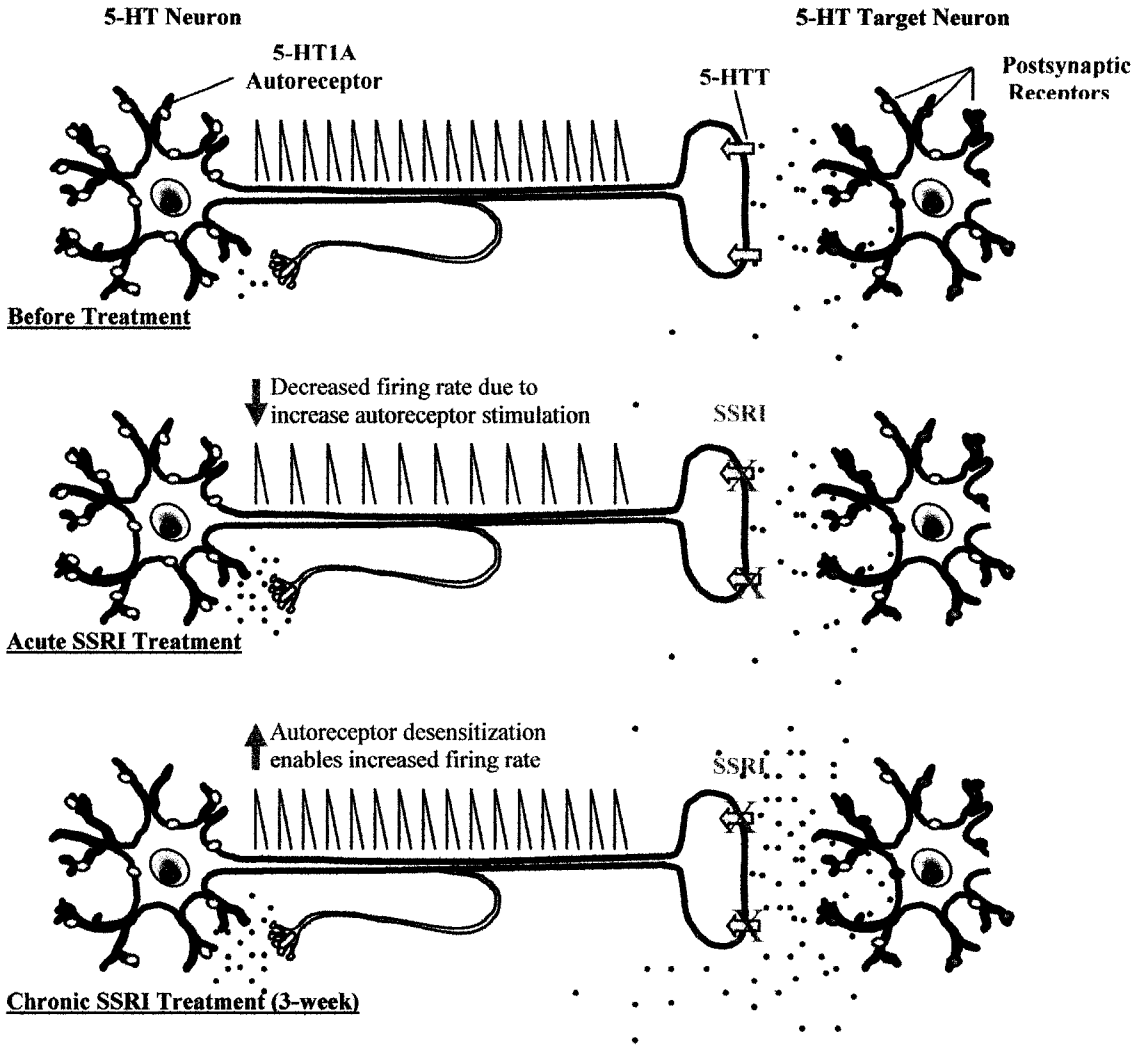


Figure I-11: Chronic versus Acute SSRI Treatment

Before SSRI treatment, activation of 5-HT1A autoreceptors by 5-HT released from axon collaterals or by diffusion from the synaptic cleft provides an efficient mechanism for modulating serotonergic firing. Upon acute SSRI treatment, the blockage of 5-HTT increases free 5-HT, as well as 5-HT1A autoreceptor inhibition, decreasing overall firing. After chronic (3-week) SSRI treatment, homologous desensitization of 5-HT1A receptors disinhibits serotonergic firing, increasing 5-HT release and producing antidepressant effects. Modified from (Albert and Lemonde, 2004).

2 B(ii): Dopamine Receptors: focus on D2

The five dopamine receptors are subdivided into two subfamilies with distinct pharmacology and biochemistry, the D1-like (D1 and D5) and the D2-like (D2, D3 and D4) receptors (Missale et al., 1998; Vallone et al., 2000). Generally, the dopamine D1-like receptors are considered to be stimulatory, Gαs coupled receptors (Sibley, 1999), although there is some evidence they can couple to Gαo in some cell lines (Kimura et al., 1995). D2-like receptors, on the other hand, are inhibitory Gαi/o-coupled receptors that inhibit AC and Ca^{2+} channels and increase K^{+} conductance (Missale et al., 1998). Alternative splicing of exon 6 of the D2 receptor gene leads short (D2S) and long (D2L) isoforms of the receptor, which differ by 29 amino acids in the third intracellular loop (Monsma et al., 1989). This relatively small change results in a number of distinctions between the two isoforms, not the least of which is the predominance of the D2S isoform as a presynaptic autoreceptor and of the D2L isoform in postsynaptic regions (Usiello et al., 2000; Centonze et al., 2002). Fundamentally, D2S and D2L are pharmacologically very similar; however, they display subtle differences in G protein coupling selectivity (Albert, 2002; Albert and Robillard, 2002; Banihashemi and Albert, 2002), post-translational processing and intracellular trafficking (Fishburn et al., 1995), agonist-induced sequestration and endocytosis (Itokawa et al., 1996), desensitization (Stone et al., 2007), uncoupling (Liu et al., 1992) and intracellular signalling (Takeuchi and Fukunaga, 2004; Van-Ham et al., 2007). One example of the latter is the differential activation of ERK1/2 by D2S and D2L. In rat pituitary GH4 cells stably transfected with the dopamine D2S receptor or D2L receptor, D2S inhibits ERK1/2 signalling by coupling to Gαo/Gαi3 (Banihashemi and Albert, 2002), whereas Gαi/Gαo coupling by D2L does not lead to

ERK1/2 inhibition (Van-Ham et al., 2007). In fact, in non-neuronal Balb/c-3T3 cells or NG108 neuroblastoma cells, D2S and D2L stimulate ERK1/2 activation (Albert and Robillard, 2002; Takeuchi and Fukunaga, 2004), while D2 stimulation in primary striatal cultures inhibits K^+-stimulated ERK1/2 signalling (Van-Ham et al., 2007). In general, activation of the D2L receptors leads to ERK activation, while the effects of D2S activation are cell-type dependent, highlighting an important subtlety in the signalling of the two D2 variants (Luo et al., 1998).

Another signalling pathway that is important to mention is the DARPP-32 pathway, for which Dr. Paul Greengard was awarded the Nobel Prize in 2000 (Greengard, 2001) (Figure I-12). DARPP-32 plays a central role in integrating signals from a range of neurotransmitter receptors. It is expressed in a number of brain regions that receive strong dopaminergic input, including the caudate and putamen, Acb, olfactory tubercle, BST, and amygdaloid complex, a finding which initially made DARPP-32 of interest as a potential key regulator of dopaminergic signalling (Ouimet et al., 1984). The now famous paradigm focuses on the striatum, where receptors of virtually all neurotransmitter systems are expressed, including dopamine (D1 and D2), glutamate (NMDA and AMPA), GABA (GABAA), adenosine (A2A), opioid peptide (MOR and DOR) and serotonin (i.e. 5-HT4, 5-HT6) (Fienberg et al., 1998; Greengard et al., 1999; Nishi et al., 2000; Svenningsson et al., 2000; Greengard, 2001; Flores-Hernandez et al., 2002; Svenningsson et al., 2002). Phosphorylation of DARPP-32 by the cAMP- and cGMP-dependent protein kinases PKA and PKG (respectively) renders it a potent inhibitor of protein phosphatase-1 (PP-1), a serine/threonine protein phosophatase that can dephosphorylate and modify the activity of a number of receptors and ion channels (Greengard et al., 1998; Hsieh-Wilson et al., 1999). Synergistic actions of protein phosphatase-2A and -2B (PP-2A and -

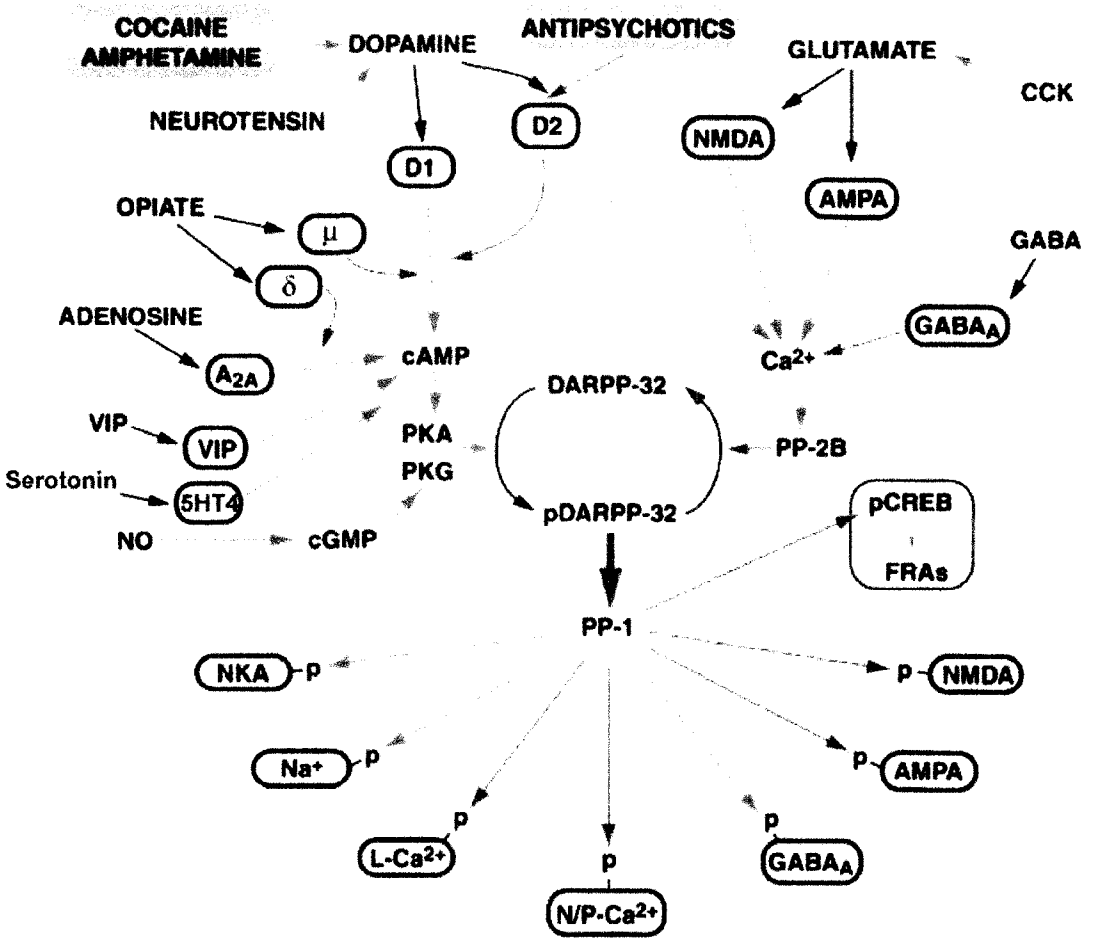


Figure I-12: DARPP-32 Signal Transduction Pathway in Medium Spiny Neurons

DARPP-32 phosphorylation is modulated by a large number of neurotransmitter signalling pathways, including nitric oxide (NO), peptide neurotransmitters such as CCK, opioid peptides and vasoactive intestinal peptide (VIP) and classical neurotransmitters such as GABA and glutamate and adenosine, DA and 5-HT. Phosphorylated DARPP-32 is a potent PP-1 inhibitor, preventing PP-1 from phophorylating multiple intracellular targets. DARPP-32 can be dephosphorylated by PP-2A (not shown) or PP-2B, a Ca^{2+}/calmodulin dependent protein phosphatase. Modified from (Greengard et al., 1999).

2B, respectively) can dephosphorylate DARPP-32, disinhibiting PP-1 (Nishi et al., 1999; Nishi et al., 2003). In this way, DARPP-32 serves as an integrative signalling molecule that balances the physiological outcomes of a vast number of intracellular signalling pathways.

The dopamine D1 receptor is the most highly expressed and widely distributed of the DA receptors, being expressed in the striatum, Acb, hippocampus, amygdala, hypothalamus and thalamus (Missale et al., 1998; LaHoste et al., 2000; Maltais et al., 2000; Chao et al., 2002; Fuxe et al., 2003a). Dopamine D2 autoreceptors are expressed in the SN and VTA, and postsynaptically they are found in the Acb, olfactory tubercle, Ce and medium spiny neurons of the striatum (Missale et al., 1998; Jansson et al., 1999; Jansson et al., 2001b; Rezayof et al., 2002).

2 C: GPCRs in Disorders of the CNS Fear Circuitry

With one third (Robas et al., 2003) to one half (Flower, 1999) of all drugs currently on the market targeting GPCRs, their relevance to the study of human disease is imminently clear. That being said, however, these drugs target only about 10% of the ~1000 GPCRs in the human genome (Vassilatis et al., 2003), a number that is destined to increase with our understanding of the diverse physiological responses controlled by these receptors. The focus of this review will be specifically on the roles of dopamine and serotonin in disorders of the fear circuitry. These include virtually all affective disorders: ADHD, bipolar disorder, eating disorders, general anxiety disorder, MDD, OCD, panic disorder, posttraumatic stress disorder, and others; however, in order to fully appreciate the etiology of fear circuitry disorders, we will also examine diseases that involve a dysregulation of the fear circuitry but are not, strictly speaking affective disorders, such as PD (Hudson and Pope, 1990; Hudson et al., 2003). These diseases are enormously

costly to society, affecting not only the individual, but the work environment (lost productive time, greater health related absenteeism, etc) and the medical system at large. Total anxiety disorder, according to the Canadian Journal of Psychiatry, has a lifetime prevalence of 16.6%, and is twice as prevalent in women (Somers et al., 2006). US workers with depression, which has a lifetime prevalence rate of 5-12% for men and 10-25% for women (Waraich et al., 2004), have been estimated to cost 31 billion dollars per year more than workers without depression in lost productive time (Stewart et al., 2003).

2 C(i): Serotonin in Disorders of the Fear Circuitry

Although the essential role of the serotonin system in affective disorders is of little debate, the inferential nature of disorders such as anxiety and depression, which have both psychological and neurobiological considerations in their diagnoses, makes pinpointing the precise role somewhat challenging (Millan, 2003). The primary serotonergic pathways arising from the DRN and median raphe nucleus (MRN) innervate the frontal cortex, hippocampus, amygdala, Acb, septum and hypothalamus are activated by anxiogenic stimuli and are implicated in fear conditioning paradigms (Silva et al., 2002; Abrams et al., 2004; Abrams et al., 2005; Hensler, 2006; Waselus et al., 2006). Furthermore, a decrease in serotonergic activity is associated with depression (Stockmeier, 1997; Ninan, 1999). The contribution of the serotonin system to anxiety and depression is possibly the most thoroughly studied; however, dysregulation of serotonergic signalling has been observed for many other affective disorders. Altered serotonergic functioning as well as polymorphisms in *TPH2* and *5-HTT* have been associated with bipolar disorder and ADHD(Mansour et al., 2005; Brookes et al., 2006; Grevet et al., 2007), SSRIs are preferentially used in the treatment of OCD (Math and Janardhan Reddy, 2007), many addictive drugs achieve their neurochemical and

behavioural effects by modulating the activity of serotonergic receptors (Koob, 2003a; Thompson et al., 2004; Alex and Pehek, 2007), and serotonin has likewise been linked to aggression (de Boer and Koolhaas, 2005), suicide (Stockmeier, 1997), and several other affective phenotypes.

The availability of knockout mice has been extremely important in our understanding of the serotonergic modulation of affect. 5-HT1A knockout mice, for example, display a high anxiety phenotype in a number of behavioural tests, whereas 5-HT1B knockout mice are more aggressive and less anxious (Zhuang et al., 1999; Gross et al., 2000). In the 5-HT1A -/- background, a tissue-specific, conditional rescue strategy of the 5-HT1A receptor expression in forebrain serotoninoceptic brain regions further identified a critical embryonic and early postnatal period in development (until postnatal day 21) in which 5-HT1A receptor expression is critical for normal affective phenotype (Gross et al., 2002; Santarelli et al., 2003). Rescuing adult 5-HT1A expression at postnatal day 21 did not improve anxiety behaviour, and shutting off receptor expression at postnatal day 80 did not result in anxiety behaviour, underscoring the developmental role of 5-HT1A in affective phenotype. Polymorphisms in the 5-HT1A receptor have been associated with anxiety, panic disorder, suicide and depression (Lemonde et al., 2003; Strobel et al., 2003; Albert and Lemonde, 2004; Del Tredici et al., 2004; Huang et al., 2004; Serretti et al., 2004; David et al., 2005; Freitag et al., 2006). One especially well-characterized polymorphism found in the promoter of the human 5-HT1A receptor gene is the C(-1019)G single nucleotide polymorphism (SNP), which associates with suicide and depression in some populations, and which results in altered gene regulation by a number of transcription factors (Lemonde et al., 2004b; Czesak et al., 2006; Hong et al., 2006a; Wasserman et al., 2006; Kraus et al., 2007) (See section 3). SSRIs increase 5-

HT neurotransmission by blocking 5-HTTs, and are prescribed commonly for treating anxiety, depression, OCD, eating disorders, chronic pain and bipolar disorder (Charney et al., 1990a; Charney et al., 1990b; Coyle and Duman, 2003; Millan, 2003; Saxena et al., 2003; Math and Janardhan Reddy, 2007). Other antidepressants, including monoamine oxidase (MAO) inhibitors, which inhibit the MAO-dependent breakdown of monoamines, and TCAs, which can act as adrenergic and serotonergic reuptake inhibitors as well as targeting other systems such as DA, also function by modifying serotonergic neurotransmission, and have been used successfully in the treatment of a range of affective disorders (Bonhomme and Esposito, 1998). Some atypical antipsychotic drugs that do not induce extrapyramidal motor symptoms (i.e. clozapine, ziprasidone), used to treat schizophrenia, also target serotonin receptors such as 5-HT1A, 5-HT2A and 5-HT2C, in addition to blocking dopamine D2 receptors (Stockmeier et al., 1993; Ishibashi et al., 1999; Chlan-Fourney et al., 2002; Terranova et al., 2005; Alex and Pehek, 2007; Bardin et al., 2007). Likewise, some benzodiazepines, which modulate GABAA receptors and are used in the treatment of anxiety and related disorders, modulate 5-HT signalling (Millan, 2003; Langen and Rundfeldt, 2007). High concentrations of GABA receptors on NE neurons of the LC have lead to the suggestion that benzodiazepines mediate their anxiolytic properties by inhibiting noradrenergic activity, a system that is tightly linked with the 5-HT system (Ninan, 1999; Delgado and Moreno, 2000). 5-HT1A receptor agonists, such as 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin), are also widely used in treating anxiety disorders (Millan, 2003; De Vry et al., 2004), and some evidence suggests that they may also protect from or delay neurodegeneration in patients with PD (Bezard et al., 2006).

In isolation, the 5-HT system may appear to be the most important single signalling system in the development and treatment of affective disorders; however, it is critical to understand how the 5-HT system interacts with other neurotransmitter systems, particularly other monoamines (Bonhomme and Esposito, 1998; Esposito, 2006). Stimulation of 5-HT1A receptors with 8-OH-DPAT, for example, causes NE release in a number of brain regions, including the VTA, hippocampus and frontal cortex, an effect that can be reversed by the 5-HT1A antagonists WAY 100635 and WAY 100135 (Suzuki et al., 1995; Barnes and Sharp, 1999). 5-HT1A agonist stimulation also increases levels of the immediate early gene, *c-fos*, in the LC, and activates NE neurotransmission from the LC (Hajos-Korcsok et al., 1999; Hajos-Korcsok and Sharp, 1999; Hajos et al., 1999). Serotonin also regulates dopaminergic neurotransmission. The 5-HT1A, 5-HT1B, 5-H2A, 5-HT3 and 5-HT4 receptors have all been shown to facilitate 5-HT stimulated DA release, whereas 5-HT2C receptors inhibit 5-HT stimulated DA release (Alex and Pehek, 2007). This interaction may underlie the efficacy of atypical antipsychotics. Agonist stimulation of 5-HT1A receptors increases the firing of VTA DA neurons and DA release in the prefrontal cortex and Acb, and this can be blocked by WAY 100635 (Arborelius et al., 1993b; Arborelius et al., 1993a; Rollema et al., 2000; Alex and Pehek, 2007). Though the exact mechanisms have not yet been fully elucidated, 5-HT1A receptors play an important role in mediating DA signalling in the mesocortical and mesolimbic pathways. Furthermore, the 5-HT-induced DA release has been shown to be critical for 'fast onset' antidepressant treatment (Dremencov et al., 2004). In addition, factors that increase extracellular 5-HT (i.e. antidepressants), can lead to uptake by the DAT in dopamine terminals of the striatum, allowing DA and 5-HT to be co-released from DA terminals (Zhou et al., 2005), and 5-HT mediates striatal DA release through 5-HT1A, 5-HT1B and

5-HT3 receptors, as well as through 5-HT2 heteroreceptors localized to striatal DA terminals (Ng et al., 1999). These neurotransmitter system interactions are highly relevant in the understanding and development of therapeutics for treating affective and related disorders.

2 C(ii): Dopamine in Disorders of the Fear Circuitry

As with the serotonin system, the role of dopamine in disorders of the CNS, and specifically in disorders affecting the fear circuitry, is extensively documented. PD, bearing the name of James Parkinson, who first described the PD as a "shaking palsy" in 1817 (Parkinson, 2002), is a neurodegenerative disease predominantly considered a movement disorder (Lang and Lozano, 1998b; Vallone et al., 2000). PD, however, is frequently co-morbid with affective disorders such as depression and anxiety, as well as cognitive (i.e. memory, dementia, paranoia, hallucinations, delusions) impairment, sleep disruptions and psychosis, leading many to suggest that PD would more accurately be considered a neuropsychiatric disease (Lang and Lozano, 1998a; Weintraub and Stern, 2005; Lieberman, 2006). PD is characterized by the progressive degeneration of the midbrain dopamine neurons and their projections to the patch matrix of the striatum (Albanese et al., 1986; Gerfen, 1989, 2006), as well as of other neurotransmitter neuron populations, including 5-HT, NE and acetylcholine (Lang and Lozano, 1998b). (Figure I-13).. In addition to the degradation of DA neurons in the SN and the loss of DA in the striatum, which represent the primary dopaminergic 'motor control' pathway, losses are seen in DA projections from the VTA to the entorhinal cortex, olfactory tubercle, cingulate gyrus and frontal cortex; in LC NE projections to the spinal cord, cerebellum, central gray of the midbrain, amygdala, substantia innominata (which contains the nucleus basalis of Meynert, a major source of cholinergic projections), thalamus, limbic

cortex and neocortex; and in 5-HT raphe projections to the spinal cord, SN, amygdala, striatum and cortex [reviewed in (Lang and Lozano, 1998b, a)]. The majority of drugs used to treat PD target the DA system, including DA precursors, such as Levodopa [L-DOPA (3,4-dihydroxy-L-phenylalanine)], the most effective treatment for PD since its discovery in the 1950 by Arvid Carlsson. (Nobel Prize, 2000) (Anden et al., 1970; Carlsson, 2001, 2002). Dopamine receptor agonists are also commonly used in treating PD; however, as with L-DOPA, there are a number of adverse effects of stimulating the dopamine system at large, such as tardive dyskinesia or psychiatric disturbances (Lang and Lozano, 1998a; Gerfen, 2006). Drugs designed to reduce DA breakdown or recycling have also been employed with some success, such as MAO inhibitors to inhibit intracellular MAO (as with affective disorders), Catechol-O-methyltransferase (COMT) inhibitors to inhibit extracellular breakdown by COMT and DAT inhibitors to increase synaptic DA (Djaldetti and Melamed, 2002; Hauser and Zesiewicz, 2007). Both D1 and D2 dopamine receptors are thought to be involved in the dopaminergic control of movement (Vallone et al., 2000), and agonists targeting all 5 subtypes have been used in treating PD (Hauser and Zesiewicz, 2007).

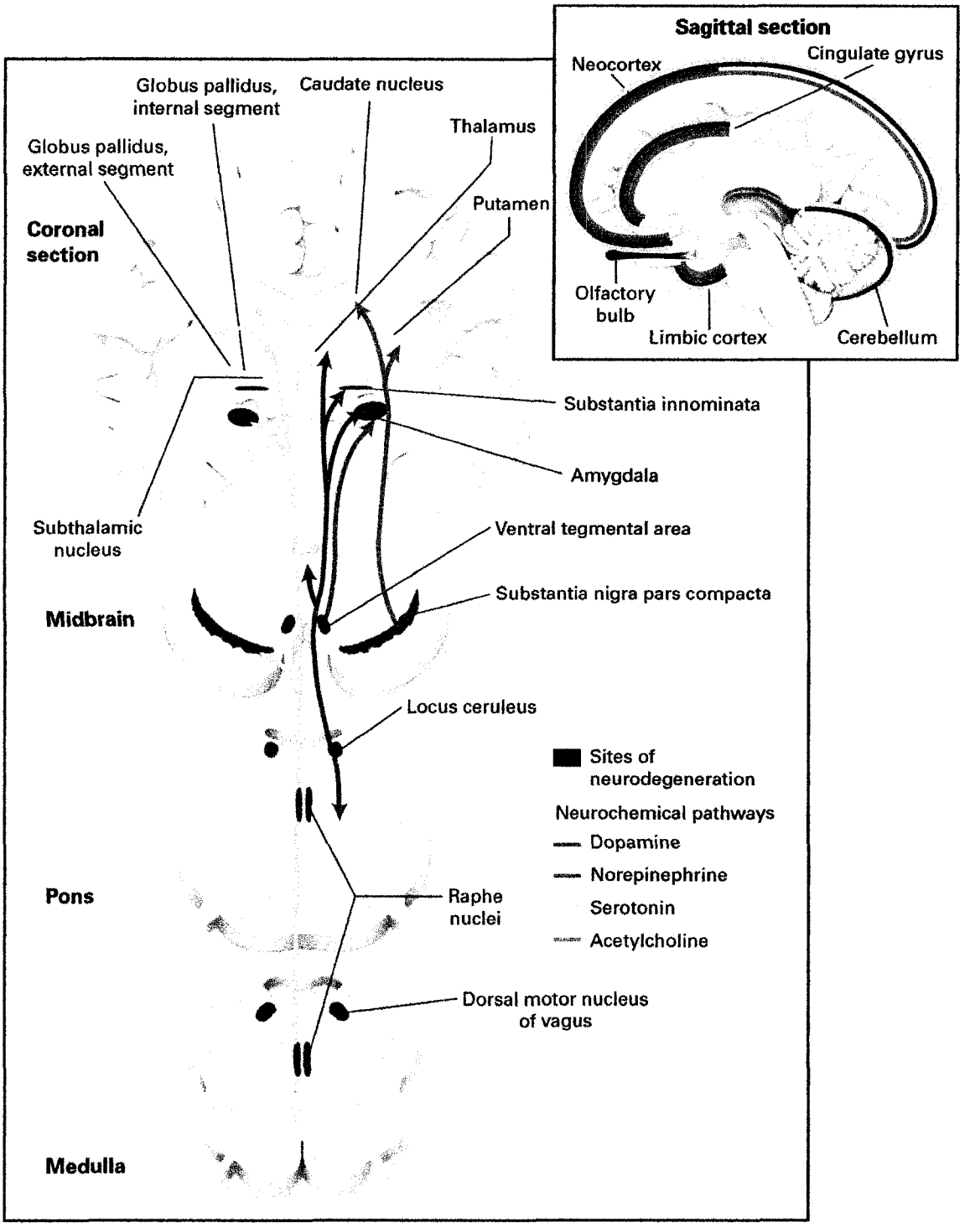

Sagittal section
Neocortex
Cingulate gyrus
Olfactory bulb
Limbic cortex
Cerebellum
Coronal section
Globus pallidus, internal segment
Caudate nucleus
Thalamus
Globus pallidus, external segment
Putamen
Substantia innominata
Amygdala
Subthalamic nucleus
Ventral tegmental area
Substantia nigra pars compacta
Midbrain
Locus ceruleus
Sites of neurodegeneration
Neurochemical pathways
Dopamine
Norepinephrine
Serotonin
Acetylcholine
Pons
Raphe nuclei
Dorsal motor nucleus of vagus
Medulla

Figure I-13: Neurochemical Pathways and Neurodegeneration in Parkinson's Disease

Sites of pathological change (dark blue) and neurochemical pathways affected in PD (coloured arrows) are indicated, including their destinations shown in axial 'sections'. Sagittal inset shows schematic of neurotransmitter projections in the brain. The majority of the classical motor symptoms of PD are accounted for by the degeneration of the DA nigrostriatal pathway; however, well-established disturbances in other neurotransmitter systems may contribute to various motor and non-motor features of PD. Modified from (Lang and Lozano, 1998b).

Interestingly, dopamine D2-receptor knockout mice have a phenotype analogous to PD, and the majority of DA agonists used clinically in treating PD target the D2-like receptors specifically (Lang and Lozano, 1998a; Hauser and Zesiewicz, 2007; Seeman, 2007). DA depletion studies also link the dopamine D2 receptor in the mechanisms of PD (Stromberg et al., 2000; Day et al., 2006; Gerfen, 2006), further underscoring the important role of the dopamine D2 receptor in motor function.

Schizophrenia, a mental illness characterized by positive (altered behaviour, hallucinations, delusions, extreme emotions, paranoia) and negative symptoms (lack of emotion, social interaction, speech), has long been described by the dopamine hypothesis, which simply states that the brain of a person affected by schizophrenia produces more DA than an unaffected person, although the mechanism of the DA increase is the subject of much debate (Vallone et al., 2000; Stone et al., 2007). This hypothesis is validated by a number of lines of evidence, for example, the psycho-stimulant amphetamine increases extracellular DA by targeting DAT and vesicular monoamine transporter 2 (VMAT2) (Fleckenstein et al., 2007), the bulk of clinically used typical and atypical antipsychotic drugs specifically block the dopamine D2-like receptors (Stockmeier et al., 1993; Ishibashi et al., 1999; Chlan-Fourney et al., 2002; Seeman, 2006; Bardin et al., 2007; Stone et al., 2007), and polymorphisms in several DA or DA-interacting protein genes associate with schizophrenia (Talkowski et al., 2007). Further studies link the DA and DA receptors to Tourette's syndrome (Vallone et al., 2000), ADHD (Terranova et al., 2005), fear and anxiety (primarily D1-like receptors) (Lamont and Kokkinidis, 1998; McGrath et al., 1999; de la Mora et al., 2005; de la Mora et al., 2007), and of course, addiction and reward paradigms (Hurd et al., 1999; Cowen and Lawrence, 2001; Hyman and Malenka, 2001; Rezayof et al., 2002; Solinas et al., 2002; Koob, 2003b, a; Alex and Pehek, 2007).

Taken together, monoamines, and particularly 5-HT and DA are fundamental regulators of the fear circuitry whose disruption is associated with a spectrum of neuropsychiatric disorders.

3: Regulation of Gene Expression

The mechanism by which DNA replication occurs became clear shortly after the structure of DNA was deciphered by James Watson, Francis Crick, Maurice Wilkins and their little-credited crystallographer, Rosalind Franklin (Watson and Crick, 1953a, b; Levinthal, 1956).

> *It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.*
>
> (Watson and Crick, 1953b)

Almost a century before Watson, Crick and Wilkins won the Nobel Prize, Johan Friedrich Miescher isolated nucleic acids ("nuclein") from the nuclei of white blood cells (Buess, 1953; Harbers, 1969) and Gregor Johann Mendel published his findings on the heritability of traits by peas, outlining a set of laws that provided the foundation for modern-day genetics (Babcock, 1921). The idea that genes code for enzymes and proteins that regulate biochemical events was first proposed George Beadle and Edward Tatum in the early 1940s (Beadle and Tatum, 1941; Tatum and Beadle, 1942). Beadle and Tatum shared the Nobel Prize in 1958 with Joshua Lederberg for work examining genetic recombination and the organization of hereditary material in microorganisms (Schade, 1959; Sulek, 1969). The missing link in the relationship between genes and proteins was fully elucidated when Vernon Ingram identified a Glu-Val substitution in the 6^{th} amino acid of β-globin that results in Sickle Cell Anaemia (Ingram, 1956, 1957b, a). Since these

pioneering discoveries, our understanding of the multiple layers of control that exist between DNA replication and protein action has grown exponentially. Within the nucleus, where DNA replication and RNA transcription occur in eukaryotes, gene 'expression' can be regulated several ways, including at the level of transcription, RNA processing, nuclear export, and even chromatin packaging (i.e. histone acetylation/deacetylation, etc) (Moran et al., 1994; Lewin, 1997). In the cytoplasm, RNA translation into proteins can also be regulated, protein activity can be altered by countless types of modification, and the ability of proteins to reach their cellular targets controlled. In this way, following a gene to the biochemical outcome of its product involves an in-depth examination of each of these steps, the first of which is transcription.

3 A: Overview of Transcription

In eukaryotes, DNA is wound around a core complex of histones (H2A, H2B, H3 and H4), forming an octameric structure called the nucleosome (Wegel and Shaw, 2005). An additional 'linker' histone (H1) locks the DNA in place around the nucleosome and allows higher order structures to form. The basic structure of DNA is on the order of 2 nm in diameter, but when tightly complexed with histones ("beads on a string", 10 nm), nucleosome interactions enable the formation of the secondary chromatin structure (30 nm) (Figure I-14). Within the chromosome, the chromatin can be highly condensed into transcriptionally inactive (silenced) heterochromatin or decondensed, potentially transcriptionally active euchromatin (Elgin and Grewal, 2003; Wegel and Shaw, 2005). Regulation of the chromatin structure is largely controlled by posttranslational modifications of both the core and linker histone tails, which protrude from the nucleosome. (Fukuda et al., 2006; Shilatifard, 2006). Acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation and citrullination

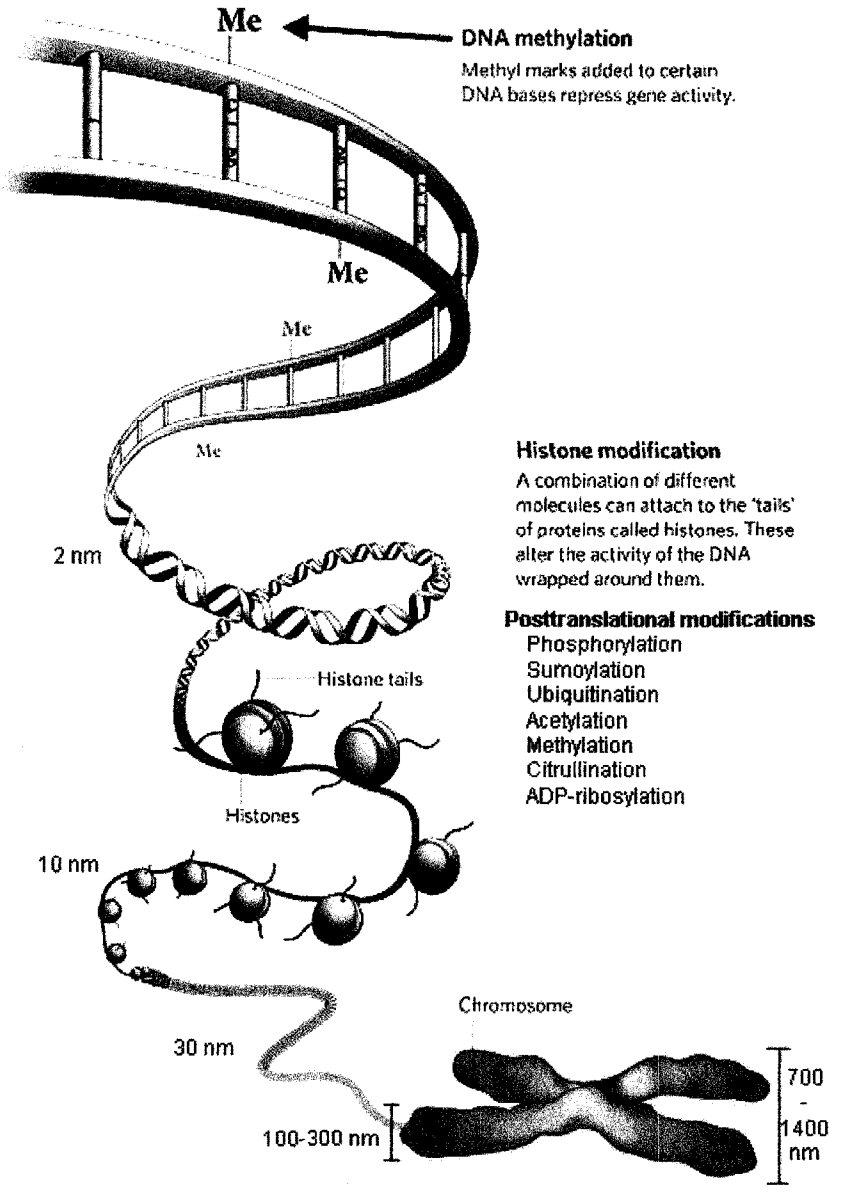


Figure I-14: Breaking Down Chromosome Structure

Chromosomes are made up of genomic DNA tightly wound around histone octameres called nucleosomes. Interactions between the nucleosome "beads on a string" enable the DNA to be wound into chromatin fibres, and further condensed into chromosomes. Modifications of both the DNA and the histone tails, which protrude from the nucleosomes, provide important mechanisms for regulating gene expression. Modified with permission from the Chromatin-/Epigenomics Lab, Copyright © 2006.

(deiminated arginine) of histone tails have all been documented to modify chromatin organization and, therefore, gene expression (Fukuda et al., 2006; Shilatifard, 2006; Thompson and Fast, 2006; Houben et al., 2007; Ito, 2007; Sato, 2007; Wisniewski et al., 2007). Histones can be acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs), and this modification represents a critical component of chromatin remodelling (Fukuda et al., 2006). While acetylation (of lysine residues) of histones is thought to open the chromatin structure and enable gene expression, methylation of histones (at lysine or arginine residues) is considered a relatively stable modification that is generally associated with transcriptional repression (Shilatifard, 2006). Regulation of transcription and chromatin structure can also be modulated by DNA methylation at cytosine residues within CpG sites (cytosine-phosphate-guanosine), a heritable modification that is catalyzed by DNA methyltransferases (DNMTs) (Robertson). The pattern of DNA methylation is thought to play an important role in embryonic development, and hypermethylated DNA is commonly associated with silenced genes.

Transcription in eukaryotes is mediated by three classes of RNA polymerases: RNA polymerase I (large rRNA), RNA polymerase II (mRNA) and RNA polymerase III (tRNA and other small RNAs) (Archambault and Friesen, 1993; Lewin, 1997; Russell and Zomerdijk, 2006). For the purposes of this thesis, we will focus on the transcription of mRNA. RNA polymerase II itself is able to unwind the double stranded DNA template, synthesize a RNA copy and rewind the DNA, but is incapable of promoter recognition or transcription initiation (Kornberg, 2007a; Kornberg, 2007b). For this, RNA polymerase requires a number of other proteins, including the general transcription factors (TFIIA, B, D, E, F and H). These factors ensure accurate promoter targeting and

initiation in addition to assisting with promoter melting and DNA unwinding (Naar et al., 2001).

The promoter of a gene, usually located proximal to the transcription start site, contains transcription factor binding motifs that enable the basal transcription machinery to be recruited and initiate transcription (Suzuki et al., 2001). The classical paradigm outlining the steps of transcription initiation, elongation an termination usually begins with recognition of the TATA box, a promoter element located 25-35 nucleotides upstream of the transcription start site, by TFIID (Andel et al., 1999; Naar et al., 2001) (See Figure I-15). It is important to note, however, that a great number of promoters do not contain TATA boxes. In a study characterizing the major promoter elements of over 1000 human genes, Suzuki and colleagues identified TATA boxes in 32%, initiator motifs in 85%, GC boxes in 97% and CAAT boxes in 64% of promoters examined. They also identified CpG islands in 48% of promoters.

For TATA-containing promoters, the TATA binding protein (TBP) and TATA associated factors (TAFs), which are collectively referred to as TFIID, bind the TATA box (Stargell and Struhl, 1996; Latchman, 2004). TFIID binding is stabilized by TFIIA on one side and TFIIB on the other, encompassing the TATA box in a large molecular complex (Andel et al., 1999). TFIIB can recruit RNA polymerase II, which enters the initiation complex with TFIIF, followed by TFIIE. TFIIH represents a large complex of proteins and is the last to enter the complex. It has a number of important functions: it contains a DNA helicase which unwinds the DNA and ensures the appropriate strand is transcribed; it contains a kinase which phosphorylates the C-terminus of RNA polymerase II, allowing transcription initiation to occur; and it has a nucleotide excision

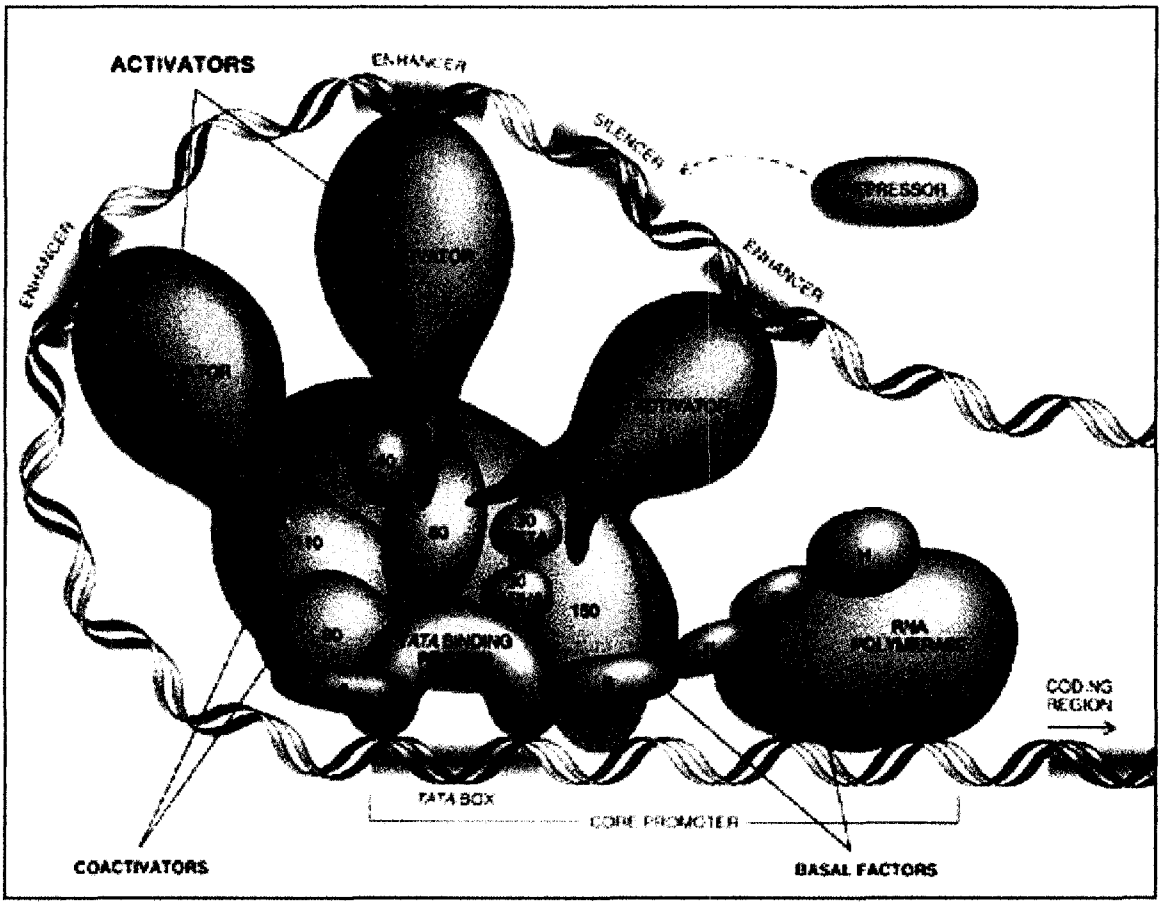


Figure I-15: Transcription Machinery at a TATA-Containing Promoter

General transcription factors TFIID (TBP and TAFs), followed by TFIIA, B, F, E and H are required for promoter recognition and transcription initiation. The activation of this core promoter can be facilitated by activator-binding to distally located enhancer sequences, where the activator complex serves as an interface for protein-protein interactions that may help recruit the transcriptional machinery to the promoter and enhance gene expression. Activators and repressors may also function by recruiting HDACs and HATs to modulate chromatin packaging, and therefore gene expression. Based on (Naar et al., 2001). Copyright © Robert Tjian.

repair function that is essential for preventing DNA damage (Naar et al., 2001; Latchman, 2004). See Figure I-15 for review. The TFIID complex is still required for promoter recognition in the absence of the TATA box. Under these conditions, the TBP can interact with factors bound to the initiator element (core promoter element downstream of the TATA box), although there are examples of TATA-less, initiator-less promoters (Sandelin et al., 2007). The prevalence of the GC box in gene promoters studied (nearly 100%), which is recognized by Sp1 and Myc-associated zinc finger protein (MAZ), is also suggestive of a more fundamental role for these promoter elements (Pugh and Tjian, 1990; Parks and Shenk, 1996, 1997; Suzuki et al., 2001).

The core promoter of a gene, which is rarely larger than 150 bp (Sandelin et al., 2007), is essential for gene transcription; however, sequences located distally can also influence gene expression (Latchman, 2004). Enhancer or silencer elements located sometimes more than a Kb away can bind to activators or repressors (respectively) and modulate gene expression, providing mechanisms for such things as tissue specific gene expression (Naar et al., 2001; Kadonaga, 2004). The bulk of evidence suggests that activator complexes, for example, serve as protein-protein interaction interfaces, enabling recruitment of the transcriptional machinery to the promoter (pictured in Figure I-15).

Although the primary point of control of gene expression is transcription initiation, virtually every step between genomic DNA to protein can also be regulated. Growing evidence points to important regulatory mechanisms involved in transcription elongation (Sims et al., 2004); the stages of RNA maturation (addition of the 5' cap, polyadenylation, intron splicing) as well as the transport of the mature RNA to the cytoplasm (Lewin, 1997; Latchman, 2004); and of course, the stages of translation (initiation, elongation and termination) as well as a wide range of posttranslational

modifications permit a comparatively rapid mechanism of control (Archambault and Friesen, 1993; Paule and White, 2000; Kozak, 2001; Kadonaga, 2004). Considering the multitude of stages at which eukaryotic gene expression can be regulated, accounting for every possible outcome on the journey from DNA to protein might be considered an exercise in pansophy. For this reason, it is necessary to focus on individual points within this cascade of regulatory events, one of the most important of which is the transcriptional modulation of the tens of thousands of protein-coding genes in eukaryotic organisms by sequence-specific factors.

3 B: Transcriptional Regulation of the 5-HT1A Receptor

Transcription of the 5-HT1A receptor gene is driven by a TATA-less promoter that contains several GC-rich sequences proximal to the start codon (Parks and Shenk, 1996). See Figure I-16 for a summary of the transcriptional regulation of the 5-HT1A receptor gene. These sequences are targeted by the zinc finger proteins Sp1 and MAZ which control the expression of a variety of genes by interacting with the same GC-rich DNA-binding sites (Song et al., 2001; Song et al., 2003). Of the four GC-elements identified in the 5-HT1A promoter, three were targeted by both Sp1 and MAZ, and the most proximal site, which spans the start codon, was determined to act as an initiator site (Parks and Shenk, 1996).

Corticosteroid hormones have also been shown to negatively regulate 5-HT1A expression in the hippocampus (but not the raphe via several NF-κB response elements in the 5-HT1A promoter (Zhong and Ciaranello, 1995; Meijer et al., 2000). 5-HT1A expression is positively regulated by NF-κB, but corticosteroids,

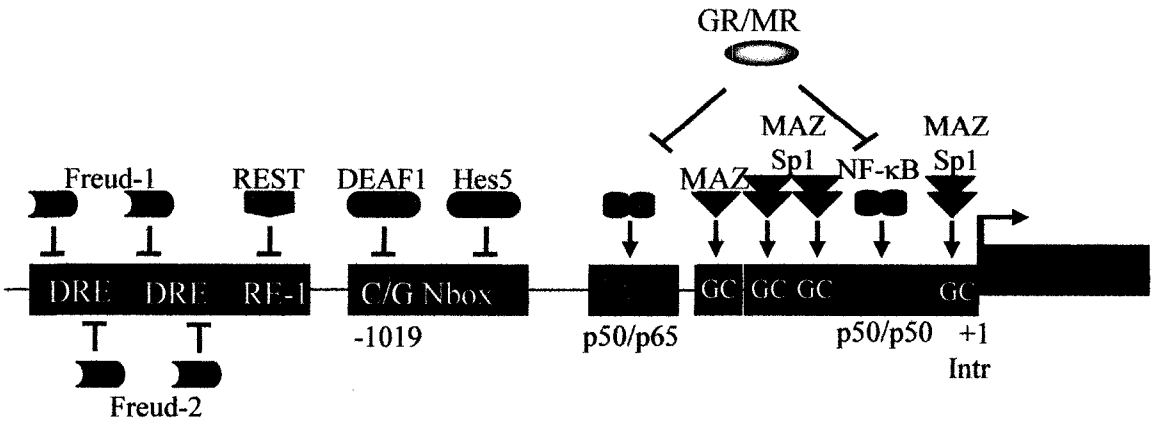


Figure I-16: Transcriptional Regulation of the 5-HT1A Receptor Gene

The 5-HT1A receptor gene contains a TATA-less promoter and several GC-rich core promoter elements that are regulated by MAZ and SP1. The most proximal GC-box in fact spans the start codon (here designated +1) and can act as an initiator site (Intr). In addition, the 5-HT1A receptor gene is positively regulated by NF-κB homo- (p50/p50) and heterodimers (p50/p65), and NF-κB-induced transcription can be repressed by the glucocorticoid receptor (GR), although the role of the mineralocorticoid receptor (MR) is not as well established. Not shown is a novel negative glucocorticoid response element (nGRE) that is synergistically regulated by MR and GR (Ou et al., 2001). Upstream of these elements are a number of transcriptional repressors including DEAF1 and Hes5, which regulate transcription through a palindromic element (in humans) at the C-, but not G-allele of a polymorphic element associated with suicide and depression [C(-1019)G]. In addition, homologous proteins, Freud-1 and -2, repress 5-HT1A receptor transcription through the 5' and 3' portions of the DRE (respectively). Finally, there is also a REST responsive element (RE-1) which silences 5-HT1A transcription in non-neuronal cells. Relative positions indicated only. Based on unpublished data and (Parks and Shenk, 1996; Wissink et al., 2000; Albert and Lemonde, 2004).

primarily the glucocorticoids, inhibit this NF-κB-induced transcription (Wissink et al., 2000; Abdouh et al., 2001). Glucocorticoid response elements have also been identified within the 5-HT1A promoter that appear to mediate the inhibition of 5-HT1A expression by corticosteroids (Ou et al., 2001). Previous work in our lab further identified a repressor element 1 (RE-1) element in the 5-HT1A promoter, which binds to the zinc finger protein, REST/NRSF [repressor element 1 (RE-1) silencing transcription factor/neuron-restrictive silencer factor] (Lemonde et al., 2004a). REST/NRSF-mediated repression is a key regulator of cell type specific genes, silencing neuronal gene expression in non-neuronal cells (Schoenherr and Anderson, 1995a, b). Immediately upstream of the RE-1 element, two copies of a novel dual repressor element (DRE) were identified (Ou et al., 2000). The DRE is comprised of 5'- and 3'-repressor elements (FRE and TRE) which bind Freud-1 (five' repressor element under dual repression binding protein-1) (Ou et al., 2003) and Freud-2 respectively (Hajighassem and Albert, unpublished data).

A great number of studies have examined the 5-HT1A C(-1019)G promoter polymorphism, which in some populations associates with suicide, depression, schizophrenia, panic attacks, substance abuse and even responsiveness to antidepressants (Lemonde et al., 2003; Strobel et al., 2003; Huang et al., 2004; Lemonde et al., 2004b; Serretti et al., 2004; Hong et al., 2006a; Parsey et al., 2006; Wasserman et al., 2006; Kraus et al., 2007). Work in our lab identified two transcription factors whose ability to repress is inhibited in the presence of the G-allele (Lemonde et al., 2003). Deformed epidermal autoregulatory factor (DEAF-1), whose binding element includes the C(-1019)G polymorphic site, and Hes5, which represses through a 26 bp palindromic element containing the polymorphic site, were both able to repress transcription of the 5-

HT1A receptor in the presence of the C-allele, but not the G-allele (Lemonde et al., 2003; Czesak et al., 2006). DEAF-1 contains HLH and SAND domains (Sp100, AIRE-1, NucP41/75 and DEAF-1), that latter of which is thought to mediate DNA binding and chromatin association (Wojciak and Clubb, 2001). Hes5, on the other hand, belongs to a family of bHLH proteins (see Section 1A) that mediates a host of developmental process (Kageyama et al., 2005). Specifically, Hes5, along with Hes1, Hes3 and Hes6 are essential regulators of neurogenesis.

Finally, work by the Deneris lab has uncovered a critical regulator of serotonin neuron development, Pet-1 (see Section 1C), which has binding elements in a number of serotonergic genes, including the promoter of the 5-HT1A receptor (Hendricks et al., 1999; Hendricks et al., 2003). Given the critical developmental role of the 5-HT1A receptor in establishing normal affective phenotype (Gross et al., 2002), it is especially important to understand how 5-HT1A expression is modulated during this critical developmental period. For this reason, this review will focus on developmental regulators of neurogenesis (the Hes family of proteins) and of serotonin neurogenesis (Pet-1).

3 B(i): Pet-1/FEV

As discussed in Section 1C and illustrated in Figure I-6, Pet-1 is part of a larger cascade of transcription factors that regulates the differentiation and phenotype specification of serotonin neurons. The induction site for 5-HT neurons is specified by Shh, FGF4 and FGF8 (Ye et al., 1998), down stream of which the homeobox factors *Nkx2.2* and *Nkx6.1* (Briscoe et al., 1999; Cheng et al., 2003), zinc finger proteins *GATA2* and *GATA3* (van Doorninck et al., 1999; Craven et al., 2004), LIM homeodomain protein *Lmx1b*(Cheng et al., 2003; Ding et al., 2003), bHLH proteins *Ascl1/Mash1*(Pattyn et al., 2004) and *Pet-1* (Hendricks et al., 1999) are all involved in 5-HT specification. The essential role of each

one of these regulators in 5-HT neuron development is underscored by the fact that mutation of any single member of this cascade results in disruption of serotonergic phenotype. *Pet-1*, however, is unique in its restricted localization exclusively to 5-HT neurons, being expressed spatiotemporally one day before 5-HT in developing raphe neurons (Hendricks et al., 1999; Pfaar et al., 2002; Maurer et al., 2004; Iyo et al., 2005). ETS transcription factors, such as *Pet-1* and the polyomavirus enhancer activator 3 (*PEA3*), regulate a large variety of developmental processes and are frequently transcriptional activators (Xin et al., 1992; Peter et al., 1997). Interestingly, it was the ability of the then unknown (rat) Pet-1 to bind to and enhance transcription through a PEA3 ETS DNA-binding motif in the 3'-untranslated exon of the neuronal nicotinic acetylcholine receptor that lead to its discovery (Fyodorov et al., 1998). The serotonergic localization of Pet-1 and ability to bind Pet-1 elements in multiple serotonergic genes, however, led to the hypothesis that *Pet-1* plays an important role in determining serotonergic fate (Hendricks et al., 1999). Indeed, in the absence of Pet-1, there are gross deficits in serotonin content (70-80%), as well as disruptions in the expression of *5-HTT, AADC, TPH,* and *VMAT2*. Pet-1 null mice display high anxiety-like and aggressive behaviour, emphasizing the importance of serotonin in the development of affective behaviours (Hendricks et al., 2003).

3 B(ii): Hairy/Enhancer-of-Split

Seven mammalian *Hes* transcription factors (Section 1A, Figure I-1) have been identified to date, all of which are important developmental regulators and have varying affinities for the GC-rich N-box (CACNAG), E-box (CANNTG) and related C-site (CACGCG) [for review see (Kageyama et al., 2006)]. Of these, *Hes1, Hes3* and *Hes5* are involved in maintaining neuronal progenitors, while *Hes6* promotes neuronal differentiation

(Ishibashi et al., 1995; Bae et al., 2000; Kimura et al., 2004). Hes proteins belong to a unique subclass of bHLH proteins that contain an adjacent Orange domain (bHLH-O) that mediate dimerization and DNA binding (bHLH) as well as the specificity of protein-protein interactions (Orange) (Davis and Turner, 2001) See Figure I-17A for an overview. A defining feature of bHLH-O factors is their ability to bind DNA and recruit transcriptional co-repressors. In the case of Hes family members, a conserved C-terminal WRPW motif enables the recruitment of co-repressors such as Groucho(Gro)/TLE (Paroush et al., 1994; Jennings et al., 2006) which can recruit the HDAC Rpd3 and therefore actively repress transcription by modifying chromatin structure (Chen et al., 1999). This interaction with Gro/TLE represents one of the primary mechanisms by which the inhibitory Hes proteins mediate repression of a host of gene targets, including pro-neural bHLHs such as *Mash1* (Figure I-17B, Mechanisms 1). Hes1, Hes3 and Hes5 can also inhibit gene activation by proneural bHLHs, such as E47, by forming non-functional heterodimers with E47, competing for DNA binding or by as yet unknown protein-protein interactions (Figure I-17B, Mechanisms 2-3) (Akazawa et al., 1992; Sasai et al., 1992; Hirata et al., 2002).

Hes1 and *Hes3* are both highly expressed in neural stem cells during the initial neuroepithelial stage of nervous system development in a largely overlapping pattern (Hatakeyama et al., 2004). Coincident with the up-regulation of *Notch* and *Delta* expression, *Hes3* is downregulated and *Hes5* is upregulated, with *Hes1* and *Hes5* expression domains being mostly complimentary to each other. Ryoichiro Kageyama and colleagues have spent more than a decade examining the essential role of *Hes* genes in neural stem cells maintenance, largely with the help of Hes knockout mice (Ohtsuka et al., 2001). Hes1 null mice, for example, display severe neurulation defects and die during

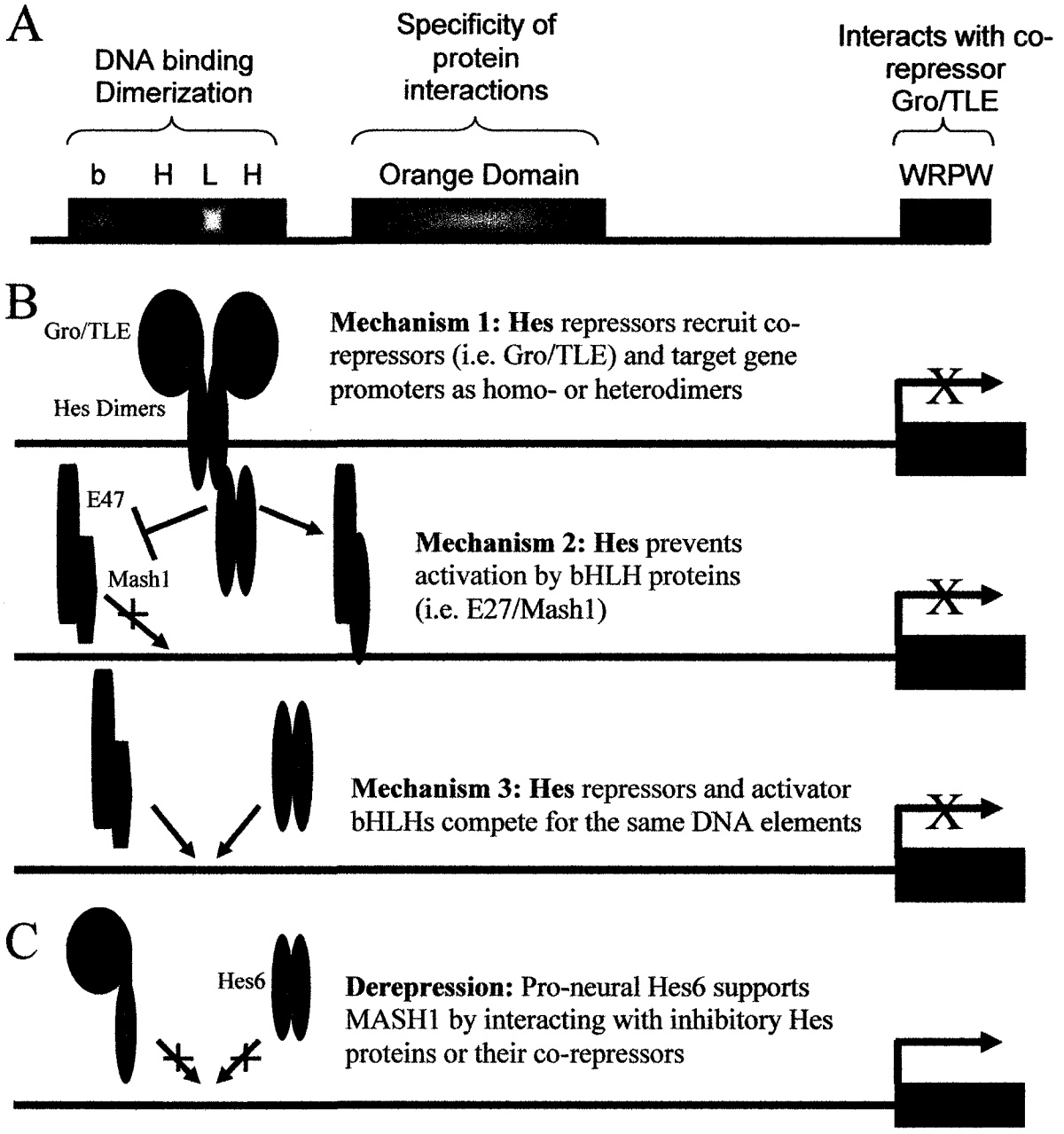


Figure I-17: Hes Structure and Mechanisms of Transcriptional Regulation

(A) Structure of bHLH-O proteins based on (Kageyama et al., 2006). (B) Mechanisms of Hes repression (i.e. by Hes1 and Hes5) based on (Davis and Turner, 2001). (C) Model of Hes6 mediated derepression of inhibitory Hes proteins (i.e. Hes1) based on (Gratton et al., 2003).

gestation (Ishibashi et al., 1995). Despite the severe deficits in Hes1 null mice, *Hes3* and *Hes5* are able to partially compensate in the absence of Hes1 (Baek et al., 2006), and double and triple knockout studies have illustrated additional roles for these genes in regulating the timing of neuronal differentiation, promoting progenitor cell proliferation and maintenance of organizer centers and boundary formation (Hirata et al., 2001; Hatakeyama et al., 2004; Kageyama et al., 2005; Murata et al., 2005; Baek et al., 2006).

While *Hes1*, *Hes3* and *Hes5* are expressed exclusively in neural progenitors, a closely related family member, *Hes6*, is expressed during embryogenesis and maintained into adulthood (Bae et al., 2000). Hes6 promotes neuronal differentiation by inhibiting Hes1, although its role in regulating the other inhibitory bHLHs has not been studied. As with other Hes proteins, Hes6 contains a C-terminal WRPW motif, enabling it to interact with Gro/TLE and potentially prevent Hes1 from interacting with its co-repressors (Figure I-17C) (Gratton et al., 2003). An additional mechanism, which requires the WRPW motif, enables Hes6 to target Hes1 to the proteosome for degradation, de-repressing Hes1-mediated inhibition (Gratton et al., 2003; Kang et al., 2005; Jhas et al., 2006). Given the overall homology of the bHLH dimerization domains within the Hes-family, and reliance of the inhibitory bHLHs on common co-repressors also targeted by Hes6, it is probable that similar Hes6-dependent mechanisms exist for Hes5 as well, although this possibility has not yet been tested.

3 C: Transcriptional Regulation of Dopamine D2 Receptor Signalling

Like 5-HT1A, the dopamine D2 receptor gene promoter is TATA-less and contains an initiator-like sequence as well as several GC-rich Sp1 sites (Minowa et al., 1992, 1994). While many ambiguities remain in our characterization of the D2 promoter, the identification of several other regulatory sites upstream of the start site adds credence to

there being a non-classical promoter similar to that of the 5-HT1A receptor. Unlike the 5-HT1A, which is an intron-less gene (Albert et al., 1990), however, the D2 receptor contains 7 introns (8 exons), the second of which contains a DRE element and is repressed by Freud-1 (Rogaeva et al., 2007), suggesting that the D2 receptor is regulated by proximal upstream as well as distally located sequence elements. In order to examine the etiology of dopaminergic diseases, which largely display abnormalities in dopaminergic signalling, this review will focus on NURR1, a transcription factor that is required for normal dopaminergic phenotype (Eells et al., 2002; Hermanson et al., 2003; Smits et al., 2003).

3 C(i): NURR1

NURR1 (Nur-related factor 1) was first cloned from a mouse cDNA library, and its rat [Regenerating liver nuclear receptor (*RNR-1*)] and human [nuclear receptor of T cells (*NOT*)] homologues were identified later (Law et al., 1992; Scearce et al., 1993; Mages et al., 1994). *NURR1* (a.k.a. NR4A2) belongs to the Nerve Growth Factor 1B-like subfamily of nuclear receptors which also includes *Nurr77* (a.k.a. NGFIB/NR4A1) and Neuron-derived orphan receptor 1 (*NOR1*, a.k.a. NR4A3). The restricted localization of *NURR1* primarily in the SN and VTA was the first clue to the important role of this transcriptional regulator in the development and function of DA neurons (Zetterstrom et al., 1996). This role was confirmed with the production of NURR1-deficient mice, who fail to generate midbrain dopamine neurons and display severe locomotor deficits, dieing shortly after birth (Zetterstrom et al., 1997). Even NURR1 heterozygotes had reduced levels of DA, strongly implicating *NURR1* in DA neuron development and the etiology of diseases such as PD. Further studies have demonstrated that *NURR1* is essential for the survival, differentiation and maturation of DA precursors of the ventral mesencephalon (Saucedo-

Cardenas et al., 1998; Witta et al., 2000; Chung et al., 2002). NURR1 is expressed in the mouse at E10.5, one day before TH appears at E11.5 (Saucedo-Cardenas et al., 1998) and can transcriptionally activate TH through a NURR1-binding motif (NBRE) in the TH promoter (Sakurada et al., 1999; Kessler et al., 2003; Kim et al., 2003). In addition to *TH*, induction of several other dopaminergic genes requires NURR1, including *DAT*, *AADC* and *VMAT2* (Chung et al., 2002; Hermanson et al., 2003; Smits et al., 2003), and putative NBREs have been identified in *DAT* (Sacchetti et al., 1999). NURR1 may also target other important regulators of DA neuron maturation such as brain derived neurotrophic factor (*BDNF*), which is a direct target of NURR1 transcriptional regulation (Volpicelli et al., 2007). See Figure I-18 for summary.

The identification of *NURR1* as an important regulator of dopaminergic development quickly lead to a search for NURR1 ligands that might be of clinical use in the treatment of PD. It was soon discovered that the apparent constitutive activity of NURR1 and failure to identify specific ligands was due to the unique structural conformation of the receptor. The crystal structure of NURR1 revealed no ligand binding cavity at all due to the presence of 6 bulky hydrophobic residues that occupy this space and maintain the NURR1 ligand binding domain (LBD) in an agonist-bound conformation (Wang et al., 2003; Flaig et al., 2005). Despite this ligand-independence, the activity of NURR1 can be modified in a number of ways. Structural studies illustrated that transcriptional activation by NURR1 is associated with the LBD adopting a more stable conformation, and the C-terminal AF-2 (activation function 2) core is thought to contribute to this ligand-independent activation (Castro et al., 1999; Wang et al., 2003). The AF-2 region, which lies within the LBD, in ligand-dependent nuclear receptors interacts with co-activators in order to mediate transcriptional activation, although

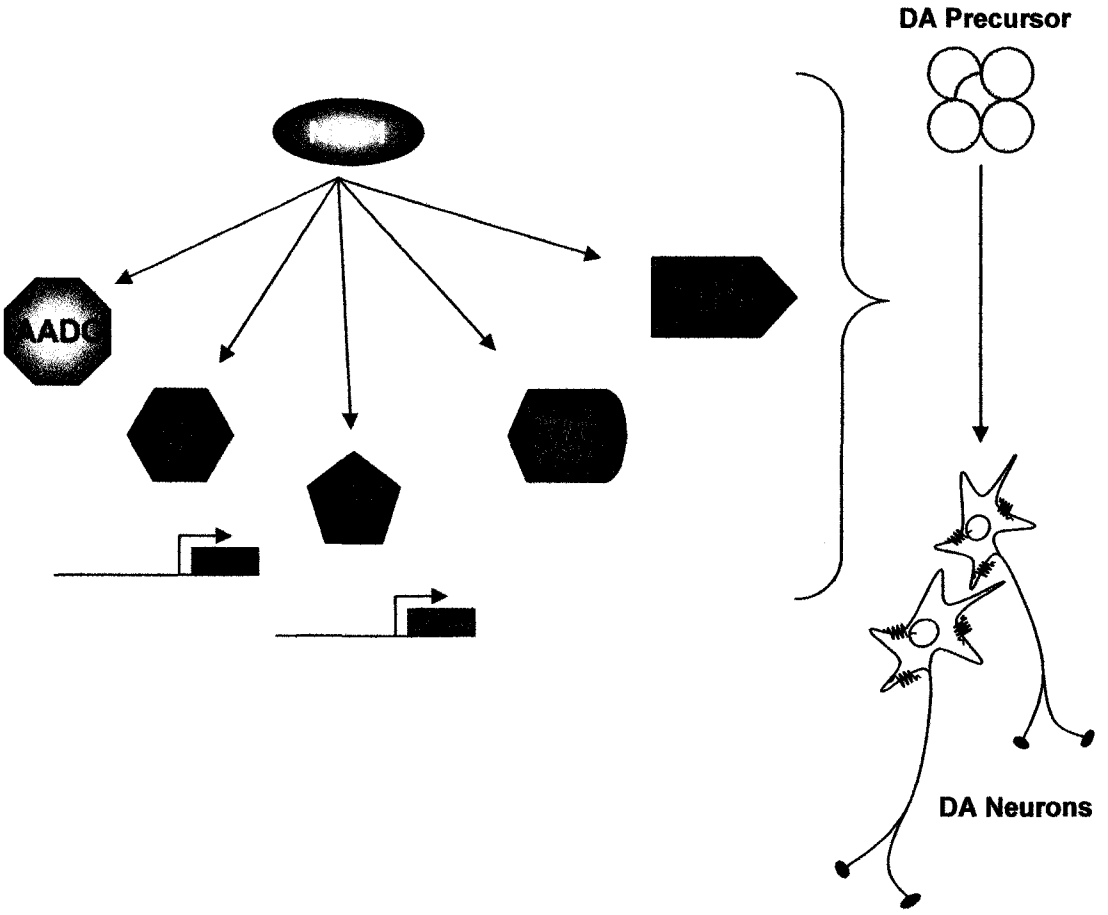


Figure I-18: Nurr1 Regulates Expression of Dopaminergic Genes

Nurr1 is required for DA neuron development and regulates the expression of *TH, DAT, AADC* and *VMAT2* which are markers of mature dopamine neurons. Nurr1 binding elements have been identified in the promoters of *TH* and *DAT*, suggesting that Nurr1 may transcriptionally regulate the expression of these genes.

NURR1 does not interact with several known nuclear receptor co-activators. NURR1 also contains an N-terminal AF-1 domain which is thought to play a more important regulatory role in NURR1 activation, and can recruit a number of known co-activators (Flaig et al., 2005). The AF-1 domain further contains a core region that is positively regulated by ERK phosphorylation, and putative MAPK sites have been identified in this region (Kovalovsky et al., 2002; Nordzell et al., 2004). In fact, D2 receptor signalling has been shown to activate NURR1 via ERK signalling, and this is thought to be a primary mechanism by which D2 receptors regulate the development of dopaminergic neurons (Kim et al., 2006).

As a critical factor in determining DA neuron fate, the role of *NURR1* has been widely examined in dopaminergic diseases of the fear circuitry. It has been studied especially in relation to PD and a number of *NURR1* variations that affect *NURR1* expression or transcriptional activity have been associated with PD (Le et al., 2003). Several other *NURR1* variants have been identified in patients with PD whose functional implications remain to be addressed (Xu et al., 2002a; Grimes et al., 2006). In addition, *NURR1* levels in the substantia nigra were found to be decreased in autopsies of patients with PD (Jankovic et al., 2005). Ironically, while dopaminergic development requires *NURR1*, overexpression of *NURR1* increases the sensitivity of mouse neural stem cells to neurotoxins such as 6-OHDA and methyl phenylpyridinium, indicating that *NURR1* may in fact be involved in the susceptibility of DA neurons to degeneration in PD (Lee et al., 2002b). *NURR1* mutations have also been found in patients with schizophrenia (Eells et al., 2002) and most typical and atypical antipsychotic drugs induce *NOR1* and *Nurr77* expression in dopaminoceptive areas, and *NURR1* expression in the SN and VTA (Maheux et al., 2005). Another interesting study of cocaine abusers and control subjects

found that the levels of NURR1 and DAT were substantially reduced in abusers (Bannon et al., 2002), further linking NURR1 to the regulation of the DA system, and the manifestation of dopaminergic diseases.

Hypotheses

1) The function of the monoamine fear circuitry is tightly controlled by a series of gating structures that resemble the intercalated cell masses and are interposed between input and output structures along the rostrocaudal axis of the extended amygdala. These gating structures are modulated by important effectors of fear and emotion such as dopamine and opioid peptides. The proposal that fear circuitry may involve both volume (mismatch) and wiring (match) mechanisms of neurotransmission suggests that both overlap and mismatch between neurotransmitters and their receptors may occur in brain regions implicated in fear circuitry. Since the amygdala is strongly implicated in fear transmission, I hypothesize that in the amygdala:
    a) The localization of terminal-like input of dopamine (detected by tyrosine hydroxylase staining in the absence of dopamine β-hydroxylase staining) and target dopamine receptors (dopamine-D1 staining) is both co-localized and mismatched.
    b) The localization of opioid terminals (β-endorphin and enkephalin) and of target opioid receptors (μ-opioid) is both co-localized and mismatched.

   By identifying these dopaminergic and opioid receptor/transmitter mismatches and their localization, a model of how the intercalated cell masses regulate fear circuitry activity- and time-dependent output can be developed.
2) The function and dysfunction of the monoamine fear circuitry is largely controlled by the developmental regulation of the major neurotransmitter systems involved in mood and emotion, which includes dopamine and serotonin. I hypothesize that alterations

in key transcriptional regulators, such as Nurr1 or Pet-1, lead to altered neurotransmitter or receptor expression in these systems.

a) Dopamine development is transcriptionally regulated by the orphan nuclear receptor, NURR1, which is an important regulator of tyrosine hydroxylase expression and can be activated by ERK 1/2 signalling. A Ser125Cys mutation in Nurr1 has been identified in a Parkinson's disease patient, which may contribute to the dopamine deficiency. I hypothesize that

   i) The Ser(125)Cys mutation, which is located in a putative ERK1/2 phosphorylation site, prevents ERK 1/2 activation of NURR1 and inhibits the transcriptional activation of TH.

   Understanding the role of ERK1/2 in TH expression may provide insights into mechanisms that regulate dopamine levels in the fear circuitry.

b) Serotonin development is transcriptionally regulated by the Ets transcription factor, Pet-1/FEV, and neural differentiation is regulated by the bHLH transcription factors, Hes1, Hes5 and Hes6. The 5-HT1A receptor, an important regulator of serotonergic activity, contains both putative Pet-1 and Hes elements in its promoter, hence I hypothesize that:

   i) Pet-1/FEV is able to modulate expression of 5-HT1A receptors by acting as a transcriptional enhancer for 5-HT1A autoreceptors through multiple Pet-1/FEV response elements in the 5-HT1A promoter.

   ii) In the absence of Pet-1, 5-HT1A autoreceptor expression will be inhibited.

   iii) Since Pet-1 directs serotonin neuron development, the 5-HT deficit in Pet-1 null mice may also result in altered expression of both pre- and post-synaptic 5-HT1A receptors.

iv) Hes1 and Hes5 transcriptionally repress 5-HT1A receptor expression through an N-box proximal to the C(-1019)G promoter polymorphism, and this repression is inhibited by Hes6.

v) In the absence of developmental Hes1-mediated repression of 5-HT1A, 5-HT1A mRNA will be prematurely expressed.

An understanding of the role of Pet-1 in regulation of the 5-HT1A receptor may provide insight into the development of anxiety and aggression in mice lacking Pet-1. The role of Hes proteins in regulating 5-HT1A receptor expression may contribute to the association of the 5-HT1A C(-1019)G polymorphism with depression, panic attack and suicide.

Approaches

In order to address how the monoamine fear circuitry controls emotion and behaviour, we examined the anatomy of the emotional 'center' of the fear circuitry, the extended amygdala. Specifically, we used immunofluorescent analysis and a combination of light- and confocal microscopy to examine the neurotransmitter composition of the gating structures that line the rostrocaudal axis of the extended amygdala. Using these data we proposed a model describing how emotional responses might be modulated by the monoamine fear circuitry and the neurotransmitter systems that interact with it, which has been validated by a number of collaborative studies.

In order to investigate how the monoamine fear circuitry is regulated through development, as well as how perturbations in the regulatory mechanisms controlling this development might be related to neuropsychiatric disorders, we used a range of *in vitro* and *in vivo* molecular tools, including both Pet-1 and Hes1 null mice.

PART ONE: Mapping the Monoamine Fear Circuitry

Background and Relevance – Chapters II and III

A 2002 survey by the Public Health Agency of Canada reported that 1/5 Canadians met the criteria for a mood or anxiety disorder at some point during their lifetime (24% of women, 17 % of men) (Health_Canada, 2002). These diseases carry with them a great deal of stigma, with more than half of those surveyed reporting embarrassment about their conditions and having faced discrimination related to their mental health problems. The serious stigma associated with mental illnesses causes affected individuals to avoid or delay seeking medical help, alienates them from society and perpetuates the symptoms of the illness itself (Government_of_Canada, 2006). One major cause for this social stigma relates to lack of education, and the perception that mental illness is not a 'disease' in the same sense as more tangible affectations. Worldwide anti-stigma campaigns are tackling this issue by raising awareness using unique advertising strategies that makes our lack of understanding of mental illness pointedly clear (Figure I-19). Intriguingly, making mental illness "tangible" is an issue which continues to dog current research on the subject. That virtually every neurotransmitter system and brain region has been associated with this set of diseases in some ways contributes to this misperception, as the absence of a clear cause (or set of causes) means that psychiatric disorders must be diagnosed based on symptomology. As a primary step in linking brain chemistry, tangibly, to mental illness, it is critical to understand how emotion is 'wired' in the brain. Not only is this essential for determining how the 'circuitry' controlling emotion might be dysregulated in mental illness, but also for elucidating how and where to manipulate the circuitry in the treatment of such disorders. For this reason, the first part of this thesis will discuss some important features of the "fear circuitry", responsible for modulating mood and emotion.

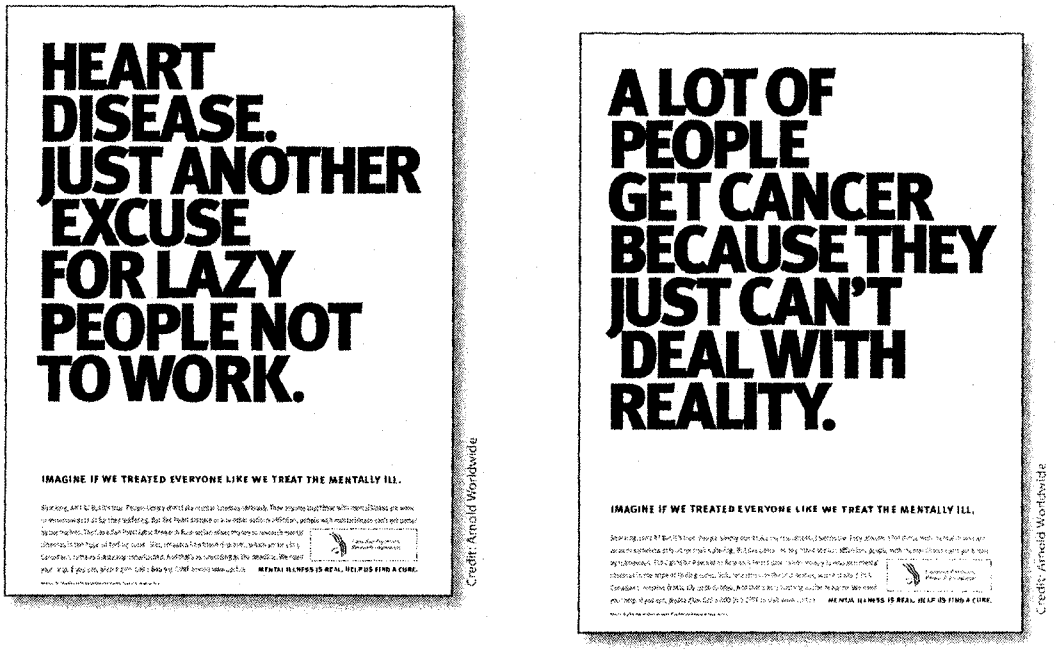


Figure I-19: Canadian Psychiatric Research Foundation Anti-Stigma Campaign

Examples of advertisements by the CPRF raising awareness about mental illness. The poignancy of the campaign lies in suggesting that people affected with commonly 'accepted' diseases such as heart disease and cancer are somehow at fault for their affliction. Advertisements reproduced from the Ottawa Citizen.

Specifically, the following chapters will discuss a unique feature within the fear circuitry that enables a single neurotransmitter system to modulate emotion through a combination of fast synaptic connections and slower, more graded connections that may provide mechanisms to spatially and temporally measure emotional responses.

CHAPTER II: The dopamine D1 receptor-rich main and paracapsular intercalated nerve cell groups of the rat amygdala: relationship to the dopamine innervation

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Contribution of Co-Authors

All of the animal work, imaging and figures were done by Kirsten X. Jacobsen.

Tables containing image quantification of image data were done by Luigi F. Agnati.

First author was Kjell Fuxe, with substantial written contribution from Kirsten X. Jacobsen and Malin Höistad.

Abstract

The intercalated cell masses are GABAergic neurons interposed between the major input and output structures of the amygdala. Dopaminergic projections to the main and paracapsular intercalated islands were examined by determining the relationship of the dopamine nerve-terminal networks to the D1-receptor immunoreactive staining of cells within the intercalated islands, using double-fluorescence immunolabelling procedures in combination with confocal laser microscopy. The relationship of terminals positive for both tyrosine hydroxylase and dopamine β-hydroxylase (noradrenaline and/or adrenaline) to terminals positive for tyrosine hydroxylase but negative for dopamine β-hydroxylase (dopamine terminals) was studied in relation to the D1-receptor immunoreactivity in adjacent sections at various rostrocaudal levels. The microscopy and image analysis revealed that there was only a minor dopaminergic innervation of the D1 receptor-immunoreactive cells in the rostromedial and caudal component of the main intercalated island, suggesting volume transmission as the main communication mode for dopamine in these regions. In contrast, the D1 receptor-immunoreactive areas in the rostrolateral part of the main island and also the paracapsular intercalated islands showed a high degree of dopaminergic innervation, indicating that synaptic and perisynaptic dopamine transmission plays a dominant role in these regions.

It is known that amygdala neurons are involved in the elicitation and learning of fear-related behaviours. We suggest that slow dopaminergic volume transmission in the rostromedial and caudal parts of the main intercalated island may have a role in tonic excitatory modulation in these parts of the main island, allowing GABAergic activity to develop in the central amygdaloid nucleus and thereby contributing to inhibition of fear-related behavioural and autonomic responses. In contrast, a faster synaptic and

perisynaptic dopaminergic transmission in the rostrolateral part of the main intercalated island and in the paracapsular intercalated islands may have a role in allowing a more rapid elicitation of fear-related behaviours.

Introduction

The intercalated islands of the amygdala are cell clusters interposed between the basolateral complex and the central nucleus of the amygdala, receiving glutamatergic input from the basolateral complex and projecting GABAergic fibres to the central nucleus. This has been shown in the rat (Millhouse, 1986; Nitecka and Ben-Ari, 1987), the cat (Pare and Smith, 1993b, a), the guinea-pig (Royer et al., 1999) and in the monkey (McDonald and Augustine, 1993; Pitkanen and Amaral, 1994). Thus, these interconnected clusters of neurons represent an inhibitory interface between the input and output nuclei of the amygdala (Royer et al., 1999, 2000b, a).

A number of studies have been performed on the dopaminergic input to the amygdala (Fuxe, 1965b; Fallon et al., 1978; Emson et al., 1979; Takada, 1990; Roder and Ciriello, 1993; Revay et al., 1996; Asan, 1997b; Brinley-Reed and McDonald, 1999) and to the intercalated cell masses (Asan, 1997a, 1998). Both the main intercalated (Im) and the paracapsular (Ip) intercalated islands (nomenclature according to (Alheid et al., 1994) are rich in D1-receptor binding sites compared with other amygdaloid nuclei (Scibilia et al., 1992) and D1 mRNA is expressed in cells in the main intercalated island (Maltais et al., 2000). D2-like receptor immunoreactivity (IR) is also found in the intercalated islands, albeit at substantially lower levels, where it is colocalized with D1 mRNA, in accordance with data demonstrating significant D2 binding in the Im (Scibilia et al., 1992; Maltais et al., 2000). Therefore, dopaminergic mechanisms may play an important role in the functions of the intercalated islands of the amygdala, influencing emotional learning and memory.

The involvement of synaptic versus volume transmission (VT) in dopamine communication has been the focus of numerous studies in other regions of the brain, e.g. the striatum, the entopeduncular nucleus and the prefrontal cortex (Fuxe et al., 1988b; Fuxe et al., 1988a; Levey et al., 1993; Yung et al., 1995; Descarries et al., 1996; Jansson et al., 2002). Sixty to seventy percent of neostriatal dopamine (DA) terminals have been shown to be non-junctional (Descarries et al., 1996). However, the involvement of volume transmission in the intercalated islands of the amygdala remains to be clarified. Volume transmission is defined as a widespread mode of brain communication in the ECF and the cerebrospinal fluid involving diffusion and/or convection of informational substances, such as transmitters, ions and neuromodulators (Fuxe and Agnati, 1991; Agnati et al., 2000).

Regarding the putative existence of synaptic transmission of dopamine in these islands, tyrosine hydroxylase (TH) IR boutons have been found to form symmetric synapses on the dendrites and spines of the Ip (Asan, 1997a) but the Im has not been analyzed at the ultrastructural level. A putative synaptic dopaminergic innervation appears to exist in the rat basolateral amygdala in the form of TH-immunoreactive terminal baskets, enveloping certain parvalbumin-immunoreactive non-pyramidal cells (Brinley-Reed and McDonald, 1999). However, an involvement of dopaminergic volume transmission in the amygdala is indicated in the work of Garris and Wightman (Garris and Wightman, 1994) showing evoked extracellular dopamine release in the basolateral amygdaloid nucleus from DA terminals.

In the present study, the relationship between the D1 receptor IR-intercalated islands and its DA terminals (TH-positive but dopamine β-hydroxylase (DBH)-negative, TH+DBH−) and the noradrenaline (NA) and/or adrenaline (A) terminals (TH positive and DBH positive, TH+DBH+) was analyzed in the rat amygdala using double immunolabelling procedures to better understand the DA communication processes in the intercalated island system of the rat amygdala.

Methods

Animals

Pathogen-free adult male Sprague-Dawley rats were used. The animals were kept under standardized lighting. Efforts were made to reduce the number of animals used (n=3) and to minimize their suffering. Local protocol number was A-121, General Neurohistology. Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80-23) revised 1996. The animals were anaesthetized with a high dose of pentobarbital (100 mg/kg) and perfused through a cannula inserted in the ascending aorta with 50 mL phosphate-buffered saline (PBS, 10-mM phosphate in 0.9% saline, pH 7.4) at room temperature followed by 100 mL fixation fluid (4 °C). This fixative consisted of 4% paraformaldehyde (w/v) and 0.2% picric acid (w/v) in 0.1-M sodium phosphate buffer (pH 6.9). The brains were removed and kept in fixative at 4 °C for 30 min and rinsed for 48 h in 10% sucrose in 0.1-M phosphate buffer containing 0.01% (w/v) sodium azide.

Antibodies

A rabbit polyclonal D1 receptor antibody was used at 1:800 dilution (Jansson et al., 1999). This antibody was raised to a fusion protein comprising the C-terminus of the rat DA D1 receptor (amino acid residues 356–446). The sera from rabbits immunized with the DA D1 receptor fusion protein was analyzed on immunoblots (Western blots) for their activity to recognize the DA D1 receptor. The immunoblot obtained from striatal extracts contained a protein band (72,000 M_W) corresponding to the DA D1 receptor. This band was not detectable in the immunoblot from the cerebellum, suggesting that it recognizes the D1 receptor. Furthermore, this antiserum strongly labels the strionigral and strioento-peduncular GABA pathway, where the D1 receptors are selectively localized, and fails to

demonstrate any staining in the nigral DA cell bodies which express D2 (but not D1) receptors. Thus, this D1 antiserum does not cross-react with D2 receptors. For further control experiments, see (Jansson et al., 1999).

The TH antibody was a mouse monoclonal antibody used at a 1:500 dilution (Incstar, Stillwater, MN, USA). The dopamine β-hydroxylase (DBH) antibody was a rabbit polyclonal antibody used at 1:1000 dilution (Eugene Tech, Ridgefield Park, NJ, USA). The TH and DBH antibodies showed the well-known pattern of catecholamine nerve-terminal systems in the forebrain as previously reported (Geffen et al., 1969; Fuxe et al., 1970; Hartman and Udenfriend, 1970; Hokfelt et al., 1976).

Immunohistochemistry

Coronal cryostat sections (5 or 10 μm thick) from bregma level −1.8 to −2.6 mm according to the atlas of (Paxinos and Watson, 1986) were incubated in a humidified chamber with the primary antibody at 4 °C overnight. Three adjacent sections out of 10 were collected throughout these bregma levels. All antibodies were diluted in PBS containing 0.3% Triton X-100. After washing in PBS, the D1 receptor- or DBH-stained adjacent sections were incubated at 37 °C for 40 min with Cy3-conjugated affinity-purified donkey anti-rabbit immunoglobulins (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After washing again, the D1- or DBH-immunostained sections intended for double immunofluorescence were incubated overnight at 4 °C with a second primary antibody (mouse anti-TH antibodies). The sections were washed and incubated with fluorescein isothiocyante (FITC)-conjugated sheep-anti-mouse immunoglobulin (1:20, Amersham, Baie d'Urfe, Quebec, Canada) at 37 °C for 40 min. All sections were mounted in antifading medium (0.05% *p*-phenylenediamine in PBS with 80% glycerol) after being carefully rinsed in PBS.

Image analysis

Specific IR was visualized using the fluorophores Cy3 or FITC based on excitation at a green wavelength (545±20 nm) or a blue wavelength (490±20 nm), respectively. In the images shown, D1 IR is red, TH IR is green, and DBH IR is in pseudocolour blue, the original color being yellow by frequent overlaps of red (Cy3-DBH) and green (FITC-TH) immunofluorescence. Confocal images were generated by using an inverted, confocal laser scanning microscope (Zeiss Axiovert 100 LSM 410, Toronto, Ontario, Canada) using an air-cooled krypton/argon laser.

The image analyzer KS 400 (Zeiss Kontron, Zeiss, Arese, Italy) was used for the quantitative evaluation of the areas of immunoreactivity (Agnati et al., 1985). Measurements were made in pooled "rostral" (Br −1.8 mm to −2.1 mm) and "caudal" (Br −2.3 mm to −2.6 mm) levels and expressed as means±S.D. For the TH+D1 or DBH+D1 overlap measurements (Table II-1), after the acquisition an automatic discrimination procedure was performed based on the function threshold "RGB" (red, green, blue). The field areas (FA) in pixels was determined for each antigen (red, green or blue). Subsequently, by means of the Boolean function "AND", the FA overlaps between the TH+D1 or DBH+D1 IR areas were determined. For this procedure, the specific staining for each antigen in the same (TH versus D1 IR areas) or adjacent (DBH versus D1 IR areas) sections was used. The FA of these overlaps was determined and expressed in percent of the corresponding D1 IR field area.

Table II-1: Catecholamine terminal overlaps in the D1 IR Im

	FA(pixels)	
	Rostral	Caudal
D1	109 ± 14	87 ± 31
TH	51 ± 11	18 ± 7.0
TH+D1	27.2 ± 4.0	2.1 ± 0.9
TH+D1 (%)	26.3 ± 4.4	4.4 ± 3.3
D1	97 ± 32	42 ± 7.0
DBH	28 ± 6.0	29 ± 10
DBH+D1	4.6 ± 1.4	2.9 ± 1.7
DBH+D1 (%)	5.1 ± 1.4	6.6 ± 4.0

The FA (in pixels) is a measurement of the density of immunoreactive terminals, and values are expressed as means±S.D. (n=3–5). The regions selected for measurement were within the D1 IR Im, with rostral sections being from Br −1.8 mm to −2.1 mm and caudal sections being from Br −2.3 to −2.6 mm. The FA of either D1, TH or DBH IR is expressed as means±S.D. (n=3–5 measurements). The D1+TH+DBH− (DA terminals) or D1+TH+DBH+ (NA/A terminals) FA overlaps were evaluated as the FA (in pixels) within the D1 island that was positive both for D1 and for TH, or both D1 and DBH. These FA overlaps were then expressed as a percentage of the respective D1 IR FA: [(TH+D1)/D1×100] and [(DBH+D1)/D1×100] respectively. See Experimental Procedures for further image-analysis details.

For measurements of dopamine fibre (TH+DBH−) distribution inside versus outside the D1 IR main intercalated island (Table II-2), a field area of 500×500 pixels was selected (about 20% of the original size of the microphoto) and this field was placed at the latero-dorsal border of the D1 IR island in such a way that about 50% of this sampled field (TH IR) was inside the island and about 50% outside the D1-positive island. A zoom magnification was applied (×2), the RGB discrimination procedure was carried out on the sampled field, and the thresholds (red, green and blue) were interactively selected. After discrimination of the D1 IR island (red), the "fill-hole procedure" was applied; hence a compact profile of the island inside the sampled field was obtained. The discrimination of the terminals was performed. The Boolean operator AND applied to the D1 island versus the TH IR terminals allowed visualization of the terminals inside the island. The Boolean operator xOR applied to terminals inside the island versus the entire population of terminals allowed visualization of the terminals outside the island.

Table II-2: TH-terminal innervation pattern (TH+DBH− terminals) inside versus outside the Im

TH (n=s)	FA (Pixels)	
	Rostral	Caudal
Inside	105 ± 23	13 ± 8.0
Outside	26 ± 4.0	28 ± 11
In/out (%)	410 ± 68	45 ± 18

The FA (in pixels) is a measurement of the density of TH IR terminals, and values are expressed as means±S.D. (n=3). The regions selected for measurement were placed with half of the FA inside the D1 IR area and half of the FA outside the D1 IR area of the Im. Rostral sections were from Br −1.8 to −2.1 mm and caudal sections were from Br −2.3 to −2.6 mm. The inside versus outside ratio expresses the FA of the TH IR terminals that is within the FA of the D1 IR island as compared to outside the island, and is expressed as percentage of the respective TH IR inside value. See Experimental Procedures for further image-analysis details.

Results

D1 receptor immunoreactive intercalated islands

In Figure II-1, the main intercalated island and the paracapsular intercalated islands rich in D1-receptor IR are shown at rostrocaudal levels of the rat amygdala from Br −1.8 mm to Br −2.5 mm. A higher magnification at Br −2.5 mm is shown in Figure II-2, where a large D1 receptor-immunoreactive patch is found representing the main intercalated island (Im) located ventromedial to the basolateral amygdaloid nucleus. Small D1 receptor-immunoreactive patches surround the basolateral and lateral amygdaloid nuclei, representing the paracapsular intercalated islands (Ip).

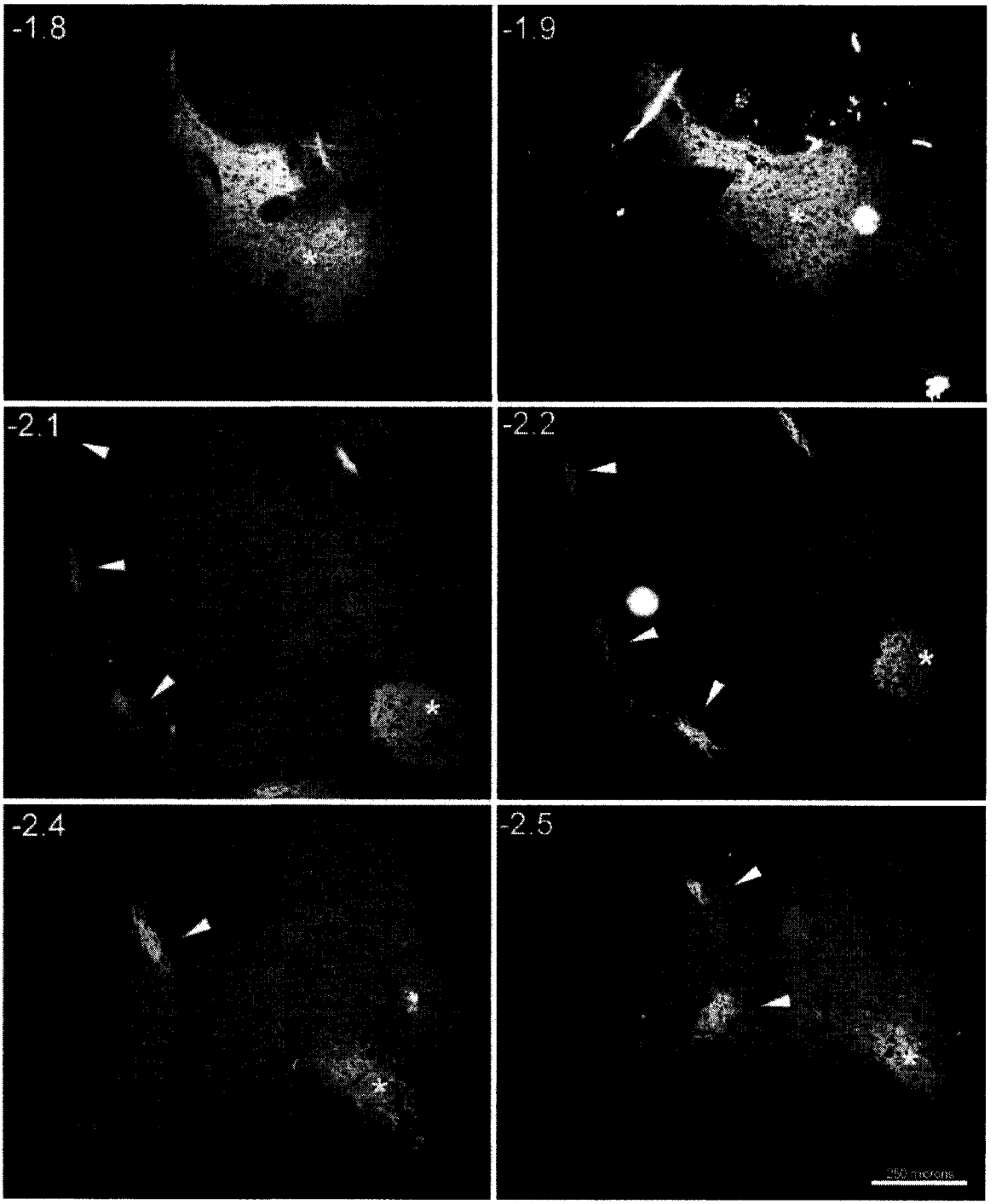

Figure II-1: D1-receptor IR in the Im and Ip.

D1-receptor immunoreactivity in the main (asterisk) and paracapsular (arrowheads) intercalated islands at various rostrocaudal levels ranging from Br −1.8 mm to −2.5 mm (10 μm-thick sections and 100 μm distance between sections) as indicated in the panels. Bregma levels according to (Paxinos and Watson, 1986) and lateral is to the left.

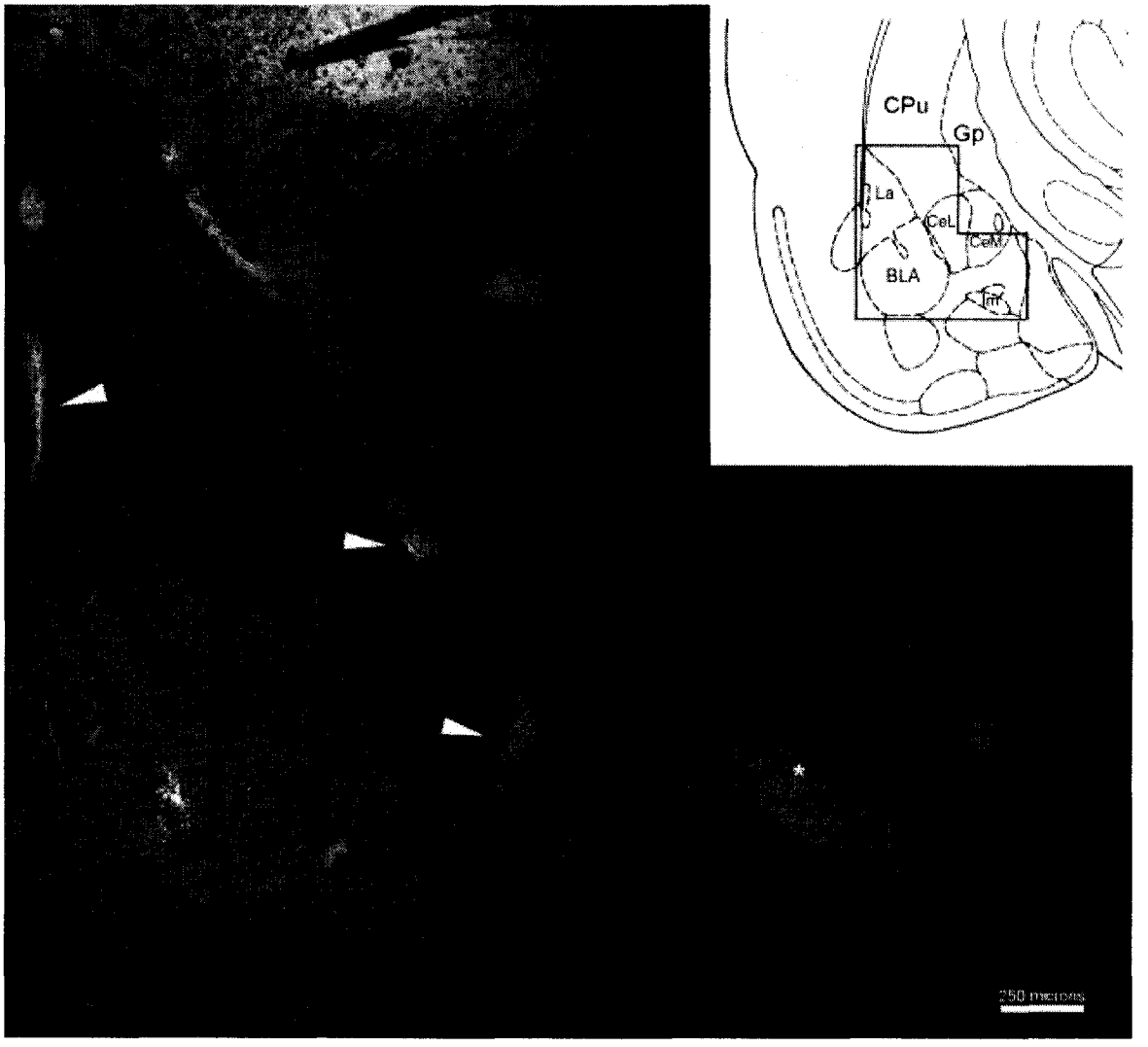

Figure II-2: D1 IR in the amygdala.

A digital photo-montage of immunoreactivity for the D1 receptor in the amygdala. The region depicted is shown in the inset from the atlas of (Paxinos and Watson, 1986) at Br level −2.56 mm. The asterisk indicates the main Im, and arrowheads indicate the small Ip surrounding the lateral and basolateral amygdaloid nuclei. For conventional cytoarchitectonics, see (Alheid et al., 1994). Im - main intercalated island of the amygdala, La - lateral amygdaloid nucleus, BLA - basolateral amygdaloid nucleus, CeM - medial central amygdaloid nucleus, CeL - lateral central amygdaloid nucleus, Gp - globus pallidus, CPu - caudate putamen.

D1 versus TH immunoreactivity in the main intercalated island

As seen in Figure II-3, the D1 IR cell bodies of the Im are closely packed and individual nerve cells cannot be distinguished at any of the rostrocaudal levels analyzed. At the rostral levels (from Br −1.8 mm to −2.1 mm) there is a dense innervation by TH IR nerve terminals of the lateral parts of the D1 IR-rich main island. The overlapping TH+D1 IR areas are shown in yellow and based on image analysis, this overlap area was 26% (in percent of the respective D1 IR area), Table II-1. In contrast, there is only a sparse innervation by TH IR nerve terminals at the caudal levels (from Br −2.3 mm to −2.6 mm) with a TH+D1 overlap area of 4% of the D1 area. In fact, at the caudal level, the TH-terminal density inside the Im was even lower than that found outside the main island (Table II-2).

At caudal levels, there were focal regions of higher D1 IR within the Im (Figure II-4). Confocal laser microscopy showed that at the caudal level many individual D1 IR nerve cells were not directly contacted by TH IR terminal-like fibres, since only scattered TH IR varicosities or terminal-like fibres were found (Figure II-5), often located 100 μm away from the D1 IR nerve-cell body.

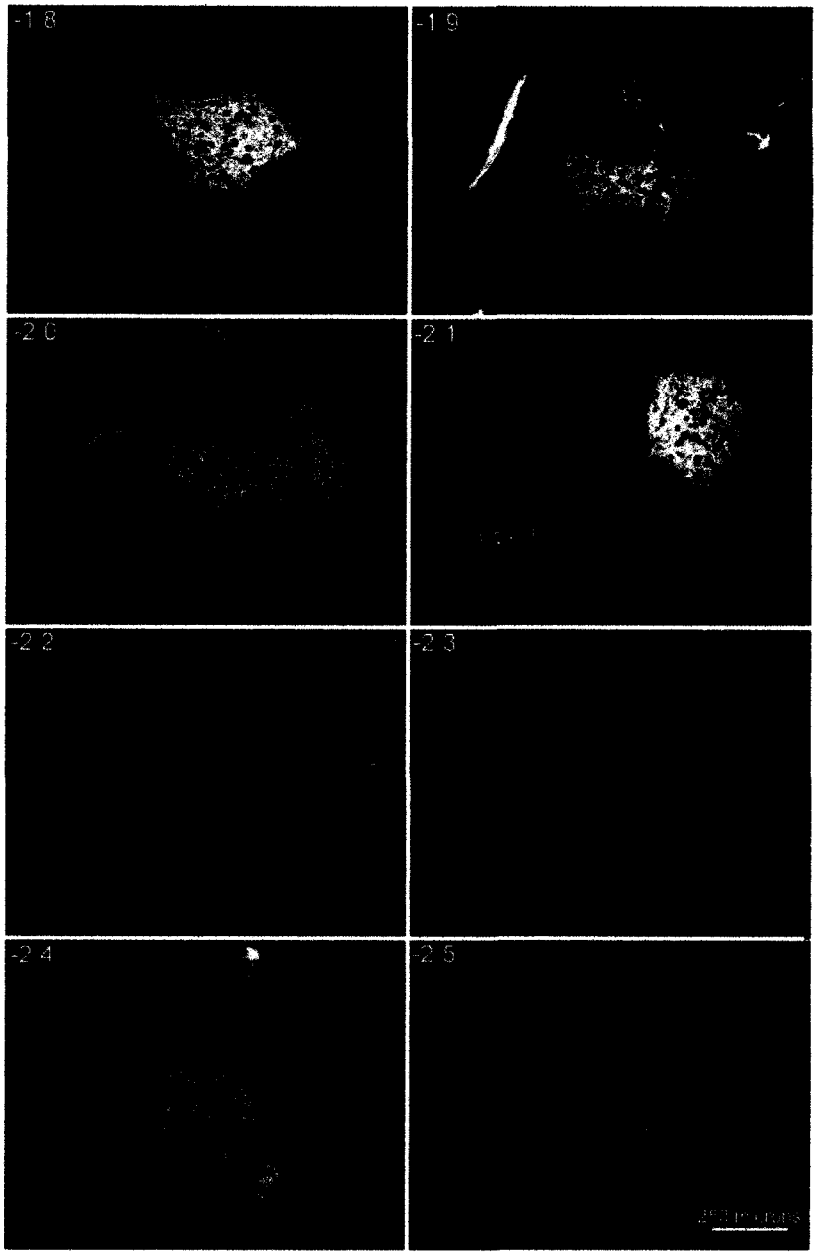

Figure II-3: DA D1 and TH IR in the Im.

Double immunolabelling of DA D1 receptors (red) and TH (green) in the amygdala at various rostrocaudal levels ranging from Br −1.8 mm to −2.5 mm as indicated in the panels (10 µm-thick sections and 100 µm distance between sections, same animal as in Figure II-2). A major overlap of TH and D1 immunoreactivity is found in the rostrolateral parts of the main intercalated island (Br −1.8 mm to −2.1 mm) but not in the rostromedial or caudal parts (Br −2.2 mm to −2.5 mm). Bregma levels according to (Paxinos and Watson, 1986) and lateral is to the left.

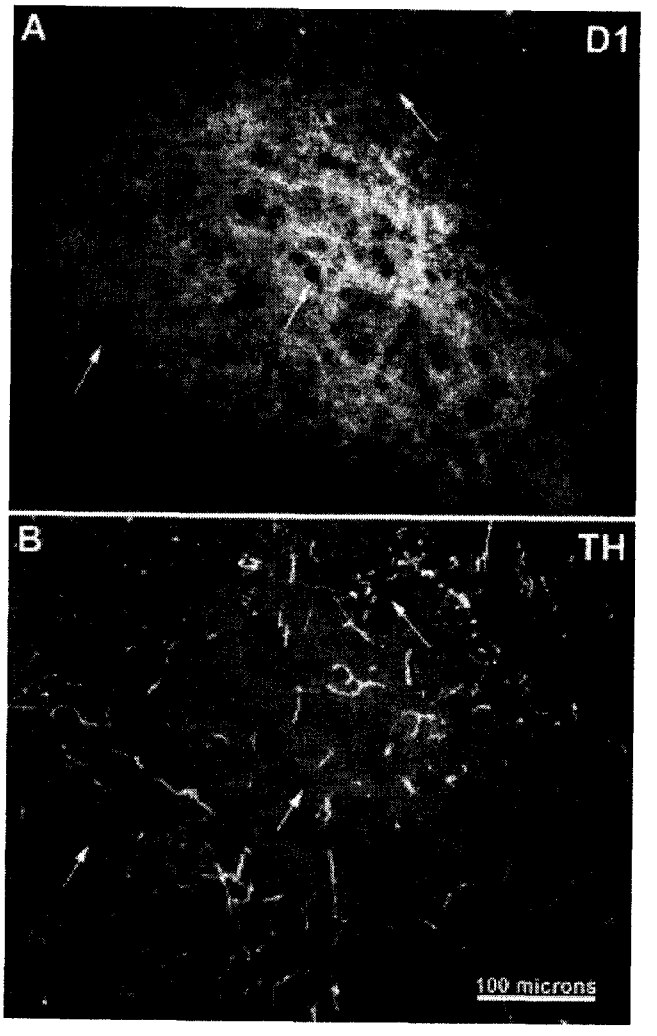

Figure II-4: D1 and TH IR in the caudal Im.

Immunoreactivity for the dopamine D1 receptor (A) and TH (B) in cell bodies and nerve terminals respectively, in a 5 μm section through the caudal part of the main intercalated island of the amygdala (Br −2.5 mm). Arrows indicate corresponding landmarks in the two microphotographs. Bregma level according to (Paxinos and Watson) and lateral is to the left.

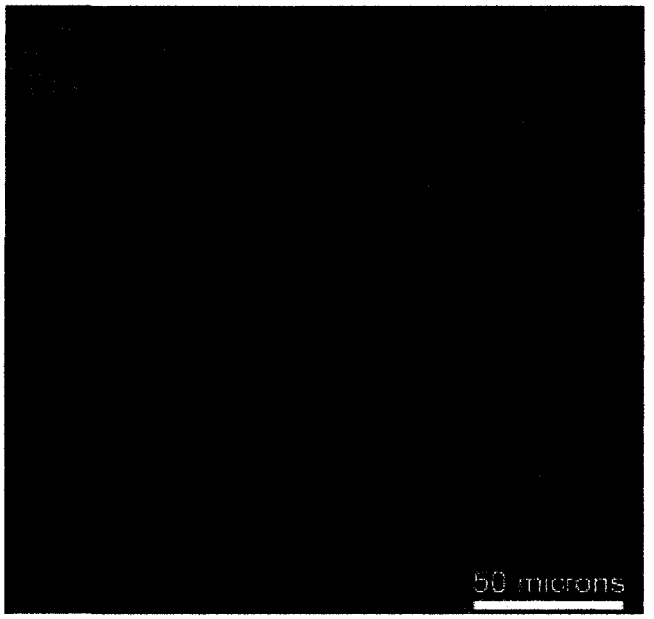

Figure II-5: Confocal image of D1 and TH IR in the Im.

A confocal laser microphotograph of double immunolabelling of the DA D1 receptor-positive cell bodies (red) and TH-positive nerve terminals (green) from the caudal part of the main intercalated island (Br −2.5 mm) in a 5 μm-thick section. Bregma level according to (Paxinos and Watson, 1986) and lateral is to the left.

TH-versus DBH-immunoreactive terminal networks in the main intercalated island

As seen in Figure II-6, at rostral levels there is a marked difference in the distribution patterns of the DBH and TH IR terminals, with dopamine (TH+DBH−) in green and noradrenaline/adrenaline terminals (TH+DBH+) in blue. Within the Im, the DA innervation in the rostrolateral part is dense, whereas caudally it is sparse throughout the Im. In regions surrounding the Im, there is a similar low DA innervation. By contrast, there is a low and uniform innervation of NA/A terminals at all levels, both within as well as surrounding the Im (see also Table II-1 and Table II-2).

D1 versus DBH immunoreactivity in the main intercalated island

As seen in Figure II-7, the D1-rich main intercalated island shown in red immunofluorescence (from an adjacent section) is within a network of noradrenaline/adrenaline terminals (TH+DBH+) shown in blue. There is a low density of DBH IR terminals inside and outside the Im at both rostral and caudal level, with an overlap of 5–6% of D1 IR area (Table II-1).

In Figure II-8, at the caudal level, indications are again obtained that the blue TH+DBH+IR (from an adjacent section) and the green TH+DBH−IR terminals form a sparse plexus inside as well as outside the D1 IR island (see also Table II-2).

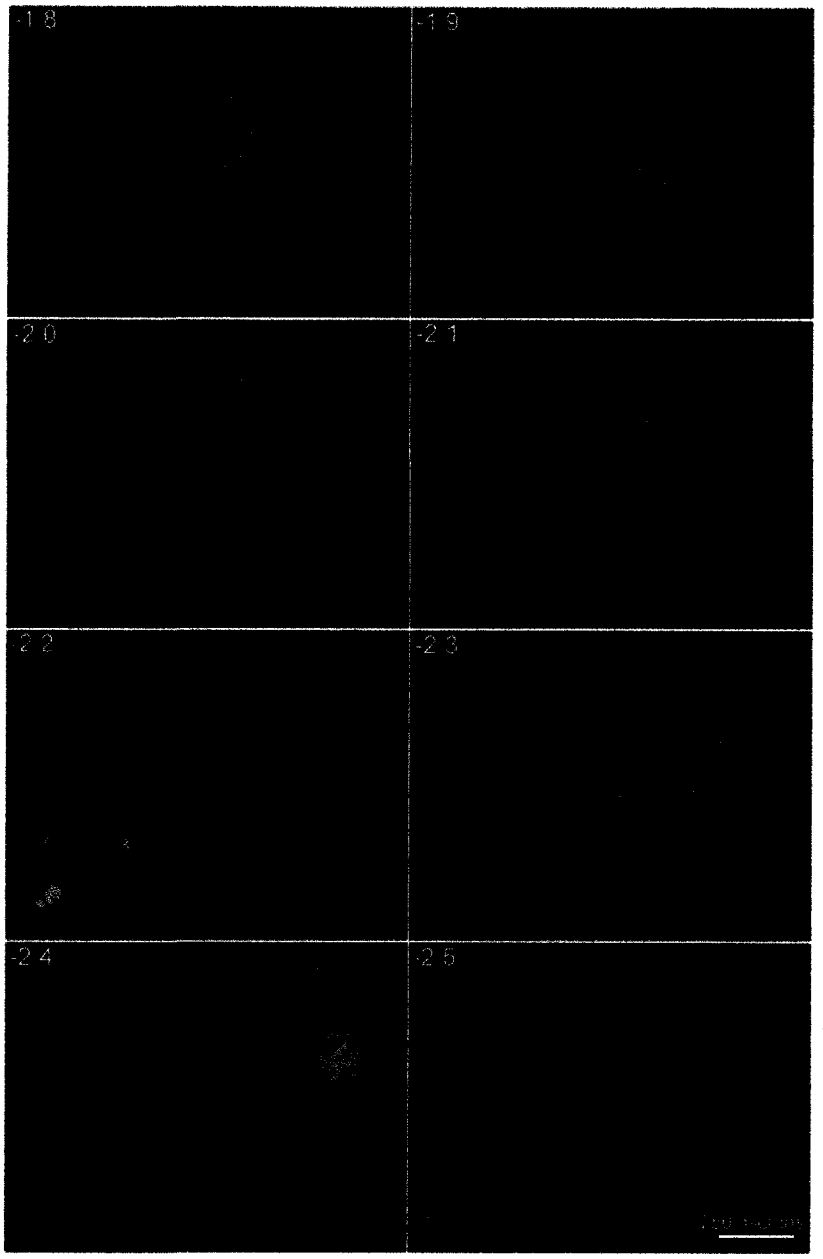

Figure II-6: TH and DBH IR in the Im.

Distribution patterns of green TH+DBH−IR terminals (DA terminals) and blue TH+DBH+IR terminals (NA/A terminals) at various rostrocaudal levels ranging from Br −1.8 mm to −2.5 mm as indicated in the panels (10 μm-thick sections and 100 μm distance between sections, same animal as in Figure II-1). The high degree of DA innervation in the rostrolateral part is seen at Br −1.8 mm to −2.1 mm. Bregma levels according to (Paxinos and Watson, 1986) and lateral is to the left.

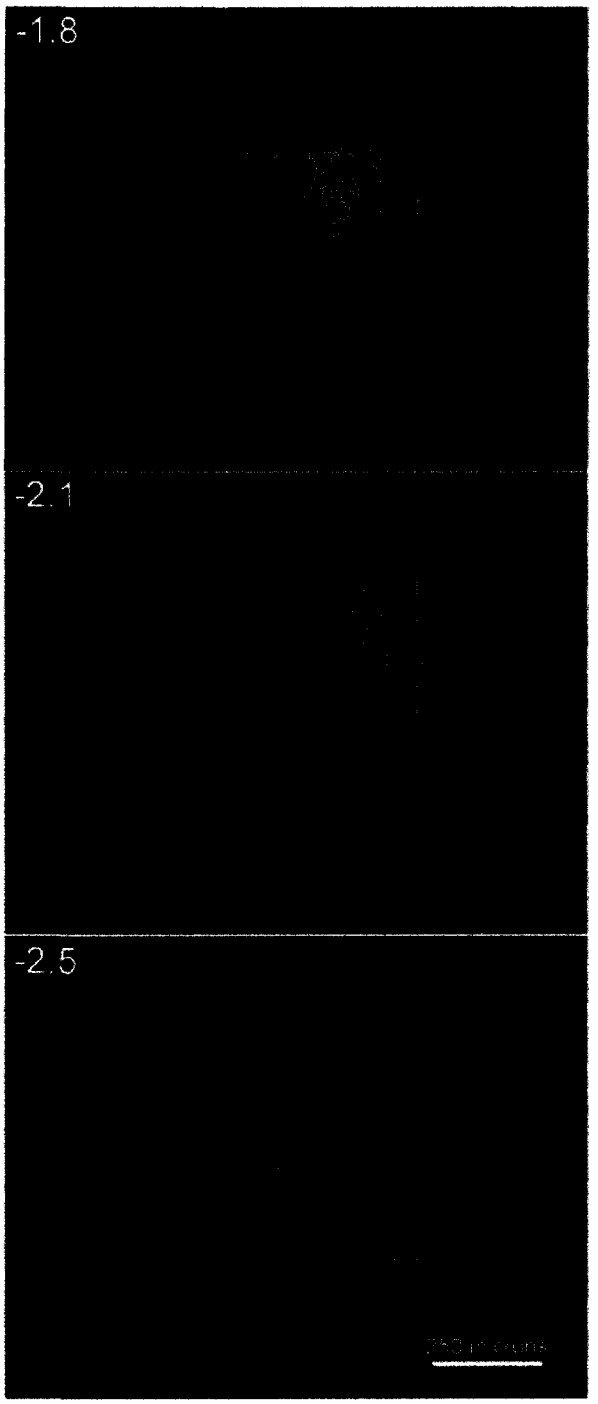

Figure II-7: Relationship of D1, TH + DBH IR in the Im.

The relationship of the red D1 IR to the blue TH + DBH + IR terminals from an adjacent section of the main intercalated island at Br −1.8 mm, −2.1 mm and −2.5 mm, as indicated in the panels (10 μm-thick sections, same animal as in Figure II-1). Bregma levels according to (Paxinos and Watson, 1986) and lateral is to the left.

Figure II-8: Relationship between DA and NA/A terminals with D1 in the Caudal lm. Microphotograph from the caudal part of the main intercalated island (Br −2.5 mm) showing immunoreactivity from a double-immunostained section, with TH+DBH− in green (DA terminals) and TH+DBH+ in blue (NA/A terminals). This image has been superimposed on a microphotograph with the DA D1 receptor immunoreactivity in red from the corresponding region of an adjacent section (5 µm-thick section). Bregma level according to (Paxinos and Watson, 1986) and lateral is to the left.

The paracapsular intercalated islands

As seen in Figure II-9, at various rostrocaudal levels, most of the D1-rich paracapsular islands show a high density of dopamine terminals (green TH+DBH−). Only the most ventral Ip, close to the lateral part of the main intercalated island, shows a rostrocaudal gradient, with a disappearance of DA terminals at the caudal level. The other Ips maintained a high D1 IR and DA innervation throughout the amygdala. The noradrenaline/adrenaline innervation of the D1-immunopositive paracapsular islands and the regions that surround them is at the same low to medium density as the Im.

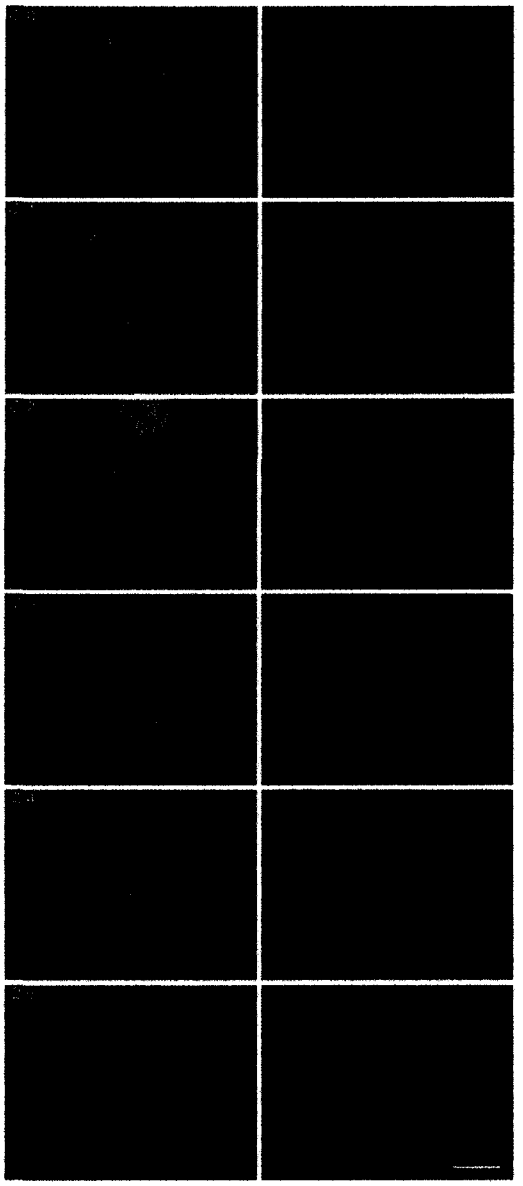

Figure II-9: D1, TH and DBH IR in the Ip.

The D1 receptor-positive (red) paracapsular intercalated islands and their distribution patterns of green TH+DBH−IR terminals (DA terminals) and blue TH+DBH+IR terminals (NA/A terminals) at various rostrocaudal levels of the, ranging from Br −2.0 mm to −2.5 mm as indicated in the panels. Each image pair of the respective Br level shows adjacent sections (10 μm-thick sections, same animal as in Figure II-1). A substantial DA innervation of the paracapsular intercalated islands appears to exist, except at the two caudal levels (Br −2.4 mm and −2.5 mm). Bregma levels according to (Paxinos and Watson, 1986) and lateral is to the left.

Discussion

The present findings demonstrate that the main and paracapsular intercalated islands of the amygdala contain densely packed D1-receptor IR cell bodies, in accordance with previous findings on high D1-receptor binding and D1 mRNA levels in the intercalated cell masses (Scibilia et al., 1992). The major new finding is the very low density of innervation by DA terminal-like fibres of the rostromedial and of the entire caudal region of the Im. While NA/A terminal density was low both within the Im and in the surrounding neuropil, DA appeared to form a 'shell' of terminal-like fibres of medium-density innervation around the rostromedial part of the Im, with almost no innervation in the caudal region of the Im itself. Thus, there is no enrichment of the DA terminal innervation over the rostromedial and caudal parts of the Im, in spite of the high density of D1 receptors. It therefore seems likely that the DA terminals in these parts of the Im and its surroundings operate mainly via volume transmission, with DA reaching the distant D1 receptors via local diffusion (in the range of 10–100 μm) in the extracellular compartment. Morphological indications of DA VT have previously been observed in special horizontal columns of the nucleus accumbens, in the zona reticulata of the substantia nigra, in the entopeduncular nucleus and in the islands of Calleja (Fuxe et al., 1988b; Fuxe et al., 1988a; Sokoloff et al., 1992; Diaz et al., 1995; Jansson et al., 1999). Also, electron microscopic studies have shown that in the striatum and in the prefrontal cortex DA receptors are mainly found extrasynaptically (Levey et al., 1993; Yung et al., 1995; Caille et al., 1996). In contrast, the rostrolateral component of the Im is densely innervated by DA terminals, but with only a sparse NA/A innervation. The TH+D1 IR overlap area was therefore substantial in this region. Also, the paracapsular intercalated islands showed indications of a high DA innervation but sparse NA/A innervation at

almost all rostrocaudal levels. Thus, synaptic and/or perisynaptic dopaminergic transmission may play a more important role in the rostrolateral Im and in the Ip (Asan, 1997a). It is worth noting that perisynaptic transmission of DA has in fact been viewed as a rapid form of VT (Gonon et al., 2000).

It is known that the GABAergic intercalated islands receive glutamatergic projections from the basolateral complex and project to the centromedial amygdala (Millhouse, 1986; Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993; Pare and Smith, 1993b, a; Pitkanen and Amaral, 1994). In this interposed position between the major input and output structures of the amygdala, the islands are able to gate the impulse traffic between these two structures (Royer et al., 1999). Royer and colleagues found that in the guinea-pig, these inhibitory cell clusters have polarized interneuronal connections, such that laterally located cells inhibit more medial ones. They proposed that the activation of the medial intercalated islands can counteract the elicitation of stereotyped fear responses induced from the central amygdala (Royer et al., 2000b, a). In the rat, it may be that the main intercalated island forms a long rostrocaudal column (Paxinos and Watson, 1986) in a way that the direction of polarized cells would be not only lateral to medial but also rostral to caudal.

We hypothesize that there may be a need for an efficient, more rapid modulatory dopaminergic transmission in the Ip and the rostrolateral part of the Im, versus a slower tonic dopaminergic VT in the rostromedial and caudal part of the Im (see Figure II-10). At the rostral level (Figure II-10A), the laterally located intercalated islands are activated by glutamate inputs from the lateral and basolateral nuclei (Royer et al., 1999) of the amygdala that, for example, respond to visual and auditory inputs. The activation of the rostrolateral Im may become strongly enhanced by the synaptically and perisynaptically

activated D1 receptors in this region. This may for instance be achieved by D1-receptor enhancement of glutamate release in this part of the Im (Bouron and Reuter, 1999) or by facilitatory postjunctional D1-NMDA-receptor interactions (Schoffelmeer et al., 2000; Lee et al., 2002a; Marti et al., 2002), as well as postsynaptic D1-(AMPA)-receptor interactions resulting in augmentation of AMPA receptor-mediated glutamate transduction (Chao et al., 2002). Accordingly, the inhibitory GABAergic activity in the rostrolateral Im would be increased. Since the lateral intercalated clusters project to the medial (Royer et al., 2000b) and caudal intercalated clusters, the rostromedially and caudally located cells would be inhibited, which would lead to a disinhibition of fear responses from the central amygdala. This would lead to a disinhibition of the central amygdaloid output, and subsequently to fear-related behaviours (LeDoux, 1995; Damasio, 1998). Such a model would explain the preferential DA innervation of the rostrolateral main intercalated island. At the rostromedial and caudal part of the Im (Figure II-10B), a tonic dopaminergic VT may allow a sustained activation of the intercalated GABAergic cells, allowing a tonic inhibition of the central amygdaloid nucleus in order to block unnecessary fear-related behaviours (Royer et al., 2000b). It has been found that a slowly deinactivating K^+ current may be involved in this tonic hyperexcitability state of these GABAergic intercalated cells (Royer et al., 2000a). Again, it may be that the DA VT signalling in these regions may enhance excitation via D1 glutamate-receptor interactions discussed above, but it should be noticed that D1 receptors can also excite low-threshold-spike GABA interneurons independent of glutamate receptors (Centonze et al., 2002). However, when true danger is present, the tonic inhibition of the central nucleus can be overridden by a preceding glutamate (from e.g. the basolateral nucleus) and/or synaptic/perisynaptic dopamine activation on the

rostrolateral GABAergic cells, which in turn inhibit the rostromedial and caudal GABAergic cells with disinhibition of the activity of the central nucleus (see Figure II-10).

McGrath and colleagues (McGrath et al., 1999) found increased anxiety in a transgenic mice model of cortical-limbic neurostimulation. These mice express a neuropotentiating transgene encoding a neuropotentiating enzymatically active intracellular subunit of the cholera toxin. This subunit transactivates the D1 receptor G_s subunit and increases the production of cAMP in D1-expressing neurons within the intercalated islands and in regions of the cerebral cortex that project to the orbitofrontal cortex and the striatum (McGrath et al., 1999). Based on the present work, it seems possible that the increased anxiety observed in this strain could be caused by a preferential and strong activation of the rostrolateral part of the intercalated islands. In addition to the already chronically overexcitable intercalated neurons (increased D1 signal transduction), the increased glutamatergic input would increase the inhibition of the rostromedial and caudal GABAergic intercalated neurons, resulting in a disinhibition of the central amygdaloid nucleus and thus to the development of pathological fear (anxiety) (McGrath et al., 1999). Such a mechanism could also explain the ability of microinjected intra-amygdaloid D1 antagonists to block conditioned fear (Lamont and Kokkinidis, 1998; Guarraci et al., 1999).

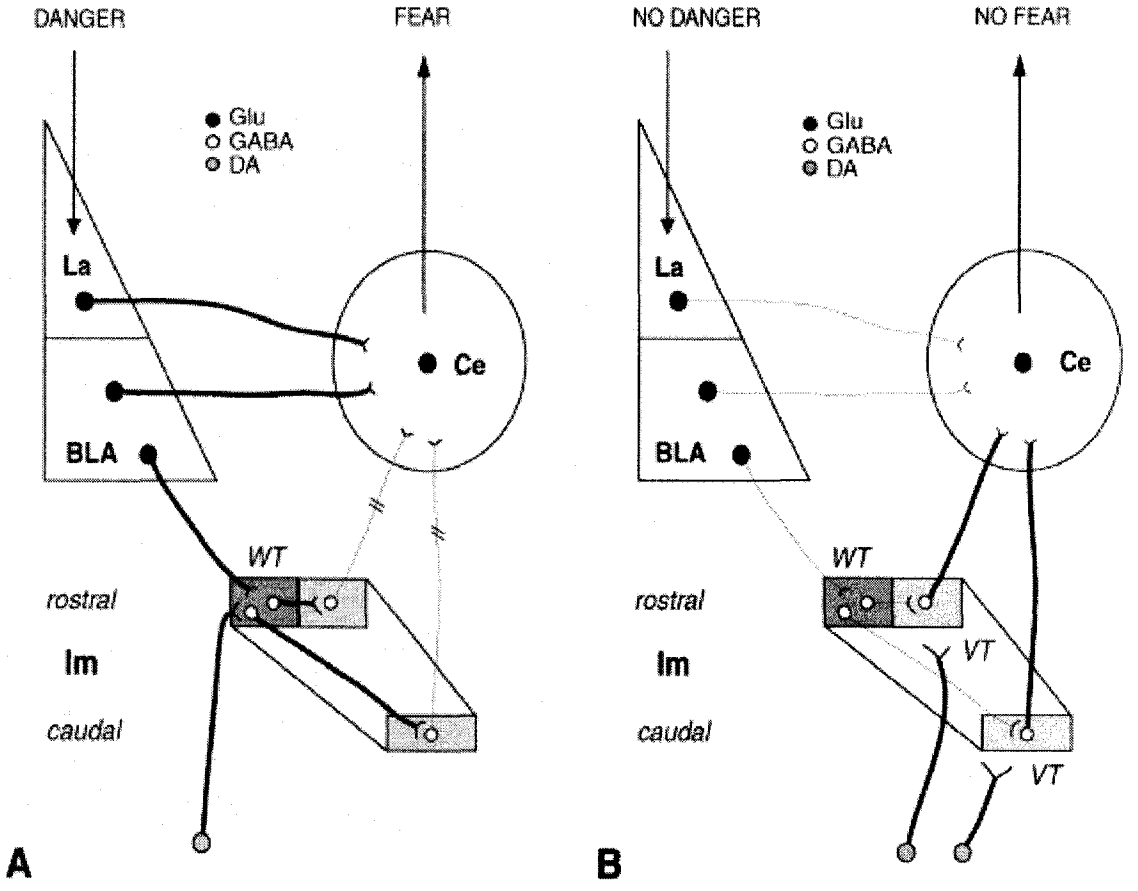

Figure II-10: Proposed model for gating and control of fear-related behaviours.

(A) During danger input. At the rostral level, glutamatergic input to the basolateral complex (La - lateral nucleus and BLA basolateral nucleus) is relayed to the rostrolateral part (dark-grey shading) of the Im. Rostrolateral Im neurons also receive putative synaptic/perisynaptic dopaminergic projections from the midbrain that may enhance glutamatergic transmission (see text). The rostrolateral GABAergic neurons of the Im project to and inhibit the rostromedial and caudal (light-gray shading) GABAergic neurons of the Im, thereby disinhibiting the output of the central amygdaloid nucleus (Ce) to the brainstem, resulting in the elicitation of fear and fear-related behaviours. (B) During no danger input. At the caudal level, in the absence of glutamatergic input to the basolateral complex and to the Im, the rostromedial and caudal GABAergic neurons of the Im are tonically activated by diffusing DA from another pathway, resulting in a sustained inhibition of the central amygdaloid output (no fear reaction). VT - volume transmission, WT - wiring transmission, rostral refers to Br −1.8 mm and caudal refers to Br −2.5.

It is known that glutamate and D1 receptors have an important role in LTP (Calabresi et al., 2000). In our model, it seems likely that the combined activation of synaptic glutamatergic and dopaminergic inputs to the rostrolateral intercalated nuclei can lead to LTP in the intercalated islands. In this way, learning and memory processes can take place in the intercalated islands that allow establishment of adequate fear responses. It seems likely that the more efficient and fast synaptic/perisynaptic DA transmission can strongly favour learning and formation of long-term memory in the rostrolateral intercalated nerve-cell clusters (Agnati et al., 2003).

The present study adds to previous morphological evidence for the volume transmission theory (Fuxe and Agnati, 1991; Descarries et al., 1997; Agnati et al., 2000). The true functional and physiological relevance of these findings and the validity of our fear model remains to be elucidated in further investigations on the amygdala.

Acknowledgements

This work was funded by the Swedish Medical Research Council and the Canadian Institutes of Health Research.

CHAPTER III: The distribution of dopamine D1 receptor and μ-opioid receptor 1 receptor immunoreactivities in the amygdala and interstitial nucleus of the posterior limb of the anterior commissure: Relationships to tyrosine hydroxylase and opioid peptide terminal systems

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Contribution of Co-Authors

All of the animal work, imaging and analysis were performed by Kirsten Jacobsen.

The manuscript was written by Kirsten Jacobsen with editorial input from Kjell Fuxe and Malin Höistad.

Abstract

Mismatches between dopamine innervation and dopamine D1 receptor (D1) distribution have previously been demonstrated in the intercalated cell masses of the rat amygdala. Here the distribution of enkephalin and β-endorphin immunoreactive (IR) nerve terminals with respect to their μ-opioid receptors is examined in the intercalated cell masses, along with a further immunohistochemical analysis of the dopamine/D1 mismatches. A similar analysis is also made within the extended amygdala.

A spatial mismatch in distribution patterns was found between the μ-opioid receptor-1 immunoreactivity and enkephalin IR in the main intercalated island of the amygdala. Discrete cell patches of dopamine D1 receptor and μ-opioid receptor-1 IR were also identified in a distinct region of the extended amygdala, the interstitial nucleus of the posterior limb of the anterior commissure, medial division (IPACM), which displayed sparse tyrosine hydroxylase or enkephalin/β-endorphin IR nerve terminals. Furthermore, distinct regions of the main intercalated island that showed dopamine/D1 receptor matches (the rostral and rostrolateral parts) were associated with strong dopamine and cyclic AMP regulated phosphoprotein, 32 kDa-IR in several D1 IR neuronal cell bodies and dendrites, whereas this was not the case for the dopamine/D1 mismatch areas (the rostromedial and caudal parts) of the main intercalated island.

The lack of correlation between the terminal/receptor distribution patterns suggests a role for volume transmission for μ-opioid receptor- and dopamine D1 receptor-mediated transmission in distinct regions of the amygdala and extended amygdala. This may have implications for amygdaloid function, where slow long lasting responses may develop as a result of volume transmission operating in opioid peptide and dopaminergic communication.

Introduction

The amygdala functions along with other limbic regions to control a variety of aspects of emotional processing. It has been studied especially for its role in the perception of emotionally relevant stimuli and production of appropriate responses (Heimer and Alheid, 1991; Alheid et al., 1995; Pitkanen et al., 1997; Aznar et al., 2004).

The largest of amygdaloid nuclei is the basolateral amygdaloid nucleus (BLA) which receives sensory information and projects to the centromedial group, consisting of the central and medial nuclei (Ce and Me respectively) (Pitkanen et al., 1997). The BLA, particularly the magnocellular division, also has direct connections with the cortex (i.e. prefrontal and insular cortex), as well as with the striatum (Kita and Kitai, 1990; Alheid, 2003)). Interposed between the BLA and central nucleus are several small intercalated cell masses (ICM). In the rat, the ICM that surround the BLA are called paracapsular intercalated islands (Ip), while the larger more medial "island" is called the main intercalated island (Im) (Asan, 1997b, a). The Im has been shown to project substantially to the medial nucleus (Pare and Smith, 1993b). The ICMs are GABAergic clusters of cells that receive excitatory glutamatergic collaterals from the BLA, and have also been found to inhibit the Ce (Millhouse, 1986; Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993; Pare and Smith, 1993b, a; Pitkanen and Amaral, 1994; Royer et al., 1999). In the guinea-pig it has been observed that these cell masses display an intrinsic inhibition of more medially located GABA cell clusters within the ICM themselves (Royer et al., 2000a). These findings have suggested that the overall function of the ICM appears to be to gate impulse traffic between the input (BLA) and output (Ce, Me) structures of the amygdala (Pare and Smith, 1993b; Royer et al., 1999, 2000b, a).

A host of immunohistochemical, tract tracing and electrophysiological data has led to the rostral extension of the borders of the amygdala, denominated the "extended amygdala" (Alheid and Heimer, 1988; Swanson and Petrovich, 1998; Alheid, 2003; McDonald, 2003). The extended amygdala consists of two corridors of neurons extending from the medial and central amygdaloid nuclei to the bed nucleus of the stria terminalis (BST) (de Olmos and Heimer, 1999; McGinty, 1999; Heimer, 2000; Alheid, 2003). The corridor of neurons connecting the medial nucleus of the amygdala to the medial BST is referred to as the medial extended amygdala (MEA), while the corridor connecting the Ce and lateral BST represents the central division of the extended amygdala (CEA). The MEA projects to the medial hypothalamus, while the CEA projects to the lateral hypothalamus and brainstem regions (Alheid et al., 1999; Shammah-Lagnado et al., 1999; Shammah-Lagnado et al., 2000; Koob, 2003b, a; McDonald, 2003).

Within the CEA lies a nucleus called the "interstitial nucleus of the posterior limb of the anterior commissure" (IPAC), which lies at the junction of the striato-pallidal system and the bed nucleus–central amygdala continuum (Alheid et al., 1995) and displays features of both the striatum and the extended amygdala (Beltramino et al., 1996; Shammah-Lagnado et al., 1999, 2001). While the IPAC is rich in tyrosine hydroxylase (TH) and acetylcholinesterase, much like the adjacent striatum (Beltramino et al., 1996; Shammah-Lagnado et al., 2000; Shammah-Lagnado et al., 2001), it is also intimately associated with the CEA by virtue of its expression of a variety of peptides and proteins that are seen in the extended amygdala but not the striatum (Alheid et al., 1999; Shammah-Lagnado et al., 1999; Alheid, 2003; McDonald, 2003). Tract-tracing and histochemical data have led to the subdivision of the IPAC into two functionally distinct medial (IPACM) and lateral (IPACL) parts (Alheid et al., 1995; de Olmos and Heimer,

1999; Shammah-Lagnado et al., 1999). Based on their differential connectivity and neurochemical marker expression, the IPACL has been affiliated with the striatum, while the IPACM, which shares substantial projections with the CEA, has been affiliated with the central extended amygdala (Shammah-Lagnado et al., 1999, 2001). The IPAC, along with the entire extended amygdala complex, has been suggested to be relevant in affective or motivated behaviours (Otake and Nakamura, 2003).

Within the rat amygdala, it has been demonstrated that dopamine terminals densely innervate the rostral ICM, the central and lateral divisions of the Ce, and to a lesser degree the BLA (Fuxe, 1965a; Lindvall and Bjorklund, 1978; Mennicken et al., 1992; Asan, 1997b, a, 1998; Brinley-Reed and McDonald, 1999; Fuxe et al., 2003b). The remaining amygdaloid nuclei contain mainly a plexus of noradrenergic (NA) terminals of low to moderate density (Fuxe, 1965b; Asan, 1997b, a, 1998). In the extended amygdala, dopamine innervation has been found to be very dense mainly in the dorsolateral part of the interstitial nucleus of the stria terminalis (Fuxe, 1965a, b; Abbracchio et al., 1987; Freedman and Cassell, 1994). A moderate to high density of enkephalin (ENK) immunoreactive (IR) nerve cell bodies and terminals is found in many nuclei of the amygdala and extended amygdala (Finley et al., 1981; Merchenthaler et al., 1986; Petrusz, 1986). The β-endorphin IR terminals are found in high densities in the Ce and in the BST, with the remaining parts of the amygdala and extended amygdala having low-to-sparse β-endorphin innervation (Bloom et al., 1978; Khachaturian, 1986; Mansour et al., 1988).

Mu-opioid receptor-like IR in nerve cell bodies and fibres have also been found in the amygdala and extended amygdala, including the ICM (Mansour et al., 1995a; Mansour et al., 1995b) and their distribution usually matches the distribution of mu-

opioid receptor mRNA expression and binding (Mansour et al., 1994). Delta-opioid receptors have also been mapped out in the amygdala and extended amygdala and both these receptors can mediate the actions of ENK and β-endorphin (Mansour et al., 1994; Mansour et al., 1995a; Mansour et al., 1995b); however, their relationships to the ENK and beta-endorphin terminals are unknown.

In a previous report we characterized the relationship between the dopamine D1 receptor (D1) and its dopaminergic innervation in the ICMs, showing a mismatch in rostromedial and caudal regions of the Im which were intensely IR for D1 but relatively devoid of dopamine terminals (Fuxe et al., 2003a). In contrast, there was a dense dopaminergic innervation of the rostrolateral Im. These results suggested a role for volume transmission (VT) (Agnati et al., 1995) in the rostromedial and caudal Im, and synaptic and/or perisynaptic transmission in the rostrolateral Im, representing a short distance, rapid component of the VT mode of communication (Fuxe et al., 2005). The presence of perisynaptic or extrasynaptic receptors has been well documented for a number of neurotransmitter receptors including dopamine, opioid, adenosine, GABA and NMDA receptors (Abbracchio et al., 1987; Garzon and Pickel, 2001; Hara and Pickel, 2005; Yashiro et al., 2005; Ciruela et al., 2006; Villalba et al., 2006). We further proposed a mechanism whereby the combined action of synaptic transmission and VT in the ICM could differentially regulate the GABAergic inhibition and disinhibition of the Ce, resulting in opposite behavioural responses (Fuxe et al., 2003a).

In this report, the relationship between the μ-opioid receptor 1 (MOR1) IR and ENK and β-endorphin IR innervation patterns in both the ICMs and the IPAC was examined. In addition, we extended into the IPAC our previous studies of D1 and dopamine innervation pattern, being TH IR positive but dopamine β hydroxylase (DBH)

IR negative. The location of dopamine and cyclic AMP regulated phosphoprotein, 32 kDa (DARPP-32) IR in these regions was also studied, as it is enriched in dopamine-innervated regions and has previously been mapped out in the amygdala (Ouimet et al., 1984). The phosphorylation state of DARPP-32 can be modulated especially by D1 and adenosine A2A receptors, but also by other receptors like MOR1 and D2 receptors, and DARPP-32 represents an important signalling mechanism in dopamine innervated neurons (Greengard et al., 1998).

Methods

Tissue preparation

All animal experiments were carried out in accordance with the requirements of the Canadian Council on Animal Care Guide (Policy 31, 1993), the Animals for Research Act (R.S.O., 1990, c.22) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 1996). All efforts were made to minimize the number of animals used and their suffering. Sprague–Dawley rats (n=6: Charles River, Wilmington, MA, USA) were anaesthetized with sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Cambridge, Ontario, Canada) and perfused transcardially with 50 mL phosphate-buffered saline (PBS, 10 mM phosphate in 0.9% saline, pH 7.4) followed by 100 mL 4% paraformaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9. Brains were removed and placed in the same fixative for an additional 90 min and then placed in 10% sucrose containing 0.01% sodium azide for storage at 4 °C. Brains were rapidly frozen with dry ice and cryostat sections were cut at 10 μm and thaw mounted onto chrome alum gelatin-coated glass slides. Sections were allowed to air dry and then rinsed in PBS prior to incubation with antibodies.

Immunohistochemistry

Polyclonal primary antibodies used in these experiments were guinea-pig anti-MOR-1 (1:3000, AB1774, Chemicon, Temecula, CA, USA), rabbit ant-ENK (1:400, R153H antiserum from Dr. R. Elde, Dept. of Neuroscience, University of Minnesota, Minneapolis, MN, USA; see (Graybiel and Illing, 1994)), rabbit anti-β-endorphin (1:20,000; from Dr. R. E. Lang, Dept. of Physiology, Philipps University, Marburg, Germany; (Lang et al., 1982; Bruckner et al., 1984)), rabbit anti-dopamine β-hydroxylase (1:1000, Eugene Tech, Ridgefield Park, NJ, USA), rabbit anti-DARPP32 (1:2000,

AB1656, Chemicon), mouse monoclonal anti-TH (1:500, Incstar, Stillwater, MN, USA) and rabbit polyclonal D1 antibody (1:800, from Dr. M. Goldstein, Dept. of Psychiatry, New York University Medical Center, New York, NY, USA; (Jansson et al., 1999)). The D1 antibody was raised against a C-terminal fusion protein from the rat dopamine D1 (residues 356–446) (Jansson et al., 1999). The β-endorphin antibody was raised in rabbits using the antigen coupled to thyroglobulin by the carboimide method (Lang et al., 1982; Bruckner et al., 1984). Cross-reactivity with β-lipoprotein was about 4% and with Met- and Leu-ENK less than 5%.

Primary antibodies were diluted in PBS containing 0.3% Triton-X 100 and incubated for 3 h at room temperature under gentle vibration (Jacobsen and Staines, 2004). Secondary antibodies used were goat anti-mouse and goat anti-guinea-pig Alexa Fluor 488 (1:200, Molecular Probes, Oregon, USA), goat anti-guinea-pig biotin (1:100, Amersham, Baie d'Urfe, Québec, Canada), donkey anti-goat FITC (1:80, Chemicon) and goat anti-rabbit Cy3 (1:400, Jackson Immunoresearch, West Grove, PA, USA). Secondary antibody incubations were performed at 37 °C for 45 min. Streptavidin-Texas Red was used to bind biotin (1:100, Amersham).

Sections were examined using a Zeiss Axiophot II microscope equipped with standard filter sets for blue excitation 450–490 (composed of excitation filters BP450-490 and emission filters FT510 and LP520) and green excitation H546 (composed of excitation filters BP546/12 and emission filters FT580 and LP590). Image data were acquired using Northern Eclipse imaging software (EMPIX, Toronto, ON, Canada) and a Retiga Qimage CCD camera (QIMAGING, Burnaby, BC, Canada).

Results

The architecture of the regions examined is schematically shown in Figure III-1, adapted from the atlas of Paxinos and Watson (Paxinos, 1997). Note that rostro-caudally, these regions span up to 5 mm in the rat brain.

D1 versus TH IR

At the level of the extended amygdala, D1 IR was found in the lateral IPAC and in discrete patches within the medial IPAC, as well as in the adjacent lateral stripe of the striatum (LSS), the ventral pallidum (VP) and the striatum (caudate putamen, CPu) (Figure III-2A; see also Table III-1).

At the level of the amygdala, D1 IR was found in the entire Im and Ip, as previously reported (Figure 3-2B) (Fuxe et al., 2003a). While the D1 IR rich lateral IPAC and LSS were both rich in TH IR terminals, the D1 IR patches in the medial IPAC were relatively devoid of TH IR terminals, resulting in a mismatch with D1 IR (Figure III-3A–C). In the Im, the TH IR terminals were concentrated to the rostrolateral part (Figure II-1E–F) as previously described (Fuxe et al., 2003a). All regions showing dense TH IR displayed low density of dispersed DBH IR nerve terminals, indicating their dopaminergic identity (data not shown).

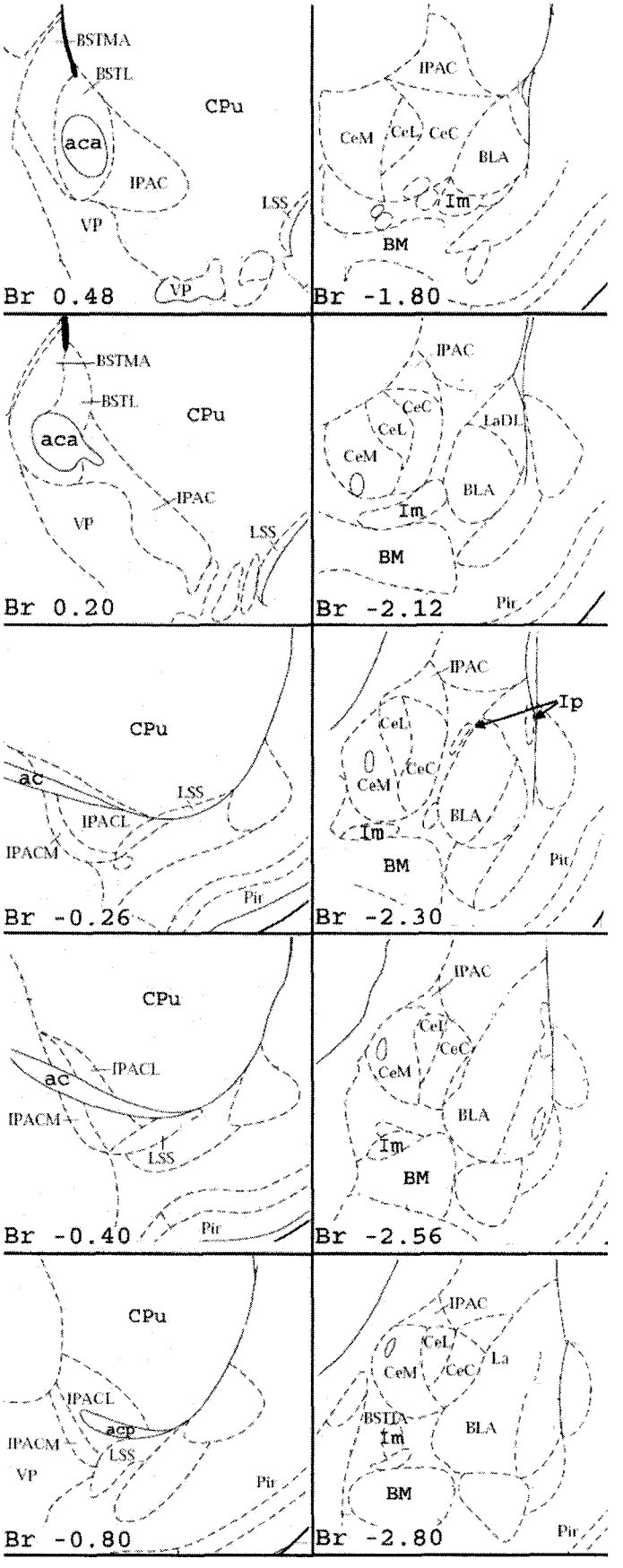
BSTMA
BSTL
CPu
aca
IPAC
VP
LSS
Br 0.48
IPAC
CeM
CeL
CeC
BLA
Im
BM
Br -1.80
BSTMA
BSTL
CPu
aca
IPAC
VP
LSS
Br 0.20
IPAC
CeC
CeL
CeM
LaDL
BLA
Im
BM
Pir
Br -2.12
CPu
ac
LSS
IPACL
IPACM
Pir
Br -0.26
IPAC
Ip
CeL
CeC
CeM
BLA
Im
BM
Pir
Br -2.30
CPu
IPACL
ac
IPACM
LSS
Pir
Br -0.40
IPAC
CeL
CeC
CeM
BLA
Im
BM
Br -2.56
CPu
IPACL
acp
IPACM
LSS
VP
Pir
Br -0.80
IPAC
CeL
CeM
CeC
La
BSTLP
Im
BLA
BM
Pir
Br -2.80

Figure III-1: Structure of the rat amygdala and extended amygdala.

Structures of the rat amygdala and extended amygdala at different brain levels with respect to bregma (Br, in mm) as adapted from the atlas of Paxinos and Watson (Paxinos, 1997). Lateral is to the right and medial is to the left, and this orientation is maintained in all figures. ac, anterior commissure (a, anterior, p, posterior); BST (L, lateral, MA, medial anterior part, IA, intraamygdaloid divisions); Ce (L, lateral, M, medial and C, central divisions); IPAC (L, lateral, M, medial divisions); Pir, piriform cortex.

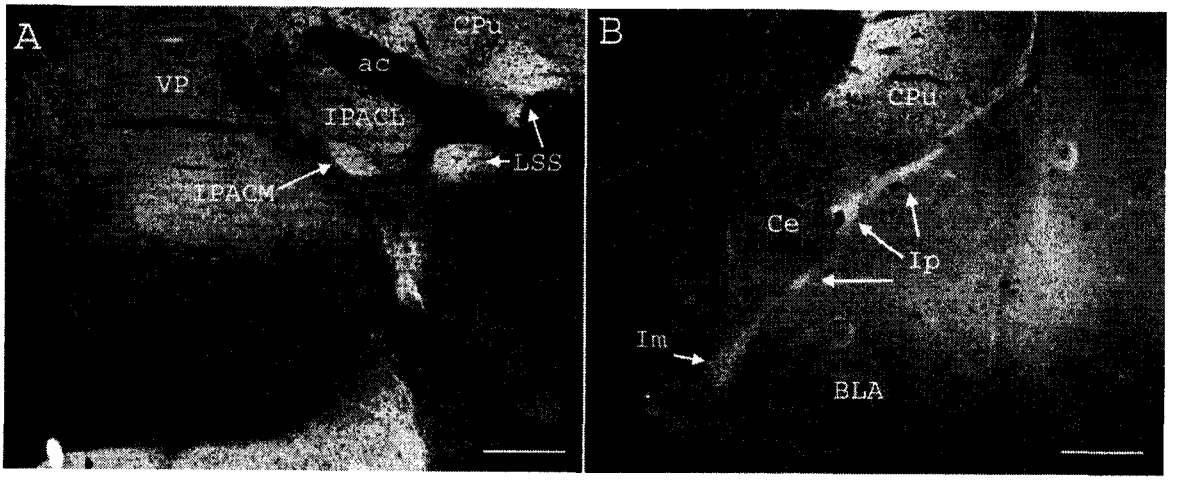

Figure III-2: D1 IR in the amygdala and extended amygdala.

Representative images of dopamine D1 IR in the rostral regions (A, Br −0.48 mm) showing D1 IR in the lateral and medial IPAC, as well as in the adjacent LSS, VP and CPu. In caudal regions (B, Br −2.56 mm) D1 IR is found in the Im and Ip. Scale bar=250 μm. Lateral is to the right.

Table III-1: Distribution of dopamine and opioid nerve terminals and DARPP32 IR in the Im and Ip, the IPAC and the LSS in relation to D1 and MOR1 receptor IR

Terminal	Amygdala intercalated islands		Extended amygdala	"Striatal" territory	
	Im	Ip	IPACM	IPACL	LSS
D1	+++ 	+++	Discrete patches ++	+++	+++
TH	Rostrolateral +++ 	+++	+	+++	+++
DARPP32	Rostrolateral ++ 	+++	+	++	n/a
MOR1	+++ 	+++	Discrete patches ++	_	+++
ENK	Rostromedial ++ 	Lateral ++	_	_	_
β-END	_ 	_	_	_	_

Density/intensity of immunoreactivity is represented on a qualitative scale of low (+) to very high (+++), with (−) representing very low or absent immunoreactivity. The distribution patterns of immunoreactivity in the Im have also been depicted in a schematic, with black and white areas representing regions of high and low IR within the Im (shown as a large oval), respectively. Lateral is to the right.

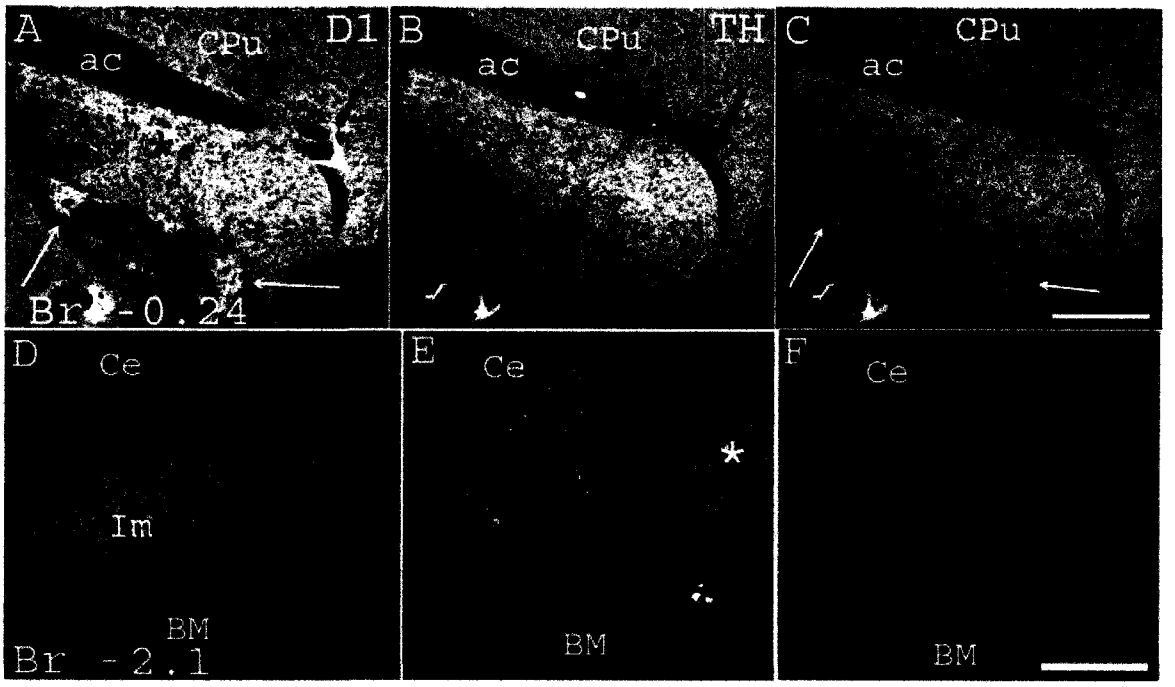

Figure III-3: D1 and TH IR in the IPAC and Im.

(A–C) D1 IR (red) was intensely localized to the lateral IPAC (*), the LSS (arrowhead), as well as to small discrete patches in the medial IPAC (arrows). TH IR (green) terminals were present throughout the lateral IPAC and LSS, but are very sparse in the medial IPAC. (D–F) In the ICM, D1 IR was intense throughout the main island, while TH IR was localized primarily in the rostrolateral Im (*). Scale bars=500 μm (A–C) and 250 μm (D–F). Lateral is to the right.

DARPP-32 IR

In the Im, the DARPP-32 IR followed a rostro-caudal gradient similar to that seen previously for the dopamine terminal innervation of the Im (Fuxe et al., 2003a). The DARPP-32 IR and D1 IR cell bodies were concentrated in the rostral and rostrolateral parts of the Im (Figure III-3, Figure III-4). Figure III-4B illustrates a level of the Im that has not been well characterized previously. At −1.80 mm from bregma (see Figure III-1), the main arm of the Im is situated dorso-medially to the BLA. Between −1.80 and −2.1 mm from bregma, the Im gives rise to a more laterally situated transverse arm, such that sections taken between these levels can sometimes give the appearance of two nuclei (as in Figure III-4B). Serial sectioning has revealed that this short transverse arm is contiguous with the longer main arm (not shown). The DARPP-32 IR mirrored the pattern of TH IR in this region (see Figure III-3E–F). Interestingly, DARPP-32 IR was also very intense in the neighbouring Ce, which contained a high density of TH IR terminals but only low levels of D1 IR (see Figure III-2B). Many D1 IR cell bodies within the Ip were also DARPP-32 IR (results not shown), matching their dense innervation by TH IR terminals in this region (Fuxe et al., 2003a). See also Table III-1.

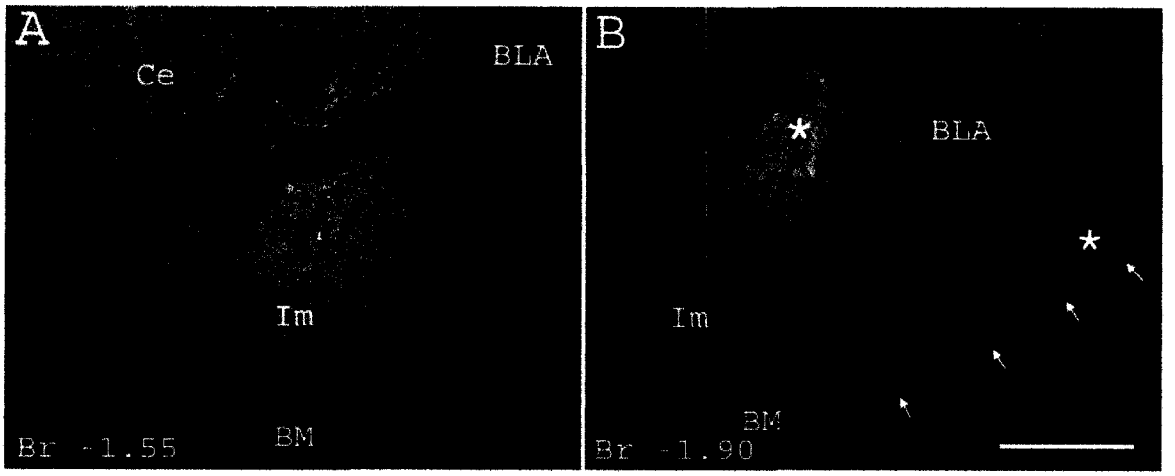

Figure III-4: DARPP-32 (green) and D1 (red) receptor IR in the Im of the amygdala. DARPP-32 IR cell bodies were found in distinct subpopulations within the Im, concentrated to the rostral (A) and rostrolateral (B, *) regions. Note that in panel B, the rostral Im has a main arm (labelled Im) and a short rostro-lateral transverse arm (arrows) which join at more caudal levels, and that the rostrolateral regions of both arms display concentrated DARPP-32 IR (B, *). A high DARPP-32 expression was also observed in the Ce. Scale bar=250 μm. Lateral is to the right.

MOR1 versus ENK IR

The Im and Ip were examined for MOR1 IR, which was found to be intensely localized to both the Im and the Ip. While MOR1 IR was intense throughout the Im, ENK IR terminals were localized to the rostromedial part of the Im, while the rostrolateral part was relatively devoid of ENK IR terminals (Figure III-5A–C). β-Endorphin IR terminals in the Im were very sparse (Figure III-5D–F). In the Ip, the ENK IR terminals were mainly localized to the lateral aspects of the LA-BLA, including the rostrolateral aspects of the Ip, with only sparse ENK IR in the medial Ip (Figure III-6A–B) and there were virtually no β-endorphin IR terminals (data not shown).

In the IPAC, discrete patches of MOR1 IR were found throughout the medial IPAC (IPACM) that were relatively devoid of ENK IR terminals (Figure III-7, arrows). β-Endorphin IR terminals were virtually absent from this region (not shown). MOR1 IR was also intense in the LSS, which were relatively devoid of ENK IR terminals (Figure III-7, arrowheads). Both ENK IR and MOR1 IR in the lateral IPAC was virtually absent (Figure III-7C, F, G). See also Table III-1.

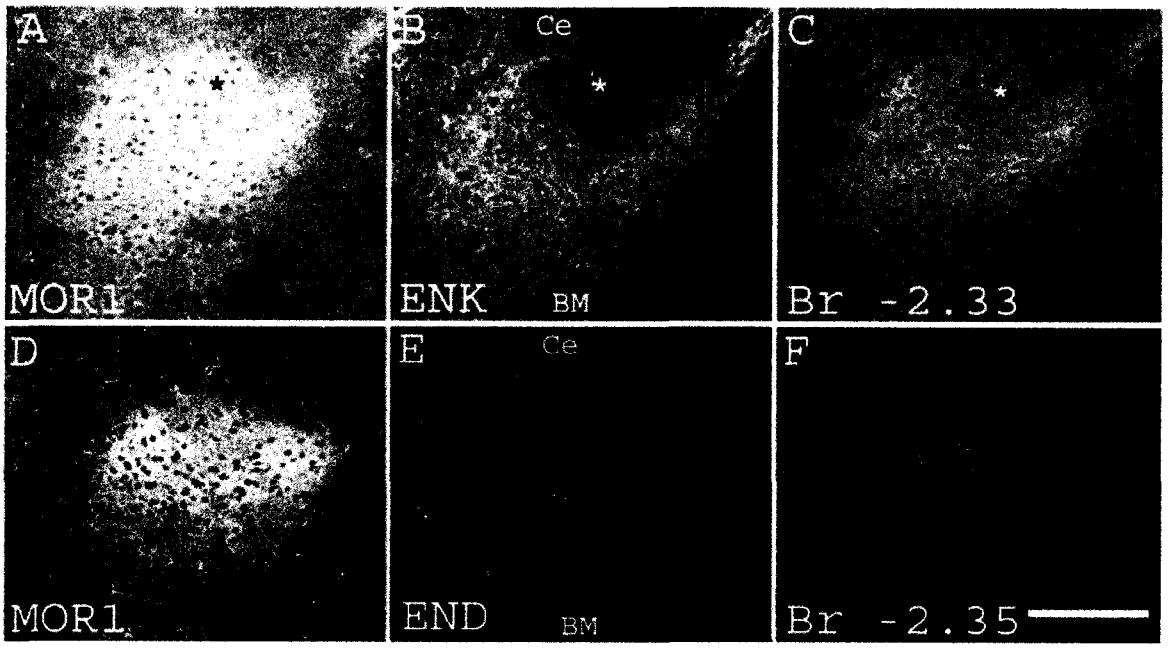

Figure III-5: MOR1, ENK and β-endorphin IR in the Im.

MOR1 IR was intensely localized to the Im, while ENK IR was localized primarily to the medial aspects of the Im (B), with very sparse ENK IR terminals in the lateral (C, *) and caudal Im. β-Endorphin IR was very sparse in the Im region (E). Also shown is the overlay of MOR1 (red) and ENK (green, C) and β-endorphin (green, F). Scale bar=250 μm. Lateral is to the right.

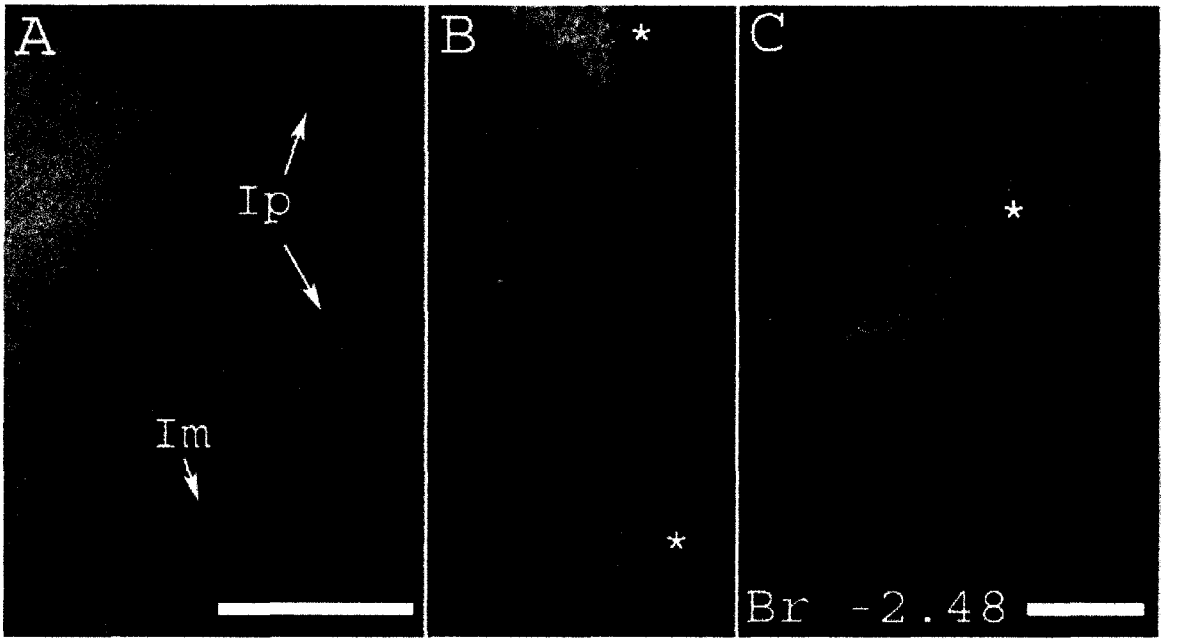

Figure III-6: MOR1 and ENK IR in the rostral amygdala.

MOR1 (red) and ENK (green) IR in the Im and Ip ICM at Br −2.48 mm. MOR1 IR was intense throughout the main and paracapsular islands, while ENK IR terminals were preferentially localized to the rostrolateral aspects of the LA-BLA, including the rostral and lateral regions of the Ip (B, *). By contrast, the rostrolateral Im is relatively devoid of ENK IR (C, *). Scale bars=500 μm (panel A) and 150 μm (panels B and C). Lateral is to the right.

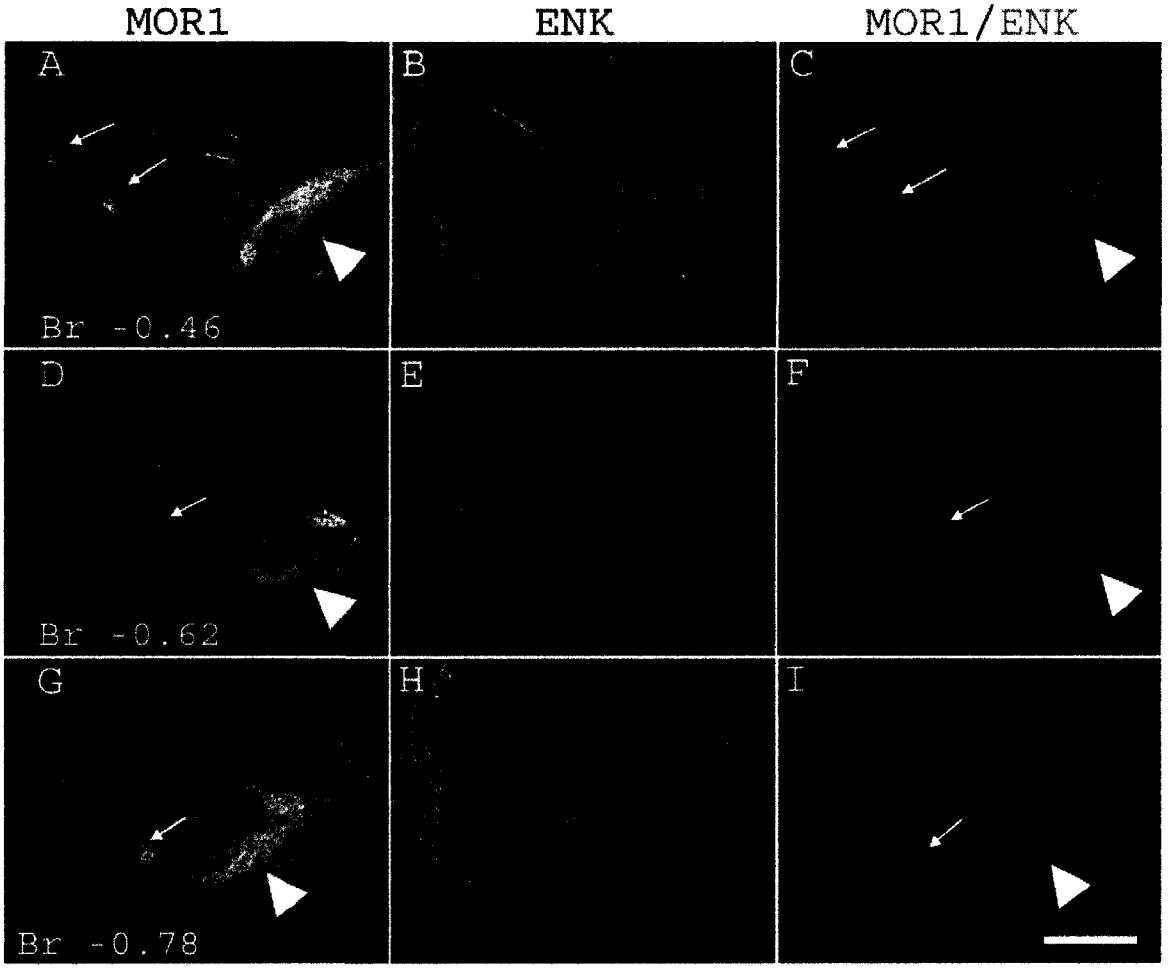

Figure III-7: MOR1 and ENK IR in the IPAC.

MOR1 IR was localized to discrete cell patches within the medial IPAC (arrows) and to the LSS (arrowhead). These regions are virtually devoid of ENK IR terminals (B). ENK IR was intensely localized to the VP (*), where MOR1 IR is weak. Overlay of MOR1 IR (red) and ENK IR (green) can be seen in panels C, F and I. Scale bar=500 μm. Lateral is to the right.

Discussion

Mismatches in dopamine and opioid peptide systems

The present findings demonstrate transmitter–receptor mismatches in the MOR mediated ENK and β-endorphin transmission in distinct subregions of the amygdala and the extended amygdala. Furthermore, the present findings confirm and extend previous observations regarding such mismatches in dopaminergic neuronal systems in the amygdala (Fuxe et al., 2003a).

The neurotransmitter–receptor mismatch phenomena have previously been extensively discussed and analyzed (Agnati et al., 1986; Fuxe and Agnati, 1991; Agnati and Fuxe, 2000), and mismatches in monoamine and peptide systems have been reviewed (Jansson et al., 2001b). For instance, a dopamine–dopamine receptor mismatch has been observed in the nucleus accumbens shell and in the islands of Calleja, the substantia nigra zona reticulata and the entopeduncular nucleus (Voorn et al., 1986; Fuxe et al., 1988b; Mengod et al., 1991; Caille et al., 1996; Jansson et al., 1999). Opioid mismatches have previously been reported in the striatum, the entopeduncular nucleus, the hypothalamus, the thalamus and the hippocampus (Agnati et al., 1986; Hamel and Beaudet, 1987; McLean et al., 1987; Herkenham and McLean, 1988; Elde et al., 1995; Commons and Milner, 1996; Wang et al., 1996).

Mismatches in the ICM

In the present study, we have examined the ICM which gate communication between the BLA and the Ce. In the Im, strong MOR1 IR and D1 IR was present throughout the entire nucleus, while dopamine and ENK terminals were only present in distinct subregions. Specifically, the ENK IR terminals were localized primarily to the rostro*medial* Im, while dopamine nerve terminals were concentrated to the rostro*lateral* aspect of the Im. These

results suggest a combined role of VT and synaptic (and/or perisynaptic) transmission of ENK and dopamine in the Im. The complimentary nature of the patterns of ENK (rostromedial) and dopamine (rostrolateral) innervation of the rostral Im suggests a specific action for each of these two neurotransmitter systems in regulating amygdaloid output via the ICM, where D1 and MOR1 receptors may interact (Fuxe et al., 2005). In contrast, β-endorphin IR terminals were very sparse or absent throughout the entire amygdala. Any potential neurotransmission via β-endorphin may therefore primarily occur via VT (Höistad et al., 2005), due to the sparse density of β-endorphin IR terminals and the high density of MOR1 IR.

In the Ip, there was a mismatch between MOR1-IR islands and the ENK and β-endorphin IR terminals. A high density of ENK IR terminals was observed only in the rostrolateral parts of the Ip, while strong MOR1 IR was found throughout the Ip islands, suggesting a role of VT also for MOR1-mediated ENK and β-endorphin transmission in the medial parts of the Ip.

Mismatches in the IPAC

In the extended amygdala, MOR1-IR was concentrated to discrete patches in the medial IPAC that were almost devoid of ENK and β-endorphin IR terminals. The MOR1 IR patches within the medial IPAC were also intensely IR for D1, with only a sparse innervation by TH IR terminals. In contrast, in the lateral IPAC (a striatal-like region) there was a good match between MOR1 and ENK IR. The lateral IPAC was also rich in D1 IR and TH IR terminals as previously reported (Alheid et al., 1995); however, ENK IR terminals were also absent from the MOR1-rich LSS, a region which has been described as being part of the patch system of the striatum (Gerfen, 1988, 1989).

These data suggest that neurotransmission in the discrete D1 and MOR1 IR patches in the medial IPAC, lacking their respective neuronal inputs of transmitters, may be regulated by VT, with dopamine and opioid peptides acting as long distance signals to influence the IPACM function. This may also be true for the MOR1-mediated ENK transmission in the LSS. Interestingly, retrograde tracing studies have shown that the IPAC receives substantial afferent connections from the ICM, although the relationship between these projections and the patches in the IPACM is not known (Shammah-Lagnado et al., 1999). The intercalated cells alongside the anterior BLA may strongly inhibit the output from the central amygdala and especially IPAC, in this way routing the information from the anterior BLA exclusively to the CPu and nucleus accumbens. This may result in increases in motivation and explorative motor activity without any inhibition produced by activation of fear mechanisms via the central amygdala and IPAC. As with the striatum, the presence of small cell clusters with unique binding sites and neurotransmitter receptor phenotypes in the amygdala and extended amygdala is not a new concept (Pare and Smith, 1993b; Veinante and Freund-Mercier, 1997). Studies of the extended amygdala have, for example, demonstrated a complimentary distribution of oxytocin and patch vasopressin binding sites throughout the amygdala, including in discrete cell clusters in the IPAC and BST, where oxytocin binding sites appear in cell regions devoid of oxytocin terminals, suggesting existence of oxytocin VT (Veinante and Freund-Mercier, 1997; Jansson et al., 2001b). While the regions examined by Veinante and Freund-Mercier differ from those examined in the present study, the ordered yet complex morphology of the extended amygdala has obvious implications for amygdaloid functions such as fear, anxiety and emotional responses. The present results provide a preliminary insight into potential gating functions in this part of the extended amygdala,

involving D1 and MOR1 interactions in patches in the IPACM where D1 and MOR1 operate mainly by responding to dopamine and ENK migrating in the ECF.

DARPP-32 distribution correlates with dopamine "match" regions

DARPP-32 is an important signalling molecule that can be regulated by several different receptors, especially D1 and adenosine A2A receptors, but also by MOR1 and D2 receptors (Greengard et al., 1998; Greengard et al., 1999). DARPP-32 has been extensively studied with respect to dopamine signalling which, by activating either D1 or D2 subclasses of receptors, can either increase or decrease DARPP-32 phosphorylation at threonine-34, respectively (Greengard, 2001). Phosphorylated DARPP-32 is a potent inhibitor of protein phosphatase 1 (PP-1) which has an array of downstream targets including receptors, ion channels and transcription factors. To elucidate potential signalling mechanisms involved in D1- and MOR1-mediated VT, DARPP-32 IR in the amygdala was examined, as DARPP-32 IR has been shown to be strong in regions innervated by dopamine terminals, including parts of the amygdala (Ouimet et al., 1984).

In the ICM, strong DARPP-32 IR was found to be localized only in the rostrolateral Im, matching the pattern of dopamine innervation of the Im. This suggests that the synaptic (and/or perisynaptic) dopamine D1-mediated transmission in the Im involves signalling through DARPP-32, resulting in enhanced phosphorylation of receptors, ion channels and transporters, and in strong excitatory actions of D1-mediated transmission on the inhibitory GABAergic interneurons. It is important to note that dopamine D2 receptor binding has also been seen in main intercalated cell group (Scibilia et al., 1992), and thus may inhibit DARPP-32 phosphorylation at threonin-34 by inhibiting adenylate cyclase and activating protein phosphatase 2B (PP-2B, calcineurin). This would disinhibit PP-1 and lead to dephosphorylation of downstream effectors of PP-

1. This architecture is probably true for all richly dopamine-innervated regions (Ouimet et al., 1984). It is also worth noting that heterogeneous populations of neurons seem to exist within the rostral Im with regard to DARPP-32 IR, although all rostral Im neurons seem to be D1 IR. Some neurons were strongly DARPP-32 IR, while others displayed either a weak or no DARPP-32 IR. It is possible that the strongly IR neurons have dopaminergic synapses on them operating via D1, while the ones with *weak* DARPP-32 IR are activated via *perisynaptic* DA transmission, which may in fact represent a short distance, rapid VT process. This could also be true for many other brain regions that are rich in dopamine nerve terminals and DARPP-32 IR cell bodies, where both strongly and weakly DARPP-32 IR cell bodies can be found, the latter especially in the cerebellar cortex where almost only DA VT exists (Ouimet et al., 1984; Fuxe et al., 1988a; Fuxe et al., 1993). In line with these observations was the fact that the caudomedial part of the D1 IR Im lacked dopaminergic innervation and showed only weak or no DARPP-32 IR, suggesting that the neurons in this region may be reached by dopamine from surrounding dopaminergic terminals (Fuxe et al., 2003a). Thus, D1 activation by dopamine via VT would result in an increase in protein kinase A–mediated phosphorylation but largely without the amplification provided by PP-1 inhibition mediated by Thr34 phosphorylation of DARPP-32 (Greengard et al., 1998; Greengard et al., 1999). Likewise, the dopamine-innervated Ip displayed many nerve cell bodies intensely IR for DARPP-32, again suggesting an important role of DARPP-32 signalling in these regions. In the rostro*medial* Im (displaying low DARPP-32 IR and low TH IR), there was a high degree of ENK innervation. This suggests a dominance of synaptic (and/or perisynaptic) ENK signalling via MOR1 in this region, which is likely DARPP-32 independent. Signalling via MOR1 receptors in the rostro*lateral* Im, either by ENK or β-endorphin, likely

operates via longer distance VT. MOR1 inhibits adenylate cyclase activity (Sharma et al., 1975) and it seems likely, therefore, that MOR1 and D1 exert antagonistic interactions in the intercalated islands at the level of adenylate cyclase. D1-mediated signalling may dominate in DA-innervated regions enriched in DARPP-32 IR, while MOR1-mediated signalling may dominate in the ENK-innervated regions, where D1-mediated VT occurs and DARPP-32 IR is low. A direct D1/MOR1 receptor–receptor interaction at the level of the membrane remains to be established. It should also be noted that D2 receptors exist in the Im, but their relationship to the D1 and MOR1 IR cells, as well as the DA and ENK nerve terminals, remains to be determined.

Functional implications

The ENK and dopamine inputs to specific structures of the amygdala and extended amygdala may serve several functions. For instance, the opposing ENK and dopamine inputs to the Im, containing GABA cell clusters which innervate the central and medial amygdaloid nuclei, may modulate the inhibition of these nuclei, either by increasing or reducing activity in the Im. The ENK and dopamine inputs to the paracapsular GABA islands, which also innervate the BLA, may modulate the inhibition of the BLA (Marowsky et al., 2005). The ICM may also be important players in the dual projections of the BLA to the central and medial nuclei of the amygdala, as well as to non-limbic areas such as the striatum. In particular, the Ip neighbouring the anterior BLA could inhibit Ce or CEA outputs in favour of communication with the basal ganglia (see above). Thus, the balance of activity in the ENK and dopamine systems controlling the GABAergic ICM may have an important role in gating and regulating fear responses, and possibly also the development of anxiety (McGrath et al., 1999; Bauman and Amaral, 2005; de la Mora et al., 2005).

It is unknown whether the discrete D1 and MOR1 IR patches of GABAergic cell bodies in the medial IPAC gate output from the extended amygdala, but it is possible that an integrated system of inhibitory GABAergic interneurons serves to modulate extended amygdala outputs in a similar manner as in the ICM. A combined action of longer distance, slow VT and shorter distance rapid synaptic and/or perisynaptic transmission may allow a single neurotransmitter system to regulate tonically or phasically different components of the same pathway, thereby creating a relatively simple mechanism for achieving a high degree of complexity (Agnati and Fuxe, 2000; Bloom, 2000). The research results presented here emphasize this combined role of slow VT and fast synaptic transmission in D1- and MOR1-mediated communication in the amygdala and the extended amygdala, which may have an impact on internal processing mechanisms. D1- and MOR1-mediated activity in these GABAergic interneuronal cell clusters may have implications for functions such as stress reactivity, fear, anxiety and addiction (McGinty, 1999). It has been postulated, based on the DA/D1 mismatches discovered in the nucleus accumbens shell, that the different velocities of wiring transmission (WT) and VT could provide a mechanism for the prediction of future reward responses (Montague et al., 1996; Agnati, 2006). The spatio-temporal relationship between WT and slower VT signals reaching a particular receptor could account for the sensitization of a neuron to the exact time of delivery of a reward following a predictive sensory cue (Montague et al., 1996). In the same way, it may be postulated, based on the discovered DA/D1 and Enk/MOR1 mismatches in the intercalated islands and IPAC, that the different velocities of WT and VT in this region may allow the prediction of future fear events. Understanding the connectivity and modes of communication within the amygdala and

extended amygdala will therefore be important for a further understanding of amygdaloid function and its modulation.

Acknowledgments

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PART TWO: Critical Developmental Regulators of the Monamine Fear Circuitry

Even though the details of the fear circuitry anatomy are still being elucidated, many of the major players in the regulation of mood and emotion have been extensively studied. In this way, the fear circuitry might be seen as a canvas, largely filled in, but with some important portions of the parchment left blank. For example, in the case of the monoamines, whose role in modulating affect is well documented, the point of origin of the developing neurons, induction cues for differentiation, timeframe and targets of the extending axons, and many of the events regulating synaptic (or non-synaptic) connections and communication have been identified. Despite these advances, there are still many pieces of the proverbial puzzle left to be filled in. In many instances, critical regulators have been identified, as is the case for Nurr1 in the development of the DA system and Pet-1 in the development of the 5-HT system (Hendricks et al., 2003; Jankovic et al., 2005); however, exactly how they achieve this regulation and how they might contribute to disorders of the fear circuitry, remain to be determined.

In order to make conclusive links between the developmental regulation of the DA and 5-HT systems and diseases related to their dysfunction, a more in depth analysis of this critical timeframe is necessary. Since virtually all disorders affecting the fear circuitry are heterogeneous, with multiple genetic and environmental variables, it is especially important to understand that each regulatory event cannot be considered in isolation. Rather, the degree of circuitry crosstalk, common signalling molecules, and even common developmental origins suggests that even a minor alteration in the development of a single neurotransmitter system would impact the entire fear circuitry. As a result, the following chapters focus on determining how a select number of critical factors regulate the development of the fear circuitry. In particular, the following chapters

focus on two monoamines, DA and 5-HT, for their unequivocal roles in affective function and dysfunction, and we will attempt to delineate the subtle differences in the regulatory events that are unique to each system.

Background and Relevance – Chapter IV

From the initial specification by Shh and FGFs to the transcriptional regulators essential for differentiation and acquisition of a mature phenotype, the serotonin and dopamine systems are tightly linked (Ye et al., 1998; Smidt et al., 2000; Cheng et al., 2003; Zhao et al., 2006). In many ways, this makes it challenging to delineate the developmental contributions of each of these systems to monoamine fear circuitry function and dysfunction. For this reason, it is important to study the regulators that are unique to the DA system, for their potential role in dopaminergic function and as therapeutic targets. To date, the only factor identified that is primarily localized to the DA system and is critical for DA neuron differentiation and phenotype acquisition is Nurr1 (see Figure VII-2) (Smidt and Burbach, 2007). For this reason, examining how Nurr1 might contribute to DA development and function, and by extension, DA dysfunction, will provide important insight into the role of developmental events in the manifestation of DA-specific diseases. This will be especially relevant for understanding the genetic events that might predispose an individual to neuropsychiatric disorders such as schizophrenia and Parkinson's disease. The following chapter examines this link between the developmental regulation of DA neuron identity and the onset of disease.

CHAPTER IV: A NURR1 point mutant, implicated in Parkinson's disease, uncouples ERK1/2-dependent regulation of tyrosine hydroxylase transcription.

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Contribution of Co-Authors

The manuscript was written by Kirsten Jacobsen with editorial input from Dr. Paul Albert and Dr. Denis Bulman.

The NBRE constructs were cloned by Dr. Sylvie Lemonde and Mireille Daigle.

The NURR1 constructs were cloned by Heather MacDonald.

All other experimental procedures and data analysis were performed by Kirsten Jacobsen.

Abstract

The orphan nuclear receptor NURR1 is critical for the development of mesencephalic dopamine neurons and directly regulates tyrosine hydroxylase (TH) via specific NGFI-B response elements (NBRE). We identified a Parkinson's disease patient with a *NURR1* mutation, resulting in a p.Ser125Cys change, immediately adjacent to the putative ERK1/2 phosphorylation site. Here we show, in dopaminergic SK-N-AS human neuroblastoma cells, that this substitution markedly attenuated NURR1-induced transcriptional activation through a human TH promoter NBRE. Furthermore, in SK-N-AS cells co-transfected with the dopamine-D2S receptor and NURR1, the D2 agonist quinpirole stimulated ERK1/2 phosphorylation and enhanced transcriptional activation by wild-type NURR1 but not the p.Ser125Cys NURR1 mutant, and these actions were blocked by the specific MEK1 inhibitor, PD98059. These results indicate that Ser125 is critical for basal and ERK1/2-induced NURR1 activity and suggests a role for this and other *NURR1* mutations in the regulation of dopamine synthesis and predisposition to Parkinson's disease.

Introduction

NURR1 (Nur-related factor 1, a.k.a. NR4A2/NOT1/RNR-1/HZF-3/TINUR) (Mages et al., 1994) is an orphan nuclear receptor transcriptional activator that is required for the development of mesencephalic dopamine (mDA) neurons (Law et al., 1992; Wang et al., 2003; Jorgensen et al., 2006). As with other NR4A orphan nuclear receptors, NURR1 lacks a cavity for ligand binding, and adopts a transcriptionally active conformation owing to the presence of several large hydrophobic residues that occupy this space (Wang et al., 2003). NURR1 contains N- and C-terminal activation function (AF-1 and AF-2, respectively), regions thought to regulate its transcriptional activity (Castro et al., 1999; Wang et al., 2003; Nordzell et al., 2004; Martinez-Gonzalez and Badimon, 2005). NURR1 activity is positively regulated by mitogen-activated protein kinase (ERK1/2) signalling via the N-terminal AF-1 region, and putative ERK1/2 phosphorylation sites have been identified proximal to the AF-1 core of NURR1 (Kovalovsky et al., 2002; Nordzell et al., 2004; Kim et al., 2006).

NURR1 (NR4A2) is highly expressed in midbrain dopamine neurons of the substantia nigra (Zetterstrom et al., 1996; Maheux et al., 2005; Martinez-Gonzalez and Badimon, 2005; Jorgensen et al., 2006), and the progressive degeneration of these neurons is implicated in Parkinson's disease (Missale et al., 1998; Vallone et al., 2000; Jankovic et al., 2005). NURR1 is implicated in the differentiation, survival, connectivity and migration of dopamine neurons, and in mice lacking NURR1, mesencephalic precursors fail to undergo terminal differentiation and adopt a mature dopaminergic phenotype, dying as development progresses (Saucedo-Cardenas et al., 1998; Witta et al., 2000; Eells et al., 2002). Importantly, NURR1 directly induces transcription of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of dopamine (Sakurada et al.,

1999; Kessler et al., 2003; Kim et al., 2003), as well as other important dopaminergic markers, including the dopamine transporter (DAT) and vesicular monoamine transporter 2 (Smits et al., 2003). Taken together, these studies provide compelling evidence for an essential regulatory role of NURR1 in dopamine neuron development.

The important role *NURR1* plays in dopaminergic development has been underscored by the identification of several mutations in this gene that associate with Parkinson's disease (PD) (Xu et al., 2002a; Le et al., 2003; Jankovic et al., 2005; Grimes et al., 2006). We previously identified a novel mutation in a patient (P4) with non-familial PD that induced a C(709)G missense mutation in exon 3 of *NURR1*, resulting in a serine to cysteine substitution at a site immediately next to the putative ERK1/2 phosphorylation site (Figure IV-1) (Nordzell et al., 2004; Grimes et al., 2006). Patient P4, last examined in February 2004, was diagnosed in his early 60s and had no family history of Parkinson's or other related neurodegenerative diseases. The p.Ser125Cys mutation was postulated to inhibit the ERK1/2 activation of NURR1, which would inhibit the transcriptional activation of *TH* and *DAT* by NURR1. To address the regulation of NURR1 and p.Ser125Cys-NURR1 by ERK1/2 activation, we examine the basal and ERK1/2-mediated transcriptional regulation by NURR1 of an NGFI-B response element (NBRE) identified in the TH promoter using reporter assays in SK-N-AS cells, a D2-, TH- and NURR1-positive cell line that models DA neurons (Kim et al., 2003; Michelhaugh et al., 2005; Wang and Bannon, 2005).

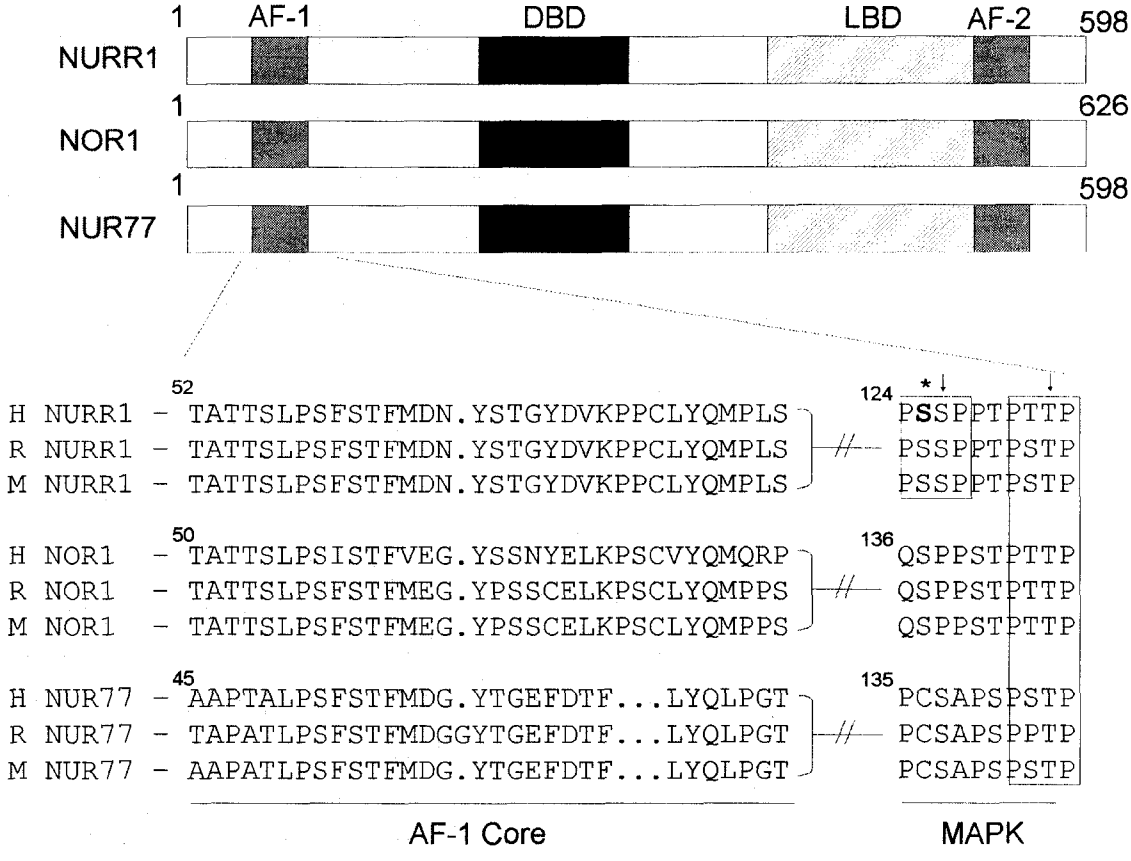

Figure IV-1: Schematic representation of NURR1 and related family members.

The structural domains of NURR1, NOR1 and NUR77 are shown (top), including the highly conserved amino acid sequence (bottom) of the human (H), rat (R) and mouse (M) AF-1 Core region and putative MAPK phosphorylation sites identified in the rat NURR1 (arrows) (Nordzell et al., 2004). Also shown is the site of the Ser125Cys mutation (*) identified in an individual with Parkinson's disease (Grimes et al., 2006).

Materials and Methods

Plasmid Constructs

We previously reported a p.Ser125Cys NURR1 variant in a patient with Parkinson's disease is thought to reduce its ERK-dependent and transcriptional activation of the TH NBRE (Grimes et al., 2006). In order to examine the activity of NURR1 on the NBRE, we designed a transient transfection approach in dopaminergic SK-N-AS cells to study the activity of the wild type and variant NURR1 isoforms at TH NBRE. The NBRE wt and NBRE mt luciferase reporter plasmids were obtained by cloning into the NheI/XhoI sites of pGL3-Promoter (pGL3-P, Promega) annealed primers designed to include the NBRE NL3 site sequence identified in the TH promoter that produces the greatest induction and binding NURR1 (Kim et al., 2003). The forward primer sequences were: NBRE wt: 5'- gctagcgaaaagggtcacggctcgag; NBRE mut: 5'-gctagcgaattcctcacggctcgag). The human dopamine-D2S receptor cDNA was subcloned into EcoR1 sites of the pcDNA3 expression construct (Liu et al., 1992).

The NURR1-pcDNA3.1 expression constructs obtained by isolating RNA from patient (P4) lymphocytes with TriReagent (Sigma) using the protocol suggested by the manufacturer. One microgram of total RNA was reversed transcribed using Superscript II (Invitrogen, Burlington, Ontario, Canada) and random hexamers (Amersham Piscataway, NJ), according to the suggested protocol provided by Invitrogen. Two overlapping PCR products were amplified: 5' amplicon contained the forward and reverse primers 5'-gtcaagcttctgtccacctttaatttcctcgaaaacgcc-3' and 5'-gttcatggggacgtgca-3', while the 3' amplicon contained the forward and reverse primers 5'-cacatgatcaagcagaggaa-3' and 5'-gtcaagcttagtgcttgggaggaggtctt-3'. The PCR products were combined and amplified using the 5' primer from the first amplicon and the 3' primer from the second amplicon. The

resulting PCR product was digested with *HindIII* (Amersham) and subcloned into pcDNA3.1 and transformed into *Escherichia coli* XL1-Blue cells. Sequence errors introduced during amplification were corrected using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX). Both the wild type and the p.Ser125Cys NURR1 variants were generated and their respective sequences verified.

Cell Culture

5-HT1A-expressing human SK-N-SH neuroblastoma cells (kind gift from Dr. Lakshmi Devi, New York University (Fricker et al., 2005) and dopaminergic SK-N-AS human neuroblastoma cells (from Dr. Michael Bannon, Wayne State University, Detroit, USA) (Michelhaugh et al., 2005; Wang and Bannon, 2005) were cultured on polystyrene plates (Fisher, Ottawa, Ontario, Canada) in DMEM (Wisent, St. Bruno, Quebec, Canada), supplemented with 8% v/v heat inactivated foetal bovine serum (FBS) at 37°C in 5% CO_2.

Transfections

Cells were split into 12-well plates (Fisher) to a final confluence of 50 %, 16 hours prior to transfection. All transfections were done using the Lipofectamine Plus Kit (Invitrogen) and 0.5 μg total DNA was transfected per well. A ratio of 1:1:1 DNA:lipofectamine:plus reagent was maintained for all transfections and the plasmid ρCMV-β-gal was used to control for transfection efficiency. Cells were transfected in serum-free OptiMEM (Invitrogen) for 3 hours, following which medium was replaced with DMEM containing 8% FBS. After 48 hours, luciferase and β-galactosidase activity were assessed using the

L-MaxII and SpectroMax M2 (Molecular Devices, Woodbridge, Ontario, Canada), respectively. In all cases, transfections were performed in triplicate and repeated at least three times. Statistical analysis, and 2-tailed students T-tests were performed in Excel (Microsoft), and error bars represent standard error of the mean (S.E.).

Drug Treatments

SK-N-AS cells were co-transfected with *NURR1* (wt or mut) expression constructs, and the NBRE reporter construct in OptiMEM for 3 hours, and allowed to recover in DMEM containing 8% FBS for 36 hours. For ERK1/2 inhibition studies, growth medium was replaced with serum-free OptiMEM and cells were serum-starved for 1 hour, followed by pre-treatment with the specific MEK1/2-inhibitor PD98059 (10 μM, NEB, Ipswich, MA, USA) for 30 minutes, conditions which have been shown previously to be sufficient to abolish DA-induced ERK activation (Pumiglia and Decker, 1997; Luo et al., 1998). Cells not treated with PD98059 remained in the same serum-free OptiMEM for 30 minutes. Following pre-treatment with PD98059, 1 μM quinpirole (Sigma, St. Louis, MO, USA), a selective D2 agonist (Chen et al., 1988), was added for 6 hours, after which plates were immediately placed on ice, rinsed with ice cold PBS and either 1X reporter lysis buffer (for reporter assays) or 50 μl of IP Buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM CaCl, 1% Triton X-100) containing PMSF (Sigma), Protease Inhibitors (Roche, Basel, Switzerland) and DTT (Fisher, Ottawa, ON, Canada) for western blotting. Although higher concentrations are often used, 1 μM of quinpirole has been shown to be sufficient to stimulate the D2-dependent activation of the MAPK/ERK cascade (Guerrero et al., 2002).

Western Blot

Western blot analysis was performed as described previously (Banihashemi and Albert, 2002) using (1:1000) anti-phospho-p44/42 ERK1/2 (NEB) to detect ERK1/2 activation. Specific bands were detected by BM Chemiluminescence Blotting Substrate (Roche). The membranes were re-probed with anti-β-actin (1:1000) to control for loading.

Results

Mutant NURR1 Fails to Activate Transcription through the TH NBRE

In order to compare the transcriptional activity of the wild type NURR1 (NURR1 wt) and the Ser(125)Cys NURR1 variant (NURR1 mut) on the NBRE element identified in the *TH* promoter, we co-transfected dopaminergic SK-N-AS cells with the NURR1 wt or NURR1 mut expression constructs and the NBRE luciferase reporter construct (Figure IV-2). Compared to the empty pGL3-P vector, co-transfection of the NBRE reporter construct with empty pcDNA3 vector resulted in a small but significant increase in reporter activity, in line with the presence of endogenous NURR1 in SK-N-AS cells (Wang and Bannon, 2005). Co-transfection of the NBRE construct with NURR1 wt, however, resulted in a substantial increase in reporter activity, demonstrating its strong enhancer activity. When co-transfected with the NURR1 mut construct, on the other hand, there was no significant difference in reporter activity when compared to the NBRE alone. The modest enhancement by endogenous NURR1 remained even in the presence of over-expressed mutant NURR1, further suggesting that this NURR1 variant is not able to compete for binding to the NBRE. Importantly, in parallel experiments performed in which the NBRE core (AAGG) had been mutated, no transcriptional activation was observed by either NURR1 isoform (Figure IV-2). Furthermore, this effect was cell type specific, with the dopamine D2-receptor-negative, NURR1-negative, SK-N-SH cells likewise showing no transcriptional activation by NURR1, suggesting that a signalling cascade unique to SK-N-AS cells is essential for activation by NURR1 (not shown).

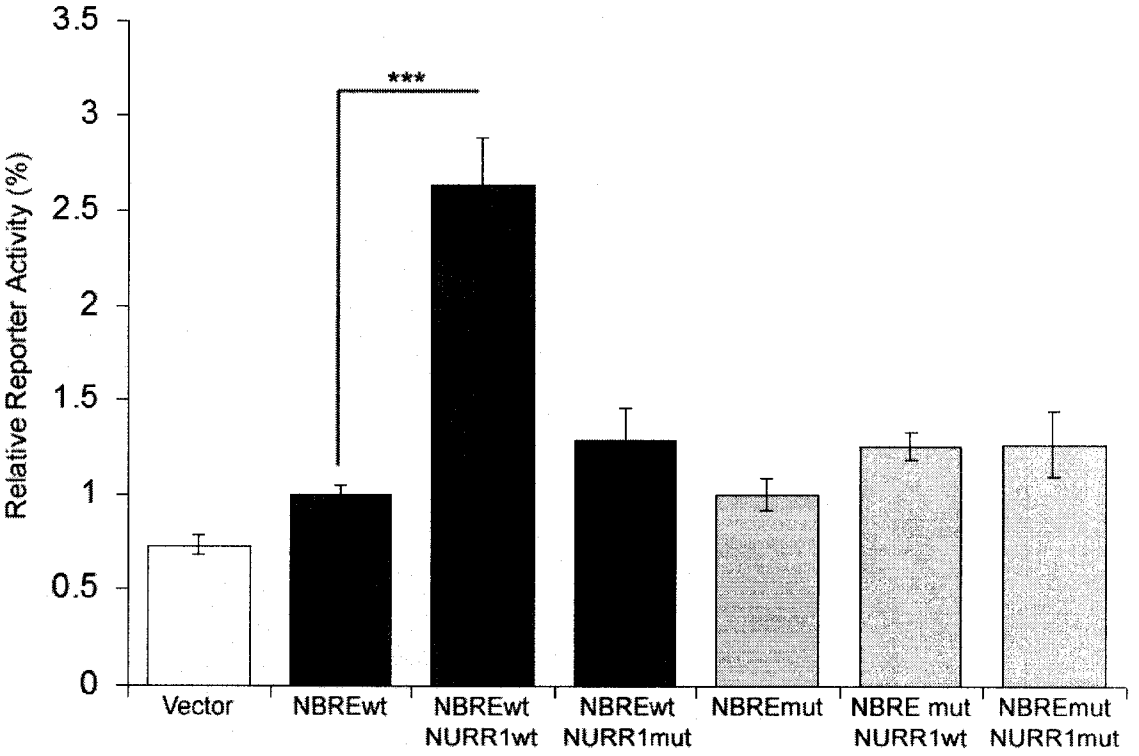

Figure IV-2: Activity of NURR1 at the TH NBRE in SK-N-AS cells.

Dopaminergic SK-N-AS cells were co-transfected with wild type (NURR1wt) or Ser125Cys NURR1 (NURR1mut) and the TH NBRE (NBREwt) or a mutant NBRE element in which the AAGG core has been mutated (NBREmut) and reporter activity normalized to β-galactosidase activity. NURR1wt but not NURR1mut potently activated transcriptional activity of the TH NBRE (black bars), and this effect was lost in the mutant NBRE (grey bars). Reporter activity is normalized to the respective NBRE control; shown is the mean ± S.E. of three independent experiments, each done in triplicate. *** $p < 0.0001$.

NURR1 Activation through the TH NBRE requires ERK1/2 activation

In order to determine whether the p.Ser125Cys mutation in NURR1 prevents its activation by ERK1/2, we measured NBRE-dependent transcriptional activity in SK-N-AS cells co-transfected with a dopamine-D2S receptor expression plasmid upon treatment with the selective D2 agonist quinpirole or specific MEK1 inhibitor PD98059. Despite the similarity between the short and long dopamine-D2 receptor isoforms, which differ by alternate splicing of a sequence encoding 29 amino acids, the dopamine-D2S receptor serves primarily as a presynaptic autoreceptor *in vivo* (Usiello et al., 2000) and regulates TH activity in dopaminergic neurons (Khan et al., 1998; Usiello et al., 2000; Lindgren et al., 2003). Thus, the dopamine-D2S receptor was transfected into dopaminergic SK-N-AS cells in order to better reflect the *in vivo* environment in presynaptic dopamine neurons.

In cells co-transfected with the human dopamine-D2S receptor, NURR1 wt or mut, and the NBRE reporter, quinpirole treatment induced transcriptional activation through the NBRE by NURR1 wt, but not by NURR1 mut (Figure IV-3A). Pretreatment with the MEK1 inhibitor PD98059 significantly reduced basal activation by NURR1 wt and prevented quinpirole-induced activation of the NBRE. Unlike NURR1 wt, quinpirole treatment of SK-N-AS cells transfected with NURR1 mut did not increase basal transcriptional activity, and PD98059 treatment did not inhibit it. A modest enhancement was observed with the combined treatment with quinpirole and PD98059, which may signify roles of additional D2-dependent signalling pathways, although the strong inhibition of NURR1 wt activation by PD98059 indicates that the ERK1/2 pathway is predominant.

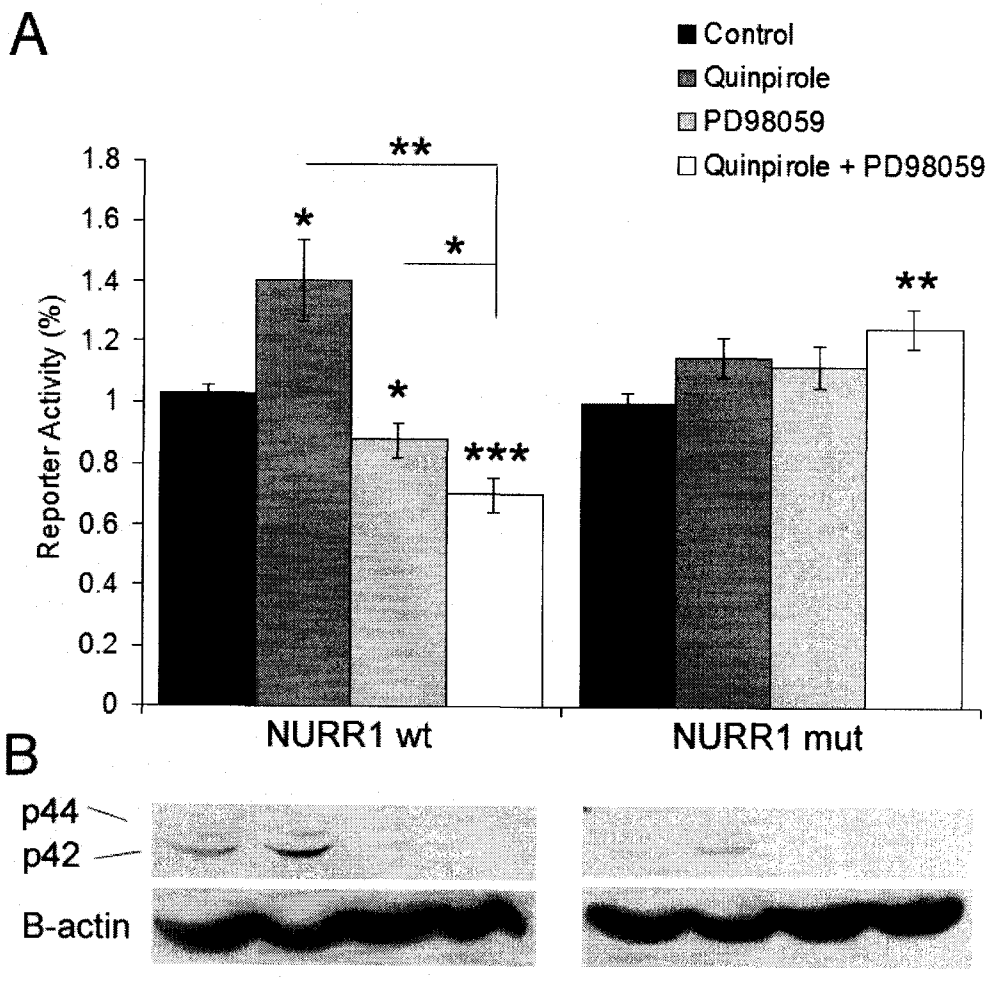

Figure IV-3: ERK1/2-dependent regulation of NURR1 activity is blocked in Ser125Cys NURR1. (A) Wild type or Ser125Cys NURR1 (wt and mut, respectively) were co-transfected in SK-N-AS cells with a dopamine-D2S receptor expression plasmid and the TH NBRE reporter construct. Quinpirole treatment enhanced NURR1wt-induced activation of the TH NBRE, which was blocked completely by pre-treatment with the MEK1 inhibitor PD98059. The Ser125Cys NURR1 mutant failed to respond to quinpirole. Reporter activity is normalized to the respective NBRE control untreated cells. Shown is the mean ± S.E. of three independent experiments, each done in triplicate.*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$ compared to untreated control. (B) Dopamine-D2S mediated phosphorylation of ERK1/2 is inhibited by the MEK1 inhibitor PD98059 in both NURR1wt- and NURR1mut-transfected cells. Shown is a representative blot that was repeated three times with similar results.

In order to further examine the role of the ERK1/2 signalling cascade in dopamine-D2 induced NURR1 activation of TH through the NBRE, we examined ERK1/2 phosphorylation by Western blot analysis (Figure IV-3B). In SK-N-AS cells co-transfected with the dopamine-D2S receptor, NURR1 wt or mut and the NBRE reporter construct, quinpirole treatment increased the levels of phospho-ERK1/2, the immediate downstream target of MEK1. Pre-treatment with PD98059 reduced basal ERK1/2 phosphorylation to less than basal levels, and also prevented activation by quinpirole. These results demonstrate that a dopamine D2S-stimulated MEK1-ERK1/2 signalling cascade triggers the transcriptional activation of NURR1, and that in the presence of the p.Ser125Cys mutation in NURR1, the D2 receptor-induced ERK1/2-dependent transcription activation of *TH* through the NBRE is inhibited.

Discussion

NURR1-Ser(125) is critical for NURR1 function

The results presented here illustrate that the p.Ser125Cys NURR1 variant previously identified in a patient (P4) with Parkinson's disease (Grimes et al., 2006) prevents its transcriptional activation by NURR1 through the NBRE identified in the promoter of the TH gene. We further show that this transcriptional activation can be stimulated by dopamine-D2S receptor activation, and is largely dependent on the MEK1-ERK1/2 signalling pathway. The evidence that wild type, but not the p.Ser125Cys variant form of NURR1, can activate transcription, strongly supports a role for this NURR1 variant in the development of Parkinson's disease. The ability of the specific D2 agonist, quinpirole, to stimulate this activation through dopamine-D2S receptors, and of the specific MEK1 inhibitor, PD98059, to inhibit it, provides mechanistic insight into the NURR1-dependent regulation of TH in dopaminergic cells (Figure IV-3). Importantly, although dopamine-D2S receptor activation induced ERK1/2 phosphorylation, D2S-induced NBRE activation was inhibited for the p.Ser125Cys NURR1 variant, suggesting that this mutation blocks ERK1/2-mediated NURR1 activation. While the involvement of other signalling cascades on the D2-mediate regulation of NURR1 transcriptional activity cannot be ruled out, the complete loss of NURR1 transcriptional activation through the NBRE in the presence of the specific MEK1 inhibitor PD98059 strongly suggest that the MAPK/ERK pathway is a dominant signalling mechanism in this system.

The site of the p.Ser125Cys mutation in NURR1 that abolishes ERK1/2-mediated activation is located within an ERK1/2 phosphorylation consensus sequence that likely blocks ERK1/2-induced phosphorylation of NURR1. Figure 1 shows an alignment of the human, mouse and rat AF-1 domains and proximal putative ERK1/2 phosphorylation

sites (arrows) identified previously for NURR1 (Nordzell et al., 2004). Additionally shown are the alignment of analogous regions of related orphan nuclear receptors NUR77 and NOR1. The putative ERK1/2 phosphorylation sites (based on the minimal PXS/TP consensus) are well conserved in all species, as well as in the analogous regions of NUR77 and NOR1 (Slagsvold et al., 2002). NGF-induced ERK kinase-dependent phosphorylation Ser105 of NGF1-B (corresponding to Ser140 of NUR77) has been reported to be required for nuclear export (Katagiri et al., 2000), and other studies have demonstrated a role for MEK1, JNK and Akt in this process (Han et al., 2006). Interestingly, the Ser(125) that is mutated in individual P4 with Parkinson's disease is conserved in NOR1 but not NUR77, consistent with the greater degree of identity between NURR1 and NOR1 compared with NURR1 and NUR77 (58.9% and 53.8% at the protein level, respectively). The ERK1/2 pathway has been previously implicated in the transcriptional activity of both NURR1 and NUR77 (Kovalovsky et al., 2002). Taken together, these observations suggest that the NURR1 transcriptional activation of TH is mediated by a signalling cascade that is largely dependent on ERK1/2 phosphorylation at or near Serine 125.

NURR1/NBRE-dependent TH transcription

The presence of the minimal NBRE identified in the promoter of the rat TH gene as the activation site for NURR1 (Kim et al., 2003) was sufficient for a strong activation of transcription by NURR1 (Figure IV-2), an effect that was lost when the GGAA core of this element was mutated. Sequence alignment of the rat and human TH promoters revealed a nearly identical site in the human TH promoter (rat NBRE at -909 to -901: 5'-AAAGGTCA; human NBRE -884 to -876: 5'-AAAGGTCt) implying that the NURR1 Ser(125)Cys mutation prevents transcriptional activation of TH through the NBRE.

Patient P4 was identified as being heterozygous for the p.Ser125Cys mutation in NURR1 (Grimes et al., 2006); however, the reduced levels of TH RNA resulting from reduced NURR1-induced transcriptional activation of the NURR1 mutant may result in an overall decrease in TH expression and dopamine synthesis, which, in combination with other genetic or developmental alterations, could exacerbate the development of idiopathic Parkinson's disease by accelerating dopamine depletion.

Dopamine-D2S induced ERK1/2-NURR1 regulation

Our findings demonstrate that the dopamine D2S receptor stimulates ERK1/2 phosphorylation in SK-N-AS cells, a model of presynaptic dopamine neurons (Michelhaugh et al., 2005). Previous work in our lab has demonstrated that in BALB/c-3T3 cells stably transfected with the dopamine D2S receptor, D2S stimulation with apomorphine increases phosphorylation of ERK1/2 (Ghahremani et al., 2000) as has been seen previously in other cell lines (Luo et al., 1998; Welsh et al., 1998). Treatment with pertussis toxin (PTX) completely prevented this activation, demonstrating the coupling to Gαi/o proteins, although the exact signalling mechanism has yet to be determined, and is likely cell-type specific (Albert, 2002; Albert and Robillard, 2002; Banihashemi and Albert, 2002). For example, in GH4 pituitary cells, a neuroendocrine model of dopamine signalling, the dopamine-D2S receptor inhibited ERK1/2 activation (Banihashemi and Albert, 2002), a pathway that mediates dopaminergic inhibition of prolactin gene transcription (Liu et al., 2005).

The present findings indicate that D2S-induced ERK1/2 phosphorylation activates NURR1 to induce TH expression, which is consistent with the key developmental role demonstrated for D2S-induced ERK1/2 activation of NURR1 in dopaminergic

differentiation and development in vivo (Kim et al., 2006). Paradoxically, in adult dopamine neurons, the D2S receptor mediates inactivation of TH (Lindgren et al., 2003), possibly through PKA or ERK1/2 (Lindgren et al., 2002). Thus, D2S-induced stimulation of ERK1/2 may represent an early developmental signalling pathway that is converted to inhibition of ERK1/2 in the adult. Consistent with this, we recently found that quinpirole inhibits ERK1/2 phosphorylation in primary rat striatal cultures, which could mediate in part dopamine-D2 induced inhibition of TH activity (Van-Ham et al., 2007). Further studies of D2 receptor mediated regulation of ERK1/2 and TH gene transcription *in vivo* are required to address the role of this inhibitory pathway; however the importance of dopamine-D2S induced ERK1/2 activation in dopaminergic differentiation is clear (Kim et al., 2006). Our results provide a plausible mechanism of how ERK1/2 activation may induce NURR1-dependent transcription of the key dopaminergic marker, TH. It would be important to determine whether the mutation in NURR1 does indeed reduce the expression of TH *in vivo* through the generation of an animal model incorporating the mutation.

Mutations in *NURR1* have previously been implicated in the development of Parkinson's disease (Xu et al., 2002a; Jankovic et al., 2005; Chu et al., 2006; Grimes et al., 2006) and other dopaminergic diseases such as cocaine abuse, manic depression and schizophrenia (Buervenich et al., 2000; Bannon et al., 2002). While the mutation identified in patient P4 has not previously been identified, it provides an important model further linking NURR1 to the development of Parkinson's disease, as well as for an increased understanding of the mechanism of the NURR1 regulation of downstream targets such as the *TH* gene.

Acknowledgements

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Background and Relevance – Chapter V

In order to understand how the developmental regulation of 5-HT differentiation and phenotype acquisition relates to serotonergic function and dysfunction, it is important to study 5-HT-specific regulators. As with the DA system, there are few regulators that are exclusively localized to 5-HT neurons in addition to being critical for 5-HT differentiation and maturation. Pet-1 is one such 5-HT-specific transcriptional regulator which in many ways parallels the role of Nurr1 in DA neurons. As with Nurr1, Pet-1 is required for the expression of factors involved in neurotransmitter synthesis (TPH and AADC) and signalling (5-HTT and VMAT2), and has Pet-1 binding elements in a number of serotonergic genes (Hendricks et al., 1999). Furthermore, Pet-1 null mice display an aggressive, anxious phenotype, reminiscent of the phenotypes of the 5-HT1B and 5-HT1A null mice strains, respectively (Heisler et al., 1998; Zhuang et al., 1999; Hendricks et al., 2003). Due to the established importance of the 5-HT1A receptor in anxiety behaviour, in the following chapter we focus our attention on the role of Pet-1 in regulating the 5-HT1A receptor. As Pet-1 is a transcription factor and is also essential for the generation of 5-HT neurons, we focus both on the role of Pet-1 as a transcriptional regulator and on the role of 5-HT in directing developmental events.

CHAPTER V: Region-specific regulation of 5-HT1A receptor expression by direct and adaptive Pet-1-dependent mechanisms *in vivo*

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Submitted to *J. Neurochem.*

Contribution of Coauthors

The Pet-1 null mice were obtained from Dr. Evan Deneris and bred by Kirsten Jacobsen with assistance from technician Christine Boudreau and summer student Heather Kooiman.

All experiments were done by Kirsten Jacobsen

The paper was written by Kirsten Jacobsen with editorial help from Dr. Paul Albert.

Abstract

Serotonin (5-HT) neurotransmission is negatively regulated by 5-HT1A autoreceptors on raphe neurons, and is implicated in mood disorders. Pet-1/FEV is an ETS transcription factor expressed exclusively in serotonergic neurons and is essential for serotonergic differentiation, although its regulation of 5-HT receptors has not yet been studied. Here we show that recombinant Pet-1/FEV binds directly to multiple Pet-1 elements of the 5-HT1A receptor promoter to enhance its transcriptional activity. To address the effect of Pet-1 on 5-HT1A receptor regulation *in vivo*, we compared the expression of 5-HT1A receptor RNA and protein in Pet-1 null and wild-type littermate mice. Paradoxically, in the hindbrain and midbrain of Pet-1 null mice, 5-HT1A receptor RNA and protein was greatly up-regulated, suggesting an adaptive change to the reduced 5-HT neurotransmission in these mice, despite the absence of Pet-1-mediated transcriptional enhancement of 5-HT1A autoreceptors in serotonergic neurons. Interestingly, 5-HT1A receptor expression was up-regulated in the hippocampus, but down-regulated in the striatum and cortex. These data indicate that, in addition to direct transcriptional regulation by Pet-1, 5-HT1A receptor expression is regulated by alterations in 5-HT neurotransmission in a region-specific manner that may contribute to the aggressive/anxiety phenotype observed in Pet-1 null mice.

Introduction

The serotonin (5-hydroxytryptamine, 5-HT) system, which originates from neurons of the midbrain raphe nuclei and projects widely throughout the brain (Dahlstrom and Fuxe, 1964), has been implicated in behavioural and physiological disorders including schizophrenia, obsessive-compulsive disorder, attention deficit disorder, major depression, anxiety and aggression (Ninan, 1999; Faraone and Khan, 2006; Heyman et al., 2006; Horacek et al., 2006; Levinson, 2006). Gene knockout mouse models have demonstrated contrasting roles for 5-HT1A and 5-HT1B receptors in anxiety and aggressive behaviours, with 5-HT1B null mice having an aggressive phenotype and 5-HT1A null mice having an anxious phenotype (Zhuang et al., 1999; Gross et al., 2000). Tissue-specific, conditional rescue of the 5-HT1A receptor in the 5-HT1A-/- background has identified a critical timeframe of forebrain 5-HT1A receptor expression during embryonic and early postnatal development to establish a normal anxiety phenotype in the adult (Gross et al., 2002; Lanfumey and Hamon, 2004). In addition, the 5-HT1A receptor is expressed pre-synaptically on serotonergic neurons where it functions as a negative regulator of neuronal activity and differentiation (Sotelo et al., 1990; Rumajogee et al., 2004). Thus, the developmental regulation of the 5-HT1A receptor is a critical determinant in the activity of the serotonin system and of the behavioural phenotype in the adult.

In response to elevation of 5-HT using serotonin-selective reuptake blockers (SSRI's) the presynaptic 5-HT1A autoreceptor appears to preferentially desensitize compared to post-synaptic 5-HT1A receptors (Le Poul et al., 2000), and this correlates with disinhibition of raphe neurons and clinical improvement in depression in patients (Pineyro and Blier, 1999). Similarly, in 5-HT transporter (5-HTT) null mice, a

preferential downregulation of 5-HT1A RNA and protein was observed in the dorsal raphe nucleus compared to post-synaptic sites (Fabre et al., 2000; Li et al., 2000). These data suggest that adaptive changes in 5-HT1A receptor expression are implicated in depression and response to antidepressants.

Several transcription factors have been implicated in the development of the serotonin system, including Pet-1/FEV (pheochromocytoma 12 *Ets* (E26 transformation-specific) factor/Fifth Ewing Variant), an ETS-domain factor that is exclusively localized in 5-HT neurons (Maurer et al., 2004; Iyo et al., 2005). Pet-1 is expressed approximately one day before 5-HT (E11 in the mouse) (Fyodorov et al., 1998; Hendricks et al., 1999). Pet-1 null mice (Hendricks et al., 2003) have a severe deficit in the serotonin system, with a 70-80% reduction in 5-HT levels, and display an anxious-aggressive phenotype that is reminiscent of the combined phenotypes of 5-HT1A and 5-HT1B knockout mice (Zhuang et al., 1999; Gross et al., 2000). These mice further display reduced expression of a number of Pet-1 target serotonergic genes that contain consensus Pet-1 elements (Hendricks et al., 1999), including the vesicular monoamine transporter (VMAT), amino acid decarboxylase (AADC), serotonin transporter (5-HTT) and tryptophan hydroxylase (TPH); however, the expression of 5-HT receptors in these mice has not been examined.

The presence of a Pet-1 consensus DNA element identified in the 5-HT1A receptor promoter suggests its role in regulation of presynaptic 5-HT1A receptor expression (Hendricks et al., 1999). Pet-1 was shown to enhance transcription of a reporter containing four copies of the 5-HT1A Pet-1 element, but the role of PET-1 in regulation of the 5-HT1A promoter and its transcription has not been addressed. In this study we examined the role of PET-1 (1) as a direct regulator of the 5-HT1A receptor gene and (2) in the postnatal expression of 5-HT1A receptors *in vivo*. Our results

demonstrate that PET-1 acts by direct and indirect mechanisms to regulate the expression of pre- and post-synaptic 5-HT1A receptors *in vivo*, which could contribute to the mature affective phenotype.

Methods

Cell Culture

Human SK-N-AS neuroblastoma cells (from Dr. Michael Bannon, Wayne State University, Detroit, MI), human SK-N-SH neuroblastoma cells (from Dr. Lakshmi Devi, New York University (Fricker et al., 2005) and human HEK293 cells were cultured on polystyrene plates (Fisher, Ottawa, ON) in Dulbecco's Modified Eagle's Medium (DMEM, Wisent, St. Bruno, QC), supplemented with 8% v/v heat inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2. Human retinoblastoma Y79 cells were cultured in suspension in RPMI-1640 medium (Moore and Howard, 1982) supplemented with 1.5 g/L $NaHCO_3$, 10 mM HEPES, 1 mM sodium pyruvate, 20% FBS and 4.5 g/L glucose (Wisent).

Nuclear Fractionation

Nuclear extracts from HEK 293 cells were obtained as described previously (Czesak et al., 2006). Successful nuclear fractionation was validated by western blotting using monoclonal mouse antibodies raised against Histone H1 (1:2,500, Upstate, Lake Placid, NY) as a nuclear marker and against c-raf (1:1000, BD Biosciences, Mississauga, ON) as a cytosolic marker.

Plasmid constructs

The human 5-HT1A promoter pGL3-Basic luciferase reporter constructs have been described previously (Lemonde et al., 2004a). Human Pet-1 (FEV) was PCR-amplified from human SK-N-AS cells and cloned into the *EcoRV* sites of pcDNA3.1. The accuracy of the cloned cDNA products was confirmed by DNA sequence analysis. Human Pet-1 was also subcloned into the *Kpn1* and *EcoRV* sites of pTriEx-4 for bacterial expression.

The QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) was used to mutate the core AAGG nucleotides of the Pet-1 consensus motif. Complementary primers were designed against each of the putative elements following QuickChange guidelines: (111-101: 5'-ggtctctgcattccctAGctccgaaacttcccaggagaagg; 136-126: 5'-cgccgaagcagtaagaactAGctgcttgggtctctgc; 449-439: 5'-ggagagagggaaggaagCTaataggg agaggagggtc; 532-522: 5'-ggaagagggagactgaaTCggaaggcaggtggggag; 931-921: 5'ccattcaggctccctatgctAGcttttctacatctcctattgc; 1406-1396: 5'-catatgcaaaatattCGcatccctgaa ttgactagccacaaagc; 111-101: catatgcaaaatattCGcatccctgaattgactagccacaaagc). A pGL3-Basic reporter plasmid containing 1517 bp of the 5-HT1A promoter (-1517 wt) was used as a template, and each of the sites was mutated separately by PCR following manufacturer specifications. The resultant PCR product was digested with DpnI and transformed into XL10-Gold Ultracompetent Cells. DNA was isolated from the resultant colonies and sequenced to verify successful mutagenesis.

Transfections

HEK293 cells were split into 12-well plates (Fisher) 16 hours prior to transfection. All transfections were done using the Lipofectamine Plus Kit (Invitrogen, Burlington, ON) and 0.5 μg total DNA was transfected per well. A ratio of 1:1:1 DNA:lipofectamine:plus reagent was maintained for all transfections and the plasmid pCMV-β-gal was used to control for transfection efficiency. Cells were transfected in serum-free OptiMEM (Invitrogen, Burlington, ON) for 3 hours, following which medium was replaced with DMEM containing 8% FBS. After 48 hours, plates were placed on ice, rinsed with ice cold 1X phosphate buffered saline (PBS, pH 7.4) and cell membranes lysed with 200 μl 1X reporter lysis buffer (Promega, Madison, WI). Luciferase and β-galactosidase activity

were assessed using the L-MaxII and SpectroMax M2 (Molecular Devices, Woodbridge, ON), respectively. One-way ANOVA was performed for each experiment using the Newman-Keuls Multiple Comparison *post hoc* test (*: $P < 0.05$; **: $P<0.005$; ***: $P<0.0005$).

Protein Expression

Pet-1:pTriEx-4 was transformed into BL21 (DE3) E. Coli and grown over night at 37 °C. 500 mL of pre-warmed LB/Amp was then inoculated with the overnight culture and grown at 37 °C with vigorous shaking until an OD_{600} of 0.6 was reached. Expression was then induced with 1 mM isopropyl-β-D-thioglactopyranoside (IPTG, Wisent, St-Bruno, QC) and cultures were incubated for an additional 3.5 hours. Cells were harvested by centrifugation and Pet-1 protein was isolated under denaturing conditions following the QIAexpressionist protocol (Qiagen, Mississauga, ON) and using Ni-NTA beads to select for HIS-tagged proteins. All fractions were collected and analysed for protein expression by western blotting using anti-HIS-tag (Cell Signaling, Danvers, MA) and anti-S-tag antibodies (Novagen, San Diego, CA). Fractions with the highest expression were combined and dialyzed against DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% Glycerol, pH 7.9) containing 2 mM DTT for use in Electrophoretic Mobility Shift Assay. Protein was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides corresponding to known 5-HT1A Pet-1 binding elements (5-HT1A -137), were annealed and end-labelled with [α-^{32}P]dCTP using 2.5U Klenow (New England Biolabs, Pickering, ON) and purified using a Sephadex G-50 column (GE

HealthCare, Baie D'Urfe, QC). Bacterially-expressed, purified human Pet-1 was pre-incubated with or without annealed competitor probes: PEA3 (polyomavirus enhancer activator 3) wt (5'- gggatccaggaagtga), PEA3 mut (5'- gggatccatcaagtga), E2F (5'-tatagtgtactctactattctgctc), 5-HT1A -111/101 (5'- ggtgcattcccttcctccgaaac), 5-HT1A -136/126 (ggagtaagaacttcctgcttggg), 5-HT1A -450/440 (5'-ggggaaggaaggaaatagggaga), 5-HT1A -532/522 (5'-ggaaataaagggaagtgaggagg), 5-HT1A -931/921 (5'-ggtccctatgcttccttttctca), 5-HT1A -1406/1396 (5'-gggcaaaatatttccatccctga), 5-HTT -1154/-1144 (5'-gggaacgataggaagtagaagac), 5-HTT -1172/-1162 (5'-gggaccgaaaggaaatagcagtg), some of which have been described previously (Hendricks et al., 1999; Rogaeva et al., 2007). Binding was done in 25-μL reaction containing DNA binding buffer, 250 ng of herring sperm DNA (Roche, Laval, QC) incubated at room temperature for 30 minutes prior to addition of the ^{32}P-labelled 5-HT1A -137 probe (50,000 cpm/reaction), followed by incubation for an additional 20 minutes at room temperature. DNA/protein complexes were separated by electrophoresis on a 5% native polyacrylamide gel at 4 °C, vacuum-dried and exposed to film. Band intensity was quantified in Adobe Photoshop (Adobe Systems, San Jose, CA), normalizing to the specific Pet-1 + ^{32}P-136/126 band (in percent) in order to combine multiple experiments and to calculate SEM.

Animals

Pet-1 null (B6.129-Pet1$^{Tm\ Eds}$/SJL) mice were generously provided by Dr. Evan S. Deneris, Case Western Reserve University, Cleveland OH (Hendricks et al., 2003). Limb or tail DNA was isolated in DNA lysis buffer, pH 8.0 (5 mM EDTA, 0.2% SDS, 200 mM NaCl) containing 10 mg/mL proteinase K and PCR amplified for Pet-1 genotyping: Forward primer (wild type) 5'-gcgacttgggggtcattatcac, reverse primer 5'-

gcctgatgttcaaggaagacctcgg, forward primer (knockout) 5'-cggtggatgtggaatgtgtgcg. Results were analyzed by running on a 3% agarose gel (wild type band: 209 bp; knock out band: 361 bp). The University of Ottawa Animal Care Committee approved all animal care procedures, in accordance with requirements of the Canadian Council on Animal Care Guide (Policy 31, 1993), the Animals for Research Act (R.S.O., 1990, c.22) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). Animals were sacrificed with a lethal IP injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Cambridge, ON). Brain regions immediately isolated and placed in ice cold sterile phosphate buffered saline (pH 7.4) prior to tissue isolation for Real Time reverse transcription PCR (Q RT-PCR) and western blotting. For immunofluorescence, mice were perfused transcardially with 25 mL phosphate-buffered saline (PBS, 10 mM phosphate in 0.9% saline, pH7.4) followed by 50 mL 4% paraformaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9. Brains were removed and placed in the same fixative for an additional 90 minutes and then placed in 10% sucrose containing 0.01% sodium azide for storage at 4°C. Brains were rapidly frozen with dry ice and cryostat sections were cut at 10 μm and thaw mounted onto chrome alum gelatin-coated glass slides. Sections were allowed to air dry and then rinsed in PBS prior to incubation with antibodies.

Immunohistochemistry

Primary antibodies used were rabbit polyclonal anti-5-HT (1:1000, Protos Biotech Corp, New York, NY) and rabbit polyclonal D1 receptor antibody (1:800, from Dr. M. Goldstein, New York, USA; (Jansson et al., 1999). The D1 antibody was raised against a C-terminal fusion protein from the rat dopamine D1 receptor (residues 356-446) (Jansson

et al., 1999). Primary antibodies were diluted in PBS containing 0.3 % Triton-X 100 and incubated for 3 hours at room temperature under gentle vibration (Jacobsen and Staines, 2004). The secondary antibody (goat anti-rabbit Alexa Fluor 488, 1:200, Molecular Probes, OR) was diluted in PBS and incubated at 37 °C for 45 minutes. Sections were examined using a Zeiss Axiophot II microscope equipped with standard filter sets for blue excitation 450-490 nm (composed of excitation filters BP450-490 and emission filters FT510 and LP520) and green excitation H546 (composed of excitation filters BP546/12 and emission filters FT580 and LP590). Image data was acquired using Northern Eclipse imaging software (EMPIX, Toronto, ON, Canada) and a Retiga Qimage CCD camera (QIMAGING, Burnaby, BC, Canada).

RNA Isolation

For Q RT-PCR, whole brains were removed from adult mice and rinsed in sterile ice cold PBS. Midline hindbrain (HB), midline midbrain (MB), hippocampus (Hip), striatum (CPu) and cortex (Cx) were dissected and placed in ice cold microcentrofuge tubes, and total RNA was extracted by trituration in 800 µl TRIzol (Invitrogen, Burlington, ON). For RT-PCR, 800 µl TRIzol (Invitrogen) was added directly to cells pre-rinsed in sterile PBS. 160 µl chloroform was then added and centrifuged for 25 min at 4 °C and the aqueous phase precipitated using isopropanol. RNA was treated with 1 µl TURBO DNase to remove genomic DNA contamination in 50 µl, for 30 min at 37 °C, using the TurboDNA free™ kit (Ambion, Austin, TX). The DNase treatment was stopped using 5 µl DNase Inactivation Reagent. DNase-free RNA was converted to cDNA by incubating 10 µl of the RNA with dNTPs and Random Decamers, followed by 10X RT Buffer, the M-MLV Reverse Transcriptase and RNase Inhibitors using the Cells-to-cDNA™ II kit (Ambion)

and following manufacturer specifications. Control samples, as obtained by following all above steps except the addition of the M-MLV Reverse Transcriptase, did not amplify, demonstrating the cDNA specificity of probes and successful removal of genomic DNA contamination.

Q RT-PCR

Real time PCR of cDNA prepared as described above was done using a Rotor-Gene RG-3000 (Corbett research, Sydney, Australia) and with TaqMan Gene Expression Assay and probes obtained from Applied Biosystems (Foster City, CA). Where possible, probes were designed to discriminate between genomic DNA and cDNA. Probes used were as follows GAPDH (Mm99999915_g1) and Htr1a (Mm00434106_s1). A standard was included for each gene, and compared to respective standard curves in order to compare sequential experiments, with GAPDH as an internal control. 1.5 µl of cDNA was combined 0.5 µl 20X specific probe, 5 µl 2X TaqMan Universal PCR Master Mix and 3 µl nuclease free ddH_2O for a final volume of 10 µl. For each probe, no-template controls and no-reverse transcription controls were included in sample runs and demonstrated no amplification. Reactions were carried out in a Rotor Gene 3000 cycler (Corbett Research). The cycling program used was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Data were analysed using Rotor Gene v6.0 software which compares the Ct values of the standards for each probe included in duplicate on the run with a standard curve for each gene in order to calculate the concentrations of samples included in each run. These values were then normalized against GAPDH concentrations performed analogously in each run for each sample in order to obtain normalized concentration values that could be compared across multiple experiments. At least 5

different animals of each genotype were analyzed for each gene, and SEM calculated. Two tailed, paired students T-test scores appear on the graphs, where *<0.05, **<0.005, ***<0.0005.

Western Blot

For western blotting, tissue was homogenized on ice in 50 µl of IP Buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100) containing PMSF (Sigma, Oakville, ON, Canada), Protease Inhibitors (Roche, Basel, Switzerland) and DTT (Fisher, Ottawa, ON, Canada). Western blot analysis was performed as described previously (Banihashemi and Albert, 2002) using (1:4000) anti-5-HT1A (Millipore, Mississauga, ON). Specific bands were detected by BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland). The membranes were re-probed with anti-β-actin (1:1000) to control for loading. Band intensity of 4 independent experiments was quantified using Adobe Photoshop (Adobe Systems).

RNA Isolation

For Q RT-PCR, whole brains were removed from adult mice and rinsed in sterile ice cold PBS. Midline hindbrain (HB), midline midbrain (MB), hippocampus (Hip), striatum (CPu) and cortex (Cx) were dissected and placed in ice cold microcentrofuge tubes, and total RNA was extracted by trituration in 800 µl TRIzol (Invitrogen, Burlington, ON). For RT-PCR, 800 µl TRIzol (Invitrogen) was added directly to cells pre-rinsed in sterile PBS. 160 µl chloroform was then added and centrifuged for 25 min at 4 °C and the aqueous phase precipitated using isopropanol. RNA was treated with 1 µl TURBO DNase to remove genomic DNA contamination in 50 µl, for 30 min at 37 °C, using the TurboDNA free™ kit (Ambion, Austin, TX). The DNase treatment was stopped using 5 µl DNase

Inactivation Reagent. DNase-free RNA was converted to cDNA by incubating 10 µl of the RNA with dNTPs and Random Decamers, followed by 10X RT Buffer, the M-MLV Reverse Transcriptase and RNase Inhibitors using the Cells-to-cDNA™ II kit (Ambion) and following manufacturer specifications. Control samples, as obtained by following all above steps except the addition of the M-MLV Reverse Transcriptase, did not amplify, demonstrating the cDNA specificity of probes and successful removal of genomic DNA contamination.

Q RT-PCR

Real time PCR of cDNA prepared as described above was done using a Rotor-Gene RG-3000 (Corbett research, Sydney, Australia) and with TaqMan Gene Expression Assay and probes obtained from Applied Biosystems (Foster City, CA). Where possible, probes were designed to discriminate between genomic DNA and cDNA. Probes used were as follows GAPDH (Mm99999915_g1) and Htr1a (Mm00434106_s1). A standard was included for each gene, and compared to respective standard curves in order to compare sequential experiments, with GAPDH as an internal control. 1.5 µl of cDNA was combined 0.5 µl 20X specific probe, 5 µl 2X TaqMan Universal PCR Master Mix and 3 µl nuclease free ddH_2O for a final volume of 10 µl. For each probe, no-template controls and no-reverse transcription controls were included in sample runs and demonstrated no amplification. Reactions were carried out in a Rotor Gene 3000 cycler (Corbett Research). The cycling program used was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Data were analysed using Rotor Gene v6.0 software which compares the Ct values of the standards for each probe included in duplicate on the run with a standard curve for each gene in order to calculate the concentrations of samples

included in each run. These values were then normalized against GAPDH concentrations performed analogously in each run for each sample in order to obtain normalized concentration values that could be compared across multiple experiments. At least 5 different animals of each genotype were analyzed for each gene, and SEM calculated. Two tailed, paired students T-test scores appear on the graphs, where *<0.05, **<0.005, ***<0.0005.

Western Blot

For western blotting, tissue was homogenized on ice in 50 µl of IP Buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100) containing PMSF (Sigma, Oakville, ON, Canada), Protease Inhibitors (Roche, Basel, Switzerland) and DTT (Fisher, Ottawa, ON, Canada). Western blot analysis was performed as described previously (Banihashemi and Albert, 2002) using (1:4000) anti-5-HT1A (Millipore, Mississauga, ON). Specific bands were detected by BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland). The membranes were re-probed with anti-β-actin (1:1000) to control for loading. Band intensity of 4 independent experiments was quantified using Adobe Photoshop (Adobe Systems).

Results

Pet-1 regulates 5-HT1A receptor transcription through multiple sites

Based on the consensus Pet-1 binding element (Hendricks et al., 1999), we searched the human 5-HT1A promoter to identify putative Pet-1 binding elements using the Transcription Element Search Software (TESS) (Schug, 2003). In addition to the Pet-1 element previously identified in the 5-HT1A promoter (1A-136/126), we identified 5 other putative Pet-1 elements within the first 1.5 Kb of the 5-HT1A promoter (Table 1). Many of these putative Pet-1 elements are highly conserved between human, rat and mouse 5-HT1A promoters (Table 2). In order to examine whether Pet-1 binds to these putative Pet-1 elements, we cloned and bacterially expressed human Pet-1 for use in EMSA experiments with radio-labelled 1A-136/126 as probe, competing with excess unlabeled primers (Figure V-1). A major Pet-1/DNA complex (arrow) was identified that was competed by primers designed against the related PEA3 element, 5-HTT Pet-1 elements or unlabelled 1A -136/126 primers, but not by non-specific competitors (PEA3 mut and E2F). The other putative 5-HT1A Pet-1 elements all competed, but more weakly than the 1A -136/126 site, with 1A -931/921, -449/439 and -532/522 being the most effective. Addition of specific, but not non-specific, competitors, further lead to enrichment of a low mobility species (Figure V-1, asterisk) that may represent a low affinity complex between Pet-1 elements and dimeric, partially degraded or modified Pet-1 that forms at higher Pet-1 primer concentration. We next isolated nuclear extract from HEK-293 cells, which express endogenous Pet-1 (Figure V-3), for use in EMSA experiments (Figure V-2). In the presence of labelled 1A-136/126 and HEK-293 nuclear extract, a single band was observed (arrowhead). This experiment revealed a strong competition by all 5 putative Pet-1 elements, with 1A-449/439 and 1A-533/522

competing better than the 1A-136/126 element. We again observed a low mobility complex with specific competitors, but not non-specific competitors (Figure V-2, asterisk, and data not shown). Together, these data indicate that like recombinant Pet-1, a Pet-1-like nuclear component binds to multiple Pet-1 response elements in the 5-HT1A promoter, suggesting a role for Pet-1 in the regulation of 5-HT1A receptor transcription.

Table V-1: Multiple Pet-1 Elements in Serotonergic Genes

Gene	Site	Sequence
h5-HTT	-1154/1144	GAT**AGGAA**GTA
h-5HTT	-1172/1162	GAA**AGGAA**ATA
m-5HTT	-2424/2414	CCC**AGGAA**ATG
m-5HTT	-2024/2014	GGG**AGGAA**ATG
hTPH	-1154/1144	ATA**CGGAA**ATT
mTPH	-790/780	TAC**AGGAT**ATA
h-AADC	Intron	TTC**AGGAA**ATT
h5-HT1A	-136/126 R	AGC**AGGAA**GTT
h5-HT1A	-111/101 R	CGG**AGGAA**GGG
h5-HT1A	-449/439 F	GGA**AGGAA**ATA
h5-HT1A	-532/522 F	GAA**GGGAA**AGT
h5-HT1A	-931/921 R	AAA**AGGAA**GCA
h5-HT1A	-1406/1396 R	GGA**TGGAA**ATA
PEA3		TCC**AGGAA**GTG
Consensus		RRM**AGGAA**RTR

Alignment of consensus Pet-1 elements previously identified (Hendricks et al., 1999), as well as putative additional elements in the 5-HT1A promoter, including their respective positions with respect to the start ATG and presence on the coding (F) or non-coding (R) strand. 5-HTT, serotonin transporter; AADC, amino acid decarboxylase; TPH, tryptophan hydroxylase; PEA3, polyomavirus enhancer activator 3. R = G or A, M = A or C.

Table V-2: Multiple Conserved Pet-1 Elements in the 5-HT1A Promoter

Species	Site (human)	Sequence
Human	-136/126 R	AGCAGGAAGTT
Rat		AGCGGGAAGTT
Mouse		AGCGGGAAGTT
Human	-111/101 R	CGGAGGAAGGG
Rat		CAGAGGGAGGG
Mouse		CGAAGGGAGGG
Human	-449/439 F	GGAAGGAAATA
Rat		AGAAGCAACCA
Mouse		CGAAGGAACTA
Human	-532/522 F	GAAGGGAAAGT
Rat		GAAGGGAAGGT
Mouse		GGAAGGAAGGT
Human	-931/921 R	AAAAGGAAGCA
Rat		TAAACAGAGCC
Mouse		TAAATAGAGCC
Human	1406/1396 R	GGATGGAAATA
Rat		GGAAAGAAATC
Mouse		GGAAAGAAATT

Comparison of putative Pet-1 elements in the promoter of the 5-HT1A receptor. Position of the human element with respect to the start ATG is indicated as well as presence on the coding (F) or non-coding (R) strand. Highlighted is the position of the minimal GGAA Pet-1 binding core.

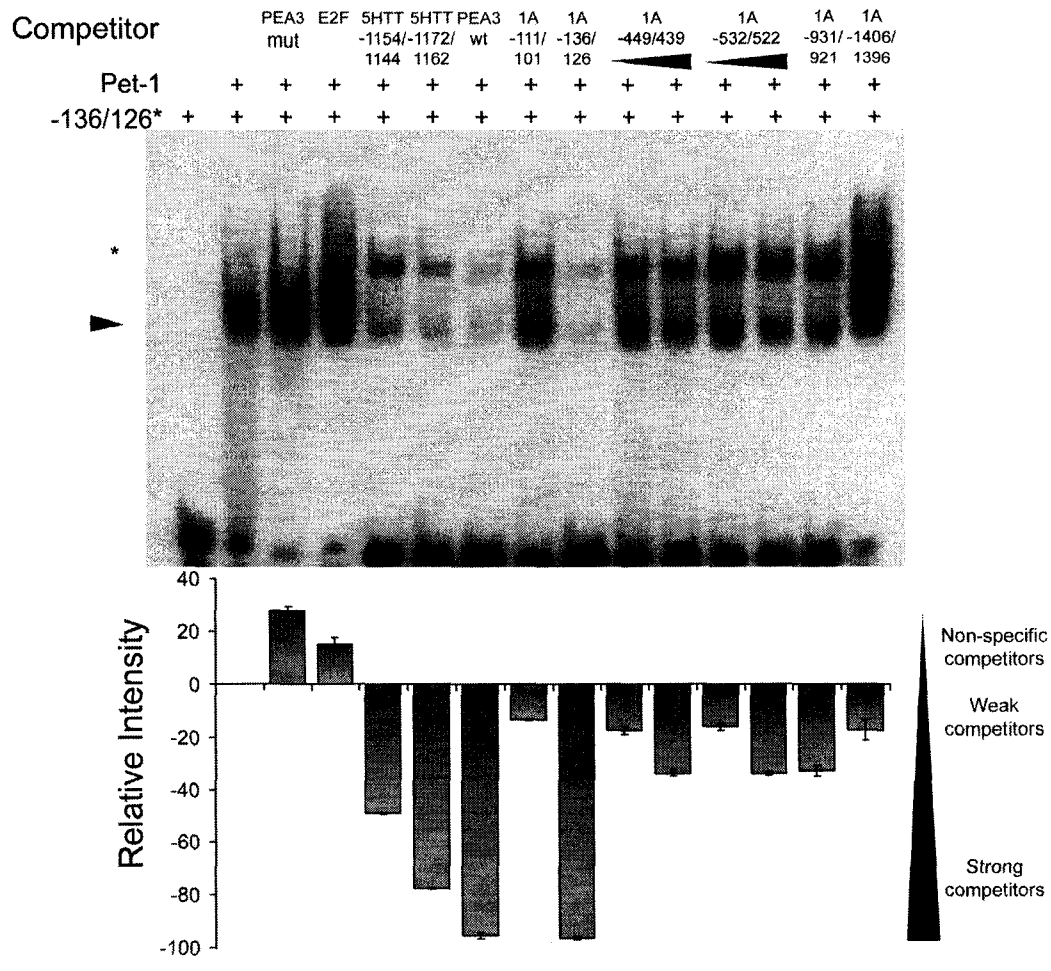

Figure V-1: Human Pet-1 directly binds multiple Pet-1 elements in the human 5-HT1A promoter.

EMSA experiment showing Pet-1 binding to the previously identified Pet-1 element in the 5-HT1A promoter (-136/126, arrow) and subsequent competition by known specific probes (5-HTT -1154/1144, 5-HTT -1172/1162, PEA3 wt, 1A -136/126) but not known non-specific probes (PEA3 mut, E2F). Putative 5-HT1A Pet-1 elements (1A -111/101, -450/440, -532/522, -931/921 and -1406/1396) were also able to compete for Pet-1 binding, although to a smaller extent than the 5-HT1A -136/126 itself. In all cases, 200X molar excess competitor was used, and where a concentration range was tested, the lower concentration of competitor was 100X molar excess. Band intensity is quantified below each lane and expressed as intensity relative to the baseline Pet-1 interaction with the radiolabelled 1A -136/126 element. (*) Low affinity interaction between second Pet-1 complex and radio-labelled probe in the presence of specific, but not non-specific, competitors. Error bars represent standard error of the mean for band intensity quantified for multiple experiments.

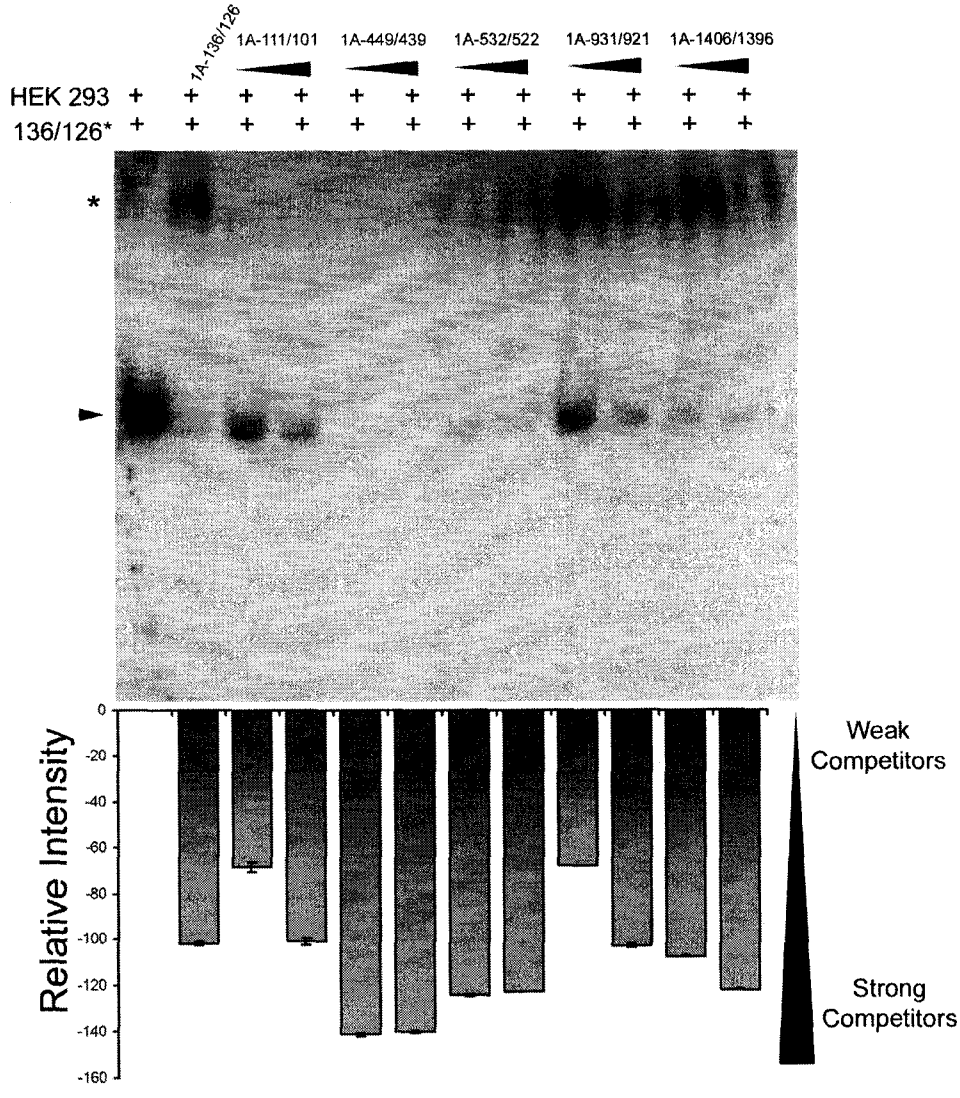

Figure V-2: Specific binding of HEK 293 nuclear protein and 5-HT1A promoter Pet-1 elements.

EMSA experiment showing complex formed between nuclear protein isolated from HEK 293 cells and previously identified Pet-1 element in the 5-HT1A promoter (-136/126, arrow) and competition by putative 5-HT1A promoter Pet-1 elements (1A -111/101, -450/440, -532/522, -931/921 and -1406/1396). In all cases, a concentration range of 100X to 200X molar excess competitor was used, with 200X excess unlabelled 1A-136/126. Band intensity is quantified below each lane and expressed as intensity relative to the baseline Pet-1 interaction with the radiolabelled 1A -136/126 element. (*) Low affinity interaction between second Pet-1 complex and radio-labelled probe in the presence of specific, but not non-specific, competitors. Error bars represent standard error of the mean of band intensity quantified for multiple experiments.

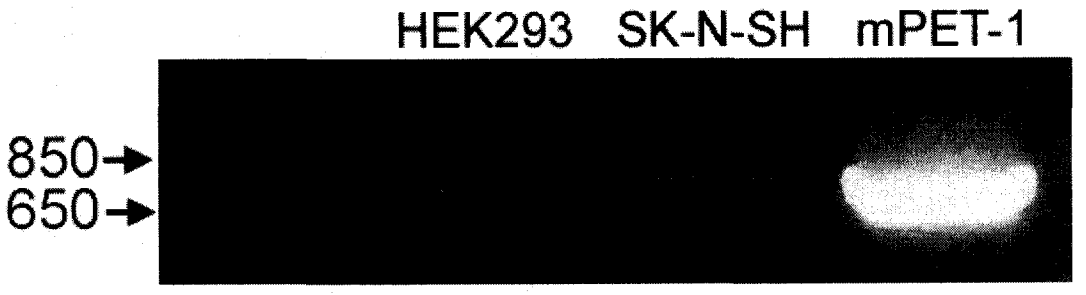

Figure V-3: Pet-1 mRNA is expressed in multiple cell lines.

RT-PCR of RNA extract from human 5-HT1A-negative HEK293 kidney and 5-HT1A positive SK-N-SH neuroblastoma cells reveals expression of Pet-1 mRNA. Mouse Pet-1 cloned into the pSLAX expression vector was used as a PCR control (m PET-1).

In order to address the activity of Pet-1 at the 5-HT1A promoter, we screened for a Pet-1-deficient cell line for use in expression studies. Unexpectedly, RT-PCR of extracts from human retinoblastoma Y79 cells, and dopamine-D2 or 5-HT1A receptor-expressing neuroblastoma cells (SK-N-AS and SK-N-SH, respectively) all revealed the presence of Pet-1 transcripts (Figure V-3, data not shown). Since HEK293 cells, unlike SK-N-SH cells, lack detectable 5-HT1A receptor expression, this suggests that the presence of Pet-1 is not sufficient to induce 5-HT1A receptor expression. Hence, HEK293 cells were used to test the activity of exogenous Pet-1. In order to determine the role of Pet-1 sites of the 5-HT1A receptor gene, HEK293 cells were co-transfected with 5-HT1A promoter-luciferase reporter constructs containing different lengths of promoter sequence (denoted -139, -224, -391, -1128 and -1517, Figure V-4A) with human Pet-1 expression plasmid or vector, and luciferase activity was measured (Figure V-4B). All constructs except the -139 construct displayed significant enhancement relative to pGL3-Basic. The shorter constructs (-139, -224 and -391), containing Pet-1 binding elements 1A-111/101 and 1A-136/126, all displayed a modest but significant activation by Pet-1. The low level of activation by transfected Pet-1 may reflect elevated basal activity of the 5-HT1A reporter constructs due to the presence of endogenous Pet-1 in these cells (Figure V-3). The longer -1128 1A construct, which includes the palindromic repressor elements for HES1, HES5 and DEAF1 (Lemonde et al., 2003; Jacobsen et al., 2007), was not further activated by co-transfection with Pet-1; however, the longest 5-HT1A promoter construct (-1517), which contains all of the identified Pet-1 elements, displayed the greatest activation by Pet-1. These data suggest that there is a combined activation by Pet-1 through multiple Pet-1 responsive elements in the 5-HT1A promoter.

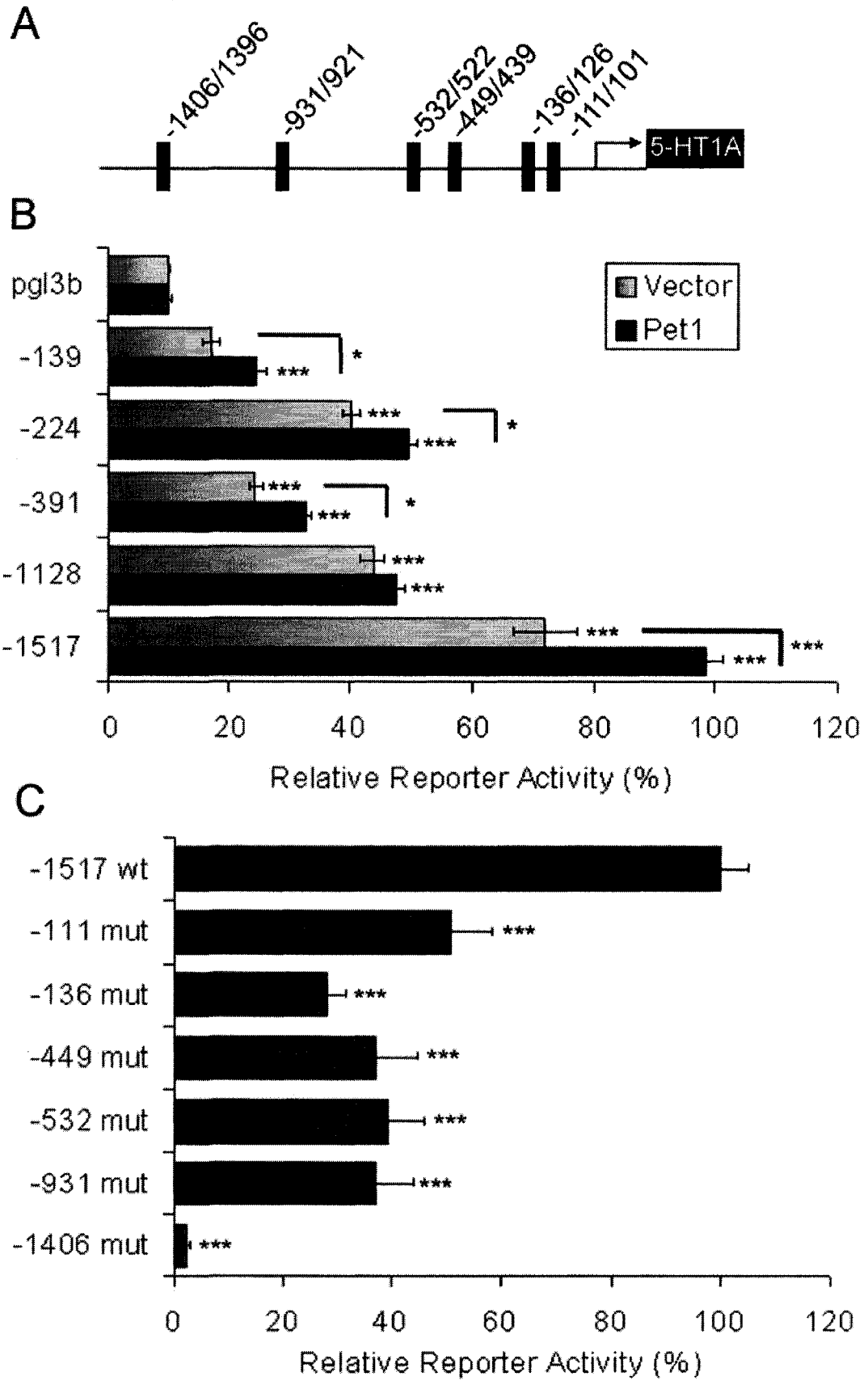
A
-1406/1396
-931/921
-532/522
-449/439
-136/126
-111/101
5-HT1A
B
pgl3b
-139
-224
-391
-1128
-1517
Vector
Pet1
*
***
0
20
40
60
80
100
120
Relative Reporter Activity (%)
C
-1517 wt
-111 mut
-136 mut
-449 mut
-532 mut
-931 mut
-1406 mut
0
20
40
60
80
100
120
Relative Reporter Activity (%)

Figure V-4: Human Pet-1 activates transcription through multiple Pet-1 elements in the human 5-HT1A promoter.

(A) Map showing position of Pet-1 elements in the 5-HT1A promoter. (B) Transcriptional activity of Pet-1 on 5-HT1A promoter constructs containing increasing lengths of promoter sequence in HEK293 cells. Pet-1 activation is strongest on the longest construct that contains all of the putative Pet-1 elements (-1517). Luciferase activity was normalized to β-galactosidase activity and expressed as percent of vector control. Error bars represent standard error of the mean. *** $p < 0.0005$, ** $p < 0.005$, * $p < 0.05$. N=4.

In order to further examine whether endogenous Pet-1 enhances 5-HT1A transcription through multiple Pet-1 sites, we mutated the AAGG core of each putative Pet-1 element in the -1517 5-HT1A promoter construct that displayed enhancement by Pet-1 (Figure V-4C). These constructs were co-transfected with the Pet-1 construct in HEK293 cells and each mutant construct displayed significantly reduced reporter activity relative to the non-mutated 5-HT1A promoter construct. This result indicates that all of the Pet-1 consensus elements identified in this study contribute to Pet-1 mediated 5-HT1A activation. Interestingly, the greatest decrease in transcriptional activity occurred with the -1406 mutant, suggesting a major role for this distally-located Pet-1 element in the regulation of 5-HT1A receptor expression. The importance of the -1406 site is supported by the stronger transcriptional activation by Pet-1 observed with the longest 5-HT1A reporter construct, which contains this site (Figure V-4B).

Pet-1 is critical for long term 5-HT1A receptor expression

Based on our data suggesting a role for Pet-1 in the regulation of 5-HT1A promoter activity *in vitro* and in HEK-293 cells, we addressed whether Pet-1 regulates the expression of 5-HT1A receptor expression *in vivo*, using Pet-1 null mice as a model system. Pet-1 null mice display a 70-80% reduction in serotonin levels as well as a disruption in serotonergic genes expression (Hendricks et al., 2003). To verify this phenotype we performed immunofluorescent staining for 5-HT on brainstem sections of adult male Pet-1 null mice and wild type littermates and observed significant reductions in 5-HT-immunoreactive cells in all regions of the dorsal raphe (Figure V-5). In wild-type mice, 5-HT-immunoreactive cells were clearly visualized (inset), while in PET-1-/- brain sections the staining was weak. Especially notable is the nearly complete reduction in 5-HT-stained cells in the interfascicular and ventrolateral parts of the dorsal raphe (DRI and DRVL respectively) in Pet-1 null mice compared with the very high 5-HT-immunoreactivity in wild type mice.

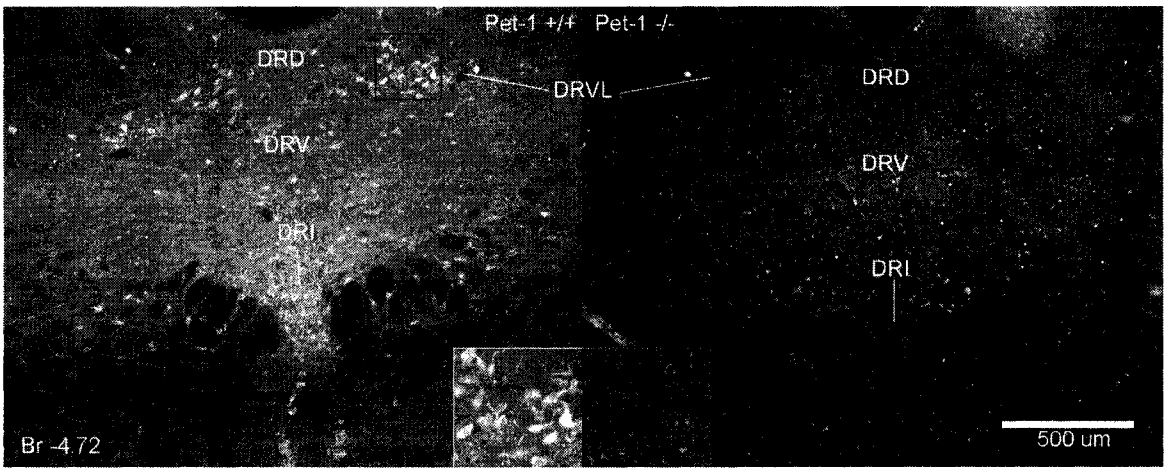

Figure V-5: 5-HT immunoreactivity is substantially reduced in Pet-1 null mice.

5-HT immunoreactive cell bodies are intensely stained in Pet-1 +/+ mice, especially in the ventrolateral (VL) and interfascicular (I) parts of the dorsal raphe (DR), with lower levels in the dorsal (D) and ventral (V) divisions. In Pet-1 null mice, 5-HT immunoreactivity is extremely low or absent. Size bar represents 500 μm. Inset is a 4X magnification of boxed region of the DRVL.

To address whether Pet-1 regulates the expression of 5-HT1A receptors in *vivo*, Pet-1 knock out mice and wild type littermates were compared for levels of 5-HT1A mRNA (Figure V-6A) and protein (Figure V-6B) expression in various brain regions by Q RT-PCR and Western blotting, respectively. Regions examined included: 5-HT1A-negative cerebellum (Cerb) as a negative control; medial hindbrain (HB) and midbrain (MB), which express pre-synaptic 5-HT1A receptors; and hippocampus (Hip), striatum (CPu, including caudate, putamen and globus pallidus), and cortex (Cx), which express post-synaptic 5-HT1A receptors. In the HB of Pet-1 -/- mice, 5-HT1A mRNA and protein was significantly increased. 5-HT1A protein was also substantially elevated in the MB of Pet-1 -/-, although the mRNA increase did not reach statistical significance. In Pet-1 -/- hippocampus, 5-HT1A protein, but not mRNA was also elevated, while in the striatum and cortex, 5-HT1A (protein and mRNA respectively) was reduced compared to wild type controls. These data suggest that, while Pet-1 may act as a transcriptional enhancer of 5-HT1A receptor activity, compensatory mechanisms that could involve the serotonergic deficit in Pet-1 -/- mice, lead to region-specific alterations 5-HT1A receptor expression in pre-synaptic and target locations.

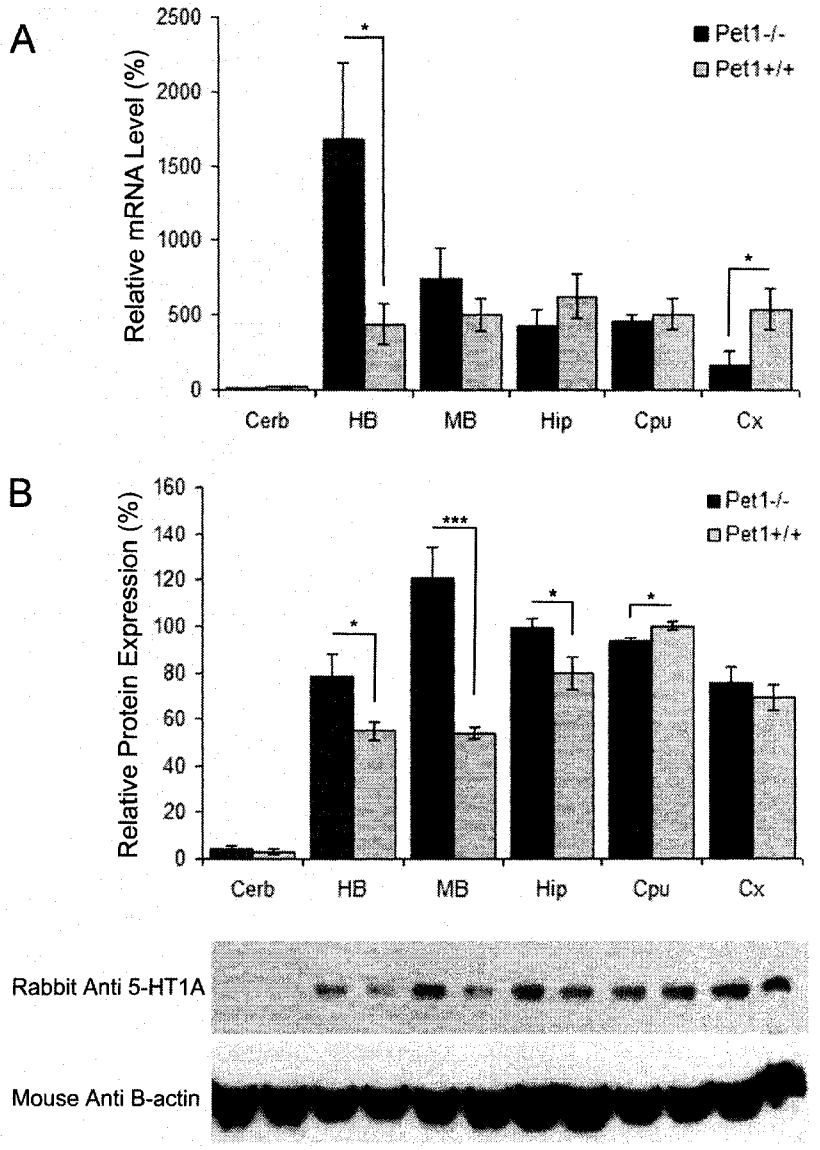

Figure V-6: Pet-1 knock out mice display altered 5-HT1A receptor expression.

Levels of 5-HT1A mRNA (A) and protein (B) in indicated brain regions were measured by Q-RT-PCR and Western blot, respectively. (A) 5-HT1A mRNA was significantly elevated in the medial hindbrain (HB) and reduced in the cortex (Cx) of Pet-1 null mice. GAPDH was used as an internal control. No template and no reverse transcription controls displayed no amplification. N=6. (B) 5-HT1A protein is elevated in the medial midbrain (MB), HB and hippocampus (HIP), and reduced in the striatum (CPu). Band intensity of 4 independent experiments was quantified. β-actin was used as a loading control. In cerebellum (Cerb) no signal for 5-HT1A RNA or protein was detected. Error bars represent standard error of the mean. *** $p < 0.0005$, ** $p < 0.005$, * $p < 0.05$.

Discussion

Direct regulation of 5-HT1A receptor gene transcription by Pet-1

Our data illustrate a role for Pet-1 as a direct transcriptional enhancer of 5-HT1A transcription that binds multiple Pet-1 elements in the 5-HT1A promoter (Figure V-1, Figure V-2) to enhance its transcription (Figure V-4). In EMSA experiments performed with recombinant human Pet-1, the 5-HT1A -136/126 element displayed the greatest competition, with most of the other 5-HT1A Pet-1 elements displayed weaker but detectable competition (Figure V-1); however, in HEK293 nuclear extracts, all of the 5-HT1A elements identified competed as well or better than the 5-HT1A -136/126 element (Figure V-2). While HEK293 cells express Pet-1, it is not known whether other ETS transcription factors are also expressed in this cell line; however, the enhancement provided by co-transfection of Pet-1 with 5-HT1A promoter constructs (Figure V-4) suggests a direct role for Pet-1 in transcriptional regulation of the 5-HT1A receptor. The loss of enhancement observed when any one of the Pet-1 elements is mutated suggests that all of the Pet-1 elements identified in the 5-HT1A promoter contribute to transcriptional regulation of this receptor. The greater degree of enhancement seen with the longest -1517 5-HT1A promoter construct and the nearly complete loss of transcriptional activity upon mutation of the -1406 Pet-1 site, further suggest a key role for this site in transcriptional activation of the gene. Since Pet-1 is expressed in the brain exclusively in 5-HT neurons (Hendricks et al., 1999), these data suggest that it is an important regulator of 5-HT1A autoreceptor expression. Although previous studies have demonstrated a potential enhancer activity of Pet-1 using an expression construct containing concatemerized Pet-1 elements (Hendricks et al., 1999), this study provides the first evidence of Pet-1 enhancer activity at a 1.5 Kb construct that contains the

majority of known 5-HT1A promoter elements. Furthermore, our findings demonstrate for the first time the activity of human Pet-1 to enhance transcription of a human gene, since previous transcription studies were performed using the rat homologue which may contain an additional 103 N-terminal amino acids (Fyodorov et al., 1998), although it is possible that the smaller, more homologous transcript is also expressed. For this reason, our data examining human Pet-1 validate the role of Pet-1 in regulating human 5-HT1A receptor transcription.

Adaptive regulation of 5-HT1A receptor expression in the absence of Pet-1

In order to begin to understand the impact of Pet-1 on 5-HT1A receptor expression *in vivo*, we compared the 5-HT1A mRNA (Figure V-6A) and protein (Figure V-6B) levels in a number of different brain regions in Pet-1 knock out and wild type animals. While Pet-1 was able to bind (Figure V-1, Figure V-2) and transcriptionally activate 5-HT1A receptor expression (Figure V-4B) through a series of Pet-1 elements in the 5-HT1A promoter, Pet-1 null mice displayed a paradoxical elevation of levels of 5-HT1A mRNA or protein in the midline hindbrain and midbrain regions that contain the majority of the serotonergic raphe nuclei (Dahlstrom and Fuxe, 1964). Pet-1 null mice displayed a significant reduction in 5-HT immunoreactive cell bodies in all raphe nuclei examined (FFigure V-5, data not shown), which was especially pronounced in the interfascicular (DRI) and ventrolateral (DRVL) dorsal raphe nuclei (Figure V-5). The reduction in detectable 5-HT-positive cells would suggest that the increase in 5-HT1A receptor expression is occurring in a small population of raphe cells, making the substantial increase even more striking. It is possible that this increase in 5-HT1A autoreceptor expression reflects a sensitization to the reduced 5-HT signalling, and subsequent receptor

up-regulation; however, further studies are necessary to elucidate the precise mechanism. Alternately the increase in 5-HT1A receptor mRNA and protein in the midbrain and hindbrain could represent increases in 5-HT1A receptor expression in non-serotonergic neurons or glia that masks a decrease in 5-HT1A levels in 5-HT-deficient raphe cells, although this remains to be determined. By contrast, in 5-HTT knockout mice, which display a persistent increase in synaptic 5-HT (Schmitt et al., 2007), 5-HT1A receptor binding sites and mRNA levels were significantly decreased in the dorsal raphe (Fabre et al., 2000; Li et al., 2000), further suggesting that 5-HT tightly controls pre-synaptic 5-HT1A receptor expression. Consistent with this, the 5-HTT LPR short allele, which reduces 5-HTT expression, has been associated with a reduction in 5-HT1A receptor levels in humans (David et al., 2005).

In target regions for 5-HT neurons, which, in Pet-1 null mice, receive a substantially reduced input of functional 5-HT projections (Hendricks et al., 2003), several alterations in 5-HT1A RNA and protein were observed. In the hippocampus of Pet-1 null mice, 5-HT1A protein was significantly increased compared to wild type controls, although to a lesser extent than that observed in mid- and hindbrain. In the cortex, there was a substantial reduction in 5-HT1A RNA, but without a corresponding reduction in protein, suggesting a stabilization or reduced down-regulation of 5-HT1A receptors. Lesion of 5-HT projections to the hippocampus using the 5-HT neurotoxin, 5,7-dihydroxytryptamine, results in increased levels of 5-HT1A protein in the hippocampus (Patel et al., 1996), and thus the lack of 5-HT in the PET-1 knockout mice may also result in up-regulation of these post-synaptic 5-HT1A receptors. In agreement with this, acute treatment with 5-HT1A antagonist WAY100,635 was also shown to upregulate 5-HT1A receptor number in cortex and hippocampus (Abbas et al., 2007).

Oppositely, in 5-HTT null mice, several post-synaptic regions including septum, amygdala, and hypothalamus display decreased 5-HT1A binding sites, with no change in 5-HT1A RNA (Li et al., 2000). In contrast, 5-HT1A protein was reduced in the striatum of Pet-1 knockout mice. Since Pet-1 expression is restricted to pre-synaptic 5-HT neurons and not present in target cells, the absence of Pet-1 cannot directly regulate 5-HT1A receptor expression in these post-synaptic regions. Rather, in line with the evidence that 5-HT acts as a developmental cue (Whitaker-Azmitia et al., 1996; Beique et al., 2004; Schmitt et al., 2007), the deficit in 5-HT signalling in these regions, combined with the proposed increase in inhibitory 5-HT1A autoreceptors, likely results in altered 5-HT1A receptor expression in post-synaptic regions. The reduction in 5-HT1A RNA levels in the cortex agrees with pharmacological studies demonstrating that reduced 5-HT signalling delays expression of 5-HT type 1 receptors (Lauder, 1990; Whitaker-Azmitia et al., 1996). That this reduction is sustained into adulthood provides striking evidence that early developmental signalling of the serotonin system is critical for 5-HT1A receptor expression, which has implications for the affective phenotype of Pet-1 knock out mice. Interestingly, in Pet-1 knockout mice the increase of 5-HT1A receptor expression in the hippocampus, versus a decrease in striatum, suggests that reduced serotonergic signalling may alter receptor expression in a site-specific manner.

Adaptive regulation of 5-HT1A receptor expression and behaviour in Pet-1 null mice

Pet-1 null mice display a heightened anxiety/aggressivity phenotype (Hendricks et al., 2003). Interestingly, 5-HT1A receptor knockout studies have demonstrated that early developmental expression of the 5-HT1A receptor is critical for establishing normal anxiety-like behaviour (Gross et al., 2002). On the other hand 5-HT1B receptor knockout mice display an aggressive phenotype with reduced anxiety (Saudou et al., 1994; (Zhuang et al., 1999). The virtual absence of 5-HT in the DRI and DRVL, raphe nuclei that are implicated in peripheral immune activation (Hollis et al., 2006; Lowry et al., 2007) raises the possibility that the physiological and behavioural responses to peripheral immune activation are altered in Pet-1 null mice, responses that may contribute to pathophysiology of mood and emotion in these mice. Interestingly, a study of suicide victims with major depression revealed an increase in 5-HT1A receptor transcripts in the DRVL compared to control subjects (Stockmeier, 1997). In addition the G/G(-1019) genotype of the 5-HT1A receptor gene, which leads to its up-regulation, particularly in depressed patients (Lemonde et al., ; Parsey et al., 2006), has recently been associated with interferon-alpha induced depression, as well as major depression and suicide (Kraus et al., 2007). These results suggest a causative link between the elevated hindbrain and midbrain 5-HT1A receptor levels with the anxiety phenotype of Pet-1 null mice. In addition to 5-HT1A receptors, several other 5-HT receptors (5-HT1B, 5-HT2A, 5-HT2C, 5-HT4 and others) have been implicated in affective disorders and in the therapeutic actions of antidepressants (Conductier et al., 2006; Esposito, 2006), and may be altered in Pet-1-/- mice. It is as of yet unclear whether there is compensation by any other neurotransmitter system in Pet-1 null mice. Our preliminary data indicate an increase in tyrosine hydroxylase-positive projections (largely noradrenergic) in certain brainstem

regions of Pet-1 knock out mice, such as the dorsal raphe (data not shown). The implications of this finding have yet to be elucidated, but suggest that additional adaptive changes may contribute to the behavioural phenotype of the PET-1 null mice.

The reduction in 5-HT observed in PET-1 null mice could produce altered 5-HT1A expression and developmental changes that result in altered behaviour in the adult. For example, maternal administration of para-chlorophenylalanine (ρCPA), a competitive inhibitor of TPH that depletes embryonic 5-HT, from E12-17, reduces the postnatal expression of 5-HT type 1 receptors, including 5-HT1A, in putative target cells (Lauder, 1990; Lauder et al., 2000). Over-stimulation of 5-HT receptors with the general 5-HT1/5-HT2 receptor agonist, 5-methoxytryptamine, also reduces expression of 5-HT1A receptor transcripts (Lauder et al., 2000) and regulates neurite outgrowth and animal behaviour (Shemer et al., 1988; Shemer et al., 1991). 5-HT is thought to modulate neuronal coupling (Rorig and Sutor, 1996), plasticity (Whitaker-Azmitia et al., 1996) and differentiation (Gaspar et al., 2003), and can regulate the spatio-temporal expression of other neurotransmitter phenotypes, such as GABA, via 5-HT1A receptors (Allain et al., 2005). Thus, a disruption of 5-HT developmentally represents a deficit not only in serotonergic signalling, but potentially molecular and morphological changes throughout the brain that can result in lifelong alterations in behaviour.

Taken together, the above data demonstrate that Pet-1 can directly activate 5-HT1A receptor expression through multiple Pet-1 response elements in the 5-HT1A promoter, suggesting that Pet-1 may activate 5-HT1A autoreceptors through development. The Pet-1 null mice, however, illustrate the critical role of Pet-1 in the development of serotonin neurons, and their subsequent role as a differentiation signal for

target cells. Our data suggest that in certain regions, 5-HT may act as a developmental regulator of 5-HT1A receptor expression, such as in the cortex and striatum, while in other regions, the absence of 5-HT signalling results in upregulation of 5-HT1A receptors (such as in the hippocampus and brainstem). The Pet-1-dependent cascade that regulates 5-HT neuron development, therefore, is essential for the expression of 5-HT1A autoreceptors and for 5-HT1A receptor expression in target regions, and has inextricable implications for the role of this gene in the development of affective behaviours.

Acknowledgements

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Background and Relevance – Chapter VI

The clear importance of Pet-1 in the development of the 5-HT system provides a unique tool for understanding how 5-HT contributes to the development of the fear circuitry, as well as to mood and emotion. Developmental regulators that are not 5-HT-specific may also be central to establishing proper fear circuitry connectivity, thus impacting 5-HT function. The Hes family of bHLH transcription factors has been extensively studied for their important role in regulating neurogenesis (Kageyama and Ohtsuka, 1999; Kageyama et al., 2006). The identification of Hes5 as a transcriptional repressor of the 5-HT1A receptor gene by our lab, however, suggests that more attention should be paid to this family of proteins in relation to the developmental expression of 5-HT1A (Lemonde et al., 2003). The possibility that Hes proteins may also direct the timeframe of embryonic 5-HT1A expression is addressed in the following chapter. As alterations in the embryonic expression of 5-HT1A have direct consequences for anxiety behaviour (Zhuang et al., 1999; Overstreet et al., 2003; Gross and Hen, 2004), this would implicate the Hes transcriptional regulators in modulating affective phenotype.

CHAPTER VI: HES1 regulates 5-HT1A receptor gene transcription at a functional polymorphism: Essential role in developmental expression.

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Hes-1 mice were obtained from Dr. Ryoichiro Kageyama care of Dr. Ruth Slack.

Mice were bred by Dr. Jackie Vanderluit and Kirsten Jacobsen.

All experiments were done by Kirsten Jacobsen.

The paper was written by Kirsten Jacobsen with editorial input from Dr. Paul Albert and Dr. Jackie Vanderluit.

Abstract

Mammalian HES1 and HES5 are abundant in developing CNS and inhibit neurogenesis, while HES6 promotes neurogenesis. An early serotonergic differentiation marker, the 5-HT1A receptor, is repressed by HES5 and DEAF1 which recognize the C(-1019), but not G(-1019) allele of a 26-bp human 5-HT1A promoter palindrome associated with mood disorders. We tested whether HES1 and HES6 regulate transcriptional activity at this element. HES1 strongly repressed 5-HT1A transcription in neuronal and non-neuronal cells, while HES6 reversed HES1- and HES5-mediated repression. Mutation of a putative HES consensus site blocked HES1 and HES5, but, unlike HES5, HES1 repressed at the G(-1019) allele. To address its role *in vivo*, the temporal expression of 5-HT1A receptor RNA and protein was examined in HES1-/- mice, and elevated levels in E12.5 hindbrain and midbrain were observed. Thus, HES1 and HES6 oppositely regulate 5-HT1A receptor transcription and HES1 is required for its correct developmental expression.

Introduction

Serotonin (5-HT) regulates a variety of behavioural processes and alterations in serotonin signalling are implicated in depression and anxiety (Gaspar et al., 2003; Beique et al., 2004). Serotonin neurons develop early (E10-12 in mouse) in brainstem raphe nuclei (B1-9) (Fuxe, 1965b; Lauder, 1990) and differentiation is driven by a cascade of transcription factors, including Sonic hedgehog-induced homeobox genes *Nkx2-2/Nkx 6.1,* basic helix-loop-helix (bHLH) protein *Ascl1/Mash1* and zinc-finger gene *Gata2,* which induce serotonin specification factors *Lmx1b* and *Pet1* (Cheng et al., 2003; Craven et al., 2004; Pattyn et al., 2004). 5-HT1A receptors are also expressed during neurogenesis (E12 in rat) in 5-HT target regions (Hillion et al., 1993; Benninghoff et al., 2002; Patel and Zhou, 2005) and as somatodendritic autoreceptors in the midbrain raphe nuclei. Gene knockout studies of the 5-HT1A receptor illustrate its developmental role in affective disorders, as mice lacking the receptor during embryonic and early postnatal development display an anxiety phenotype that persists into adulthood (Gross et al., 2002).

We previously identified two transcription factors which regulate 5-HT1A receptor expression through a 26-bp palindromic element containing the 5-HT1A C(-1019)G (rs6295) promoter polymorphism that associates with suicide and major depression (Lemonde et al., 2003). In a serotonergic raphe (RN46A) cell line that expresses 5-HT1A receptors (White et al., 1994), DEAF1 (Deformed epidermal autoregulatory factor-1) (Czesak et al., 2006) and HES5 (Hairy/Enhancer-of-split-5) repressed transcription of the 5-HT1A promoter through the C-allele, but not G-allele, suggesting that reduced repression by DEAF1 and HES5 could predispose individuals with the G-allele to affective disorders. DEAF1 recognizes TTCG-containing motifs and

regulates transcription of many genes, including the 5-HT1A receptor (Huggenvik et al., 1998; Michelson et al., 1999; Lemonde et al., 2003). DEAF1 is expressed ubiquitously throughout development, and has a dual repressor/enhancer function that is cell-type specific (Czesak et al., 2006). HES5 belongs to a family of bHLH proteins known to regulate many developmental processes (Kageyama et al., 2006). HES1, HES3 and HES5 inhibit proneural genes such as *Mash1, Math3* and *Neurogenin2*, thereby maintaining neuronal progenitors (Kageyama et al., 2005). HES1 and HES5 are downstream effectors of Notch signalling, while HES3 may be subject to other regulatory mechanisms (Kageyama and Ohtsuka, 1999; Androutsellis-Theotokis et al., 2006). While gene knockout studies show that HES1/HES3/HES5 can partially compensate for each other, disruption of inhibitory HES genes leads to upregulation of proneural bHLH proteins, premature neurogenesis and disruption of developmental organizing centers (Hirata et al., 2001; Kageyama et al., 2005; Baek et al., 2006). HES6, a proneural bHLH protein, is expressed in developing and mature neurons as neurogenesis proceeds (Bae et al., 2000) and can heterodimerize with HES1 and target it to the proteosome for degradation, thus inhibiting HES1 function and stimulating neurogenesis (Gratton et al., 2003; Nuthall et al., 2004; Kang et al., 2005).

Most bHLH proteins bind the canonical CANNTG sequence (E box) (Takebayashi et al., 1995). HES1 and HES5 also have high affinity for the CACNAG site (N box), and for the class C site (CACGCG) in the case of HES1 (Akazawa et al., 1992). The first aim of this study was to determine whether HES1, HES5 and HES6 coordinately regulate transcription of the 5-HT1A receptor gene through a putative HES binding site (CACtCG) located in the 26-bp palindromic element. As neurogenesis proceeds, decreasing HES1 and HES5 levels correlate inversely with increasing 5-HT1A receptor

expression; hence, the second goal was to examine the role of HES1 in the developmental expression of 5-HT1A receptors *in vivo*.

Materials and Methods

Plasmid constructs:

The 26-bp-C(TK) pGL3-Basic and 26-bp-C(6) pGL3-Promoter reporter plasmids were obtained as described previously (Lemonde et al., 2003). The 26-bp-C, 26-bp-G, 26-bp-C:Nboxmut and 26-bp-G:Nboxmut pGL3-Promoter reporter constructs were generated by insertion of *KpnI-SacI* digested fragments of the human 5-HT1A promoter (obtained by annealing oligomers designed against the 26 bp palindrome) into the pGL3-Promoter digested with *KpnI* and *SacI* (see Figure VI-2 for sequence information). Generation of the human HES5 expression plasmid has also been described previously (Lemonde et al., 2003). Human HES1 and HES6 expression plasmids were obtained by PCR-amplification out of a human brain library using sense (5-AATGCCAGCTGATATAATGGAGAAAAAT-3) and antisense (5-GAGCCCCCTGAGTTCCGCCACG-3) primers specific to human HES1 flanking sequences and sense (5-TCCTAGCCCCTGCCGCGT-3) and antisense (5-GGCATTGGACACCAAGGCCTCCAGACAC-3) primers specific to human HES6 flanking sequences. dATPs were added to the blunt end PCR fragments using *Taq polymerase* (NEB, Pickering, Ontario, Canada), following which they were cloned into pGEM-Teasy (Promega, Madison, WI, USA). HES1 and HES6 were then excised from pGEM-Teasy by digesting with *EcoRI* and subcloned into pcDNA3 and the accuracy of the cloned cDNA products was confirmed by DNA sequence analysis.

Cell culture:

Rat L6 skeletal muscle myoblasts (5-HT1A-negative) and 5-HT1A expressing rat-mouse hybrid septal SN48 cells and human SK-N-SH neuroblastoma cells (kind gift from Dr.

Lakshmi Devi, New York University (Fricker et al., 2005)) were cultured in DMEM (Wisent, St. Bruno, Quebec, Canada) supplemented with 8% v/v heat inactivated fetal bovine serum at 37°C in 5% CO_2. Rat raphe RN46A cells (5-HT1A expressing) were cultured on surface modified polystyrene Primaria plates (BD Falcon, Franklin Lakes, NJ, USA) in neurobasal medium (Invitrogen BRL, Rockville, MD, USA) supplemented with 8% v/v heat-inactivated fetal bovine serum and L-glutamine (2 mM final, Wisent) at 33 °C in 5% CO_2.

Transfections:

Cells were split into 12-well plates (Fisher) or, in the case of RN46A cells, into 6-well Primaria plates (BD Falcon) 16 hours prior to transfection. All transfections were done using the Lipofectamine Plus Kit (Invitrogen BRL). For SK-N-SH, L6, and SN48 cells, 0.5 μg total DNA was transfected per well, and for RN46A cells, 1 μg total DNA was transfected per well. For all transfections, a ratio of 1:1:1 DNA:lipofectamine:plus reagent was maintained and the plasmid pCMV-β-gal was used to control for transfection efficiency. Cells were transfected in serum-free OptiMEM (Invitrogen BRL) for 3 hours, following which medium was replaced with appropriate growth medium. After 48 hours, luciferase and β-galactosidase activity were assessed using the L-MaxII and SpectroMax M2 (Molecular Devices, Woodbridge, Ontario, Canada) respectively. For all experiments, assays were done in triplicate and repeated at least 3 times. Luciferase activity was normalized both to β-gal activity and vector control for each experiment in order to allow comparison between experiments. This is presented as Relative Reporter Activity (%). Data were analyzed in Excel (Microsoft) and error bars represent standard

error of the mean (SE). One-way ANOVA was performed for each experiment using Dunnett's multiple comparison *post hoc* test (*: $P < 0.05$; **: $P<0.005$; ***: $P<0.0005$).

Animals

The University of Ottawa Animal Care Committee approved all animal care procedures, in accordance with requirements of the Canadian Council on Animal Care Guide (Policy 31, 1993), the Animals for Research Act (R.S.O., 1990, c.22) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). Heterozygous HES1 mice (Ishibashi et al., 1995; Ito et al., 2000) were mated overnight and E0 was determined as the day of discovery of the vaginal plug. Pregnant mothers were sacrificed with a lethal IP injection of sodium pentobarbital (Somnitol) at E12.5, and embryos were removed and placed immediately in sterile ice cold phosphate buffered saline (PBS, pH 7.4). For Real Time reverse transcription PCR (Q RT-PCR), embryonic brains were isolated and hindbrain, midbrain and forebrain regions individually dissected. For *in situ* hybridization, whole brains were placed immediately in ice cold PBS followed by PBS containing 4% paraformaldehyde, 12% sucrose, 16% sucrose and 22% sucrose, each for 24 hours. Whole brain sections were then frozen on dry ice, cryostat sectioned (12 microns) and collected on SuperFrost / Plus glass slides (Microm, Walldorf).

Genotyping

Limb or tail DNA was isolated in DNA lysis buffer, pH 8.0 (5 mM EDTA, 0.2% SDS, 200 mM NaCl) containing 10 mg/mL proteinase K and PCR amplified for genotyping (forward primer 5'-ATATATAGAGGCCGACAGGGCCTGGGATC, reverse primer

(wild type) 5'-CGCAGGTACTGTCTTACCTTTCTGTGCTCAGAGGCC, reverse primer (knockout) 5'-CGCTTCCATTGCTCAGCGGTGCTGTCCATC). Results were analyzed by running on a 1% agarose gel (wild type band: 400 bp; knock out band: 600 bp).

RNA Isolation

Wild type and knockout mouse embryos (E12.5) were euthanized using a lethal I.P. injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Cambridge, Ontario, Canada), and brain regions were dissected, placed in ice cold microcentrofuge tubes, and total RNA was extracted by trituration in 800 µl TRIzol (Invitrogen, Burlington, Ontario, Canada). 160 µl chloroform was then added and centrifuged for 25 min at 4 °C and the aqueous phase precipitated using isopropanol. RNA was then treated with 1 µl TURBO DNase to remove genomic DNA contamination in 50 µl, for 30 min at 37 °C, using the TurboDNA free™ kit (Ambion, Austin, Texas, USA). The DNase treatment was stopped using 5 µl DNase Inactivation Reagent. DNase-free RNA was converted to cDNA by incubating 10 µl of the RNA with dNTPs and Random Decamers, followed by 10X RT Buffer, the M-MLV Reverse Transcriptase and RNase Inhibitors using the Cells-to-cDNA™ II kit (Ambion) and following manufacturer specifications. Control samples for each amplification were obtained by following all above steps except the addition of the M-MLV Reverse Transcriptase and did not amplify, thus demonstrating the cDNA specificity of probes and successful removal of genomic DNA contamination.

Q RT-PCR

Real time PCR of cDNA prepared as described above was done using a Rotor-Gene RG-3000 (Corbett research, Sydney, Australia) and with TaqMan Gene Expression Assay probes obtained from Applied Biosystems (Foster City, California, USA). Where possible, probes discriminated between genomic DNA and cDNA. Probes used were as follows GAPDH (Mm99999915_g1), HES1 (Mm00468601_m1), HES5 (Mm00439311_g1), Htr1a (Mm00434106_s1) and TPH2 (Mm00557717_m1). In each run, a standard was included for each gene, and compared to respective standard curves in order to compare sequential experiments, with GAPDH as an internal control. 1.5 µl of cDNA was combined 0.5 µl 20X specific probe, 5 µl 2X TaqMan Universal PCR Master Mix and 3 µl nuclease free ddH_2O for a final volume of 10 µl. For each probe, no-template controls and no-reverse transcription controls were included in sample runs and demonstrated no amplification. Reactions were carried out in a Rotor Gene 3000 cycler (Corbett Research, Sydney, Australia). The cycling program used was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Data were analysed using Rotor Gene v6.0 software which compares the Ct values of the standards for each probe included in duplicate on the run with a standard curve for each gene in order to calculate the concentrations of samples included in each run. These values were then normalized against GAPDH concentrations performed analogously in each run for each sample in order to obtain normalized concentration values that could be compared across multiple experiments. At least 5 different animals of each genotype were analyzed for each gene, and SEM calculated. Outliers were discarded, as validated by the Q test, with at least

95% confidence (Skoog et al., 1994). Data were analyzed by two-tailed, paired Student's t-test, where *<0.05, **<0.005, ***<0.0005.

In situ hybridization

Sense and anti-sense 5-HT1A riboprobes were transcribed from the pST-Blue1 plasmid a 417-bp sequence of the mouse 5-HT1A receptor cDNA (kind gift of Dr. Alexandre Bonnin) (Bonnin et al., 2006) using the SP6 or T7 (respectively) polymerases. Probes were digoxigenin (DIG)-labelled (Roche, Laval, Quebec) following manufacturer specifications. *In situ* hybridizations were performed according to the method of Ferguson et al., 2005 (Ferguson et al., 2005).

Results

Hes regulation of 5-HT1A receptor transcription through a 26-bp palindrome.

HES5 has been shown to repress transcription of the full-length human 5-HT1A promoter or heterologous promoters through a 26-bp element that contains the C(-1019)G polymorphism associated with major depression (Lemonde et al., 2003; Czesak et al., 2006). In order to determine whether other HES family members regulate 5-HT1A receptor transcription through this element, HES expression plasmids were co-transfected with reporter constructs containing the 5-HT1A 26-bp element upstream of different promoters (Figure VI-1). To validate the activity of this element in different cellular contexts, we examined cell lines that model relevant neuronal or non-neuronal lineages including RN46A cells, a model of 5-HT1A-positive serotonergic raphe cells (White et al., 1994), and SN48 septal cells, a model of 5-HT1A-expressing target cells for serotonergic afferents (Charest et al., 1993; Shi et al., 2003). These cells also endogenously express HES factors, as detected by RT-PCR (Lemonde et al., 2003) and Western blot (not shown) and can be differentiated to express neuronal markers, making them suitable for studying the developmental regulation of the 5-HT1A receptor. In these cells, both HES1 and HES5 repressed the transcription of a luciferase reporter containing the 5-HT1A 26-bp element upstream of the thymidine kinase (TK) promoter (Lemonde et al., 2003) (Figure VI-1A). HES5 repressed activity by about 50%, while HES1 repressed by 80-90%, demonstrating a significantly stronger repressor activity than HES5 at this 26-bp palindrome. Conversely, transfection of HES6, which promotes neuronal differentiation (Bae et al., 2000), enhanced basal reporter activity. Thus, like HES5, HES1 exerts strong repressor activity at this element, while HES6 appears to de-repress its activity.

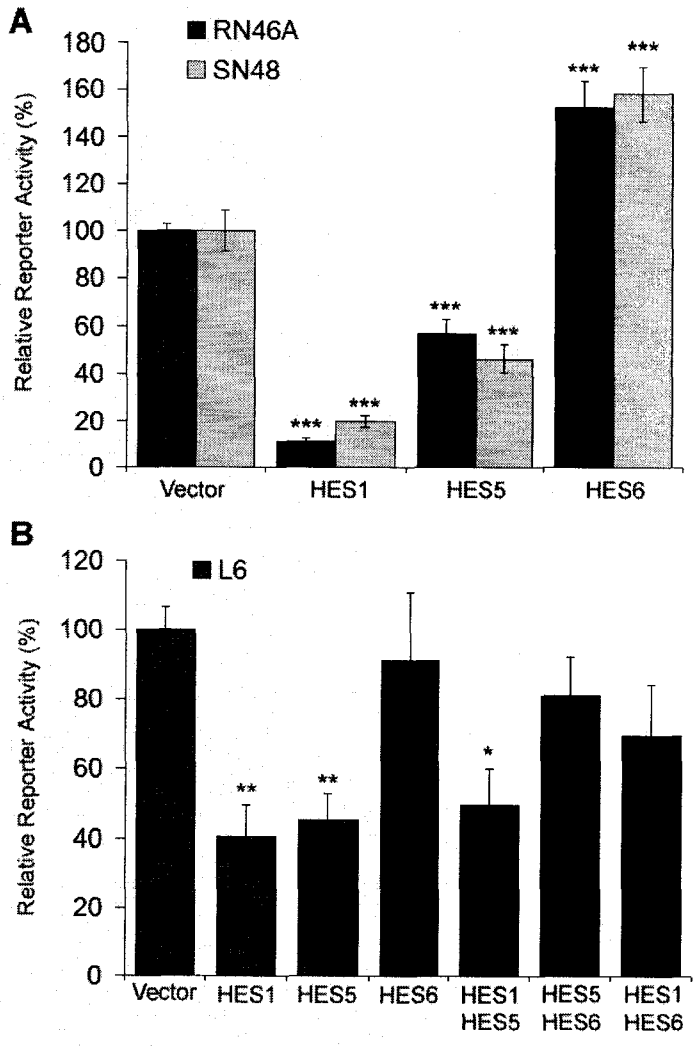

Figure VI-1: Activity of Hes family members at the 26-bp palindrome in the human 5-HT1A receptor promoter that contains the C(-1019)G polymorphism.

(A) Repressor activity of Hes proteins in 5-HT1A-positive cells. The 26-bp palindromic sequence (C-allele) was cloned into the pGL3-Basic vector under the control of the TK promoter and this luciferase reporter construct was co-transfected with expression constructs containing Hes1, Hes5 or Hes6 in 5-HT1A-expressing RN46A raphe cells or SN48 septal cells. (B) Antagonism of Hes1/HES5 repression by Hes6. Hes1, Hes5 or Hes6 expression constructs were co-transfected in non-neuronal L6 myoblasts with the 26bpC(6) reporter construct, containing six copies of the 26 bp palindrome in the pGL3-Promoter vector. Co-transfection of Hes6 with either Hes1 or Hes5 completely inhibits repression to baseline levels, suggesting Hes6 inhibits both Hes1 and Hes5-mediated repression of 5-HT1A receptor activity. Error bars represent standard error of the mean. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

HES6 interacts with and targets HES1 for proteosomal degradation, attenuating HES1 repressor activity (Bae et al., 2000; Gratton et al., 2003; Nuthall et al., 2004; Kang et al., 2005). It is not known whether HES6 is also able to inhibit HES5 repressor activity. In order to investigate the activity of HES proteins in non-neuronal precursors that lack 5-HT1A receptor expression, and to analyze the combined effects of multiple HES factors, L6 myoblasts were co-transfected with the sensitive 5-HT1A luciferase reporter construct containing six copies of the 26-bp element [26bp-C(6)] (Lemonde et al., 2003) and HES1, HES5 or HES6, alone or in combination (Figure VI-1B). As observed in neuronal cell lines, HES1 and HES5 both acted as transcriptional repressors at the 26-bp element. Co-transfection of HES1 and HES5 did not lead to a further repression, and HES6 alone had no significant effect on basal transcription. When HES6 was co-transfected with either HES1 or HES5, however, no repression was observed, indicating that HES6 inhibits both HES1 and HES5-mediated repression at the 5-HT1A 26-bp element. Thus HES6 functions as a transcriptional antagonist of both HES1 and HES5.

Hes regulation through a putative N-box in the 5-HT1A promoter

The site of HES5 action within the 26-bp 5-HT1A palindrome has not previously been addressed; however, there is a site resembling an N-box (CACtCG) that we proposed as the repressor element recognized by HES5 (Lemonde et al., 2003). Interestingly, at -1099 bp in the mouse and -1106 bp in the rat 5-HT1A upstream sequence there is a perfect N-box (CACCAG) within 20 nucleotides of the human element when the three sequences are aligned. To test the role of this site, four luciferase reporter constructs were created containing one copy of the 26-bp element with C- or G-allele in combination with wild-type or mutated N-box sequence located upstream of the SV40 promoter (Figure VI-2A-B). To examine their activity in an appropriate human background, these reporter constructs were co-transfected with HES1, -5 and -6 in human SK-N-SH neuroblastoma cells, a neuronal cell model of post-synaptic 5-HT1A receptor expression (Fricker et al., 2005). As observed in RN46A and SN48 cells, HES1 and HES5 both repressed transcription of the 26-bp(C) construct. HES6 alone had no effect on the 26-bp(C) construct in these cells. Repression by HES5 was lost in the 26-bp(G) construct consistent with previous studies (Lemonde et al., 2003); however, HES1 retained repressor activity at the 26-bp(G) reporter construct. Repression by both HES1 and HES5 was completely abolished by mutation of the N-box, indicating that this site is critical for the action of HES family proteins at the 5-HT1A palindrome region.

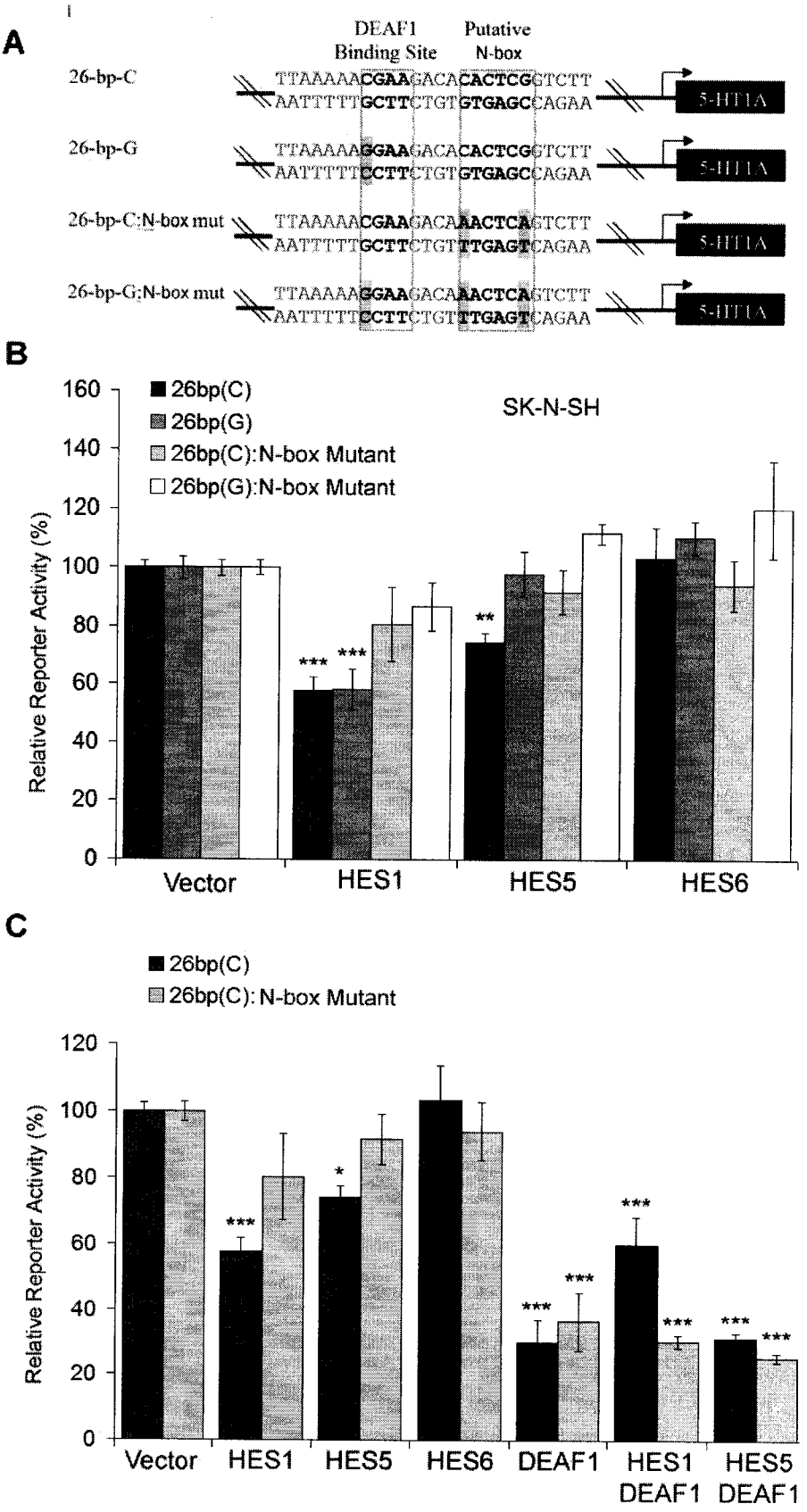
A
DEAF1 Binding Site
Putative N-box
26-bp-C
TTAAAAACGAAGACACACTCGGTCTT
AATTTTTGCTTCTGTGTGAGCCAGAA
5-HT1A
26-bp-G
TTAAAAAGGAAGACACACTCGGTCTT
AATTTTTCCTTCTGTGTGAGCCAGAA
5-HT1A
26-bp-C:N-box mut
TTAAAAACGAAGACAAACTCAGTCTT
AATTTTTGCTTCTGTTTGAGTCAGAA
5-HT1A
26-bp-G:N-box mut
TTAAAAAGGAAGACAAACTCAGTCTT
AATTTTTCCTTCTGTTTGAGTCAGAA
5-HT1A
B
26bp(C)
26bp(G)
26bp(C):N-box Mutant
26bp(G):N-box Mutant
SK-N-SH
Relative Reporter Activity (%)
160
140
120
100
80
60
40
20
0
***
***
**
Vector
HES1
HES5
HES6
C
26bp(C)
26bp(C): N-box Mutant
Relative Reporter Activity (%)
120
100
80
60
40
20
0
***
*
***
***
***
***
***
Vector
HES1
HES5
HES6
DEAF1
HES1 DEAF1
HES5 DEAF1

Figure VI-2: Activity of Hes family members at a putative N-box in the 26-bp palindrome in the 5-HT1A.

(A) Schematic diagram of 5-HT1A 26 bp pGL3-Promoter constructs depicting location of putative DEAF1 and Hes binding sites, as well as polymorphic and mutant constructs. (B) Repressor activity of Hes proteins in 5-HT1A-positive SK-N-SH cells. Four constructs containing the 26-bp palindromic sequence (C-allele, G-allele and C/G-allele/N-box mutant) were cloned into the pGL3-Promoter and co-transfected with expression constructs containing Hes1, Hes5 or Hes6. (C) Combined repressor activities of Hes1/HES5 and DEAF1 at the 26 bp palindrome in SK-N-SH cells. Co-transfection of Hes1, Hes5, Hes6 and DEAF1 with the 26 bp reporter construct (C-allele or C-allele/N-box mutant) reveals no additive repressor effect of these proteins. DEAF1 repression is not affected by the mutation of the putative Hes N-box. Error bars represent standard error of the mean. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

Hes and DEAF1 regulate 5-HT1A transcription at a functional promoter polymorphism

In raphe RN46A cells, DEAF1/NUDR and HES5 have been shown to act at the 26-bp palindrome to repress 5-HT1A receptor transcription (Lemonde et al., 2003). Since DEAF1 and HES5 are thought to act at adjacent sites on the 26-bp palindrome and the G(-1019) allele reduces their binding and function, we addressed whether coordinate transcriptional regulation by DEAF1 and other HES family members occurs through this element (Figure VI-2C). Human 5-HT1A-expressing SK-N-SH cells were co-transfected with an SV40 reporter plasmid containing a single copy of the 26-bp palindrome (C-allele), as well as human DEAF1, HES1, HES5, or HES6 expression vectors, alone or in combination. In these cells, DEAF1 mediated the strongest repression, followed by HES1 and HES5, with no effect of HES6 as observed in Figure VI-2B. Co-transfection of DEAF1 with HES1 or HES5 did not further repress the level of transcription, and this may reflect their relative binding affinity (HES1>DEAF1>HES5), although further analysis would be required to confirm this possibility. The G(-1019) allele disrupts the minimal consensus element for Deaf-1 (CGAA) to block its activity, but also reduced HES5 activity. For the HES homologue Helt, it has been demonstrated that nucleotides proximal to the minimal consensus can influence binding affinity (Nakatani et al., 2004), and the G(-1019) may be proximal enough to attenuate HES5 binding. We next sought to determine whether mutation of the putative N-box would affect DEAF1 activity. Co-transfection of the 26-bp(C):NboxMut along with HES1, HES5 and DEAF1 revealed that mutation of the N-box alone does not affect DEAF1 repressor function. Co-transfection of HES1 and DEAF1 with the N-box mutant results in repression to a level approximately equal to that of DEAF1 alone, suggesting that the loss of the dominant HES1-mediated repression enables full DEAF1 repression at this site. These data indicate that the 26-bp

palindrome is regulated by a series of transcription factors including bHLH family members and DEAF1 at distinct sites within the element.

The absence of an additive inhibitory effect of co-transfected DEAF1 and HES family members (Figure VI-2) suggests that these transcriptional regulators compete, rather than co-operatively regulate transcription of the 5-HT1A receptor. While basic HLH transcription factors are known to form homo- and heterodimers (Kageyama et al., 2005; Kageyama et al., 2006), we next tested the possibility that DEAF1 is also able to engage in protein-protein interactions with HES5 using yeast mating (Figure VI-3). When normalized to β-galactosidase activity of the positive control vector, pCL1, the DEAF1-HES5 interaction was found to be less than 4%, suggesting that the inhibitory bHLH proteins and DEAF1 may interact, but very weakly. These data support the idea that HES proteins and DEAF1 are not synergistic, but may compete for binding sites on the 26-bp palindrome.

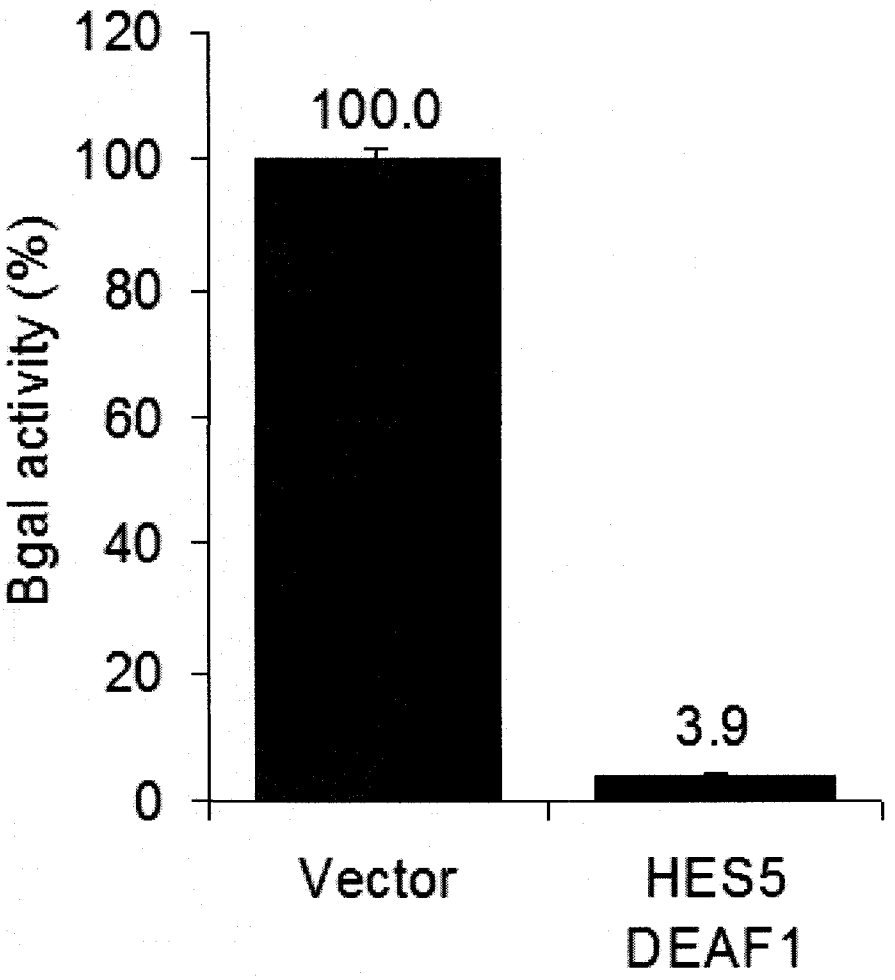

Figure VI-3: Yeast mating of Hes5 and DEAF1.

Hes5 and DEAF1 in the pACT2 and pAS2-1 yeast expression vectors do not interact significantly compared to the pCL1 positive control vector. β-galactosidase activity is normalized to positive control. Error bars represent standard error of the mean. N=3.

Enhanced 5-HT1A Receptor Expression in Hes1-/- Mice

In order to address the role of HES proteins in the regulation of 5-HT1A receptor expression *in vivo*, we examined 5-HT1A receptor expression in mice with a targeted disruption of the HES1 locus (Ishibashi et al., 1995; Ito et al., 2000). These mice exhibit severe neural defects by E13 that result in embryonic lethality (E13-E14), but provide an important tool for studying the effect of the HES1 gene deletion on early embryonic 5-HT1A receptor expression. Total RNA was extracted from E12.5 embryo hindbrain (HB), midbrain (MB) and forebrain (FB) and analyzed using quantitative real time PCR (Q RT-PCR) to determine the mRNA levels of HES1, HES5 and 5-HT1A (Figure VI-4), as well as TPH2, a marker of mature serotonin neurons that is not believed to be regulated by HES family members. HES1 mRNA was not detected in HES1-/- mice but was present in wild-type animals (Figure VI-4). HES5 mRNA levels were substantially higher in all brain regions in HES1-/- embryos, consistent with the known compensatory actions of HES family members (Hirata et al., 2001; Baek et al., 2006; Hatakeyama et al., 2006). TPH2 mRNA levels were not affected in the HES1-/- mice; however 5-HT1A mRNA was substantially higher in both the hindbrain and midbrain of HES1-/- mice, suggesting that HES1 maintains a tonic repression of 5-HT1A receptor expression that cannot be fully compensated for by HES5 or DEAF1 during early embryogenesis. In order to validate our Q RT-PCR data, we next examined 5-HT1A receptor RNA expression *in vivo* by *in situ* hybridization (Figure VI-5). As observed by Q RT-PCR, there is a substantial increase in 5-HT1A receptor mRNA in the developing hindbrain and midbrain regions of HES1-/- mice, as well as a more subtle increase in the forebrain that was not detectable by Q RT-PCR. These data demonstrate that HES1 is a critical developmental regulator of 5-HT1A receptor expression *in vivo*.

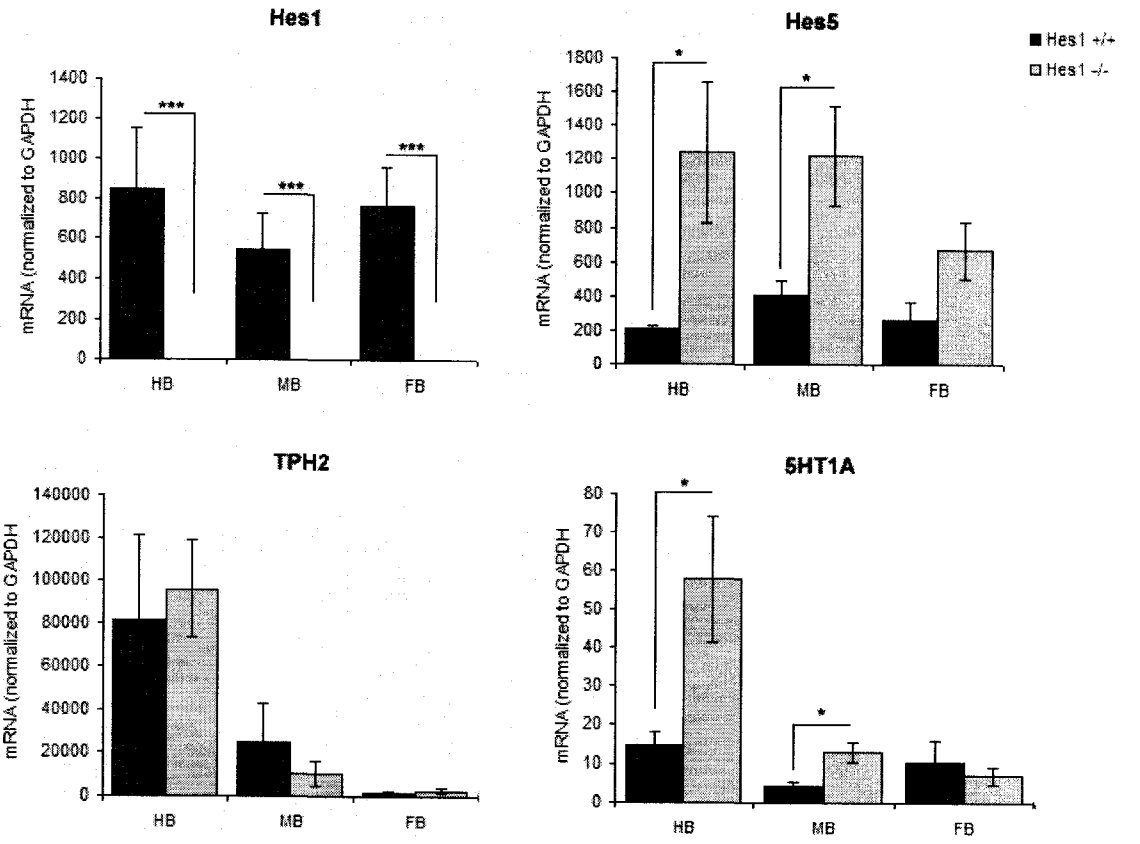

Figure VI-4: Q RT-PCR results showing mRNA expression in brain regions of E12.5 Hes1 -/- mice.

Hes1 -/- mice do not express Hes1 mRNA, confirming the genotype of these animals, and display the expected compensation by Hes5 in all brain regions. TPH2 mRNA expression is not altered in Hes1 -/- mice compared to Hes1 +/+ mice controls, and the expression is highest in the hindbrain (HB) with lower levels in the midbrain (MB) and forebrain (FB), consistent with the developmental progression of serotonin neurons at E12.5. 5-HT1A mRNA is substantially elevated in the HB and MB of Hes1 -/- mice and is not altered in the FB. In all cases, Hes1 -/- mice were compared to Hes +/+ littermate controls. Error bars represent standard error of the mean. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

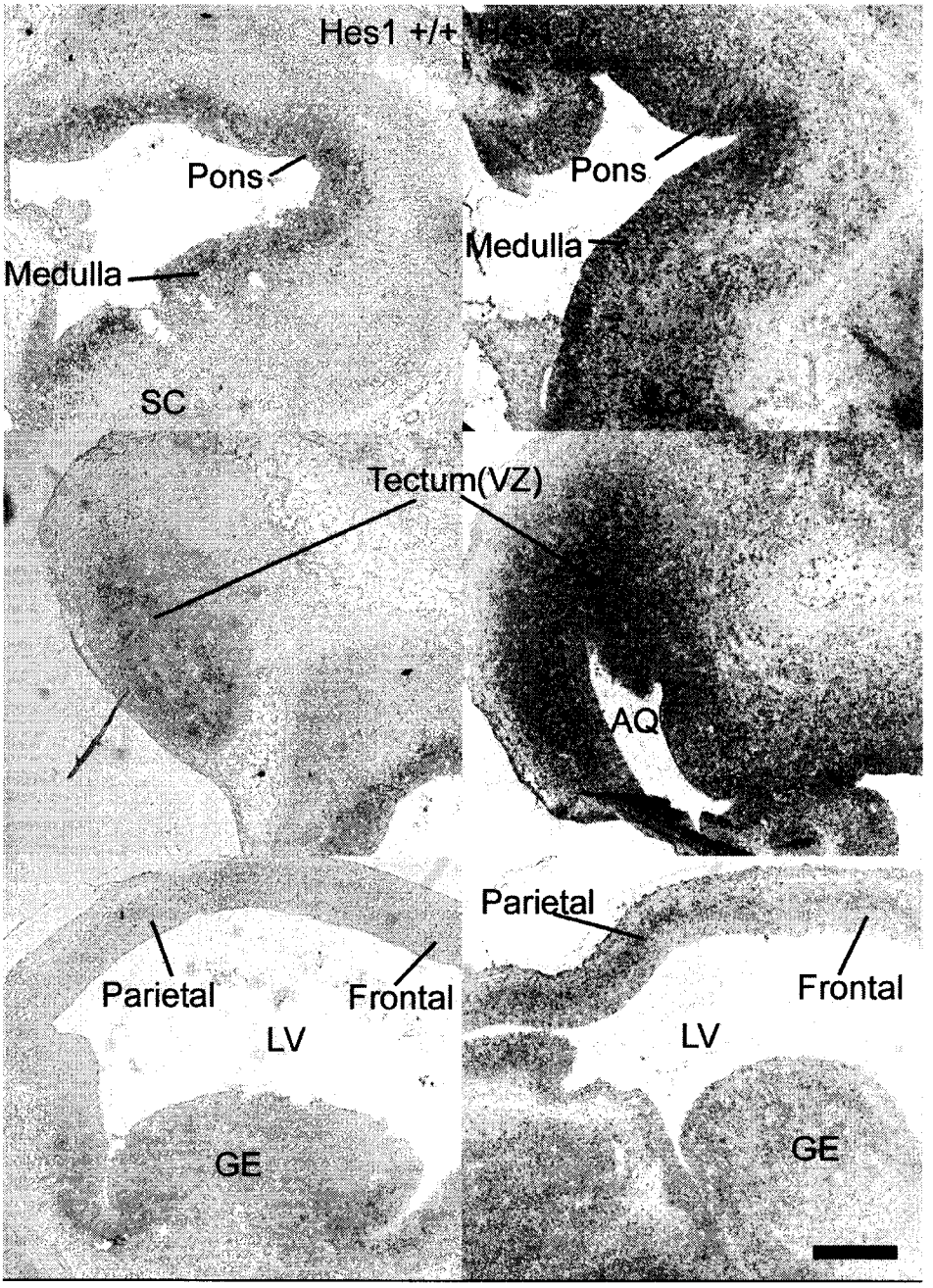

Figure VI-5: 5-HT1A mRNA in the E12.5 Hes1 -/- or +/+ mouse brain.

In situ hybridization of E12.5 sagittal sections of Hes1 -/- (right) or +/+ (left) mouse hindbrain (top panels), midbrain (middle panels) or forebrain (bottom panels) showing enhanced 5-HT1A mRNA expression in Hes1 -/- mice. Developing pons, medulla, spinal cord (SC), tectum, ventricular zone (VZ), parietal and frontal cortex, lateral ventricle (LV), cerebral aqueduct (AQ) and ganglionic eminence (GE) are labelled. Size bar represents 400 μm.

Discussion

Regulation of the 5-HT1A Receptor by Hes proteins

The findings presented here demonstrate transcriptional regulation by multiple HES family members at a functional promoter polymorphism of the 5-HT1A receptor gene. Importantly, although HES5 was initially identified to regulate this site, HES1 displayed stronger repressor activity than HES5 in the transcriptional reporter assay. HES1 repressed 5-HT1A transcription by 80-90% in 5-HT1A-expressing RN46A and SN48 cells, compared with 40-50% repression by HES5. In all cells tested, HES6 inhibited HES1- and HES5-induced repression, indicating that, as for HES1, HES6 also inhibits HES5 activity, possibly by targeting HES proteins to proteosomal degradation (Bae et al., 2000; Gratton et al., 2003; Nuthall et al., 2004; Kang et al., 2005). In RN46A and SN48 cells, HES6 alone significantly derepressed basal transcription, likely due to inhibition of endogenous HES1 and/or HES5 expressed in these cells (Lemonde et al., 2003). This is in keeping with evidence that RN46A and SN48 cells represent models of early neurogenesis (Charest et al., 1993; White et al., 1994; Shi et al., 2003), a timeframe in which HES1 and HES5 are critical for maintaining progenitors and inhibiting neurogenesis (Kageyama et al., 2006). These results suggest a possible role for HES6 in regulating 5-HT1A receptor expression during neuronal differentiation by removing HES1 or HES5 inhibition. We also observed that HES6 can block DEAF-1-induced repression (not shown), further supporting a role for HES6 in regulation of 5-HT1A gene expression, although the exact mechanism of this interaction needs to be further elucidated. Such a mechanism could be critical for proper developmental expression of 5-HT1A receptors in mid-to-late embryogenesis.

Transcriptional Regulation at a Functional Polymorphism

The C(-1019)G 5-HT1A promoter polymorphism is a well-studied polymorphism that has been associated with suicide, major depression, and anxiety disorders (Lemonde et al., 2003; Strobel et al., 2003; Huang et al., 2004; Koller et al., 2006; Wasserman et al., 2006; Kraus et al., 2007), and is associated with higher levels of presynaptic 5-HT1A receptors in depressed patients (Parsey et al., 2006). This polymorphism is embedded in a palindrome sequence, and a nuclear protein complex bound preferentially to the C-allele, suggesting a functional role of this site in regulating 5-HT1A receptor expression (Lemonde et al., 2003). Yeast-one-hybrid screening identified both DEAF1 and HES5 as proteins that specifically bind to the C(-1019) allele to repress 5-HT1A receptor transcription, but were inactive at the G(-1019) allele (Lemonde et al., 2003). The DEAF1 binding site conformed to an inverted consensus TTCG (Huggenvik et al., 1998), with the G-allele changing this to TTCC, and hence completely blocking DEAF1 binding and function; however the specific site of HES5 activity was not identified. In the current study we have defined the HES5 site, and shown that it is required for HES family protein activity. The 26-bp palindrome contains the sequence CACTCG, a sequence resembling a class C site (CACGCG) or related N-box (CACNAG) (Murata et al., 2005). Our results demonstrate that the N-box found within the 26-bp palindrome in the human 5-HT1A promoter is essential for HES1 and HES5 repressor activity. Although most bHLH transcription factors, including HES1, are known to function through E-box dependent (CANNTG) mechanisms, HES1 is known to have a stronger affinity for the class C site and HES5 for the classical N box (Nakatani et al., 2004; Kageyama et al., 2005), and transcriptional repression of the 26-bp construct is lost when the N-box is mutated. Interestingly, while HES5 repression was completely lost in the presence of the G-allele,

HES1 maintained its repressor ability at the G-allele, consistent with the distal location of the C(-1019) site relative to the N-box, and with the stronger activity of HES1 compared to HES5. Thus, the presence of the G(-1019) allele would be expected to have minimal effect on regulation of the 5-HT1A receptor by HES1/HES6, while regulation by HES5 and DEAF1 is blocked. The differential activity of HES1 and HES5 at the G(-1019) allele is interesting because it indicates 1) a stronger repression by HES1 that is not affected by the G(-1019) allele and 2) a steric inhibition of HES5 activity by the G(-1019) allele, possibly by inhibiting interaction of the HES5 heteromeric complex with the DNA. The reduced repression by HES5 and DEAF1 at the G(-1019) allele could explain the specific upregulation in raphe 5-HT1A receptor binding observed by PET imaging in depressed subjects homozygous for the G(-1019) allele (Parsey et al., 2006), as well as lowered responsiveness to antidepressant drugs such as fluoxetine (Lemonde et al., 2004b; Serretti et al., 2004; Hong et al., 2006a). The G(-1019) allele may have its greatest impact on 5-HT1A receptor expression in early post-natal to adult development when HES1 expression is down-regulated and DEAF1 remains strongly expressed. Reactivation of HES1 could provide a mechanism to overcome the effect of the G(-1019) allele to block DEAF1 or HES5 repression.

DEAF1 is a well characterized regulator of 5-HT1A receptor gene transcription, acting through a minimal consensus (forward TTCG or reverse AAGC) which contains the C(-1019)G polymorphism, and is located immediately adjacent to the N-box within the 26-bp polymorphic element (Lemonde et al., 2003). As a result, we next sought to examine coordinated regulation of 5-HT1A transcription by DEAF1 and HES proteins. The results presented here demonstrate a very strong repression by DEAF1 (extent of repression DEAF1 > HES1 > HES5) at the C-allele. Interestingly, co-transfection of

DEAF1 with HES1 leads to repression levels resembling HES1, while co-transfection with HES5 leads to levels resembling DEAF1. One explanation could be that the relative affinity for the C-allele of the 5-HT1A palindrome is HES1>DEAF1>HES5, although further studies would be required in order to determine binding site affinity. In previous studies we found that DEAF1 has strong enhancer activity in SN48 cells, which was not blocked by co-transfection of HES5 (Czesak et al., 2006). This is consistent with the greater apparent affinity of DEAF1 over HES5 for the C-allele palindrome. By contrast, at the G-allele, only HES1 displayed repression. Thus, the 5-HT1A palindrome is regulated by HES1, HES5 and DEAF1, and their relative efficacy for repression is greatly dependent on the allele.

The possibility that DEAF1 and HES family members are able to interact in order to mediate their effects was also tested. HES bHLH proteins are able to homo- or heterodimerize through their bHLH domains, with the specificity of these interactions being conferred by the Orange domain (Kageyama et al., 2006). DEAF1 contains several conserved domains including a SAND domain (also found in Sp100 proteins) containing the conserved KDWK sequence implicated in DNA binding, a zinc-binding MYND domain that mediates repression, and a myc-like HLH domain that mediates dimerization of DEAF1 (Bottomley et al., 2001; Wojciak and Clubb, 2001). Yeast mating, however, clearly demonstrated a very weak interaction between HES5 and DEAF1, which, along with the co-transfection studies, suggests that these transcriptional repressors may compete, rather than cooperatively bind to, the 26 bp palindrome. It is important to note that this interaction between HES and DEAF-1 would occur primarily in embryonic or early post-natal development, as the expression of HES family proteins is largely down-regulated in adult, with the exception of the subgranular zone of the hippocampus.

Critical Role of Hes1 in the Developmental Regulation of 5-HT1A Receptor Expression

The evidence of widespread induction of 5-HT1A receptor expression in HES1-/- mice demonstrates a role for HES1 in the regulation of 5-HT1A receptor mRNA expression during early neurogenesis. Studies by Kageyama and collaborators using single, double and triple knock outs of HES1, HES5 and HES3, have illustrated that these inhibitory HES family members are able to compensate for each other in single knock out experiments (Hirata et al., 2001; Ohtsuka et al., 2001; Baek et al., 2006; Hatakeyama et al., 2006). In line with this, we observed a substantial increase in HES5 levels in the hindbrain, midbrain and forebrain of HES1-/- compared to wild type control. We also observed a significant increase in 5-HT1A receptor mRNA levels in the hindbrain and midbrain of HES1-/- mice, suggesting a role for HES1 in the repression of 5-HT1A receptor transcription that is lost in its absence. Even the significant increase in HES5 in these regions was unable to reduce the 5-HT1A levels to that of the wild type littermates, strongly implicating HES1 as a critical regulator of 5-HT1A receptor transcription during early development.

In order to examine whether the alterations in 5-HT1A receptor mRNA levels were due to premature neurogenesis of serotonergic raphe cells in HES1 -/- mice, we also examined TPH2, a marker of mature 5-HT neurons (Walther et al., 2003; Czesak et al., 2007; Lenicov et al., 2007) and found no difference between wild type and knock out mice. This result suggests that the increase in brainstem and midbrain 5-HT1A mRNA is due to increased expression (or decreased repression) in non-serotonergic precursors, rather than premature neurogenesis of serotonin neurons. As the serotonin system is one of the earliest neuronal populations to differentiate and project throughout the brain (Lauder, 1990; Hillion et al., 1993), the premature neurogenesis observed in HES1 null

mice may not impact the developing serotonin system as substantially as later-developing cell populations. Interestingly, in addition to an increase in 5-HT1A mRNA levels observed in HES1 null mice, we further observed an apparent expansion of 5-HT1A mRNA localization in the hindbrain and midbrain (Figure 4). Given the premature neurogenesis and disruption of neural tube morphogenesis seen in HES1 null mice (Ishibashi et al., 1995), this expansion of 5-HT1A-positive cells could reflect an inappropriate expansion or migration of precursors that do not express TPH2, beyond their wild type boundaries, and a premature increase in 5-HT1A RNA due to lack of HES1-mediated repression. These data clearly demonstrate that HES1 is essential for the normal developmental expression of 5-HT1A receptors, and suggest that the decreasing expression of HES1 as neurogenesis proceeds regulates the spatio-temporal appearance of 5-HT1A receptors. Although the role of the HES proteins in direct regulation of 5-HT1A transcription via the 26-bp palindrome suggests a direct action of HES1 on 5-HT1A receptor transcription *in vitro*, indirect mechanisms via induction of other proneural regulators may also contribute. It is possible that the dysregulation of proliferation in HES1 null mice (Ishibashi et al., 1995; Hirata et al., 2001) alters the expression of other relevant regulators that may also contribute to the increase in 5-HT1A receptor expression in these mice. The importance of HES1 in the early developmental regulation of 5-HT1A receptors, however, is clearly shown by its upregulation in the HES1 null mice, suggesting a role for HES1 in the development and maintenance of a normal behavioural phenotype.

Summary

The current work demonstrates coordinated regulation of 5-HT1A receptor transcription by bHLH proteins at the 5-HT1A palindrome sequence that contains a functional polymorphism. HES1 and HES5 are potent inhibitors of 5-HT1A transcription, whose repressor activities are inhibited by HES6, while DEAF1 is a cell-specific regulator that is also blocked by HES6. While DEAF1 acts through the TTCG (CGAA) motif containing the C(-1019)G polymorphism, HES1 and HES5 act through a putative N-box also found in this 26-bp palindrome. Unlike DEAF1 and HES5, HES1 maintains its repressor ability in the presence of the G-allele, a feature that underscores overlapping and sometimes functionally redundant actions of these two HES proteins. Elevated 5-HT1A mRNA levels in HES1 null mice suggest an essential role for HES1 in the early developmental repression of 5-HT1A transcription. As 5-HT1A receptor expression in late embryonic / early postnatal development is critical for normal affective phenotype (Gross et al., 2002), a finely-tuned coordinated transcriptional regulation would be important for developmental 5-HT1A receptor expression. HES1 and HES5, which are expressed exclusively in undifferentiated neurons, may inhibit expression of 5-HT1A receptors during early neurogenesis, only allowing 5-HT1A receptor expression as neurogenesis proceeds.

Acknowledgments

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CHAPTER VII: Discussion

In Canada, mental illness touches everyone. Twenty percent of Canadians will develop a mental illness at some point in their lives, while the remaining eighty percent will have a family member who is affected (Health_Canada, 2002; Government_of_Canada, 2006). These disorders are caused by a combination of polygenic and environmental factors, making it challenging to draw meaningful conclusions from isolated molecular studies (Leonardo and Hen, 2006). For this reason, it is essential to regard the systems that regulate mood and emotion as a whole when considering affective dysfunction. This thesis attempts to address the global nature of mental illness and affective disorders by (1) examining how the circuitry that directs emotional and behavioural responses is wired, and (2) by investigating how the critical molecular determinants of this 'fear circuitry' contribute to its function and dysfunction.

PART ONE: Mapping the Monoamine Fear Circuitry

Gating Fear Responses in the Amygdala

The first part of this thesis is focussed on examining how behavioural responses might be modulated by the amygdala. Initial examination of the ICM, which are known to be GABAergic clusters of neurons responsible for gating communication between the BLA (input) and the Ce (output) (Pare et al., 2003), revealed a dense distribution of the dopamine D1 receptor in the ICM (Figure II-1). By contrast, while the Ip also displayed a high density of DA terminal IR input (TH+/DBH-), terminal input to the larger Im was restricted to the rostrolateral aspects (Figure II-3). This raised the possibility that amygdaloid behavioural responses such as fear may be modulated by a combination of fast synaptic DA signalling via the D1 receptors in the rostrolateral Im and slower

diffusion-based activation of D1 receptors in the caudal and rostromedial Im (see Figure VII-1 for illustration). We proposed a model in which unnecessary fear responses are tonically inhibited by DA VT to the caudal and rostromedial GABA neurons projecting to the Ce. In response to an appropriate danger stimulus, however, this tonic inhibition can be overridden by the strong synaptic stimulation of the rostrolateral GABA neurons. This is consistent with the finding that the ICM have slowly deinactivating K^+ channels that are hyperexcitable and may be involved in this tonic inhibition of Ce outputs (Royer et al., 2000a). Importantly, DARPP-32 IR was also restricted to the regions receiving DA-like terminal input (Figure III-4), suggesting that DARPP-32 is especially important for DA-mediated synaptic transmission, but not for DA-mediated VT.

That fear responses might be gated by differential activation of GABAergic clusters of cells within the amygdala lead us to consider that a similar mechanism might be present throughout the extended amygdala as well. The CEA is of prime interest in studying fear and emotion, and represents a rostral extension of the amygdala via the BSTL, IPACM and AcbSh (Figure I-4) (Koob, 2003a). As seen in the amygdala proper, we discovered D1-rich clusters of GABAergic cells within the IPACM, the portion of the IPAC that belongs to the extended amygdala (Figure III-3). These receptor rich patches contained very sparse DA-like terminal input, suggesting that, as with the Im, a tonic inhibitory gating mechanism may be involved in gating extended amygdala communication. Interestingly, previous work has identified similar D1-rich patches with little or no terminal input in the AcbSh (Jansson et al., 1999) and islands of Calleja (Diaz et al., 1995), both of which are important components of the fear circuitry. Although it is not known whether the D1-rich clusters within the AcbSh are GABAergic, the islands of Calleja, which are associated with the olfactory component of the limbic system, are rich

in both GABA and Glutamate receptors, and contain a dense terminal innervation by GABA (Smith et al., 1987; Bischoff et al., 1997; Riedel et al., 1998).

The presence of GABA-rich clusters throughout the extended amygdala lead to the hypothesis that an integrated system of inhibitory GABAergic interneurons serves to modulate extended amygdala outputs in a similar manner as in the ICM. We next sought to identify whether other neurotransmitter systems might be involved in modulating this gating mechanism. We identified a similar distribution of MOR1 IR throughout the Ip (Figure III-6), Im (Figure III-5), IPAC (Figure III-7), islands of Calleja and AcbSh (Figure VIII-1) with little or no β-endorphin or ENK-like terminal input. Especially interesting was the observation that, while the DA-like input to the Im was isolated to the rostrolateral aspects, ENK-like input was isolated to the rostromedial Im (Figure III-3, Figure III-5). This implies that DA and ENK play opposing roles in the Im, differentially modulating the inhibition of Ce or Me outputs. On the other hand, the presence of GABAergic clusters throughout the extended amygdala that are rich in both D1 and MOR1 is suggestive of an integrated system whereby the balance of activity of these two receptors provides a critical gating mechanism for regulating amygdaloid responses such as fear. Furthermore, the combined actions of fast synaptic transmission and slower VT may provide a simple mechanism for a single neurotransmitter system to regulate tonically and phasically different components of the same pathway. That is, tonic inhibition by VT throughout the extended amygdala, while preventing unnecessary amygdaloid outputs during basal conditions, may effectively prime the amygdala for appropriate fear-related responses by providing a relatively simple 'on switch' (disinhibition of the Ce).

Based on these studies, a spatially complex picture of the extended amygdala is beginning to emerge. Two corridors of neurons representing the MEA and CEA extend rostrally starting at the amygdala proper (Me and Ce, respectively) and ending in the nucleus accumbens (core and shell, respectively) (Alheid et al., 1994, 1995; Alheid, 2003). Based on its input from other amygdala structures, such as the BLA, as well as the thalamus and cortex, and on its projections to the hypothalamus and brainstem, the CEA is thought to be of primary importance in regulating fear and emotion (Alheid et al., 1995; Shammah-Lagnado et al., 2000; Koob, 2003b). The CEA has a large volume of DA input, as well as having a great numbers of GABAergic interneurons that project primarily within the CEA (Freedman and Cassell, 1994). In addition, there is very little DAT within the CEA, suggesting that, unlike 5-HT, which is accompanied proportional numbers of 5-HTT, DA functions primarily by VT in this region (Freedman and Shi, 2001). Within this corridor of neurons are a series of GABAergic clusters of cells that we propose receive low-level tonic VT stimulation via D1 and MOR1, and possibly other receptor systems as well. This would provide a sensitive mechanism for modulating amygdala function.

While this is only one potential model of how communication between the input and output structures of the amygdala might be gated, a number of collaborative studies have validated and expanded upon this model. Studies with the labs of Dr. Miguel Perez de la Mora and Dr. Kjell Fuxe illustrated that microinjection of a specific dopamine D1 receptor antagonist near the rostal Im decreased anxiety, presumably by blocking the fast synaptic GABA-mediated disinhibition of the Ce (see Figure VII-1) (de la Mora et al., 2005). More recently, we examined the CCK/Gastrin-2 receptors, which are densely localized to the GABAergic neurons in the ICM (de la Mora et al., 2007). CCK is a neuropeptide that co-localizes to DA neurons and has been shown to modulate dopamine

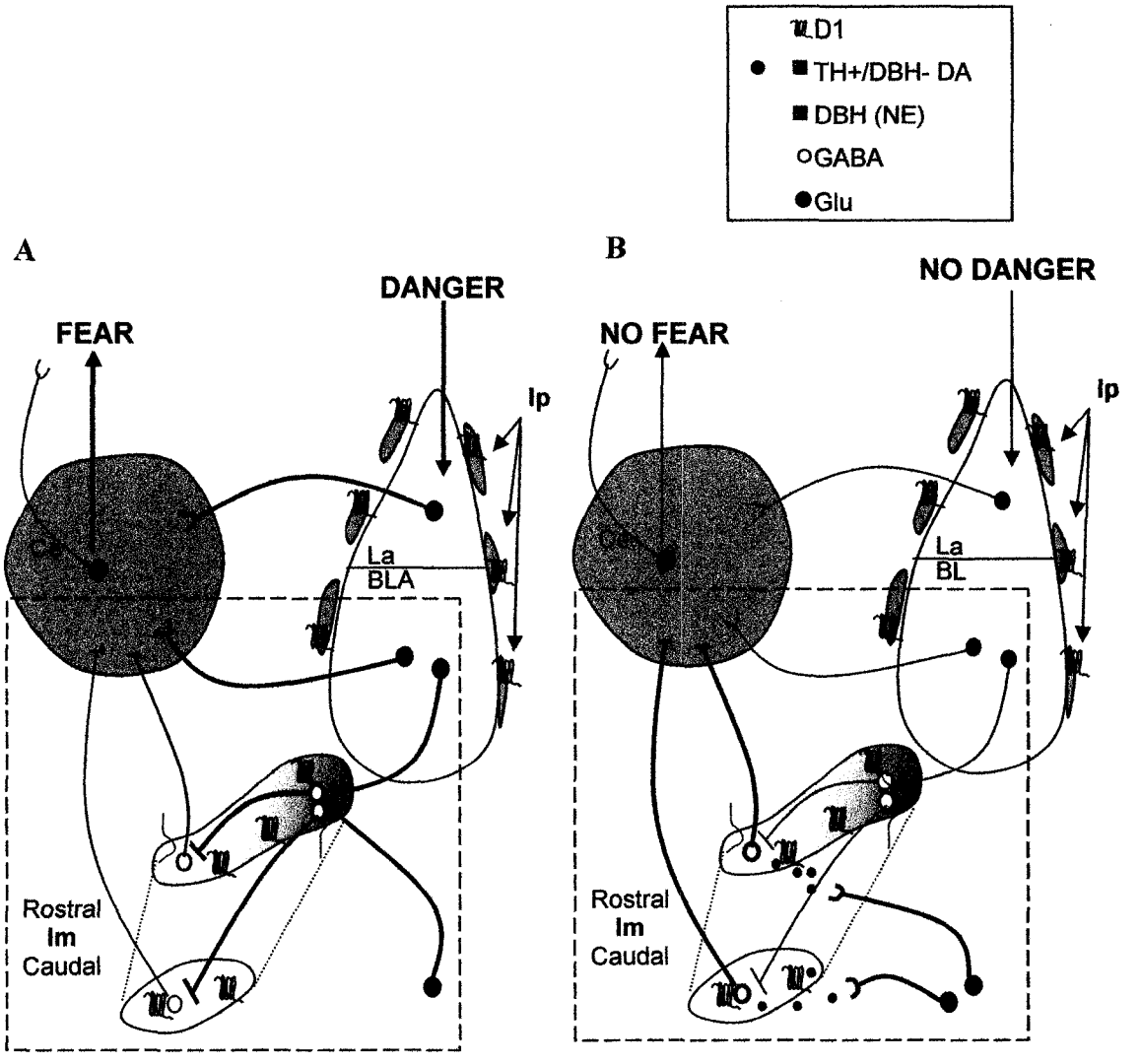


Figure VII-1: Gating of Fear Responses in the Amygdala

Model depicting the location of the dopamine D1 receptors in relation to TH+/DBH- nerve terminals in the ICM of the amygdala. (A) In response to an appropriate 'danger' stimulus, fast synaptic activation of the rostrolateral Im results in disinhibition of the Ce, which, along with direct glutamatergic stimulation of the Ce enables an immediate fear response. (B) Diffusion of DA to the rostromedial Im leads to inhibition of the Ce, tonically dampening fear output. Lateral is to the Right. Updated from (Fuxe et al., 2003a).

receptor activation, making CCK, as well as interactions between the CCK/DA receptor systems, extremely interesting in understanding and treating neuropsychiatric disorders (Agnati et al., 1985; Nair et al., 1985; Wang and White, 1985; Fuxe et al., 1995; van Megen et al., 1996). Not only does CCK IR mimic the pattern of DA IR terminals in the Im, but microinjection of either CCK-4 or CCK-8S in this region reduced anxiety (de la Mora et al., 2007). This anxiolytic affect is blocked both by a specific CCK/Gastrin-2 receptor antagonist and by a glutamatergic AMPA [(±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid]/kainate receptor antagonist, suggesting that CCK modulates the glutamate-GABA interaction in the amygdala. A further examination of the metabotropic glutamate receptor 5 (mGluR5), which was localized to the BLA and Ce, demonstrated that microinjection of the mGluR5 antagonist MPEP (2-methyl-6(phenylethenyl) pyridine) in the rostral amygdala also had anxiolytic effects, most likely by reducing the direct activation of Ce outputs by glutamate (Perez de la Mora et al., 2006). See Table VIII-1 in the appendix for a summary of the fear circuitry anatomy within the amygdala and extended amygdala.

While the anatomy is complex, the take home message of all of these studies is that there are multiple levels of control in the gating of emotionally relevant stimuli to the amygdala and the subsequent behavioural outputs. The bare bones of this circuitry involve glutamatergic stimulation both of the Ce (output center) and ICM (gating structures) along with multiple sets of inhibitory GABAergic interneurons and projection neurons which enable this system to be activated or deactivated in a spatio-temporal manner. The fine-tuning occurs at the level of the numerous neurotransmitter systems able to modulate the glutamate-GABA mechanism, namely the monoamines (especially DA) and neuropeptides (i.e. CCK, ENK). Taking into account the extended amygdala

structures, however, a number of questions arise. Within the extended amygdala (Acb, IPAC, islands of Calleja, etc) do the GABAergic clusters receive glutamatergic afferents as in the ICM? The presence of mGluR5 glutamate receptors in the islands of Calleja (Bischoff et al., 1997) and our own observation that mGluR5 IR is dense within the IPACM (Perez de la Mora et al., 2006) suggests that they do, although further studies are required. Furthermore, whether the ICM are interconnected with the CEA GABA clusters is also not known. Such a network would suggest that the clusters within the AcbSh and IPACM play an analogous gating role to the ICM. Also, identifying what structures project to the GABAergic patches within the IPACM, AcbSh and islands of Calleja will also shed light on their functional significance. These questions will need to be addressed in order to fully comprehend how the communication between amygdaloid input and output structures is gated along the length of the extended amygdala as well as their functional implications in controlling fear and emotion.

Early Life Events Direct Affective Development in the Fear Circuitry

The idea that affective disorders have an underlying genetic component is based on a number of founding observations. Anxiety behaviour in humans is relatively consistent throughout an individual's lifetime, with high and low anxiety phenotypes often presenting during early childhood (Van Ameringen et al., 1998; Kagan and Snidman, 1999; Schwartz et al., 1999). Twin studies and the comorbidity of anxiety and depressive disorders further suggest that there is a common underlying genetic contribution to disorders of the monoamine fear circuitrycircuitry (Bulik et al., 2000; Sullivan et al., 2000; Hettema et al., 2001). These predisposing genetic factors are thought to interact

with their environment, meaning that early life events, both genetic and environmental, are particularly important in determining affective phenotype.

In 1951, child psychoanalyst, John Bowlby proposed what is now called the maternal deprivation hypothesis (Bowlby, 1951). He discussed the importance of a warm, intimate and continuous relationship with a permanent mother figure, and hypothesized that breaking this bond would seriously and irreversibly impact emotional, social and intellectual development.

> *Mother love in infancy and childhood is as important for mental health as are vitamins and proteins for physical health.* - John Bowlby, 1953

Inspired by John Bowlby, American psychologist Harry Harlow initiated a series of controversial studies examining the effects of maternal deprivation on rhesus monkeys between 1963 and 1968 (Harlow, 1965; Seay and Harlow, 1965; Harlow and Suomi, 1971). Infant monkeys separated from their mothers were housed in total isolation for up to 24 months, and displayed permanent emotional and behavioural problems even after 3 months of isolation. Even when housed in partial isolation, where they were able to see, hear, smell, but not touch other animals, monkeys developed severe social, emotional and behavioural deficits. Despite criticism at the time, both in terms of the shear cruelty of these experiments and their relevance to humans, there are many comparable examples of maternal deprivation syndrome in children caused by failure to provide adequate care, poverty and abuse (Rutter, 1979; Garfinkel et al., 1982; Reite, 1987). These early life traumas are commonly associated with an increased risk of developing a mood disorder in adulthood (Felitti et al., 1998; Chapman et al., 2004).

One of the primary effects of early life experiences such as maternal deprivation is on the HPA axis (Levine, 2005). In fact, of late, there is a trend towards re-labelling this stress-response system the LHPA, reflecting the substantial contribution of the limbic (L) system in the regulation of the HPA hormonal cascade. In particular, the monoamine neurotransmitters (DA, NE, 5-HT) have been implicated in the control of the LHPA axis, especially in terms of the disruption of monoamine transmission and HPA axis dysregulation that are characteristic of stress and depression (McLeod et al., 2001; Cubala and Landowski, 2006). Varying paradigms of neonatal or early life trauma have revealed that the monoamine fear circuitry is highly sensitive to environmental stimuli during the critical early developmental period. This is especially relevant for genes such as the 5-HT1A receptor, whose expression is required during embryonic and neonatal development in order to establish a normal anxiety phenotype (Gross et al., 2002) and is especially sensitive to early life trauma (Fish et al., 2000; Vazquez et al., 2000; Weller et al., 2003; van Riel et al., 2004; Vicentic et al., 2006; Arborelius and Eklund, 2007; Lee et al., 2007). Likewise, early life trauma is associated with increased DA response to stress, as well as alterations in postsynaptic dopamine D1- and presynaptic D2-receptor expression and signalling (Meaney et al., 2002; Ploj et al., 2003; Ziabreva et al., 2003). These studies indicate that the monoamine fear circuitry is responsive to environmental stimuli during defined periods of time in development. In many ways, the critical genetic determinants of mood and emotion may be viewed as susceptibility genes for fear circuitry dysfunction. It is, therefore, important to examine the regulatory mechanisms that drive the formation of the fear circuitry during this critical developmental timeframe in order to understand how early life events might predispose an individual to affective disorders.

PART TWO: Critical Developmental Regulators of the Fear Circuitry

The second part of this thesis focused on some of the key regulators that govern the monoamine fear circuitry development. We first examined *Nurr1* and *Pet-1*, which perform analogous roles in directing development and maturation of DA and 5-HT neurons, respectively (Cheng et al., 2003; Smidt and Burbach, 2007). One unique feature of Pet-1 is its apparent role as a transcriptional regulator of the 5-HT1A receptor gene, which is implicated in anxiety, depression and related affective disorders (Zhuang et al., 1999; Albert and Lemonde, 2004; Gross and Hen, 2004). We next examined another transcriptional regulator of *5-HT1A*, Hes5, as well as its closely related family members Hes1 and Hes6. Unlike *Nurr1* and *Pet-1,* which are restricted in expression to DA and 5-HT neurons, respectively, Hes family members tightly control neurogenesis, with Hes1 and Hes5 inhibiting neuronal differentiation and repressing pro-neural genes, and Hes6 promoting neurogenesis (Kageyama et al., 2006). The interconnectivity of these transcriptional regulators is summarized in Figure VII-2. In order to provide a more detailed discussion of the results presented in this thesis, we will examine each factor in isolation; however, the degree of interconnectivity between the 5-HT and DA systems is such that even a slight modification at any point in this large cascade can have implications for the entire monoamine fear circuitry.

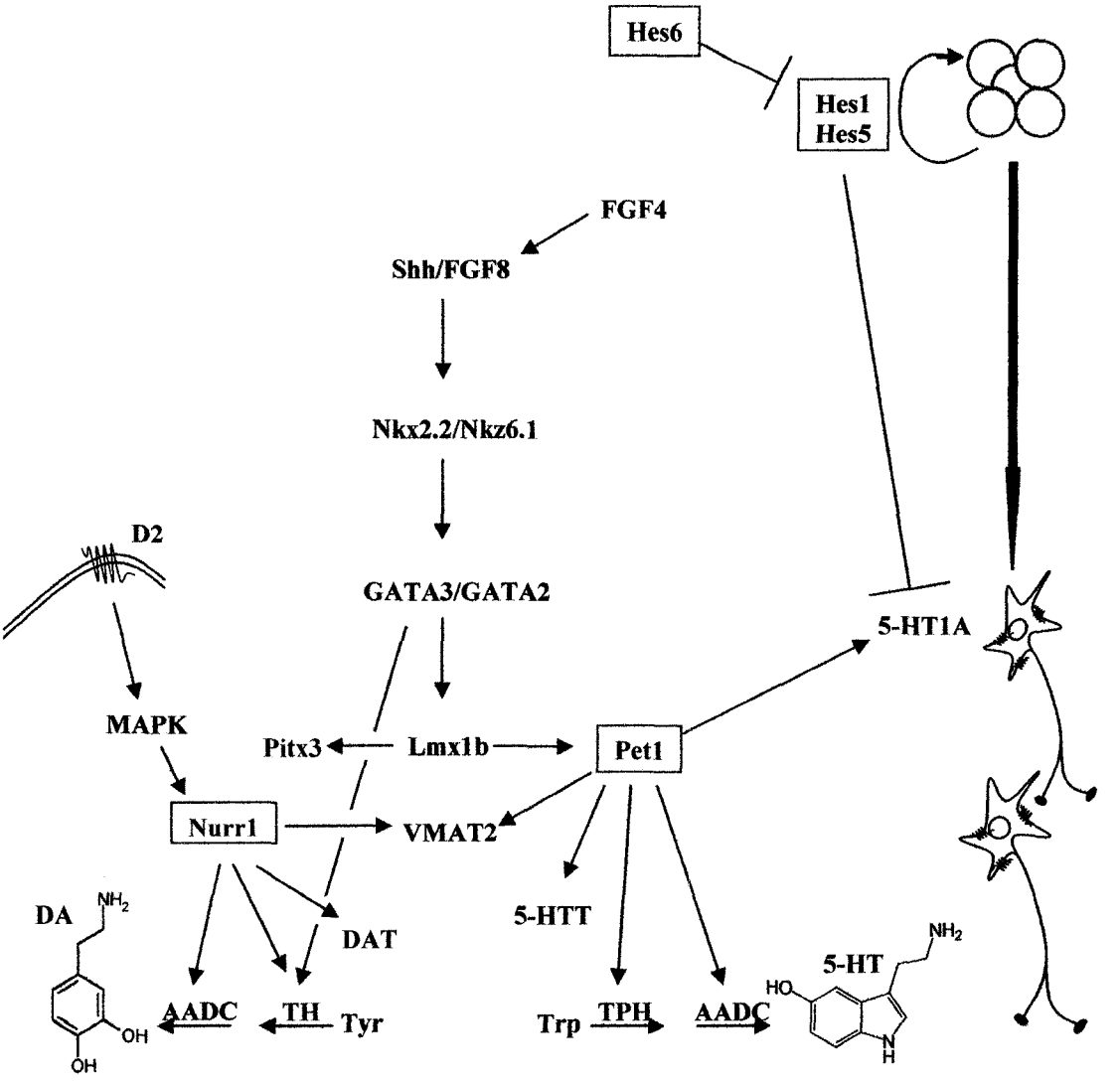


Figure VII-2: Summary of the Key Developmental Regulators of the 5-HT and DA Systems

Schematic diagram illustrating the common (black) and unique (DA=blue, 5-HT=purple) pathway components involved in directing the development and maturation of DA and 5-HT neurons. Nurr1 and Pet1 fill analogous roles in DA and 5-HT neurons, respectively, regulating expression of factors involved in regulating neurotransmitter phenotype and signalling, as well as being required for neuronal differentiation and maintenance. Hes1 and Hes5 maintain neural progenitors and inhibit *5-HT1A* receptor expression embryonically. Coincident with neuronal differentiation and decreasing levels of Hes1/Hes5, *5-HT1A* receptor expression increases along with Hes6.

NURR1

The orphan nuclear receptor, NURR1, is expressed primarily in the DA neurons of the VTA and SN, and is required for DA neuron differentiation, expression of factors involved in DA synthesis (*TH* and *AADC*), as well as DA signalling (*DAT*, *VMAT2*) (Smidt et al., 2003; Smits et al., 2003; Chinta and Andersen, 2005; Jankovic et al., 2005). Using transfected SK-N-AS cells, which are a neuroblastoma cell line that model presynaptic DA neurons, we demonstrated that NURR1 is able to activate transcription through an NBRE identified in the *TH* gene promoter (Figure IV-2). Previously, a point mutation was identified in a patient with late onset Parkinson's disease that causes a Ser125Cys amino acid change proximal to the AF-1 core of NURR1 (Grimes et al., 2006). This mutant variant of NURR1 is unable to activate transcription through the NBRE, suggesting that Serine125 residue is important for NURR1 transcriptional activity. Examination of the NURR1 protein sequence revealed that this mutation is immediately adjacent to a putative ERK phosphorylation site (Figure IV-1), and ERK activation has been shown to positively regulate NURR1 (Kovalovsky et al., 2002; Nordzell et al., 2004; Kim et al., 2006).

Previous work in our lab has examined the cell-type specific coupling of the D2S autoreceptor to ERK activation. In order to determine whether the transcriptional activity of NURR1 is regulated by ERK, we co-transfected SK-N-AS cells with vectors encoding the D2S receptor, the TH NBRE luciferase construct and NURR1 (Figure IV-3). Although SK-N-AS cells express *TH, NURR1, DAT* and the *D2S* autoreceptor (Michelhaugh et al., 2005; Wang and Bannon, 2005), our attempts to study the D2S-ERK-NUR1 pathway in cells transfected only with the TH NBRE construct were unsuccessful. As with a number of other cell types (Luo et al., 1998; Welsh et al., 1998),

in SK-N-AS cells, agonist stimulation of the D2S receptor increased ERK1/2 phosphorylation. Agonist stimulation also increased activation of transcription through the *TH* NBRE by NURR1. The specific MEK1 inhibitor, PD98059, completely blocked both the D2-stimulated phosphorylation of ERK1/2 and the transcriptional activation by NURR1, suggesting that NURR1 transcriptional activity is positively regulated by ERK signalling. While the D2-stimulated activation of ERK signalling was maintained in the presence of the Ser125Cys NURR1 mutant, agonist stimulation of the D2S receptor did not result in increased *TH* NBRE reporter transcription, nor did pre-treatment with PD98059 alter transcriptional activity, suggesting that the D2-stimulated activation of NURR1 by ERK requires Ser125. Based on these results, we stipulated that ERK activation leads to phosphorylation of Ser125, and that this is required for NURR1 transcriptional activity. Importantly, an identical NBRE sequence is present in *DAT* gene promoter (Kim et al., 2003), which suggests that an ERK-dependent mechanism may also regulate the activation of DAT by NURR1.

Although the patient identified in this study is heterozygous for the Ser125Cys NURR1 mutation and still retains one normal copy of the NURR1 gene, it is likely that this mutation contributed to the onset of PD in combination with other genetic or environmental factors. Our studies suggest that the mutant copy of NURR1 would be unable to activate *TH* transcription, decreasing the overall expression of this gene. Given that TH is the rate-limiting enzyme in the synthesis of DA, even a subtle decrease in *TH* levels might substantially reduce the production of DA. Certainly, the link between NURR1 and PD is not new. Several studies have linked NURR1 SNPs and other variants to PD (Xu et al., 2002a; Jankovic et al., 2005; Michelhaugh et al., 2005). NURR1 levels are also substantially reduced in individuals with PD and other Parkinsonian syndromes,

correlating with the loss of TH in PD, and measuring NURR1 mRNA levels in the blood may be a useful tool in the early diagnosis of such diseases (Chu et al., 2006). NURR1 may also be a potential target in the treatment of PD. Constitutive expression of *NURR1* in embryonic stem cells upregulated virtually all markers of DA neurons, including DA, *TH, DAT, AADC* and *VMAT2*, but not markers of other neuronal types such as 5-HT and GABA (Chung et al., 2002). In addition their electrophysiological and behavioural properties are as would be expected for midbrain DA neurons (Kim et al., 2002), making them extremely interesting for potential stem cell therapy strategies. In addition, our observation that D2S activation can stimulate NURR1 transcriptional activity may provide a tool for inducing *NURR1* expression in patients with PD. A number of NURR1-like agonists that can mimic NURR1 transcriptional activity are also being tested in animal models of PD (Jankovic et al., 2005). These molecules are able to cross the blood-brain barrier and remain bioavailable for prolonged periods of time, and may be of clinical importance in the near future.

In addition to PD, NURR1 has been linked to a number of other neuropsychiatric disorders. Mutations in *NURR1* have been identified in patients with schizophrenia and manic-depressive disorder that reduce the transcriptional activity of NURR1 (Buervenich et al., 2000). Long term cocaine abuse also leads to significant decreases in *NURR1* expression within DA neurons, and the involvement of NURR1 in regulating *DAT* expression suggests an adaptive response as a result of the prolonged blockage of DAT by cocaine (Bannon et al., 2002). The critical role of *NURR1* in DA neuron development also concretely links it to the regulation of the monoamine fear circuitry. While NURR1 is essential for the induction and survival of DA neurons, and mice lacking this gene die shortly after birth (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998), NURR1

heterozygous mice survive and appear normal, making them an interesting model in which to study NURR1 function (Eells et al., 2002). NURR1 +/- mice display reduced mesocortical and mesolimbic DA and increased locomotor behaviour in response to mild stress, implicating NURR1 in the dopaminergic regulation of the fear circuitry. NURR1 +/- mice further display a reduced retention of emotional memories, an elevated response to forced swim stress, and gender-specific alterations in DA turnover, providing insight into schizophrenia and related disorders of the monoamine fear circuitry (Rojas et al., 2007). Although future studies will need to specify the precise role of NURR1 in all aspects of DA neuron development, including differentiation, pathfinding and maintenance, the clearly defined role of NURR1 in maturation (i.e. expression of dopaminergic markers) makes it a valuable target for treating DA diseases.

Pet-1

We next examined Pet-1, a critical developmental regulator of the serotonin system that plays a largely analogous role to that of NURR1 in the dopamine system. Pet-1 is also important for expression of factors involved in 5-HT synthesis (TPH, AADC) and signalling (5-HTT, VMAT2), and Pet-1 binding elements have been identified in all of these genes (Hendricks et al., 1999; Hendricks et al., 2003). Knockout studies were fundamental in discovering the involvement of NURR1 in terminal differentiation and maintenance of DA neurons; however, NURR1-null mice die shortly after birth due to their inability to suckle and poor motor function, making it impossible to study the role of NURR1 in the affective component of the DA system in these mice (Witta et al., 2000; Smits et al., 2003). On the other hand, while Pet-1 null mice display analogous deficits in 5-HT neuron development, their survival is not compromised, although they do display a

highly anxious and aggressive phenotype (Hendricks et al., 2003). Their affective phenotype, along with the severe reduction in 5-HT in Pet-1 null mice (70-80%), makes them an ideal model, not only for studying the role of Pet-1 in directing serotonergic development, but also for examining the role of 5-HT in the development of affective disorders. Additionally, the presentation of anxiety and aggression in Pet-1 null mice is reminiscent of the phenotypes of 5-HT1A and 5-HT1B null mice (Zhuang et al., 1999), raising the possibility that the anxiety behaviours present in Pet-1 null mice represent an alteration of 5-HT1A receptor expression. Indeed, the initial examination of Pet-1 as a regulator of serotonergic development did reveal a Pet-1 binding site in the 5-HT1A gene promoter although the ability of Pet-1 to transcriptionally regulate 5-HT1A expression was ambiguous (Hendricks et al., 1999). For this reason, we sought to examine how Pet-1 might regulate expression of the 5-HT1A receptor gene.

In addition to the Pet-1 binding element identified in the 5-HT1A promoter by the Deneris lab (Hendricks et al., 1999), we identified 5 additional putative Pet-1 elements that were well conserved when compared with the Pet-1 consensus (Table V-1) and between species (Table V-2). EMSA experiments demonstrated that Pet-1 was able to bind all of these putative Pet-1 elements, although when using bacterially expressed Pet-1 (Figure V-1), this interaction was weaker than when Pet-1-expressing HEK 293 nuclear extracts were used (Figure V-2). It is not known whether HEK 293 cells express other ETS proteins that might also bind to the putative Pet-1 elements identified; however, our results suggest that Pet-1 interacts moderately with all 6 putative Pet-1 elements, but most strongly with the 1A-136/126 element. Previous studies were unable to conclusively demonstrate the transcriptional activity of Pet-1 on the human 5-HT1A promoter. We transfected HEK293 cells with constructs expressing human Pet-1 and between 139 and

1517 bp of the human 5-HT1A promoter, and found that Pet-1 was able to activate transcription, with the greatest activation occurring with the construct containing all 6 putative Pet-1 elements (Figure V-4). This suggested that Pet-1 activates transcription of 5-HT1A through multiple Pet-1 elements in the 5-HT1A promoter.

Since Pet-1 null mice display an anxious phenotype similar to that observed in 5-HT1A knockout mice (Gross et al., 2000; Hendricks et al., 2003), and the early developmental expression of 5-HT1A is essential for normal affective behaviour (Gross et al., 2002), we next examined whether 5-HT1A expression was altered in the absence of Pet-1. Using Pet-1 knockout mice obtained from Dr. Evan Deneris, we examined the expression of 5-HT1A mRNA and protein in the ventral hindbrain and midbrain, which contain the raphe nuclei, and in forebrain structures receiving 5-HT afferents, including the hippocampus, striatum and cortex (Figure V-6). Strikingly, we found that Pet-1 null mice displayed elevated levels of 5-HT1A mRNA and protein in the hindbrain and midbrain. Given that Pet-1 is able to transcriptionally activate 5-HT1A, this result is somewhat paradoxical. It is possible this represents an increase in postsynaptic 5-HT1A receptors located in non-raphe brainstem targets. While 5-HT neurons do project to a number of brainstem targets, 5-HT1A receptors are particularly concentrated in the raphe nuclei, especially the median and dorsal raphe, and is not expressed in some of the major 5-HT target regions, such as the SN (McQuade et al., 2004; Luna-Munguia et al., 2005; Clark et al., 2006). The 5-HT1B, 5-HT1D and 5-HT2A receptors, on the other hand, are all expressed in the SN (Sijbesma et al., 1991; Meltzer et al., 2003). 5-HT1A receptors are also expressed in non-raphe regions such as the periaqueductal gray, although even here, they are co-localized with 5-HT and have been suggested to also be 5-HT1A autoreceptors (Zhang et al., 2000). It is therefore likely that this result signifies an

adaptive increase in 5-HT1A autoreceptors resulting from the severe reduction in 5-HT neurotransmission. Interestingly, the converse has also been seen. 5-HTT null mice display a persistent elevation in synaptic 5-HT, and 5-HT1A mRNA and binding is substantially reduced in the dorsal raphe (Fabre et al., 2000; Li et al., 2000; Schmitt et al., 2007), further validating that alterations in 5-HT levels drastically alter 5-HT1A autoreceptor expression.

In post-synaptic regions, Pet-1 knock out mice had variable 5-HT1A levels compared to wild type controls (Figure V-6). In the hippocampus, Pet-1 null mice had increased levels of 5-HT1A protein, but not mRNA. This is in agreement with other studies showing increased 5-HT1A expression in the hippocampus following specific lesioning of 5-HT neurons using 5,7-dihydroxytryptamine or acute 5-HT1A antagonist treatment (Patel et al., 1996; Abbas et al., 2007). In the striatum and cortex, on the other hand, 5-HT1A protein and mRNA (respectively) were decreased, which agrees with studies showing that inhibiting embryonic 5-HT synthesis by maternal administration of ρCPA reduces the expression of post-synaptic 5-HT1A receptors (Lauder et al., 2000). While Pet-1 certainly has a role as a transcriptional regulator, the relative absence of functional 5-HT signalling during embryonic development in these mice has dramatic implications for the expression of 5-HT1A receptors, both in Pet-1 expressing serotonergic raphe cells and in non-serotonergic target regions that are not transcriptionally regulated by Pet-1.

Given the high degree of crosstalk between the monoaminergic neurotransmitter systems, it will be interesting to examine how the absence of 5-HT signalling affects the expression and cytoarchitecture of the DA and NE systems. Preliminary studies (not

shown) suggest that there is an increase in NE in the DR, which may represent an invagination of NE projections (i.e. from the LC), or possibly even a molecular switch from a serotonergic to noradrenergic phenotype, although more studies are necessary to understand the relevance of this finding. Certainly, though, the important role of Pet-1 in regulating 5-HT neuron development, and subsequently of 5-HT as a developmental cue, is clear. Pet-1 may act as a transcriptional regulator of 5-HT1A at certain points during development; however, these studies indicate that the absence of 5-HT has far more potent effects on 5-HT1A receptor expression, resulting in an upregulation of 5-HT1A autoreceptors and 5-HT1A receptors in the hippocampus, and permanently decreasing 5-HT1A receptor levels in the striatum and cortex. 5-HT1A null mice have a high anxiety phenotype, and 5-HT1A overexpressing mutants are associated with reduced anxiety behaviour, suggesting that the decreased levels of 5-HT1A in the cortex and striatum, and increase autoreceptor expression, might explain the anxiety behaviour observed in Pet-1 mice (Gross et al., 2000; Deng et al., 2007). On the other hand, in high-aggressive house mice, 5-HT1A mRNA and binding was found to be substantially elevated in the hippocampus compared to low-aggressive mice, suggesting that our observed increase in hippocampal 5-HT1A in Pet-1 null mice might in part explain their aggressive phenotype (Korte et al., 1996).

Hes1, Hes5 and Hes6

Hes1 and Hes5 are bHLH transcriptional repressors that inhibit the differentiation of pluripotent neural progenitors (Jhas et al., 2006). They recruit co-repressors such as Gro/TLE and repress the expression of a host of target genes, including the proneural genes that activate expression of neuron-specific genes such as Hes6 (see Figure I-1).

Hes6, in turn, promotes neuronal differentiation, at least in part by inhibiting the action of Hes1 (Gratton et al., 2003). We previously identified a 26 bp palindrome in the 5-HT1A promoter that contains a polymorphism that associates with suicide and depression (Lemonde et al., 2003). A yeast one-hybrid analysis of proteins with differential binding to the C- and G-alleles of this palindromic element identified DEAF1 and Hes5, both of which were also found to act as transcriptional repressors of 5-HT1A in the presence of the C- but not G-allele. We therefore sought to investigate how the Hes family of bHLHs might regulate the developmental expression of 5-HT1A. Using *in vitro* transfection of RN46A and SN48 cells, 5-HT1A expressing models of presynaptic 5-HT neurons and 5-HT target neurons, respectively, we determined that Hes1 is a strong repressor of 5-HT1A receptor transcription, in many cases acting as a stronger repressor than Hes5 (Figure VI-1). In both of these cell lines, Hes6 alone was able to enhance transcription, although it is believe this is by inhibiting endogenous Hes1 and Hes5 present in these cells (not shown) since the Hes6 alone had no effect on transcription in non-neuronal L6 myoblasts, which are 5-HT1A- and Hes-negative. On the other hand, co-transfection of Hes6 along with either Hes1 or Hes5 completely inhibited the transcriptional repression by either Hes1 or Hes5, suggesting that Hes6 acts by derepressing the inhibitory bHLHs rather than by activating transcription directly. We next demonstrated that Hes1 and Hes5 mediate their transcriptional repression through a site resembling an N-box proximal to the C(-1019)G polymorphism (Figure VI-2). In a 5-HT1A-expressing neuroblastoma cell line (SK-N-SH), mutation of the putative N-box prevented transcriptional repression by either Hes1 or Hes5. Interestingly, while Hes5 was unable to repress transcription in the presence of the G(-1019)-allele, Hes1 was still able to repress transcription of the polymorphic construct, which explains why it was not identified in our initial yeas one-

hybrid screen. Given that DEAF1 and Hes both modulate 5-HT1A receptor expression through consensus elements separated by only 4 nucleotides, we also examined whether there is an additive repression by Hes1 or Hes5 with DEAF1. When Hes1 and DEAF1 were co-transfected, levels of repression similar to that of Hes1 were seen with the 26-bp C-allele, and similar to that of DEAF1 when the N-box was mutated. Additionally, when Hes5 and DEAF1 were co-transfected, the levels of repression were always similar to that of DEAF1. These results might be suggestive of binding affinities (Hes1>DEAF1>Hes5), although further studies would be necessary to confirm this. Strikingly, we also found that DEAF1 repression could be inhibited by co-transfection with Hes6 (not shown), though the mechanism and functional implications of this finding are not yet understood.

Our transcription data suggest that Hes1, Hes5 and Hes6 are all important regulators of 5-HT1A receptor expression, with Hes1 mediating the strongest repression. Hes1 (and Hes5) levels decrease as neurogenesis proceeds, in an inversely proportional pattern to the increase in 5-HT1A and Hes6 during this same timeframe (Hillion et al., 1993; Kageyama et al., 2006). For this reason, we next investigated whether the decrease in Hes1 during neurogenesis is somehow causative to the increase in 5-HT1A using Hes1 knockout mice (Figure VI-4). Hes1 null mice display severe neural tube deficits by E13-E14 and this knockout is embryonic-lethal (Ito et al., 2000; Hatakeyama et al., 2004). As a result, we performed Q RT-PCR on mRNA extracted from hindbrain, midbrain and forebrain of E12.5 Hes1 null mice and wild type littermates in order to examine potential alterations in 5-HT1A receptor expression at a point in embryonic development where 5-HT1A receptor expression should just be detectable. Hes1 null mice show the expected compensation by Hes5, which is known to be upregulated in regions normally expressing Hes1 in Hes1 null mice (Hatakeyama et al., 2006). Also, TPH2 mRNA levels are not

affected by the Hes1 null mice, suggesting that the presynaptic 5-HT neurons are not affected in the absence of Hes1, and serving as a serotonergic control that was not expected to be regulated by Hes bHLHs. Finally, our investigation of 5-HT1A mRNA expression revealed a substantial increase in 5-HT1A levels in the hindbrain and midbrain of Hes1 null mice. We also validated this finding by *in situ* hybridization (Figure VI-5), noting a clear increase in 5-HT1A mRNA in the forebrain of Hes1 null mice that was not detectable by Q RT-PCR. We also noted an expansion of 5-HT1A mRNA away from the ventricular zone that may reflect the premature neurogenesis that is known to occur in Hes1 null mice (Ohtsuka et al., 2001).

This study highlights a novel role for Hes1 in the developmental expression of 5-HT1A receptors and is thus of great interest in understanding affective behaviours, given that altered 5-HT1A receptor expression is tightly linked to disorders such as depression, anxiety and aggression (Heisler et al., 1998; Albert and Lemonde, 2004; Kusserow et al., 2004; David et al., 2005), as well as being the target of numerous psychoactive drugs (Le Poul et al., 2000; Chen et al., 2003; McQuade et al., 2004; Bardin et al., 2007). There are, however, a number of questions that remain to be addressed in future studies. Although the bulk of the evidence we have presented points towards a direct regulation of 5-HT1A receptor expression by Hes1 and Hes5 through the N-box identified in the 26 bp polymorphic region. We have not, however, been successful in demonstrating a direct interaction to date. As far as we are aware, no EMSA experiments using mammalian Hes proteins in EMSA experiments have been published, and our attempts to perform such experiments have thus far been unsuccessful, suggesting that expression of recombinant Hes results in an unstable protein. Alternatively, it is possible that Hes binding to DNA requires co-factors such as Gro/TLE. In addition, there are currently no suitable

antibodies for use in supershift or chromatin immunoprecipitation experiments. Future work will, therefore, focus on attempting to address this issue. Despite these challenges, we did previously show by yeast one-hybrid analysis that the Hes5-GAL4AD can transactivate the LacZ gene at the 26-bp palindrome, which indirectly measures its DNA binding capacity and hence concluded that Hes5 does bind to the C-allele of the 26-bp palindrome (Lemonde et al., 2003).

Another important question relates to the mechanism of Hes6 action. Figure I-17C illustrates several of the potential mechanisms by which Hes6 might derepress Hes1 or Hes5. Indeed some studies have suggested that the primary mechanism by which Hes6 inhibits Hes1 is by interacting with Hes1 and targeting it to the proteosome for degradation, although this same study illustrated that Hes6 can also interact with Hes1 co-repressors Gro/TLE, suggesting a possible competitive mechanism (Gratton et al., 2003). It is not known whether a similar mechanism might exist for Hes5 and Hes6, although our studies demonstrating that Hes6 can inhibit Hes5-mediated repression suggest that it does. There are numerous other possibilities, however, which could be tested. For example, Hes6 might heterodimerize with Hes1 or Hes5 and prevent them from binding the DNA or from recruiting co-repressors (Jhas et al., 2006). These questions need to be addressed in order to fully appreciate how these bHLHs regulate not only 5-HT1A receptor expression during development, but neurogenesis itself.

Finally, it is now well established that the methylation state of CpG sites within CpG islands, which are present in nearly half of all mammalian genes, has important consequences for transcriptional regulation (Suzuki et al., 2001; Song et al., 2003). Methylation of the cysteine within a CpG site is an epigenetic chemical modification that is frequently associated with reduced recognition by transcription factors and gene

silencing. Preliminary studies in our lab are examining methylation of the CpG site within the Hes N-box in the 26 bp palindrome. Initial results point to an association between methylation at this with suicide and schizophrenia, although these studies are still in progress. It is probable that an increased methylation at this site would inhibit repression by Hes1 and Hes5, which, based on our results, might lead to substantial alterations in 5-HT1A receptor expression. Interestingly the C(-1019)G polymorphism also lies within a CpG site, the methylation of which associates with alcoholism and suicide in preliminary studies.

The role of 5-HT1A receptors and of the monoamine fear circuitry in regulating mood and emotion is of little debate (Gross and Hen, 2004). Widespread 5-HT projections and 5-HT1A expression throughout the monoamine fear circuitry, especially the hippocampus and extended amygdala, may largely explain how the 5-HT system, and specifically 5-HT1A, modulates emotional behaviours (Aznar et al., 2003; Palchaudhuri and Flugge, 2005; Domschke et al., 2006; Huang and Kandel, 2007). In support of this, mice lacking post-synaptic 5-HT1A receptors have increased fear/anxiety behaviours and mice overexpressing 5-HT1A receptors in serotoninoceptive brain regions have reduced anxiety behaviour (Gross et al., 2002; Kusserow et al., 2004; Klemenhagen et al., 2006). The precise mechanism by which 5-HT1A is able to regulate amygdaloid functions such as fear and anxiety, however, has not been well-characterized, although some studies are beginning to shed some light on this subject. 5-HT1A receptor agonists are commonly used in the treatment of anxiety (Overstreet et al., 2003; De Vry et al., 2004). In a study examining the response to fear conditioning, it was found the postsynaptic 5-HT1A receptors located primarily in the amygdala, but also hippocampus, mediated these anxiolytic actions, whereas 5-HT1A receptors located in the prefrontal cortex were not

involved (Li et al., 2006). 5-HT1A receptors in the BLA also modulate the early depression of synaptic transmission during fear-based long term memory formation (Huang and Kandel, 2007). In addition, 5-HT1A autoreceptor binding potential inversely correlates with amygdala reactivity in healthy human subjects, implicating the negative feedback loops regulating 5-HT neurotransmission in the amygdala in the development of affective behaviours, predisposition to amygdaloid dysfunction, and even responsiveness to psychiatric drugs (Fisher et al., 2006). In order to fully appreciate how mood and emotion are controlled, it is essential to understand how the "anxiety genes", such as 5-HT1A, modulate the emotional centers of the brain, as well as how these "anxiety genes" are themselves regulated, especially during the critical developmental timeframe in which the fear circuitry becomes hardwired.

SUMMARY

Taken together, this body of work outlines the complex and finely tuned system of regulatory mechanisms governing all levels the monoamine fear circuitry. In the adult, the communication between anxiogenic or other relevant emotional stimuli (input) and appropriate emotional behavioural responses (output) is controlled by a series of inhibitory gating structures that line the entire rostrocaudal axis of the extended amygdala. Not only do these gating structures effectively determine amygdaloid function, but representative members from virtually every class of neurotransmitter are involved in modulating this system. The development of the monoamine fear circuitry is also tightly regulated by a number of often overlapping transcription factors. Developmental regulation by a number of critical factors is essential for the spatiotemporal expression of the components of the fear circuitry that are most relevant to its modulation of mood and emotion. In this thesis we attempt to go beyond the understanding that the monoamine systems are involved the development of anxiety and affective disorders and examine the specific mechanism by which alterations in these systems might result in affective pathology.

CHAPTER VIII: Appendix

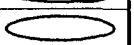
	IPACL	IPACM	Ce	BL	Ip	Im	
D1	++	+++	++	+	+++	+++	
TH	+++	~	+++	+	+++	+++	
DBH	~	~	~	~	~	~	
MOR1	+	+++	+	+	+++	+++	
ENK	~	~	++	+	++	++	
βEND	~	~	~	~	~	~	
mGluR5	+++	+	+++	++	+++	+++	
GAD	++	++	++	++	+++	+++	
VGAT	+	+++	++	+	+	+	
CCK	++	++	+++	~	++	+++	
DARPP-32	+	+++	+++	~	+++	+++	

Table VIII-1: Distribution of Neurotransmitters in the Amygdala

Summary of IR intensity and distribution in the IPAC (L-lateral and M-medial), Ce, BLA, Ip and Im. A cartoon representation of the distribution pattern of each marker in the Im is shown on the right. Note that TH, CCK and DARPP-32 are all concentrated in the rostrolateral Im, while ENK is absent in this region. Summarized from (Fuxe et al., 2003a; Jacobsen et al., 2006; Perez de la Mora et al., 2006; de la Mora et al., 2007)and unpublished data. Lateral is Right.

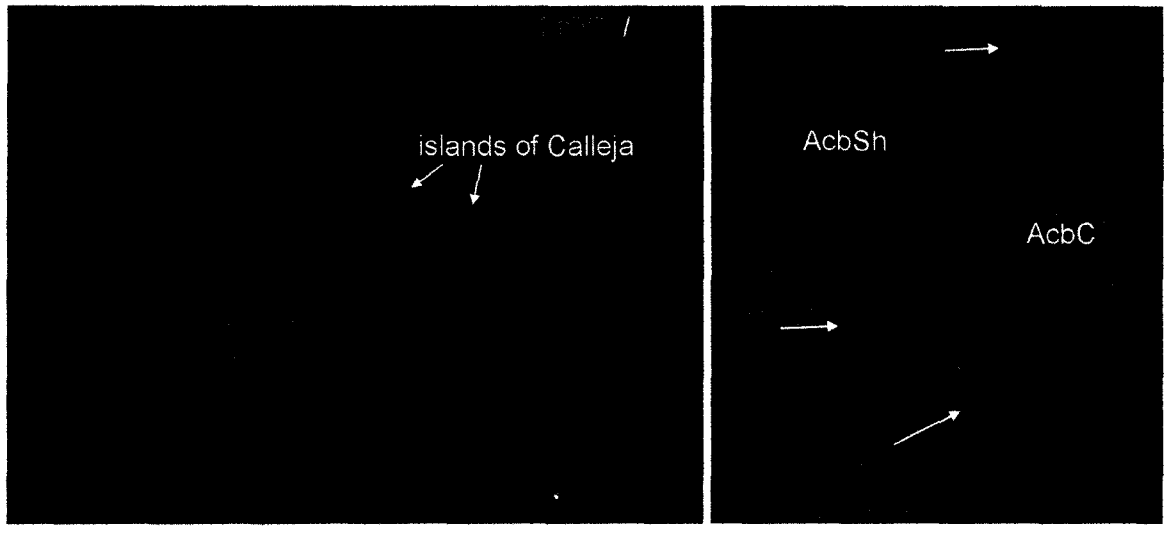


Figure VIII-1: MOR1 and ENK IR in the Islands of Calleja and the Nucleus Accumbens MOR1 IR (green) and ENK IR (red) in the islands of Calleja which adjoin the ventral pallidal extensions of the olfactory tubercle (left) and in patches within the AcbSh (right). The MOR1-rich patches are devoid of ENK-like terminal input.

CHAPTER IX: References

Abbas SY, Nogueira MI, Azmitia EC (2007) Antagonist-induced increase in 5-HT1A-receptor expression in adult rat hippocampus and cortex. Synapse 61:531-539.

Abbracchio MP, Colombo F, Di Luca M, Zaratin P, Cattabeni F (1987) Adenosine modulates the dopaminergic function in the nigro-striatal system by interacting with striatal dopamine dependent adenylate cyclase. Pharmacol Res Commun 19:275-286.

Abdouh M, Storring JM, Riad M, Paquette Y, Albert PR, Drobetsky E, Kouassi E (2001) Transcriptional mechanisms for induction of 5-HT1A receptor mRNA and protein in activated B and T lymphocytes. J Biol Chem 276:4382-4388.

Abrams JK, Johnson PL, Hollis JH, Lowry CA (2004) Anatomic and functional topography of the dorsal raphe nucleus. Ann N Y Acad Sci 1018:46-57.

Abrams JK, Johnson PL, Hay-Schmidt A, Mikkelsen JD, Shekhar A, Lowry CA (2005) Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. Neuroscience 133:983-997.

Adler B, R.R. G, G.W. P (1990) Opioid peptide receptors. In: Handbook of Chemical Neuroanatomy. Neuropeptides in the CNS Part II (Bjorklund A, Hokfelt T, M.I. K, eds), pp 359-390. Amsterdam: Elsavier.

Agnati LF, Fuxe K (2000) Volume transmission as a key feature of information handling in the central nervous system possible new interpretative value of the Turing's B-type machine. In: Volume Transmission Revisited (Agnati LF, Fuxe K, Nicholson C, Syková E, eds), pp 3-19. Amsterdam: Elsevier.

Agnati LF, Zoli M, Stromberg I, Fuxe K (1995) Intercellular communication in the brain: wiring versus volume transmission. Neuroscience 69:711-726.

Agnati LF, Fuxe K, Nicholson C, Syskova E (2000) Volume Transmission Revisited. Oxford: Elsevier Science.

Agnati LF, Fuxe K, Zoli M, Ozini I, Toffano G, Ferraguti F (1986) A correlation analysis of the regional distribution of central enkephalin and beta-endorphin immunoreactive terminals and of opiate receptors in adult and old male rats. Evidence for the existence of two main types of communication in the central nervous system: the volume transmission and the wiring transmission. Acta Physiol Scand 128:201-207.

Agnati LF, Franzen O, Ferre S, Leo G, Franco R, Fuxe K (2003) Possible role of intramembrane receptor-receptor interactions in memory and learning via formation of long-lived heteromeric complexes: focus on motor learning in the basal ganglia. J Neural Transm Suppl:1-28.

Agnati LF, Fuxe K, Giardino L, Calza L, Zoli M, Battistini N, Benfenati F, Vanderhaeghen JJ, Guidolin D, Ruggeri M, et al. (1985) Evidence for cholecystokinin-dopamine receptor interactions in the central nervous system of the adult and old rat. Studies on their functional meaning. Ann N Y Acad Sci 448:315-333.

Agnati LF, Leo, G. Zanardi, A., Genedani, S., Rivera, A., Fuxe, K., Guidolin, D. (2006) Volume transmission and wiring transmission from cellular to molecular networks: history and perspectives. Acta Physiol Scand 187:329-344.

Aizman O, Brismar H, Uhlen P, Zettergren E, Levey AI, Forssberg H, Greengard P, Aperia A (2000) Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons. Nat Neurosci 3:226-230.

Akazawa C, Sasai Y, Nakanishi S, Kageyama R (1992) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. J Biol Chem 267:21879-21885.

Albanese A, Altavista MC, Rossi P (1986) Organization of central nervous system dopaminergic pathways. J Neural Transm Suppl 22:3-17.

Albert PR (2002) G protein preferences for dopamine D2 inhibition of prolactin secretion and DNA synthesis in GH4 pituitary cells. Mol Endocrinol 16:1903-1911.

Albert PR, Robillard L (2002) G protein specificity: traffic direction required. Cell Signal 14:407-418.

Albert PR, Lemonde S (2004) 5-HT1A receptors, gene repression, and depression: guilt by association. Neuroscientist 10:575-593.

Albert PR, Zhou QY, Van Tol HH, Bunzow JR, Civelli O (1990) Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. J Biol Chem 265:5825-5832.

Alex KD, Pehek EA (2007) Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission. Pharmacol Ther 113:296-320.

Alheid GF (2003) Extended amygdala and basal forebrain. Ann N Y Acad Sci 985:185-205.

Alheid GF, Heimer L (1988) New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. Neuroscience 27:1-39.

Alheid GF, de Olmos JS, Beltramino CA (1994) Amygdala and the extended amygdala. In: The rat nervous system, 2nd edition (G. P, ed), pp 495-578. London: Academic Press.

Alheid GF, de Olmos JS, Beltramino CA (1995) Forebrain: Amygdala and extended amygdala. In: The Rat Nervous System. (Paxinos G, ed), pp 495-578. San Diego: Academic.

Alheid GF, Shammah-Lagnado SJ, Beltramino CA (1999) The interstitial nucleus of the posterior limb of the anterior commissure: a novel layer of the central division of extended amygdala. Ann N Y Acad Sci 877:645-654.

Allain AE, Meyrand P, Branchereau P (2005) Ontogenic changes of the spinal GABAergic cell population are controlled by the serotonin (5-HT) system: implication of 5-HT1 receptor family. J Neurosci 25:8714-8724.

Allen PB, Ouimet CC, Greengard P (1997) Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. Proc Natl Acad Sci U S A 94:9956-9961.

Andel F, 3rd, Ladurner AG, Inouye C, Tjian R, Nogales E (1999) Three-dimensional structure of the human TFIID-IIA-IIB complex. Science 286:2153-2156.

Anden NE, Carlsson A, Kerstell J, Magnusson T, Olsson R, Roos BE, Steen B, Steg G, Svanborg A, Thieme G, Werdinius B (1970) Oral L-dopa treatment of parkinsonism. Acta Med Scand 187:247-255.

Andersen SL, Rutstein M, Benzo JM, Hostetter JC, Teicher MH (1997) Sex differences in dopamine receptor overproduction and elimination. Neuroreport 8:1495-1498.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442:823-826.

Arborelius L, Eklund MB (2007) Both long and brief maternal separation produces persistent changes in tissue levels of brain monoamines in middle-aged female rats. Neuroscience 145:738-750.

Arborelius L, Nomikos GG, Hacksell U, Svensson TH (1993a) (R)-8-OH-DPAT preferentially increases dopamine release in rat medial prefrontal cortex. Acta Physiol Scand 148:465-466.

Arborelius L, Chergui K, Murase S, Nomikos GG, Hook BB, Chouvet G, Hacksell U, Svensson TH (1993b) The 5-HT1A receptor selective ligands, (R)-8-OH-DPAT and (S)-UH-301, differentially affect the activity of midbrain dopamine neurons. Naunyn Schmiedebergs Arch Pharmacol 347:353-362.

Archambault J, Friesen JD (1993) Genetics of eukaryotic RNA polymerases I, II, and III. Microbiol Rev 57:703-724.

Armbruster BN, Roth BL (2005) Mining the receptorome. J Biol Chem 280:5129-5132.

Asan E (1997a) Ultrastructural features of tyrosine-hydroxylase-immunoreactive afferents and their targets in the rat amygdala. Cell Tissue Res 288:449-469.

Asan E (1997b) Interrelationships between tyrosine hydroxylase-immunoreactive dopaminergic afferents and somatostatinergic neurons in the rat central amygdaloid nucleus. Histochem Cell Biol 107:65-79.

Asan E (1998) The catecholaminergic innervation of the rat amygdala. Adv Anat Embryol Cell Biol 142:1-118.

Asbreuk CH, Vogelaar CF, Hellemons A, Smidt MP, Burbach JP (2002) CNS expression pattern of Lmx1b and coexpression with ptx genes suggest functional cooperativity in the development of forebrain motor control systems. Mol Cell Neurosci 21:410-420.

Aston-Jones G, Shipley MT, Chouvet G, Ennis M, van Bockstaele E, Pieribone V, Shiekhattar R, Akaoka H, Drolet G, Astier B, et al. (1991) Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. Prog Brain Res 88:47-75.

Avshalumov MV, Chen BT, Marshall SP, Pena DM, Rice ME (2003) Glutamate-dependent inhibition of dopamine release in striatum is mediated by a new diffusible messenger, H2O2. J Neurosci 23:2744-2750.

Ayoub MA, Couturier C, Lucas-Meunier E, Angers S, Fossier P, Bouvier M, Jockers R (2002) Monitoring of ligand-independent dimerization and ligand-induced conformational changes of melatonin receptors in living cells by bioluminescence resonance energy transfer. J Biol Chem 277:21522-21528.

Aznar S, Qian ZX, Knudsen GM (2004) Non-serotonergic dorsal and median raphe projection onto parvalbumin- and calbindin-containing neurons in hippocampus and septum. Neuroscience 124:573-581.

Aznar S, Qian Z, Shah R, Rahbek B, Knudsen GM (2003) The 5-HT1A serotonin receptor is located on calbindin- and parvalbumin-containing neurons in the rat brain. Brain Res 959:58-67.

Babcock EB (1921) Gregor Mendel and the Support of Scientific Work at Brunn. Science 54:275-276.

Bae S, Bessho Y, Hojo M, Kageyama R (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development 127:2933-2943.

Baek JH, Hatakeyama J, Sakamoto S, Ohtsuka T, Kageyama R (2006) Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. Development 133:2467-2476.

Baker MW, Vohra MM, Croll RP (1993) Serotonin depletors, 5,7-dihydroxytryptamine and p-chlorophenylalanine, cause sprouting in the CNS of the adult snail. Brain Res 623:311-315.

Banihashemi B, Albert PR (2002) Dopamine-D2S receptor inhibition of calcium influx, adenylyl cyclase, and mitogen-activated protein kinase in pituitary cells: distinct Galpha and Gbetagamma requirements. Mol Endocrinol 16:2393-2404.

Bannon MJ, Pruetz B, Manning-Bog AB, Whitty CJ, Michelhaugh SK, Sacchetti P, Granneman JG, Mash DC, Schmidt CJ (2002) Decreased expression of the transcription factor NURR1 in dopamine neurons of cocaine abusers. Proc Natl Acad Sci U S A 99:6382-6385.

Bao AM, Meynen G, Swaab DF (2007) The stress system in depression and neurodegeneration: Focus on the human hypothalamus. Brain Res Rev.

Bardin L, Auclair A, Kleven MS, Prinssen EP, Koek W, Newman-Tancredi A, Depoortere R (2007) Pharmacological profiles in rats of novel antipsychotics with combined dopamine D2/serotonin 5-HT1A activity: comparison with typical and atypical conventional antipsychotics. Behav Pharmacol 18:103-118.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 50:291-313.

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38:1083-1152.

Barrera G, Hernandez A, Poulin JF, Laforest S, Drolet G, Morilak DA (2006) Galanin-mediated anxiolytic effect in rat central amygdala is not a result of corelease from noradrenergic terminals. Synapse 59:27-40.

Basch ML, Bronner-Fraser M (2006) Neural crest inducing signals. Adv Exp Med Biol 589:24-31.

Bauman MD, Amaral DG (2005) The distribution of serotonergic fibers in the macaque monkey amygdala: an immunohistochemical study using antisera to 5-hydroxytryptamine. Neuroscience 136:193-203.

Beadle GW, Tatum EL (1941) Genetic Control of Biochemical Reactions in Neurospora. Proc Natl Acad Sci U S A 27:499-506.

Beique JC, Campbell B, Perring P, Hamblin MW, Walker P, Mladenovic L, Andrade R (2004) Serotonergic regulation of membrane potential in developing rat prefrontal cortex: coordinated expression of 5-hydroxytryptamine (5-HT)1A, 5-HT2A, and 5-HT7 receptors. J Neurosci 24:4807-4817.

Beltramino CA, Forbes MS, Swanson DJ, Alheid GF, Heimer L (1996) Amygdaloid input to transiently tyrosine hydroxylase immunoreactive neurons in the bed nucleus of the stria terminalis of the rat. Brain Res 706:37-46.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mossner R, Westphal H, Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. Mol Pharmacol 53:649-655.

Benninghoff J, Schmitt A, Mossner R, Lesch KP (2002) When cells become depressed: focus on neural stem cells in novel treatment strategies against depression. J Neural Transm 109:947-962.

Bertrand N, Castro DS, Guillemot F (2002) Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3:517-530.

Bezard E, Gerlach I, Moratalla R, Gross CE, Jork R (2006) 5-HT1A receptor agonist-mediated protection from MPTP toxicity in mouse and macaque models of Parkinson's disease. Neurobiol Dis 23:77-86.

Bhatnagar A, Sheffler DJ, Kroeze WK, Compton-Toth B, Roth BL (2004) Caveolin-1 interacts with 5-HT2A serotonin receptors and profoundly modulates the signaling of selected Galphaq-coupled protein receptors. J Biol Chem 279:34614-34623.

Bhatnagar A, Willins DL, Gray JA, Woods J, Benovic JL, Roth BL (2001) The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT2A) serotonin receptors reveals differential sorting of arrestins and 5-HT2A receptors during endocytosis. J Biol Chem 276:8269-8277.

Bianchi S, Dotti MT, Federico A (2005) Physiology and pathology of notch signalling system. J Cell Physiol 3:3.

Binetruy B, Heasley L, Bost F, Caron L, Aouadi M (2007) Concise review: regulation of embryonic stem cell lineage commitment by mitogen-activated protein kinases. Stem Cells 25:1090-1095.

Bischoff S, Barhanin J, Bettler B, Mulle C, Heinemann S (1997) Spatial distribution of kainate receptor subunit mRNA in the mouse basal ganglia and ventral mesencephalon. J Comp Neurol 379:541-562.

Bissiere S, Humeau Y, Luthi A (2003) Dopamine gates LTP induction in lateral amygdala by suppressing feedforward inhibition. Nat Neurosci 6:587-592.

Bjelke B, Stromberg I, O'Connor WT, Andbjer B, Agnati LF, Fuxe K (1994) Evidence for volume transmission in the dopamine denervated neostriatum of the rat after a unilateral nigral 6-OHDA microinjection. Studies with systemic D-amphetamine treatment. Brain Res 662:11-24.

Bloom F, Battenberg E, Rossier J, Ling N, Guillemin R (1978) Neurons containing beta-endorphin in rat brain exist separately from those containing enkephalin: immunocytochemical studies. Proc Natl Acad Sci U S A 75:1591-1595.

Bloom FE (2000) Integration of wiring transmission and volume transmission. Prog Brain Res 125:21-26.

Bonhomme N, Esposito E (1998) Involvement of serotonin and dopamine in the mechanism of action of novel antidepressant drugs: a review. J Clin Psychopharmacol 18:447-454.

Bonnin A, Peng W, Hewlett W, Levitt P (2006) Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. Neuroscience 141:781-794.

Bonnin A, Torii M, Wang L, Rakic P, Levitt P (2007) Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. Nat Neurosci 10:588-597.

Bottomley MJ, Collard MW, Huggenvik JI, Liu Z, Gibson TJ, Sattler M (2001) The SAND domain structure defines a novel DNA-binding fold in transcriptional regulation. Nat Struct Biol 8:626-633.

Bourgeais L, Gauriau C, Bernard JF (2001) Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. Eur J Neurosci 14:229-255.

Bouron A, Reuter H (1999) The D1 dopamine receptor agonist SKF-38393 stimulates the release of glutamate in the hippocampus. Neuroscience 94:1063-1070.

Bowery NG, Enna SJ (2000) gamma-aminobutyric acid(B) receptors: first of the functional metabotropic heterodimers. J Pharmacol Exp Ther 292:2-7.

Bowlby J (1951) Maternal care and mental health. Bull World Health Organ 3:355-533.

Brinley-Reed M, McDonald AJ (1999) Evidence that dopaminergic axons provide a dense innervation of specific neuronal subpopulations in the rat basolateral amygdala. Brain Res 850:127-135.

Briscoe J, Sussel L, Serup P, Hartigan-O'Connor D, Jessell TM, Rubenstein JL, Ericson J (1999) Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature 398:622-627.

Broaddus WC, Bennett JP, Jr. (1990) Postnatal development of striatal dopamine function. II. Effects of neonatal 6-hydroxydopamine treatments on D1 and D2 receptors, adenylate cyclase activity and presynaptic dopamine function. Brain Res Dev Brain Res 52:273-277.

Brookes K, Xu X, Chen W, Zhou K, Neale B, Lowe N, Anney R, Franke B, Gill M, Ebstein R, Buitelaar J, Sham P, Campbell D, Knight J, Andreou P, Altink M, Arnold R, Boer F, Buschgens C, Butler L, Christiansen H, Feldman L, Fleischman K, Fliers E, Howe-Forbes R, Goldfarb A, Heise A, Gabriels I, Korn-Lubetzki I, Johansson L, Marco R, Medad S, Minderaa R, Mulas F, Muller U, Mulligan A, Rabin K, Rommelse N, Sethna V, Sorohan J, Uebel H, Psychogiou L, Weeks A, Barrett R, Craig I, Banaschewski T, Sonuga-Barke E, Eisenberg J, Kuntsi J, Manor I, McGuffin P, Miranda A, Oades RD, Plomin R, Roeyers H, Rothenberger A, Sergeant J, Steinhausen HC, Taylor E, Thompson M, Faraone SV, Asherson P (2006) The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. Mol Psychiatry 11:934-953.

Bruckner UB, Lang RE, Ganten D (1984) Release of opioid peptides in canine hemorrhagic hypotension: effects of naloxone. Res Exp Med 184:171-178.

Budnik V, Wu CF, White K (1989) Altered branching of serotonin-containing neurons in Drosophila mutants unable to synthesize serotonin and dopamine. J Neurosci 9:2866-2877.

Buervenich S, Carmine A, Arvidsson M, Xiang F, Zhang Z, Sydow O, Jonsson EG, Sedvall GC, Leonard S, Ross RG, Freedman R, Chowdari KV, Nimgaonkar VL, Perlmann T, Anvret M, Olson L (2000) NURR1 mutations in cases of schizophrenia and manic-depressive disorder. Am J Med Genet 96:808-813.

Buess H (1953) Joh. Friedrich Miescher and the contribution of Basle physicians to the biology of the nineteenth century. Yale J Biol Med 25:250-261.

Bulik CM, Sullivan PF, Wade TD, Kendler KS (2000) Twin studies of eating disorders: a review. Int J Eat Disord 27:1-20.

Bunin MA, Wightman RM (1998) Quantitative evaluation of 5-hydroxytryptamine (serotonin) neuronal release and uptake: an investigation of extrasynaptic transmission. J Neurosci 18:4854-4860.

Bunin MA, Prioleau C, Mailman RB, Wightman RM (1998) Release and uptake rates of 5-hydroxytryptamine in the dorsal raphe and substantia nigra reticulata of the rat brain. J Neurochem 70:1077-1087.

Caille I, Dumartin B, Bloch B (1996) Ultrastructural localization of D1 dopamine receptor immunoreactivity in rat striatonigral neurons and its relation with dopaminergic innervation. Brain Res 730:17-31.

Calabresi P, Centonze D, Gubellini P, Marfia GA, Pisani A, Sancesario G, Bernardi G (2000) Synaptic transmission in the striatum: from plasticity to neurodegeneration. Prog Neurobiol 61:231-265.

Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, Neve K, Fuxe K, Agnati LF, Woods AS, Ferre S, Lluis C, Bouvier M, Franco R (2003) Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J Biol Chem 278:46741-46749.

Carlsson A (2001) A paradigm shift in brain research. Science 294:1021-1024.

Carlsson A (2002) Treatment of Parkinson's with L-DOPA. The early discovery phase, and a comment on current problems. J Neural Transm 109:777-787.

Castro DS, Arvidsson M, Bondesson Bolin M, Perlmann T (1999) Activity of the Nurr1 carboxyl-terminal domain depends on cell type and integrity of the activation function 2. J Biol Chem 274:37483-37490.

Ceccatelli S, Millhorn DE, Hokfelt T, Goldstein M (1989) Evidence for the occurrence of an enkephalin-like peptide in adrenaline and noradrenaline neurons of the rat medulla oblongata. Exp Brain Res 74:631-640.

Centonze D, Usiello A, Gubellini P, Pisani A, Borrelli E, Bernardi G, Calabresi P (2002) Dopamine D2 receptor-mediated inhibition of dopaminergic neurons in mice lacking D2L receptors. Neuropsychopharmacology 27:723-726.

Chao SZ, Ariano MA, Peterson DA, Wolf ME (2002) D1 dopamine receptor stimulation increases GluR1 surface expression in nucleus accumbens neurons. J Neurochem 83:704-712.

Chapman DP, Whitfield CL, Felitti VJ, Dube SR, Edwards VJ, Anda RF (2004) Adverse childhood experiences and the risk of depressive disorders in adulthood. J Affect Disord 82:217-225.

Charest A, Wainer BH, Albert PR (1993) Cloning and differentiation-induced expression of a murine serotonin1A receptor in a septal cell line. J Neurosci 13:5164-5171.

Charney DS, Krystal JH, Delgado PL, Heninger GR (1990a) Serotonin-specific drugs for anxiety and depressive disorders. Annu Rev Med 41:437-446.

Charney DS, Woods SW, Krystal JH, Heninger GR (1990b) Serotonin function and human anxiety disorders. Ann N Y Acad Sci 600:558-572; discussion 572-553.

Chen F, Rezvani AH, Lawrence AJ (2003) Autoradiographic quantification of neurochemical markers of serotonin, dopamine and opioid systems in rat brain mesolimbic regions following chronic St John's wort treatment. Naunyn Schmiedebergs Arch Pharmacol 367:126-133.

Chen G, Fernandez J, Mische S, Courey AJ (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. Genes Dev 13:2218-2230.

Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL (1998) Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet 19:51-55.

Chen YF, Jin HK, Paul R, Nagahama S (1988) Blunted pressor responsiveness to quinpirole, a specific dopamine D2 receptor agonist, in conscious deoxycorticosterone acetate/NaCl hypertensive rats is related to atrial natriuretic peptide release. J Pharmacol Exp Ther 246:485-492.

Cheng L, Chen CL, Luo P, Tan M, Qiu M, Johnson R, Ma Q (2003) Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype. J Neurosci 23:9961-9967.

Cheung S, Ballew JR, Moore KE, Lookingland KJ (1998) Contribution of dopamine neurons in the medial zona incerta to the innervation of the central nucleus of the amygdala, horizontal diagonal band of Broca and hypothalamic paraventricular nucleus. Brain Res 808:174-181.

Childers SR (1991) Opioid receptor-coupled second messenger systems. Life Sci 48:1991-2003.

Chinta SJ, Andersen JK (2005) Dopaminergic neurons. Int J Biochem Cell Biol 37:942-946.

Chlan-Fourney J, Ashe P, Nylen K, Juorio AV, Li XM (2002) Differential regulation of hippocampal BDNF mRNA by typical and atypical antipsychotic administration. Brain Res 954:11-20.

Chrousos GP (2000) The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes. Int J Obes Relat Metab Disord 24 Suppl 2:S50-55.

Chu Y, Le W, Kompoliti K, Jankovic J, Mufson EJ, Kordower JH (2006) Nurr1 in Parkinson's disease and related disorders. J Comp Neurol 494:495-514.

Chung S, Sonntag KC, Andersson T, Bjorklund LM, Park JJ, Kim DW, Kang UJ, Isacson O, Kim KS (2002) Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. Eur J Neurosci 16:1829-1838.

Ciruela F, Casado V, Rodrigues RJ, Lujan R, Burgueno J, Canals M, Borycz J, Rebola N, Goldberg SR, Mallol J, Cortes A, Canela EI, Lopez-Gimenez JF, Milligan G, Lluis C, Cunha RA, Ferre S, Franco R (2006) Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci 26:2080-2087.

Civelli O, Bunzow JR, Grandy DK (1993) Molecular diversity of the dopamine receptors. Annu Rev Pharmacol Toxicol 33:281-307.

Clark MS, McDevitt RA, Neumaier JF (2006) Quantitative mapping of tryptophan hydroxylase-2, 5-HT1A, 5-HT1B, and serotonin transporter expression across the anteroposterior axis of the rat dorsal and median raphe nuclei. J Comp Neurol 498:611-623.

Commons KG, Milner TA (1996) Cellular and subcellular localization of delta opioid receptor immunoreactivity in the rat dentate gyrus. Brain Res 738:181-195.

Conductier G, Dusticier N, Lucas G, Cote F, Debonnel G, Daszuta A, Dumuis A, Nieoullon A, Hen R, Bockaert J, Compan V (2006) Adaptive changes in serotonin neurons of the raphe nuclei in 5-HT(4) receptor knock-out mouse. Eur J Neurosci 24:1053-1062.

Cooke BM, Hegstrom CD, Breedlove SM (2002) Photoperiod-dependent response to androgen in the medial amygdala of the Siberian hamster, Phodopus sungorus. J Biol Rhythms 17:147-154.

Cooke BM, Hegstrom CD, Keen A, Breedlove SM (2001) Photoperiod and social cues influence the medial amygdala but not the bed nucleus of the stria terminalis in the Siberian hamster. Neurosci Lett 312:9-12.

Corbett AD, Henderson G, McKnight AT, Paterson SJ (2006) 75 years of opioid research: the exciting but vain quest for the Holy Grail. Br J Pharmacol 147 Suppl 1:S153-162.

Cowen MS, Lawrence AJ (2001) Alterations in central preproenkephalin mRNA expression after chronic free-choice ethanol consumption by fawn-hooded rats. Alcohol Clin Exp Res 25:1126-1133.

Coyle JT, Duman RS (2003) Finding the intracellular signaling pathways affected by mood disorder treatments. Neuron 38:157-160.

Craven SE, Lim KC, Ye W, Engel JD, de Sauvage F, Rosenthal A (2004) Gata2 specifies serotonergic neurons downstream of sonic hedgehog. Development 131:1165-1173.

Crossley PH, Martin GR (1995) The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 121:439-451.

Cubala WJ, Landowski J (2006) [Serotoninergic system and limbic-hypothalamic-pituitary-adrenal axis (LHPA axis) in depression]. Psychiatr Pol 40:415-430.

Czesak M, Burns AM, RemesLenicov F, Albert PR (2007) Characterization of rat rostral raphe primary cultures: multiplex quantification of serotonergic markers. J Neurosci Methods 164:59-67.

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci 26:1864-1871.

Dahlstrom A, Fuxe K (1964) Localization of monoamines in the lower brain stem. Experientia 20:398-399.

Damasio AR (1998) Emotion in the perspective of an integrated nervous system. Brain Res Brain Res Rev 26:83-86.

Dang L, Yoon K, Wang M, Gaiano N (2006) Notch3 signaling promotes radial glial/progenitor character in the mammalian telencephalon. Dev Neurosci 28:58-69.

Daunais JB, Letchworth SR, Sim-Selley LJ, Smith HR, Childers SR, Porrino LJ (2001) Functional and anatomical localization of mu opioid receptors in the striatum, amygdala, and extended amygdala of the nonhuman primate. J Comp Neurol 433:471-485.

David SP, Murthy NV, Rabiner EA, Munafo MR, Johnstone EC, Jacob R, Walton RT, Grasby PM (2005) A functional genetic variation of the serotonin (5-HT) transporter affects 5-HT1A receptor binding in humans. J Neurosci 25:2586-2590.

Davis M (1992) The role of the amygdala in fear-potentiated startle: implications for animal models of anxiety. Trends Pharmacol Sci 13:35-41.

Davis M (2002) Role of NMDA receptors and MAP kinase in the amygdala in extinction of fear: clinical implications for exposure therapy. Eur J Neurosci 16:395-398.

Davis M, Myers KM (2002) The role of glutamate and gamma-aminobutyric acid in fear extinction: clinical implications for exposure therapy. Biol Psychiatry 52:998-1007.

Davis RL, Turner DL (2001) Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene 20:8342-8357.

Day M, Wang Z, Ding J, An X, Ingham CA, Shering AF, Wokosin D, Ilijic E, Sun Z, Sampson AR, Mugnaini E, Deutch AY, Sesack SR, Arbuthnott GW, Surmeier DJ (2006) Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. Nat Neurosci 9:251-259.

De Biasi S, Rustioni A (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. Proc Natl Acad Sci U S A 85:7820-7824.

de Boer SF, Koolhaas JM (2005) 5-HT1A and 5-HT1B receptor agonists and aggression: a pharmacological challenge of the serotonin deficiency hypothesis. Eur J Pharmacol 526:125-139.

de la Mora MP, Cardenas-Cachon L, Vazquez-Garcia M, Crespo-Ramirez M, Jacobsen K, Höistad M, Agnati L, Fuxe K (2005) Anxiolytic effects of intra-amygdaloid injection of the D1 antagonist SCH23390 in the rat. Neurosci Lett 377:101-105.

de la Mora MP, Hernandez-Gomez AM, Arizmendi-Garcia Y, Jacobsen KX, Lara-Garcia D, Flores-Gracia C, Crespo-Ramirez M, Gallegos-Cari A, Nuche-Bricaire A, Fuxe K (2007) Role of the amygdaloid cholecystokinin (CCK/Gastrin)-2 receptors in the modulation of anxiety in the rat. Effects of CCK/Gastrin-4 and CCK/Gastrin08 on anxiety-like behaviour and [^{3}H]GABA release. . Eur J Neurosci In Press.

de Olmos JS, Heimer L (1999) The concepts of the ventral striatopallidal system and extended amygdala. Ann N Y Acad Sci 877:1-32.

De Vry J, Schreiber R, Melon C, Dalmus M, Jentzsch KR (2004) 5-HT1A receptors are differentially involved in the anxiolytic- and antidepressant-like effects of 8-OH-DPAT and fluoxetine in the rat. Eur Neuropsychopharmacol 14:487-495.

Del Tredici AL, Schiffer HH, Burstein ES, Lameh J, Mohell N, Hacksell U, Brann MR, Weiner DM (2004) Pharmacology of polymorphic variants of the human 5-HT1A receptor. Biochem Pharmacol 67:479-490.

Delgado PL, Moreno FA (2000) Role of norepinephrine in depression. J Clin Psychiatry 61:5-12.

Deng DR, Djalali S, Holtje M, Grosse G, Stroh T, Voigt I, Kusserow H, Theuring F, Ahnert-Hilger G, Hortnagl H (2007) Embryonic and postnatal development of the serotonergic raphe system and its target regions in 5-HT1A receptor deletion or overexpressing mouse mutants. Neuroscience 147:388-402.

Descarries L, Mechawar N (2000) Ultrastructural evidence for diffuse transmission by monoamine and acetylcholine neurons of the central nervous system. Prog Brain Res 125:27-47.

Descarries L, Gisiger V, Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol 53:603-625.

Descarries L, Watkins KC, Garcia S, Bosler O, Doucet G (1996) Dual character, asynaptic and synaptic, of the dopamine innervation in adult rat neostriatum: a quantitative autoradiographic and immunocytochemical analysis. J Comp Neurol 375:167-186.

Diaz J, Levesque D, Lammers CH, Griffon N, Martres MP, Schwartz JC, Sokoloff P (1995) Phenotypical characterization of neurons expressing the dopamine D3 receptor in the rat brain. Neuroscience 65:731-745.

Ding YQ, Marklund U, Yuan W, Yin J, Wegman L, Ericson J, Deneris E, Johnson RL, Chen ZF (2003) Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci 6:933-938.

Djaldetti R, Melamed E (2002) New drugs in the future treatment of Parkinson's disease. J Neurol 249 Suppl 2:II30-35.

Domschke K, Braun M, Ohrmann P, Suslow T, Kugel H, Bauer J, Hohoff C, Kersting A, Engelien A, Arolt V, Heindel W, Deckert J (2006) Association of the functional -1019C/G 5-HT1A polymorphism with prefrontal cortex and amygdala activation measured with 3 T fMRI in panic disorder. Int J Neuropsychopharmacol 9:349-355.

Drake CT, Chang PC, Harris JA, Milner TA (2002) Neurons with mu opioid receptors interact indirectly with enkephalin-containing neurons in the rat dentate gyrus. Exp Neurol 176:254-261.

Dremencov E, Gispan-Herman I, Rosenstein M, Mendelman A, Overstreet DH, Zohar J, Yadid G (2004) The serotonin-dopamine interaction is critical for fast-onset action of antidepressant treatment: in vivo studies in an animal model of depression. Prog Neuropsychopharmacol Biol Psychiatry 28:141-147.

Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier JF (2001) Role of endogenous opioid system in the regulation of the stress response. Prog Neuropsychopharmacol Biol Psychiatry 25:729-741.

Ebner K, Singewald N (2006) The role of substance P in stress and anxiety responses. Amino Acids 31:251-272.

Eells JB, Lipska BK, Yeung SK, Misler JA, Nikodem VM (2002) Nurr1-null heterozygous mice have reduced mesolimbic and mesocortical dopamine levels and increased stress-induced locomotor activity. Behav Brain Res 136:267-275.

Eggers AE (2007) Redrawing Papez' circuit: A theory about how acute stress becomes chronic and causes disease. Med Hypotheses 69:852-857.

Eglen RM, Bosse R, Reisine T (2007) Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening. Assay Drug Dev Technol 5:425-451.

Elde R, Arvidsson U, Riedl M, Vulchanova L, Lee JH, Dado R, Nakano A, Chakrabarti S, Zhang X, Loh HH, et al. (1995) Distribution of neuropeptide receptors. New views of peptidergic neurotransmission made possible by antibodies to opioid receptors. Ann N Y Acad Sci 757:390-404.

Elgin SC, Grewal SI (2003) Heterochromatin: silence is golden. Curr Biol 13:R895-898.

Emson PC, Bjorklund A, Lindvall O, Paxinos G (1979) Contributions of different afferent pathways to the catecholamine and 5-hydroxytryptamine-innervation of the amygdala: a neurochemical and histochemical study. Neuroscience 4:1347-1357.

Encha-Razavi F, Sonigo P (2003) Features of the developing brain. Childs Nerv Syst 19:426-428.

Esposito E (2006) Serotonin-dopamine interaction as a focus of novel antidepressant drugs. Curr Drug Targets 7:177-185.

Ever L, Gaiano N (2005) Radial 'glial' progenitors: neurogenesis and signaling. Curr Opin Neurobiol 15:29-33.

Fabre V, Beaufour C, Evrard A, Rioux A, Hanoun N, Lesch KP, Murphy DL, Lanfumey L, Hamon M, Martres MP (2000) Altered expression and functions of serotonin 5-HT1A and 5-HT1B receptors in knock-out mice lacking the 5-HT transporter. Eur J Neurosci 12:2299-2310.

Fallon JH, Koziell DA, Moore RY (1978) Catecholamine innervation of the basal forebrain. II. Amygdala, suprarhinal cortex and entorhinal cortex. J Comp Neurol 180:509-532.

Faraone SV, Khan SA (2006) Candidate gene studies of attention-deficit/hyperactivity disorder. J Clin Psychiatry 67 Suppl 8:13-20.

Felitti VJ, Anda RF, Nordenberg D, Williamson DF, Spitz AM, Edwards V, Koss MP, Marks JS (1998) Relationship of childhood abuse and household dysfunction to many of

the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. Am J Prev Med 14:245-258.

Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, Allen PB, Ouimet CC, Greengard P (2000) Spinophilin regulates the formation and function of dendritic spines. Proc Natl Acad Sci U S A 97:9287-9292.

Ferguson KL, McClellan KA, Vanderluit JL, McIntosh WC, Schuurmans C, Polleux F, Slack RS (2005) A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. Embo J 24:4381-4391.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 53:1-24.

Fienberg AA, Hiroi N, Mermelstein PG, Song W, Snyder GL, Nishi A, Cheramy A, O'Callaghan JP, Miller DB, Cole DG, Corbett R, Haile CN, Cooper DC, Onn SP, Grace AA, Ouimet CC, White FJ, Hyman SE, Surmeier DJ, Girault J, Nestler EJ, Greengard P (1998) DARPP-32: regulator of the efficacy of dopaminergic neurotransmission. Science 281:838-842.

Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F, Befort K, Gaveriaux-Ruff C, Dierich A, LeMeur M, Valverde O, Maldonado R, Kieffer BL (2000) Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. Nat Genet 25:195-200.

Finley JC, Maderdrut JL, Petrusz P (1981) The immunocytochemical localization of enkephalin in the central nervous system of the rat. J Comp Neurol 198:541-565.

Fish EW, Sekinda M, Ferrari PF, Dirks A, Miczek KA (2000) Distress vocalizations in maternally separated mouse pups: modulation via 5-HT(1A), 5-HT(1B) and GABA(A) receptors. Psychopharmacology (Berl) 149:277-285.

Fishburn CS, Elazar Z, Fuchs S (1995) Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. J Biol Chem 270:29819-29824.

Fisher PM, Meltzer CC, Ziolko SK, Price JC, Moses-Kolko EL, Berga SL, Hariri AR (2006) Capacity for 5-HT1A-mediated autoregulation predicts amygdala reactivity. Nat Neurosci 9:1362-1363.

Flaig R, Greschik H, Peluso-Iltis C, Moras D (2005) Structural basis for the cell-specific activities of the NGFI-B and the Nurr1 ligand-binding domain. J Biol Chem 280:19250-19258.

Fleckenstein AE, Volz TJ, Riddle EL, Gibb JW, Hanson GR (2007) New insights into the mechanism of action of amphetamines. Annu Rev Pharmacol Toxicol 47:681-698.

Flores-Hernandez J, Cepeda C, Hernandez-Echeagaray E, Calvert CR, Jokel ES, Fienberg AA, Greengard P, Levine MS (2002) Dopamine enhancement of NMDA currents in dissociated medium-sized striatal neurons: role of D1 receptors and DARPP-32. J Neurophysiol 88:3010-3020.

Flower DR (1999) Modelling G-protein-coupled receptors for drug design. Biochim Biophys Acta 1422:207-234.

Franco R, Ferre S, Agnati L, Torvinen M, Gines S, Hillion J, Casado V, Lledo P, Zoli M, Lluis C, Fuxe K (2000) Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. Neuropsychopharmacology 23:S50-59.

Fratucci De Gobbi JI, De Luca LA, Jr., Johnson AK, Menani JV (2001) Interaction of serotonin and cholecystokinin in the lateral parabrachial nucleus to control sodium intake. Am J Physiol Regul Integr Comp Physiol 280:R1301-1307.

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63:1256-1272.

Freedman LJ, Cassell MD (1994) Distribution of dopaminergic fibers in the central division of the extended amygdala of the rat. Brain Res 633:243-252.

Freedman LJ, Shi C (2001) Monoaminergic innervation of the macaque extended amygdala. Neuroscience 104:1067-1084.

Freitag CM, Domschke K, Rothe C, Lee YJ, Hohoff C, Gutknecht L, Sand P, Fimmers R, Lesch KP, Deckert J (2006) Interaction of serotonergic and noradrenergic gene variants in panic disorder. Psychiatr Genet 16:59-65.

Fricker AD, Rios C, Devi LA, Gomes I (2005) Serotonin receptor activation leads to neurite outgrowth and neuronal survival. Brain Res Mol Brain Res 138:228-235.

Fukuda H, Sano N, Muto S, Horikoshi M (2006) Simple histone acetylation plays a complex role in the regulation of gene expression. Brief Funct Genomic Proteomic 5:190-208.

Fuxe K (1965a) Evidence For The Existence Of Monoamine Neurons In The Central Nervous System. 3. The Monoamine Nerve Terminal. Z Zellforsch Mikrosk Anat 65:573-596.

Fuxe K (1965b) Evidence For The Existence Of Monoamine Neurons In The Central Nervous System. Iv. Distribution Of Monoamine Nerve Terminals In The Central Nervous System. Acta Physiol Scand 64:SUPPL:37+.

Fuxe K, Agnati LF (1991) Volume Transmission in the Brain. Novel Mechanisms for Neural Transmission. In: Advances in Neuroscience (Fuxe K, Agnati LF, eds), pp 1-9. New York: Raven Press.

Fuxe K, Goldstein M, Hokfelt T, Joh TH (1970) Immunohistochemical localization of dopamine- -hydroxylase in the peripheral and central nervous system. Res Commun Chem Pathol Pharmacol 1:627-636.

Fuxe K, Cintra A, Agnati LF, Harfstrand A, Goldstein M (1988a) Studies on the relationship of tyrosine hydroxylase, dopamine and cyclic AMP-regulated phosphoprotein-32 immunoreactive neuronal structures and D1 in various brain regions of the male rat-mismatches indicate a role of D1 receptors in volume transmission. Neurochemistry International 13:179-197.

Fuxe K, Jacobsen KX, Höistad M, Tinner B, Jansson A, Staines WA, Agnati LF (2003a) The dopamine D1 receptor-rich main and paracapsular intercalated nerve cell groups of the rat amygdala: relationship to the dopamine innervation. Neuroscience 119:733-746.

Fuxe K, Agnati LF, Cintra A, Andersson K, Eneroth P, Harfstrand A, Zoli M, Goldstein M (1988b) Studies on central D1 receptors role in volume transmission, neuroendrocrine regulation and release of noradrenaline. Adv Exp Med Biol 235:83-119.

Fuxe K, Li XM, Tanganelli S, Hedlund P, O'Connor WT, Ferraro L, Ungerstedt U, Agnati LF (1995) Receptor-receptor interactions and their relevance for receptor diversity. Focus on neuropeptide/dopamine interactions. Ann N Y Acad Sci 757:365-376.

Fuxe K, Rivera A, Jacobsen KX, Höistad M, Leo G, Horvath TL, Staines W, De la Calle A, Agnati LF (2005) Dynamics of volume transmission in the brain. Focus on catecholamine and opioid peptide communication and the role of uncoupling protein 2. J Neural Transm 112:65-76.

Fuxe K, Agnati LF, Bjelke B, Hedlund P, Ueki A, Tinner B, Bunnemann B, Steinbusch H, Ganten D, Cintra A (1993) Novel aspects on central 5-hydroxytryptamine neurotransmission: Focus on the cerebellum. In: Serotonin, the Cerebellum and Ataxia (Trouillas P, Fuxe, K., ed), pp 1-36. New York: Raven Press Ltd.

Fuxe K, Agnati LF, Jacobsen K, Hillion J, Canals M, Torvinen M, Tinner-Staines B, Staines W, Rosin D, Terasmaa A, Popoli P, Leo G, Vergoni V, Lluis C, Ciruela F, Franco R, Ferre S (2003b) Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson's disease. Neurology 61:S19-23.

Fuxe K, Dahlstrom A, Hoistad M, Marcellino D, Jansson A, Rivera A, Diaz-Cabiale Z, Jacobsen K, Tinner-Staines B, Hagman B, Leo G, Staines W, Guidolin D, Kehr J, Genedani S, Belluardo N, Agnati LF (2007) From the Golgi-Cajal mapping to the transmitter-based characterization of the neuronal networks leading to two modes of brain communication: Wiring and volume transmission. Brain Res Rev 55:17-54.

Fyodorov D, Nelson T, Deneris E (1998) Pet-1, a novel ETS domain factor that can activate neuronal nAchR gene transcription. J Neurobiol 34:151-163.

Garfinkel BD, Froese A, Hood J (1982) Suicide attempts in children and adolescents. Am J Psychiatry 139:1257-1261.

Garris PA, Wightman RM (1994) In vivo voltammetric measurement of evoked extracellular dopamine in the rat basolateral amygdaloid nucleus. J Physiol 478 (Pt 2):239-249.

Garzon M, Pickel VM (2001) Plasmalemmal mu-opioid receptor distribution mainly in nondopaminergic neurons in the rat ventral tegmental area. Synapse 41:311-328.

Gaspar P, Cases O, Maroteaux L (2003) The developmental role of serotonin: news from mouse molecular genetics. Nat Rev Neurosci 4:1002-1012.

Geffen LB, Livett BG, Rush RA (1969) Immunohistochemical localizatio of protein components of catecholamine storage vesicles. J Physiol 204:593-605.

Gerfen CR (1988) Synaptic organization of the striatum. J Electron Microsc Tech 10:265-281.

Gerfen CR (1989) The neostriatal mosaic: striatal patch-matrix organization is related to cortical lamination. Science 246:385-388.

Gerfen CR (2006) Indirect-pathway neurons lose their spines in Parkinson disease. Nat Neurosci 9:157-158.

Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 21:90-113.

Ghahremani MH, Forget C, Albert PR (2000) Distinct roles for Galpha(i)2 and Gbetagamma in signaling to DNA synthesis and Galpha(i)3 in cellular transformation by dopamine D2S receptor activation in BALB/c 3T3 cells. Mol Cell Biol 20:1497-1506.

Ghashghaei HT, Barbas H (2002) Pathways for emotion: interactions of prefrontal and anterior temporal pathways in the amygdala of the rhesus monkey. Neuroscience 115:1261-1279.

Gilles C, Lotstra F, Vanderhaeghen JJ (1983) CCK nerve terminals in the rat striatal and limbic areas originate partly in the brain stem and partly in telencephalic structures. Life Sci 32:1683-1690.

Gines S, Hillion J, Torvinen M, Le Crom S, Casado V, Canela EI, Rondin S, Lew JY, Watson S, Zoli M, Agnati LF, Verniera P, Lluis C, Ferre S, Fuxe K, Franco R (2000) Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci U S A 97:8606-8611.

Glass MJ, Billington CJ, Levine AS (1999) Opioids and food intake: distributed functional neural pathways? Neuropeptides 33:360-368.

Gonon F, Burie JB, Jaber M, Benoit-Marand M, Dumartin B, Bloch B (2000) Geometry and kinetics of dopaminergic transmission in the rat striatum and in mice lacking the dopamine transporter. In: Volume Transmission Revisited (Agnati LF, Fuxe K, Nicholson C, Sykova E, eds), pp 291-302. Amsterdam: Elsevier Science.

Gothert M, Schlicker E, Fink K, Classen K (1987) Effects of RU 24969 on serotonin release in rat brain cortex: further support for the identity of serotonin autoreceptors with 5-HT1B sites. Arch Int Pharmacodyn Ther 288:31-42.

Government_of_Canada (2006) The Human Face of Mental Health and Mental Illness in Canada. In: (Canada MDSo, ed). Ottawa: Minister of Public Works and Government Services Canada.

Grandy DK, Marchionni MA, Makam H, Stofko RE, Alfano M, Frothingham L, Fischer JB, Burke-Howie KJ, Bunzow JR, Server AC, et al. (1989) Cloning of the cDNA and gene for a human D2 dopamine receptor. Proc Natl Acad Sci U S A 86:9762-9766.

Gratton MO, Torban E, Jasmin SB, Theriault FM, German MS, Stifani S (2003) Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. Mol Cell Biol 23:6922-6935.

Gray JA, Bhatnagar A, Gurevich VV, Roth BL (2003) The interaction of a constitutively active arrestin with the arrestin-insensitive 5-HT(2A) receptor induces agonist-independent internalization. Mol Pharmacol 63:961-972.

Graybiel AM, Illing RB (1994) Enkephalin-positive and acetylcholinesterase-positive patch systems in the superior colliculus have matching distributions but distinct developmental histories. J Comp Neurol 340:297-310.

Greengard P (2001) The neurobiology of dopamine signaling. Biosci Rep 21:247-269.

Greengard P, Allen PB, Nairn AC (1999) Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron 23:435-447.

Greengard P, Nairn AC, Girault JA, Ouimet CC, Snyder GL, Fisone G, Allen PB, Fienberg A, Nishi A (1998) The DARPP-32/protein phosphatase-1 cascade: a model for signal integration. Brain Res Brain Res Rev 26:274-284.

Grevet EH, Marques FZ, Salgado CA, Fischer AG, Kalil KL, Victor MM, Garcia CR, Sousa NO, Belmonte-de-Abreu P, Bau CH (2007) Serotonin transporter gene polymorphism and the phenotypic heterogeneity of adult ADHD. J Neural Transm.

Grimes DA, Han F, Panisset M, Racacho L, Xiao F, Zou R, Westaff K, Bulman DE (2006) Translated mutation in the Nurr1 gene as a cause for Parkinson's disease. Mov Disord 21:906-909.

Gross C, Hen R (2004) The developmental origins of anxiety. Nat Rev Neurosci 5:545-552.

Gross C, Santarelli L, Brunner D, Zhuang X, Hen R (2000) Altered fear circuits in 5-HT(1A) receptor KO mice. Biol Psychiatry 48:1157-1163.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Guarraci FA, Frohardt RJ, Kapp BS (1999) Amygdaloid D1 dopamine receptor involvement in Pavlovian fear conditioning. Brain Res 827:28-40.

Guerrero C, Pesce L, Lecuona E, Ridge KM, Sznajder JI (2002) Dopamine activates ERKs in alveolar epithelial cells via Ras-PKC-dependent and Grb2/Sos-independent mechanisms. Am J Physiol Lung Cell Mol Physiol 282:L1099-1107.

Hajos-Korcsok E, Sharp T (1999) Effect of 5-HT(1A) receptor ligands on Fos-like immunoreactivity in rat brain: evidence for activation of noradrenergic transmission. Synapse 34:145-153.

Hajos-Korcsok E, McQuade R, Sharp T (1999) Influence of 5-HT1A receptors on central noradrenergic activity: microdialysis studies using (+/-)-MDL 73005EF and its enantiomers. Neuropharmacology 38:299-306.

Hajos M, Hajos-Korcsok E, Sharp T (1999) Role of the medial prefrontal cortex in 5-HT1A receptor-induced inhibition of 5-HT neuronal activity in the rat. Br J Pharmacol 126:1741-1750.

Hamel E, Beaudet A (1987) Opioid receptors in rat neostriatum: radioautographic distribution at the electron microscopic level. Brain Res 401:239-257.

Hamilton ME, Redondo JL, Freeman AS (2000) Overflow of dopamine and cholecystokinin in rat nucleus accumbens in response to acute drug administration. Synapse 38:238-242.

Han YH, Cao X, Lin B, Lin F, Kolluri SK, Stebbins J, Reed JC, Dawson MI, Zhang XK (2006) Regulation of Nur77 nuclear export by c-Jun N-terminal kinase and Akt. Oncogene 25:2974-2986.

Hara Y, Pickel VM (2005) Overlapping intracellular and differential synaptic distributions of dopamine D1 and glutamate N-methyl-D-aspartate receptors in rat nucleus accumbens. J Comp Neurol 492:442-455.

Harbers E (1969) On the discovery of DNA by Friedrich Miescher 100 years ago. Ger Med Mon 14:517-518.

Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, Egan MF, Weinberger DR (2002) Serotonin transporter genetic variation and the response of the human amygdala. Science 297:400-403.

Harlow HF (1965) Total Social Isolation: Effects on Macaque Monkey Behavior. Science 148:666.

Harlow HF, Suomi SJ (1971) Social recovery by isolation-reared monkeys. Proc Natl Acad Sci U S A 68:1534-1538.

Hartman BK, Udenfriend S (1970) Immunofluorescent localization of dopamine beta-hydroxylase in tissues. Mol Pharmacol 6:85-94.

Hasue RH, Shammah-Lagnado SJ (2002) Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. J Comp Neurol 454:15-33.

Hatakeyama J, Sakamoto S, Kageyama R (2006) Hes1 and Hes5 regulate the development of the cranial and spinal nerve systems. Dev Neurosci 28:92-101.

Hatakeyama J, Bessho Y, Katoh K, Ookawara S, Fujioka M, Guillemot F, Kageyama R (2004) Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131:5539-5550. .

Hauser RA, Zesiewicz TA (2007) Advances in the pharmacologic management of early Parkinson disease. Neurologist 13:126-132.

Hayashi T, Mendelson B, Phelan KD, Skinner RD, Garcia-Rill E (1997) Developmental changes in serotonergic receptor-mediated modulation of embryonic chick motoneurons in vitro. Brain Res Dev Brain Res 102:21-33.

Health_Canada (2002) A Report on Mental Illness in Canada. In: (Canada H, ed). Ottawa: Health Canada Editorial Board Mental Illnesses in Canada.

Heimer L (2000) Basal forebrain in the context of schizophrenia. Brain Res Brain Res Rev 31:205-235.

Heimer L, Alheid GF (1991) Piecing together the puzzle of basal forebrain anatomy. Adv Exp Med Biol 295:1-42.

Heimer L, Harlan RE, Alheid GF, Garcia MM, de Olmos J (1997) Substantia innominata: a notion which impedes clinical-anatomical correlations in neuropsychiatric disorders. Neuroscience 76:957-1006.

Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Gotz M (2002) Glial cells generate neurons: the role of the transcription factor Pax6. Nat Neurosci 5:308-315.

Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH (1998) Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc Natl Acad Sci U S A 95:15049-15054.

Heisler LK, Pronchuk N, Nonogaki K, Zhou L, Raber J, Tung L, Yeo GS, O'Rahilly S, Colmers WF, Elmquist JK, Tecott LH (2007) Serotonin activates the hypothalamic-pituitary-adrenal axis via serotonin 2C receptor stimulation. J Neurosci 27:6956-6964.

Hendricks T, Francis N, Fyodorov D, Deneris ES (1999) The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. J Neurosci 19:10348-10356.

Hendricks TJ, Fyodorov DV, Wegman LJ, Lelutiu NB, Pehek EA, Yamamoto B, Silver J, Weeber EJ, Sweatt JD, Deneris ES (2003) Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 37:233-247.

Hensler JG (2006) Serotonergic modulation of the limbic system. Neurosci Biobehav Rev 30:203-214.

Herkenham M, McLean S (1988) The anatomical relationship of opioid peptides and opiate receptors in the hippocampi of four rodent species. NIDA Res Monogr 82:33-47.

Hermans E (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. Pharmacol Ther 99:25-44.

Hermanson E, Joseph B, Castro D, Lindqvist E, Aarnisalo P, Wallen A, Benoit G, Hengerer B, Olson L, Perlmann T (2003) Nurr1 regulates dopamine synthesis and storage in MN9D dopamine cells. Exp Cell Res 288:324-334.

Hettema JM, Prescott CA, Kendler KS (2001) A population-based twin study of generalized anxiety disorder in men and women. J Nerv Ment Dis 189:413-420.

Heyman I, Mataix-Cols D, Fineberg NA (2006) Obsessive-compulsive disorder. Bmj 333:424-429.

Heyser CJ, Roberts AJ, Schulteis G, Koob GF (1999) Central administration of an opiate antagonist decreases oral ethanol self-administration in rats. Alcohol Clin Exp Res 23:1468-1476.

Hillion J, Milne-Edwards JB, Catelon J, de Vitry F, Gros F, Hamon M (1993) Prenatal developmental expression of rat brain 5-HT1A receptor gene followed by PCR. Biochem Biophys Res Commun 191:991-997.

Hirata H, Tomita K, Bessho Y, Kageyama R (2001) Hes1 and Hes3 regulate maintenance of the isthmic organizer and development of the mid/hindbrain. Embo J 20:4454-4466.

Hirata H, Yoshiura S, Ohtsuka T, Bessho Y, Harada T, Yoshikawa K, Kageyama R (2002) Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science 298:840-843.

Hjorth S, Tao R (1991) The putative 5-HT1B receptor agonist CP-93,129 suppresses rat hippocampal 5-HT release in vivo: comparison with RU 24969. Eur J Pharmacol 209:249-252.

Hjorth S, Suchowski CS, Galloway MP (1995) Evidence for 5-HT autoreceptor-mediated, nerve impulse-independent, control of 5-HT synthesis in the rat brain. Synapse 19:170-176.

Höistad M, Samskog J, Jacobsen KX, Olsson A, Hansson HA, Brodin E, Fuxe K (2005) Detection of beta-endorphin in the cerebrospinal fluid after intrastriatal microinjection into the rat brain. Brain Res 1041:167-180.

Hokfelt T, Johansson O, Fuxe K, Goldstein M, Park D (1976) Immunohistochemical studies on the localization and distribution of monoamine neuron systems in the rat brain. I. Tyrosine hydroxylase in the mes- and diencephalon. Med Biol 54:427-453.

Hokfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M, Markey K (1980) Evidence for coexistence of dopamine and CCK in meso-limbic neurones. Nature 285:476-478.

Hollis JH, Evans AK, Bruce KP, Lightman SL, Lowry CA (2006) Lipopolysaccharide has indomethacin-sensitive actions on Fos expression in topographically organized subpopulations of serotonergic neurons. Brain Behav Immun 20:569-577.

Hong CJ, Chen TJ, Yu YW, Tsai SJ (2006a) Response to fluoxetine and serotonin 1A receptor (C-1019G) polymorphism in Taiwan Chinese major depressive disorder. Pharmacogenomics J 6:27-33.

Hong SJ, Huh Y, Chae H, Hong S, Lardaro T, Kim KS (2006b) GATA-3 regulates the transcriptional activity of tyrosine hydroxylase by interacting with CREB. J Neurochem 98:773-781.

Horacek J, Bubenikova-Valesova V, Kopecek M, Palenicek T, Dockery C, Mohr P, Hoschl C (2006) Mechanism of action of atypical antipsychotic drugs and the neurobiology of schizophrenia. CNS Drugs 20:389-409.

Houben A, Demidov D, Caperta AD, Karimi R, Agueci F, Vlasenko L (2007) Phosphorylation of histone H3 in plants--a dynamic affair. Biochim Biophys Acta 1769:308-315.

Hoyer D, Martin G (1997) 5-HT receptor classification and nomenclature: towards a harmonization with the human genome. Neuropharmacology 36:419-428.

Hsieh-Wilson LC, Allen PB, Watanabe T, Nairn AC, Greengard P (1999) Characterization of the neuronal targeting protein spinophilin and its interactions with protein phosphatase-1. Biochemistry 38:4365-4373.

Huang YY, Kandel ER (2007) 5-Hydroxytryptamine induces a protein kinase A/mitogen-activated protein kinase-mediated and macromolecular synthesis-dependent late phase of long-term potentiation in the amygdala. J Neurosci 27:3111-3119.

Huang YY, Battistuzzi C, Oquendo MA, Harkavy-Friedman J, Greenhill L, Zalsman G, Brodsky B, Arango V, Brent DA, Mann JJ (2004) Human 5-HT1A receptor C(-1019)G polymorphism and psychopathology. Int J Neuropsychopharmacol 7:441-451.

Hudson JI, Pope HG, Jr. (1990) Affective spectrum disorder: does antidepressant response identify a family of disorders with a common pathophysiology? Am J Psychiatry 147:552-564.

Hudson JI, Mangweth B, Pope HG, Jr., De Col C, Hausmann A, Gutweniger S, Laird NM, Biebl W, Tsuang MT (2003) Family study of affective spectrum disorder. Arch Gen Psychiatry 60:170-177.

Huggenvik JI, Michelson RJ, Collard MW, Ziemba AJ, Gurley P, Mowen KA (1998) Characterization of a nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related (NUDR) transcriptional regulator protein. Mol Endocrinol 12:1619-1639.

Hurd YL, Svensson P, Ponten M (1999) The role of dopamine, dynorphin, and CART systems in the ventral striatum and amygdala in cocaine abuse. Ann N Y Acad Sci 877:499-506.

Hyman SE, Malenka RC (2001) Addiction and the brain: the neurobiology of compulsion and its persistence. Nat Rev Neurosci 2:695-703.

Hynes M, Rosenthal A (1999) Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. Curr Opin Neurobiol 9:26-36.

Ingram VM (1956) A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. Nature 178:792-794.

Ingram VM (1957a) The sulphydryl groups of sickle-cell haemoglobin. Biochem J 65:760-763.

Ingram VM (1957b) Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell haemoglobin. Nature 180:326-328.

Ishibashi M, Ang SL, Shiota K, Nakanishi S, Kageyama R, Guillemot F (1995) Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev 9:3136-3148.

Ishibashi T, Tagashira R, Nakamura M, Noguchi H, Ohno Y (1999) Effects of perospirone, a novel 5-HT2 and D2 receptor antagonist, on Fos protein expression in the rat forebrain. Pharmacol Biochem Behav 63:535-541.

Ito K (2007) Impact of post-translational modifications of proteins on the inflammatory process. Biochem Soc Trans 35:281-283.

Ito T, Udaka N, Yazawa T, Okudela K, Hayashi H, Sudo T, Guillemot F, Kageyama R, Kitamura H (2000) Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. Development 127:3913-3921.

Itokawa M, Toru M, Ito K, Tsuga H, Kameyama K, Haga T, Arinami T, Hamaguchi H (1996) Sequestration of the short and long isoforms of dopamine D2 receptors expressed in Chinese hamster ovary cells. Mol Pharmacol 49:560-566.

Iyo AH, Porter B, Deneris ES, Austin MC (2005) Regional distribution and cellular localization of the ETS-domain transcription factor, FEV, mRNA in the human postmortem brain. Synapse 57:223-228.

Jacobsen KX, Staines WA (2004) Vibration enhancement of slide-mounted immunofluorescence staining. J Neurosci Methods 137:71-77.

Jacobsen KX, Hoistad M, Staines WA, Fuxe K (2006) The distribution of dopamine D1 receptor and mu-opioid receptor 1 receptor immunoreactivities in the amygdala and interstitial nucleus of the posterior limb of the anterior commissure: relationships to tyrosine hydroxylase and opioid peptide terminal systems. Neuroscience 141:2007-2018.

Jacobsen KX, Vanderluit J, Slack RS, Albert PR (2007) HES1 regulates 5-HT1A receptor gene transcription at a functional polymorphism: Essential role in developmental expression. Mol Cell Neurosci Submitted.

Jankovic J, Chen S, Le WD (2005) The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. Prog Neurobiol 77:128-138.

Jansson A, Tinner B, Bancila M, Verge D, Steinbusch HW, Agnati LF, Fuxe K (2001a) Relationships of 5-hydroxytryptamine immunoreactive terminal-like varicosities to 5-hydroxytryptamine-2A receptor-immunoreactive neuronal processes in the rat forebrain. J Chem Neuroanat 22:185-203.

Jansson A, Descarries L, Cornea-Hébert V, Riad M, Vergé D, Bancila M, Agnati LF, Fuxe K (2001b) Transmittor-Receptor Mismatches in Central Dopamine, Serotonin and Neuropeptide Systems. Further Evidence for Volume Transmission. In: The Neuronal Environment: Brain Homeostasis in Health and Disease (Walz W, ed), pp 83-108. Totowa NJ, USA.: Humana Press.

Jansson A, Descarries L, Cornea-Hébert V, Riad M, Vergé D, Bancila M, Agnati LF, Fuxe K (2002) Transmitter-receptor mismatches in central dopamine, serotonin and neuropeptide systems: further evidence for volume transmission. In: The neuronal environment: brain homeostasis in health and disease (Walz W, ed), pp 83-108. Totowa: Human Press.

Jansson A, Goldstein M, Tinner B, Zoli M, Meador-Woodruff JH, Lew JY, Levey AI, Watson S, Agnati LF, Fuxe K (1999) On the distribution patterns of D1, D2, tyrosine hydroxylase and dopamine transporter immunoreactivities in the ventral striatum of the rat. Neuroscience 89:473-489.

Jennings BH, Pickles LM, Wainwright SM, Roe SM, Pearl LH, Ish-Horowicz D (2006) Molecular recognition of transcriptional repressor motifs by the WD domain of the Groucho/TLE corepressor. Mol Cell 22:645-655.

Jhas S, Ciura S, Belanger-Jasmin S, Dong Z, Llamosas E, Theriault FM, Joachim K, Tang Y, Liu L, Liu J, Stifani S (2006) Hes6 inhibits astrocyte differentiation and promotes neurogenesis through different mechanisms. J Neurosci 26:11061-11071.

Johnston JB (1923) Further contributions to the study of evolution of the forebrain. J Comp Neurol 35:337-481.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. Nature 396:674-679.

Jorgensen JR, Juliusson B, Henriksen KF, Hansen C, Knudsen S, Petersen TN, Blom N, Seiger A, Wahlberg LU (2006) Identification of novel genes regulated in the developing human ventral mesencephalon. Exp Neurol 198:427-437.

Kadonaga JT (2004) Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell 116:247-257.

Kagan J, Snidman N (1999) Early childhood predictors of adult anxiety disorders. Biol Psychiatry 46:1536-1541.

Kageyama R, Ohtsuka T (1999) The Notch-Hes pathway in mammalian neural development. Cell Res 9:179-188.

Kageyama R, Hatakeyama J, Ohtsuka T (2006) Roles of Hes bHLH Factors in Neural Development. In: Transcription Factors in the Nervous System: Development, Brain Function, and Diseases (Thiel Ge, ed), pp 3-22. Weinheim: Wiley-VCH.

Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R (2005) Roles of bHLH genes in neural stem cell differentiation. Exp Cell Res 306:343-348.

Kang SA, Seol JH, Kim J (2005) The conserved WRPW motif of Hes6 mediates proteasomal degradation. Biochem Biophys Res Commun 332:33-36.

Katagiri Y, Takeda K, Yu ZX, Ferrans VJ, Ozato K, Guroff G (2000) Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B. Nat Cell Biol 2:435-440.

Katoh M, Katoh M (2007) WNT signaling pathway and stem cell signaling network. Clin Cancer Res 13:4042-4045.

Kehr J, Höistad M, Fuxe K (2000) Diffusion of radiolabeled dopamine, its metabolites and mannitol in the rat striatum studied by dual-probe microdialysis. Prog Brain Res 125:179-190.

Kessler MA, Yang M, Gollomp KL, Jin H, Iacovitti L (2003) The human tyrosine hydroxylase gene promoter. Brain Res Mol Brain Res 112:8-23.

Khachaturian H, Lewis, M., Tsou, K., Watson, S. (1986) Beta-endorphin, alfa-MSH, ACTH, and related peptides. In: Handbook of Chemical Neuroanatomy: GABA and Neuropeptides in the CNS (Bjorklund A, Hokfelt, T., ed), pp 216-272.

Khan ZU, Mrzljak L, Gutierrez A, de la Calle A, Goldman-Rakic PS (1998) Prominence of the dopamine D2 short isoform in dopaminergic pathways. Proc Natl Acad Sci U S A 95:7731-7736.

Khawaja X, Evans N, Reilly Y, Ennis C, Minchin MC (1995) Characterisation of the binding of [3H]WAY-100635, a novel 5-hydroxytryptamine1A receptor antagonist, to rat brain. J Neurochem 64:2716-2726.

Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50-56.

Kim KS, Kim CH, Hwang DY, Seo H, Chung S, Hong SJ, Lim JK, Anderson T, Isacson O (2003) Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. J Neurochem 85:622-634.

Kim SY, Choi KC, Chang MS, Kim MH, Kim SY, Na YS, Lee JE, Jin BK, Lee BH, Baik JH (2006) The dopamine D2 receptor regulates the development of dopaminergic neurons via extracellular signal-regulated kinase and Nurr1 activation. J Neurosci 26:4567-4576.

Kimura J, Katahira T, Araki I, Nakamura H (2004) Possible role of Hes5 for the rostrocaudal polarity formation of the tectum. Dev Growth Differ 46:219-227.

Kimura K, White BH, Sidhu A (1995) Coupling of human D-1 dopamine receptors to different guanine nucleotide binding proteins. Evidence that D-1 dopamine receptors can couple to both Gs and G(o). J Biol Chem 270:14672-14678.

Kita H, Kitai ST (1990) Amygdaloid projections to the frontal cortex and the striatum in the rat. J Comp Neurol 298:40-49.

Klemenhagen KC, Gordon JA, David DJ, Hen R, Gross CT (2006) Increased fear response to contextual cues in mice lacking the 5-HT1A receptor. Neuropsychopharmacology 31:101-111.

Kohout TA, Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. Mol Pharmacol 63:9-18.

Koller G, Bondy B, Preuss UW, Zill P, Soyka M (2006) The C(-1019)G 5-HT1A promoter polymorphism and personality traits: no evidence for significant association in alcoholic patients. Behav Brain Funct 2:7.

Koob GF (2003a) Alcoholism: allostasis and beyond. Alcohol Clin Exp Res 27:232-243.

Koob GF (2003b) Neuroadaptive mechanisms of addiction: studies on the extended amygdala. Eur Neuropsychopharmacol 13:442-452.

Kornberg R (2007a) The Molecular Basis of Eukaryotic Transcription (Nobel Lecture). Angew Chem Int Ed Engl 46:6956-6965.

Kornberg RD (2007b) The molecular basis of eukaryotic transcription. Proc Natl Acad Sci U S A 104:12955-12961.

Korte SM, Meijer OC, de Kloet ER, Buwalda B, Keijser J, Sluyter F, van Oortmerssen G, Bohus B (1996) Enhanced 5-HT1A receptor expression in forebrain regions of aggressive house mice. Brain Res 736:338-343.

Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E (2002) Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16:1638-1651.

Kozak M (2001) New ways of initiating translation in eukaryotes? Mol Cell Biol 21:1899-1907.

Krasel C, Vilardaga JP, Bunemann M, Lohse MJ (2004) Kinetics of G-protein-coupled receptor signalling and desensitization. Biochem Soc Trans 32:1029-1031.

Kraus MR, Al-Taie O, Schafer A, Pfersdorff M, Lesch KP, Scheurlen M (2007) Serotonin-1A receptor gene HTR1A variation predicts interferon-induced depression in chronic hepatitis C. Gastroenterology 132:1279-1286.

Kroeze WK, Sheffler DJ, Roth BL (2003) G-protein-coupled receptors at a glance. J Cell Sci 116:4867-4869.

Kusserow H, Davies B, Hortnagl H, Voigt I, Stroh T, Bert B, Deng DR, Fink H, Veh RW, Theuring F (2004) Reduced anxiety-related behaviour in transgenic mice overexpressing serotonin 1A receptors. Brain Res Mol Brain Res 129:104-116.

LaHoste GJ, Henry BL, Marshall JF (2000) Dopamine D1 receptors synergize with D2, but not D3 or D4, receptors in the striatum without the involvement of action potentials. J Neurosci 20:6666-6671.

Lamont EW, Kokkinidis L (1998) Infusion of the dopamine D1 receptor antagonist SCH 23390 into the amygdala blocks fear expression in a potentiated startle paradigm. Brain Res 795:128-136.

Lanfumey L, Hamon M (2004) 5-HT1 receptors. Curr Drug Targets CNS Neurol Disord 3:1-10.

Lang AE, Lozano AM (1998a) Parkinson's disease. Second of two parts. N Engl J Med 339:1130-1143.

Lang AE, Lozano AM (1998b) Parkinson's disease. First of two parts. N Engl J Med 339:1044-1053.

Lang RE, Bruckner UB, Kempf B, Rascher W, Sturm V, Unger T, Speck G, Ganten D (1982) Opioid peptides and blood pressure regulation. Clin Exp Hypertens A 4:249-269.

Langen B, Rundfeldt C (2007) ELB139 an agonist at the benzodiazepine binding site increases 5-HT in the striatum and prefrontal cortex of rats: a microdialysis study. Pharmacol Biochem Behav 86:79-85.

Latchman DS (2004) Gene Regulation: A eukaryotic perspective, Fifth Edition. New York: Taylor & Francis Group.

Lauder JM (1990) Ontogeny of the serotonergic system in the rat: serotonin as a developmental signal. Ann N Y Acad Sci 600:297-313.

Lauder JM, Liu J, Grayson DR (2000) In utero exposure to serotonergic drugs alters neonatal expression of 5-HT(1A) receptor transcripts: a quantitative RT-PCR study. Int J Dev Neurosci 18:171-176.

Law SW, Conneely OM, DeMayo FJ, O'Malley BW (1992) Identification of a new brain-specific transcription factor, NURR1. Mol Endocrinol 6:2129-2135.

Le Poul E, Boni C, Hanoun N, Laporte AM, Laaris N, Chauveau J, Hamon M, Lanfumey L (2000) Differential adaptation of brain 5-HT1A and 5-HT1B receptors and 5-HT transporter in rats treated chronically with fluoxetine. Neuropharmacology 39:110-122.

Le WD, Xu P, Jankovic J, Jiang H, Appel SH, Smith RG, Vassilatis DK (2003) Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet 33:85-89.

LeDoux JE (1995) Emotion: clues from the brain. Annu Rev Psychol 46:209-235.

Lee FJ, Xue S, Pei L, Vukusic B, Chery N, Wang Y, Wang YT, Niznik HB, Yu XM, Liu F (2002a) Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. Cell 111:219-230.

Lee JH, Kim HJ, Kim JG, Ryu V, Kim BT, Kang DW, Jahng JW (2007) Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. Neurosci Res 58:32-39.

Lee MA, Lee HS, Lee HS, Cho KG, Jin BK, Sohn S, Lee YS, Ichinose H, Kim SU (2002b) Overexpression of midbrain-specific transcription factor Nurr1 modifies susceptibility of mouse neural stem cells to neurotoxins. Neurosci Lett 333:74-78.

Lemonde S, Rogaeva A, Albert PR (2004a) Cell type-dependent recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene. J Neurochem 88:857-868.

Lemonde S, Du L, Bakish D, Hrdina P, Albert PR (2004b) Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int J Neuropsychopharmacol 7:501-506.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci 23:8788-8799.

Lenicov FR, Lemonde S, Czesak M, Mosher TM, Albert PR (2007) Cell-type specific induction of tryptophan hydroxylase-2 transcription by calcium mobilization. J Neurochem In Press.

Leonardo ED, Hen R (2006) Genetics of affective and anxiety disorders. Annu Rev Psychol 57:117-137.

Levey AI, Hersch SM, Rye DB, Sunahara RK, Niznik HB, Kitt CA, Price DL, Maggio R, Brann MR, Ciliax BJ, et al. (1993) Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. Proc Natl Acad Sci U S A 90:8861-8865.

Levine S (2005) Developmental determinants of sensitivity and resistance to stress. Psychoneuroendocrinology 30:939-946.

Levinson DF (2006) The genetics of depression: a review. Biol Psychiatry 60:84-92.

Levinthal C (1956) The Mechanism of DNA Replication and Genetic Recombination in Phage. Proc Natl Acad Sci U S A 42:394-404.

Lewin B (1997) Genes. Oxford: Oxford University Press.

Li Q, Wichems C, Heils A, Lesch KP, Murphy DL (2000) Reduction in the density and expression, but not G-protein coupling, of serotonin receptors (5-HT1A) in 5-HT transporter knock-out mice: gender and brain region differences. J Neurosci 20:7888-7895.

Li X, Inoue T, Abekawa T, Weng S, Nakagawa S, Izumi T, Koyama T (2006) 5-HT1A receptor agonist affects fear conditioning through stimulations of the postsynaptic 5-HT1A receptors in the hippocampus and amygdala. Eur J Pharmacol 532:74-80.

Lieberman A (2006) Depression in Parkinson's disease -- a review. Acta Neurol Scand 113:1-8.

Lindgren N, Goiny M, Herrera-Marschitz M, Haycock JW, Hokfelt T, Fisone G (2002) Activation of extracellular signal-regulated kinases 1 and 2 by depolarization stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat brain. Eur J Neurosci 15:769-773.

Lindgren N, Usiello A, Goiny M, Haycock J, Erbs E, Greengard P, Hokfelt T, Borrelli E, Fisone G (2003) Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites. Proc Natl Acad Sci U S A 100:4305-4309.

Lindvall O, Bjorklund A (1978) Anatomy of the dopaminergic neuron systems in the rat brain. Adv Biochem Psychopharmacol 19:1-23.

Lindvall O, Bjorklund A, Skagerberg G (1984) Selective histochemical demonstration of dopamine terminal systems in rat di- and telencephalon: new evidence for dopaminergic innervation of hypothalamic neurosecretory nuclei. Brain Res 306:19-30.

Liu JC, Baker RE, Chow W, Sun CK, Elsholtz HP (2005) Epigenetic mechanisms in the dopamine D2 receptor-dependent inhibition of the prolactin gene. Mol Endocrinol 19:1904-1917.

Liu YF, Civelli O, Grandy DK, Albert PR (1992) Differential sensitivity of the short and long human dopamine D2 receptor subtypes to protein kinase C. J Neurochem 59:2311-2317.

Lowry CA, Hollis JH, de Vries A, Pan B, Brunet LR, Hunt JR, Paton JF, van Kampen E, Knight DM, Evans AK, Rook GA, Lightman SL (2007) Identification of an immune-responsive mesolimbocortical serotonergic system: potential role in regulation of emotional behavior. Neuroscience 146:756-772.

Lumsden A, Krumlauf R (1996) Patterning the vertebrate neuraxis. Science 274:1109-1115.

Luna-Munguia H, Manuel-Apolinar L, Rocha L, Meneses A (2005) 5-HT1A receptor expression during memory formation. Psychopharmacology (Berl) 181:309-318.

Luo Y, Kokkonen GC, Wang X, Neve KA, Roth GS (1998) D2 dopamine receptors stimulate mitogenesis through pertussis toxin-sensitive G proteins and Ras-involved ERK and SAP/JNK pathways in rat C6-D2L glioma cells. J Neurochem 71:980-990.

Ma QP, Yin GF, Ai MK, Han JS (1991) Serotonergic projections from the nucleus raphe dorsalis to the amygdala in the rat. Neurosci Lett 134:21-24.

MacMillan SJ, Mark MA, Duggan AW (1998) The release of beta-endorphin and the neuropeptide-receptor mismatch in the brain. Brain Res 794:127-136.

Mages HW, Rilke O, Bravo R, Senger G, Kroczek RA (1994) NOT, a human immediate-early response gene closely related to the steroid/thyroid hormone receptor NAK1/TR3. Mol Endocrinol 8:1583-1591.

Maheux J, Ethier I, Rouillard C, Levesque D (2005) Induction patterns of transcription factors of the nur family (nurr1, nur77, and nor-1) by typical and atypical antipsychotics in the mouse brain: implication for their mechanism of action. J Pharmacol Exp Ther 313:460-473.

Maltais S, S Ct, Drolet G, Falardeau P (2000) Cellular colocalization of dopamine D1 mRNA and D2 receptor in rat brain using a D2 dopamine receptor specific polyclonal antibody. Prog Neuropsychopharmacol Biol Psychiatry 24:1127-1149.

Mansour A, Fox CA, Akil H, Watson SJ (1995a) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci 18:22-29.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ (1987) Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. J Neurosci 7:2445-2464.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ (1988) Anatomy of CNS opioid receptors. Trends Neurosci 11:308-314.

Mansour A, Fox CA, Burke S, Akil H, Watson SJ (1995b) Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. J Chem Neuroanat 8:283-305.

Mansour A, Burke S, Pavlic RJ, Akil H, Watson SJ (1996) Immunohistochemical localization of the cloned kappa 1 receptor in the rat CNS and pituitary. Neuroscience 71:671-690.

Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. J Comp Neurol 350:412-438.

Mansour HA, Talkowski ME, Wood J, Pless L, Bamne M, Chowdari KV, Allen M, Bowden CL, Calabrese J, El-Mallakh RS, Fagiolini A, Faraone SV, Fossey MD, Friedman ES, Gyulai L, Hauser P, Ketter TA, Loftis JM, Marangell LB, Miklowitz DJ, Nierenberg AA, Patel J, Sachs GS, Sklar P, Smoller JW, Thase ME, Frank E, Kupfer DJ, Nimgaonkar VL (2005) Serotonin gene polymorphisms and bipolar I disorder: focus on the serotonin transporter. Ann Med 37:590-602.

Marowsky A, Yanagawa Y, Obata K, Vogt KE (2005) A specialized subclass of interneurons mediates dopaminergic facilitation of amygdala function. Neuron 48:1025-1037.

Marti M, Mela F, Bianchi C, Beani L, Morari M (2002) Striatal dopamine-NMDA receptor interactions in the modulation of glutamate release in the substantia nigra pars reticulata in vivo: opposite role for D1 and D2 receptors. J Neurochem 83:635-644.

Martinez-Gonzalez J, Badimon L (2005) The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. Cardiovasc Res 65:609-618.

Mason HA, Rakowiecki SM, Raftopoulou M, Nery S, Huang Y, Gridley T, Fishell G (2005) Notch signaling coordinates the patterning of striatal compartments. Development 132:4247-4258. Epub 2005 Aug 4224.

Math SB, Janardhan Reddy YC (2007) Issues in the pharmacological treatment of obsessive-compulsive disorder. Int J Clin Pract 61:1188-1197.

Mathe AA (1999) Neuropeptides and electroconvulsive treatment. J Ect 15:60-75.

Matthes HW, Smadja C, Valverde O, Vonesch JL, Foutz AS, Boudinot E, Denavit-Saubie M, Severini C, Negri L, Roques BP, Maldonado R, Kieffer BL (1998) Activity of the delta-opioid receptor is partially reduced, whereas activity of the kappa-receptor is maintained in mice lacking the mu-receptor. J Neurosci 18:7285-7295.

Maudsley S, Martin B, Luttrell LM (2007) G protein-coupled receptor signaling complexity in neuronal tissue: implications for novel therapeutics. Curr Alzheimer Res 4:3-19.

Maurer P, Rorive S, de Kerchove d'Exaerde A, Schiffmann SN, Salmon I, de Launoit Y (2004) The Ets transcription factor Fev is specifically expressed in the human central serotonergic neurons. Neurosci Lett 357:215-218.

Maurice N, Deniau JM, Menetrey A, Glowinski J, Thierry AM (1998) Prefrontal cortex-basal ganglia circuits in the rat: involvement of ventral pallidum and subthalamic nucleus. Synapse 29:363-370.

McDonald AJ (2003) Is there an amygdala and how far does it extend? An anatomical perspective. Ann N Y Acad Sci 985:1-21.

McDonald AJ, Augustine JR (1993) Localization of GABA-like immunoreactivity in the monkey amygdala. Neuroscience 52:281-294.

McGinty JF (1999) Advancing from the ventral striatum to the extended amygdala. Implications for neuropsychiatry and drug abuse. Introduction. Ann N Y Acad Sci 877:xii-xv.

McGrath MJ, Campbell KM, Veldman MB, Burton FH (1999) Anxiety in a transgenic mouse model of cortical-limbic neuro-potentiated compulsive behavior. Behav Pharmacol 10:435-443.

McLean S, Rothman RB, Jacobson AE, Rice KC, Herkenham M (1987) Distribution of opiate receptor subtypes and enkephalin and dynorphin immunoreactivity in the hippocampus of squirrel, guinea pig, rat, and hamster. J Comp Neurol 255:497-510.

McLeod TM, Lopez-Figueroa AL, Lopez-Figueroa MO (2001) Nitric oxide, stress, and depression. Psychopharmacol Bull 35:24-41.

McQuade R, Leitch MM, Gartside SE, Young AH (2004) Effect of chronic lithium treatment on glucocorticoid and 5-HT1A receptor messenger RNA in hippocampal and dorsal raphe nucleus regions of the rat brain. J Psychopharmacol 18:496-501.

Meaney MJ, Brake W, Gratton A (2002) Environmental regulation of the development of mesolimbic dopamine systems: a neurobiological mechanism for vulnerability to drug abuse? Psychoneuroendocrinology 27:127-138.

Meijer OC, Williamson A, Dallman MF, Pearce D (2000) Transcriptional repression of the 5-HT1A receptor promoter by corticosterone via mineralocorticoid receptors depends on the cellular context. J Neuroendocrinol 12:245-254.

Meltzer HY, Li Z, Kaneda Y, Ichikawa J (2003) Serotonin receptors: their key role in drugs to treat schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 27:1159-1172.

Mengod G, Vilaro MT, Niznik HB, Sunahara RK, Seeman P, O'Dowd BF, Palacios JM (1991) Visualization of a dopamine D1 receptor mRNA in human and rat brain. Brain Res Mol Brain Res 10:185-191.

Mennicken F, Savasta M, Peretti-Renucci R, Feuerstein C (1992) Autoradiographic localization of dopamine uptake sites in the rat brain with 3H-GBR 12935. J Neural Transm Gen Sect 87:1-14.

Merchenthaler I, Maderdrut JL, Altschuler RA, Petrusz P (1986) Immunocytochemical localization of proenkephalin-derived peptides in the central nervous system of the rat. Neuroscience 17:325-348.

Michelhaugh SK, Vaitkevicius H, Wang J, Bouhamdan M, Krieg AR, Walker JL, Mendiratta V, Bannon MJ (2005) Dopamine neurons express multiple isoforms of the nuclear receptor nurr1 with diminished transcriptional activity. J Neurochem 95:1342-1350.

Michelson RJ, Collard MW, Ziemba AJ, Persinger J, Bartholomew B, Huggenvik JI (1999) Nuclear DEAF-1-related (NUDR) protein contains a novel DNA binding domain and represses transcription of the heterogeneous nuclear ribonucleoprotein A2/B1 promoter. J Biol Chem 274:30510-30519.

Millan MJ (2003) The neurobiology and control of anxious states. Prog Neurobiol 70:83-244.

Miller KJ, Murphy BJ, Pelleymounter MA (2004) Central G-Protein Coupled Receptors (GPCR)s as molecular targets for the treatment of obesity: assets, liabilities and development status. Curr Drug Targets CNS Neurol Disord 3:357-377.

Millhouse OE (1986) The intercalated cells of the amygdala. J Comp Neurol 247:246-271.

Milligan G (2004) G protein-coupled receptor dimerization: function and ligand pharmacology. Mol Pharmacol 66:1-7.

Minowa T, Minowa MT, Mouradian MM (1992) Analysis of the promoter region of the rat D2 dopamine receptor gene. Biochemistry 31:8389-8396.

Minowa T, Minowa MT, Mouradian MM (1994) Negative modulator of the rat D2 dopamine receptor gene. J Biol Chem 269:11656-11662.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189-225.

Monsma FJ, Jr., McVittie LD, Gerfen CR, Mahan LC, Sibley DR (1989) Multiple D2 dopamine receptors produced by alternative RNA splicing. Nature 342:926-929.

Montague PR, Dayan P, Sejnowski TJ (1996) A framework for mesencephalic dopamine systems based on predictive Hebbian learning. J Neurosci 16:1936-1947.

Moore MM, Howard BE (1982) Quantitation of small colony trifluorothymidine-resistant mutants of L5178Y/TK+/- mouse lymphoma cells in RPMI-1640 medium. Mutat Res 104:287-294.

Moran LA, Scrimgeour KG, Horton HR, Ochs RS, Rawn JD (1994) Biochemistry, Second Edition. Upper Saddle River, NJ: Prentice Hall.

Morilak DA, Barrera G, Echevarria DJ, Garcia AS, Hernandez A, Ma S, Petre CO (2005) Role of brain norepinephrine in the behavioral response to stress. Prog Neuropsychopharmacol Biol Psychiatry 29:1214-1224.

Morris MJ, Pavia JM (2004) Increased endogenous noradrenaline and neuropeptide Y release from the hypothalamus of streptozotocin diabetic rats. Brain Res 1006:100-106.

Murata K, Hattori M, Hirai N, Shinozuka Y, Hirata H, Kageyama R, Sakai T, Minato N (2005) Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. Mol Cell Biol 25:4262-4271.

Naar AM, Lemon BD, Tjian R (2001) Transcriptional coactivator complexes. Annu Rev Biochem 70:475-501.

Nair NP, Lal S, Bloom DM (1985) Cholecystokinin peptides, dopamine and schizophrenia--a review. Prog Neuropsychopharmacol Biol Psychiatry 9:515-524.

Nakatani T, Mizuhara E, Minaki Y, Sakamoto Y, Ono Y (2004) Helt, a novel basic-helix-loop-helix transcriptional repressor expressed in the developing central nervous system. J Biol Chem 279:16356-16367.

Nandigama P, Borszcz GS (2003) Affective analgesia following the administration of morphine into the amygdala of rats. Brain Res 959:343-354.

Nauta WJ, Smith GP, Faull RL, Domesick VB (1978) Efferent connections and nigral afferents of the nucleus accumbens septi in the rat. Neuroscience 3:385-401.

Ng NK, Lee HS, Wong PT (1999) Regulation of striatal dopamine release through 5-HT1 and 5-HT2 receptors. J Neurosci Res 55:600-607.

Nicholson C, Phillips JM (1979) Diffusion of anions and cations in the extracellular micro-environment of the brain [proceedings]. J Physiol 296:66P.

Nicholson C, Phillips JM (1981) Ion diffusion modified by tortuosity and volume fraction in the extracellular microenvironment of the rat cerebellum. J Physiol 321:225-257.

Ninan PT (1999) The functional anatomy, neurochemistry, and pharmacology of anxiety. J Clin Psychiatry 60:12-17.

Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM (1996) The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. J Neurosci 16:436-447.

Nishi A, Bibb JA, Snyder GL, Higashi H, Nairn AC, Greengard P (2000) Amplification of dopaminergic signaling by a positive feedback loop. Proc Natl Acad Sci U S A 97:12840-12845.

Nishi A, Snyder GL, Fienberg AA, Fisone G, Aperia A, Nairn AC, Greengard P (1999) Requirement for DARPP-32 in mediating effect of dopamine D2 receptor activation. Eur J Neurosci 11:2589-2592.

Nishi A, Liu F, Matsuyama S, Hamada M, Higashi H, Nairn AC, Greengard P (2003) Metabotropic mGlu5 receptors regulate adenosine A2A receptor signaling. Proc Natl Acad Sci U S A 100:1322-1327.

Nitecka L, Ben-Ari Y (1987) Distribution of GABA-like immunoreactivity in the rat amygdaloid complex. J Comp Neurol 266:45-55.

Noctor SC, Martinez-Cerdeno V, Kriegstein AR (2007) Contribution of intermediate progenitor cells to cortical histogenesis. Arch Neurol 64:639-642.

Nolte J (1993) The Human Brain: An Introduction to its Functional Anatomy, 3rd Edition. St. Louis: Stamathis, G.

Nordzell M, Aarnisalo P, Benoit G, Castro DS, Perlmann T (2004) Defining an N-terminal activation domain of the orphan nuclear receptor Nurr1. Biochem Biophys Res Commun 313:205-211.

North RA, Williams JT, Surprenant A, Christie MJ (1987) Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. Proc Natl Acad Sci U S A 84:5487-5491.

Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP (2003) Pitx3 is required for development of substantia nigra dopaminergic neurons. Proc Natl Acad Sci U S A 100:4245-4250.

Nuthall HN, Joachim K, Stifani S (2004) Phosphorylation of serine 239 of Groucho/TLE1 by protein kinase CK2 is important for inhibition of neuronal differentiation. Mol Cell Biol 24:8395-8407.

O'Connor JJ, Kruk ZL (1991a) Frequency dependence of 5-HT autoreceptor function in rat dorsal raphe and suprachiasmatic nuclei studied using fast cyclic voltammetry. Brain Res 568:123-130.

O'Connor JJ, Kruk ZL (1991b) Fast cyclic voltammetry can be used to measure stimulated endogenous 5-hydroxytryptamine release in untreated rat brain slices. J Neurosci Methods 38:25-33.

Oades RD, Halliday GM (1987) Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. Brain Res 434:117-165.

Ohtsuka T, Sakamoto M, Guillemot F, Kageyama R (2001) Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. J Biol Chem 276:30467-30474.

Otake K, Nakamura Y (2003) Forebrain neurons with collateral projections to both the interstitial nucleus of the posterior limb of the anterior commissure and the nucleus of the solitary tract in the rat. Neuroscience 119:623-628.

Ou XM, Storring JM, Kushwaha N, Albert PR (2001) Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene. J Biol Chem 276:14299-14307.

Ou XM, Jafar-Nejad H, Storring JM, Meng JH, Lemonde S, Albert PR (2000) Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene. J Biol Chem 275:8161-8168.

Ou XM, Lemonde S, Jafar-Nejad H, Bown CD, Goto A, Rogaeva A, Albert PR (2003) Freud-1: A neuronal calcium-regulated repressor of the 5-HT1A receptor gene. J Neurosci 23:7415-7425.

Ouimet CC, Miller PE, Hemmings HC, Jr., Walaas SI, Greengard P (1984) DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. Immunocytochemical localization. J Neurosci 4:111-124.

Overstreet DH, Commissaris RC, De La Garza R, 2nd, File SE, Knapp DJ, Seiden LS (2003) Involvement of 5-HT1A receptors in animal tests of anxiety and depression: evidence from genetic models. Stress 6:101-110.

Palchaudhuri M, Flugge G (2005) 5-HT1A receptor expression in pyramidal neurons of cortical and limbic brain regions. Cell Tissue Res 321:159-172.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289:739-745.

Papez JW (1995) A proposed mechanism of emotion. 1937. J Neuropsychiatry Clin Neurosci 7:103-112.

Pare D, Smith Y (1993a) The intercalated cell masses project to the central and medial nuclei of the amygdala in cats. Neuroscience 57:1077-1090.

Pare D, Smith Y (1993b) Distribution of GABA immunoreactivity in the amygdaloid complex of the cat. Neuroscience 57:1061-1076.

Pare D, Collins DR, Pelletier JG (2002) Amygdala oscillations and the consolidation of emotional memories. Trends Cogn Sci 6:306-314.

Pare D, Quirk GJ, Ledoux JE (2004) New vistas on amygdala networks in conditioned fear. J Neurophysiol 92:1-9.

Pare D, Royer S, Smith Y, Lang EJ (2003) Contextual inhibitory gating of impulse traffic in the intra-amygdaloid network. Ann N Y Acad Sci 985:78-91.

Parkinson J (2002) An essay on the shaking palsy. 1817. J Neuropsychiatry Clin Neurosci 14:223-236; discussion 222.

Parks CL, Shenk T (1996) The serotonin 1a receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. J Biol Chem 271:4417-4430.

Parks CL, Shenk T (1997) Activation of the adenovirus major late promoter by transcription factors MAZ and Sp1. J Virol 71:9600-9607.

Paroush Z, Finley RL, Jr., Kidd T, Wainwright SM, Ingham PW, Brent R, Ish-Horowicz D (1994) Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell 79:805-815.

Parsey RV, Oquendo MA, Ogden RT, Olvet DM, Simpson N, Huang YY, Van Heertum RL, Arango V, Mann JJ (2006) Altered serotonin 1A binding in major depression: a [carbonyl-C-11]WAY100635 positron emission tomography study. Biol Psychiatry 59:106-113.

Patel TD, Zhou FC (2005) Ontogeny of 5-HT1A receptor expression in the developing hippocampus. Brain Res Dev Brain Res 157:42-57.

Patel TD, Azmitia EC, Zhou FC (1996) Increased 5-HT1A receptor immunoreactivity in the rat hippocampus following 5,7-dihydroxytryptamine lesions in the cingulum bundle and fimbria-fornix. Behav Brain Res 73:319-323.

Pattyn A, Simplicio N, van Doorninck JH, Goridis C, Guillemot F, Brunet JF (2004) Ascl1/Mash1 is required for the development of central serotonergic neurons. Nat Neurosci 7:589-595.

Paule MR, White RJ (2000) Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Res 28:1283-1298.

Pavlovic ZW, Cooper ML, Bodnar RJ (1996) Opioid antagonists in the periaqueductal gray inhibit morphine and beta-endorphin analgesia elicited from the amygdala of rats. Brain Res 741:13-26.

Paxinos G, Watson S (1986) The rat brain in stereotaxic coordinates, 2nd Edition. London: Academic Press.

Paxinos G, Watson, C. (1997) The rat brain in stereotaxic coordinates. San Diego, CA.

Pazos A, Palacios JM (1985) Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Res 346:205-230.

Pelletier JG, Pare D (2004) Role of amygdala oscillations in the consolidation of emotional memories. Biol Psychiatry 55:559-562.

Perez de la Mora M, Lara-Garcia D, Jacobsen KX, Vazquez-Garcia M, Crespo-Ramirez M, Flores-Gracia C, Escamilla-Marvan E, Fuxe K (2006) Anxiolytic-like effects of the selective metabotropic glutamate receptor 5 antagonist MPEP after its intra-amygdaloid microinjection in three different non-conditioned rat models of anxiety. Eur J Neurosci 23:2749-2759.

Peter M, Couturier J, Pacquement H, Michon J, Thomas G, Magdelenat H, Delattre O (1997) A new member of the ETS family fused to EWS in Ewing tumors. Oncogene 14:1159-1164.

Petrusz P, Merchantaler, I., Maderdrut, J. (1986) Distribution of enkephalin-containing neurons in the central nervous system. In: Handbook of Chemical Neuroanatomy: GABA and Neuropeptides in the CNS (Bjorklund A, Hokfelt, T., ed), pp 273-334.

Peyron C, Petit JM, Rampon C, Jouvet M, Luppi PH (1998) Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. Neuroscience 82:443-468.

Pfaar H, von Holst A, Vogt Weisenhorn DM, Brodski C, Guimera J, Wurst W (2002) mPet-1, a mouse ETS-domain transcription factor, is expressed in central serotonergic neurons. Dev Genes Evol 212:43-46. .

Pickel VM, Garzon M, Mengual E (2002) Electron microscopic immunolabeling of transporters and receptors identifies transmitter-specific functional sites envisioned in Cajal's neuron. Prog Brain Res 136:145-155.

Pike VW, McCarron JA, Lammerstma AA, Hume SP, Poole K, Grasby PM, Malizia A, Cliffe IA, Fletcher A, Bench CJ (1995) First delineation of 5-HT1A receptors in human brain with PET and [11C]WAY-100635. Eur J Pharmacol 283:R1-3.

Pineyro G, Blier P (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev 51:533-591.

Pitkanen A, Amaral DG (1994) The distribution of GABAergic cells, fibers, and terminals in the monkey amygdaloid complex: an immunohistochemical and in situ hybridization study. J Neurosci 14:2200-2224.

Pitkanen A, Savander V, LeDoux JE (1997) Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends Neurosci 20:517-523.

Ploj K, Roman E, Nylander I (2003) Long-term effects of maternal separation on ethanol intake and brain opioid and dopamine receptors in male Wistar rats. Neuroscience 121:787-799.

Price JL (2003) Comparative aspects of amygdala connectivity. Ann N Y Acad Sci 985:50-58.

Pugh BF, Tjian R (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell 61:1187-1197.

Pumiglia KM, Decker SJ (1997) Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A 94:448-452.

Ramsay D, Kellett E, McVey M, Rees S, Milligan G (2002) Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. Biochem J 365:429-440.

Rapport MM, Green AA, Page IH (1948) Crystalline Serotonin. Science 108:329-330.

Raymond JR, Mukhin YV, Gettys TW, Garnovskaya MN (1999) The recombinant 5-HT1A receptor: G protein coupling and signalling pathways. Br J Pharmacol 127:1751-1764.

Redmond L, Oh SR, Hicks C, Weinmaster G, Ghosh A (2000) Nuclear Notch1 signaling and the regulation of dendritic development. Nat Neurosci 3:30-40.

Reis RA, Ventura AL, Kubrusly RC, de Mello MC, de Mello FG (2007) Dopaminergic signaling in the developing retina. Brain Res Rev 54:181-188.

Reite M (1987) Infant abuse and neglect: lessons from the primate laboratory. Child Abuse Negl 11:347-355.

Ressler KJ, Nemeroff CB (2000) Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. Depress Anxiety 12 Suppl 1:2-19.

Revay R, Vaughan R, Grant S, Kuhar MJ (1996) Dopamine transporter immunohistochemistry in median eminence, amygdala, and other areas of the rat brain. Synapse 22:93-99.

Rezayof A, Zarrindast MR, Sahraei H, Haeri-Rohani AH (2002) Involvement of dopamine D2 receptors of the central amygdala on the acquisition and expression of morphine-induced place preference in rat. Pharmacol Biochem Behav 74:187-197.

Riad M, Watkins KC, Doucet E, Hamon M, Descarries L (2001) Agonist-induced internalization of serotonin-1a receptors in the dorsal raphe nucleus (autoreceptors) but not hippocampus (heteroreceptors). J Neurosci 21:8378-8386.

Riad M, Garcia S, Watkins KC, Jodoin N, Doucet E, Langlois X, el Mestikawy S, Hamon M, Descarries L (2000) Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. J Comp Neurol 417:181-194.

Rice ME (2000) Distinct regional differences in dopamine-mediated volume transmission. Prog Brain Res 125:277-290.

Riedel A, Hartig W, Fritschy JM, Bruckner G, Seifert U, Brauer K (1998) Comparison of the rat dorsal and ventral striatopallidal system. A study using the GABA(A)-receptor alpha1-subunit and parvalbumin immunolabeling. Exp Brain Res 121:215-221.

Robas N, O'Reilly M, Katugampola S, Fidock M (2003) Maximizing serendipity: strategies for identifying ligands for orphan G-protein-coupled receptors. Curr Opin Pharmacol 3:121-126.

Robertson KD (2002) DNA methylation and chromatin - unraveling the tangled web. Oncogene 21:5361-5379.

Roder S, Ciriello J (1993) Innervation of the amygdaloid complex by catecholaminergic cell groups of the ventrolateral medulla. J Comp Neurol 332:105-122.

Rogaeva A, Ou XM, Jafar-Nejad H, Lemonde S, Albert PR (2007) Differential repression by Freud-1/CC2D1A at a polymorphic site in the dopamine-D2 receptor gene. J Biol Chem 282:20897-20905.

Rojas P, Joodmardi E, Hong Y, Perlmann T, Ogren SO (2007) Adult mice with reduced Nurr1 expression: an animal model for schizophrenia. Mol Psychiatry 12:756-766.

Rollema H, Lu Y, Schmidt AW, Sprouse JS, Zorn SH (2000) 5-HT(1A) receptor activation contributes to ziprasidone-induced dopamine release in the rat prefrontal cortex. Biol Psychiatry 48:229-237.

Rorig B, Sutor B (1996) Serotonin regulates gap junction coupling in the developing rat somatosensory cortex. Eur J Neurosci 8:1685-1695.

Rosengarten H, Friedhoff AJ (1979) Enduring changes in dopamine receptor cells of pups from drug administration to pregnant and nursing rats. Science 203:1133-1135.

Rosenkranz JA, Grace AA (2003) Affective conditioning in the basolateral amygdala of anesthetized rats is modulated by dopamine and prefrontal cortical inputs. Ann N Y Acad Sci 985:488-491.

Rosin DL (2000) Distribution of alpha 2A- and alpha 2C-adrenergic receptor immunoreactivity in the central nervous system. Methods Mol Biol 126:475-505.

Roussa E, Krieglstein K (2004) Induction and specification of midbrain dopaminergic cells: focus on SHH, FGF8, and TGF-beta. Cell Tissue Res 318:23-33.

Royer S, Pare D (2002) Bidirectional synaptic plasticity in intercalated amygdala neurons and the extinction of conditioned fear responses. Neuroscience 115:455-462.

Royer S, Martina M, Pare D (1999) An inhibitory interface gates impulse traffic between the input and output stations of the amygdala. J Neurosci 19:10575-10583.

Royer S, Martina M, Pare D (2000a) Bistable behavior of inhibitory neurons controlling impulse traffic through the amygdala: role of a slowly deinactivating K+ current. J Neurosci 20:9034-9039.

Royer S, Martina M, Pare D (2000b) Polarized synaptic interactions between intercalated neurons of the amygdala. J Neurophysiol 83:3509-3518.

Rumajogee P, Verge D, Hanoun N, Brisorgueil MJ, Hen R, Lesch KP, Hamon M, Miquel MC (2004) Adaption of the serotoninergic neuronal phenotype in the absence of 5-HT autoreceptors or the 5-HT transporter: involvement of BDNF and cAMP. Eur J Neurosci 19:937-944.

Russell J, Zomerdijk JC (2006) The RNA polymerase I transcription machinery. Biochem Soc Symp:203-216.

Rutter M (1979) Maternal deprivation, 1972-1978: new findings, new concepts, new approaches. Child Dev 50:283-305.

Sacchetti P, Brownschidle LA, Granneman JG, Bannon MJ (1999) Characterization of the 5'-flanking region of the human dopamine transporter gene. Brain Res Mol Brain Res 74:167-174.

Sacchetti P, Dwornik H, Formstecher P, Rachez C, Lefebvre P (2002) Requirements for heterodimerization between the orphan nuclear receptor Nurr1 and retinoid X receptors. J Biol Chem 277:35088-35096.

Sajdyk TJ, Fitz SD, Shekhar A (2006) The role of neuropeptide Y in the amygdala on corticotropin-releasing factor receptor-mediated behavioral stress responses in the rat. Stress 9:21-28.

Sakurada K, Ohshima-Sakurada M, Palmer TD, Gage FH (1999) Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. Development 126:4017-4026.

Sandelin A, Carninci P, Lenhard B, Ponjavic J, Hayashizaki Y, Hume DA (2007) Mammalian RNA polymerase II core promoters: insights from genome-wide studies. Nat Rev Genet 8:424-436.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Sasai Y, Kageyama R, Tagawa Y, Shigemoto R, Nakanishi S (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev 6:2620-2634.

Sato M (2007) [Citrullination, a novel post-translational modification of histone]. Seikagaku 79:686-690.

Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, Conneely OM (1998) Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci U S A 95:4013-4018.

Saxena S, Brody AL, Ho ML, Zohrabi N, Maidment KM, Baxter LR, Jr. (2003) Differential brain metabolic predictors of response to paroxetine in obsessive-compulsive disorder versus major depression. Am J Psychiatry 160:522-532.

Scarselli M, Novi F, Schallmach E, Lin R, Baragli A, Colzi A, Griffon N, Corsini GU, Sokoloff P, Levenson R, Vogel Z, Maggio R (2001) D2/D3 dopamine receptor heterodimers exhibit unique functional properties. J Biol Chem 276:30308-30314.

Scearce LM, Laz TM, Hazel TG, Lau LF, Taub R (1993) RNR-1, a nuclear receptor in the NGFI-B/Nur77 family that is rapidly induced in regenerating liver. J Biol Chem 268:8855-8861.

Schade H (1959) [Award of the 1958 Nobel prize in medicine to Beadle, Tatum and Lederberg.]. Dtsch Med Wochenschr 84:1033-1038.

Schafe GE, Doyere V, LeDoux JE (2005) Tracking the fear engram: the lateral amygdala is an essential locus of fear memory storage. J Neurosci 25:10010-10014.

Schmitt A, Benninghoff J, Moessner R, Rizzi M, Paizanis E, Doenitz C, Gross S, Hermann M, Gritti A, Lanfumey L, Fritzen S, Reif A, Hamon M, Murphy DL, Vescovi A, Lesch KP (2007) Adult neurogenesis in serotonin transporter deficient mice. J Neural Transm 114:1107-1119.

Schmitt FO (1984) Molecular regulators of brain function: a new view. Neuroscience 13:991-1001.

Schoenherr CJ, Anderson DJ (1995a) The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science 267:1360-1363.

Schoenherr CJ, Anderson DJ (1995b) Silencing is golden: negative regulation in the control of neuronal gene transcription. Curr Opin Neurobiol 5:566-571.

Schoffelmeer AN, Vanderschuren LJ, De Vries TJ, Hogenboom F, Wardeh G, Mulder AH (2000) Synergistically interacting dopamine D1 and NMDA receptors mediate nonvesicular transporter-dependent GABA release from rat striatal medium spiny neurons. J Neurosci 20:3496-3503.

Schug J (2003) Using TESS to Predict Transcription Factor Binding Sites in DNA Sequence. In: Current Protocols in Bioinformatics (Baxevanis AD, ed): J. Wiley and Sons.

Schwartz CE, Snidman N, Kagan J (1999) Adolescent social anxiety as an outcome of inhibited temperament in childhood. J Am Acad Child Adolesc Psychiatry 38:1008-1015.

Scibilia RJ, Lachowicz JE, Kilts CD (1992) Topographic nonoverlapping distribution of D1 and D2 dopamine receptors in the amygdaloid nuclear complex of the rat brain. Synapse 11:146-154.

Seay B, Harlow HF (1965) Maternal separation in the rhesus monkey. J Nerv Ment Dis 140:434-441.

Seeman P (2006) Targeting the dopamine D2 receptor in schizophrenia. Expert Opin Ther Targets 10:515-531.

Seeman P (2007) Antiparkinson therapeutic potencies correlate with their affinities at dopamine D2(High) receptors. Synapse 61:1013-1018.

Serretti A, Artioli P, Lorenzi C, Pirovano A, Tubazio V, Zanardi R (2004) The C(-1019)G polymorphism of the 5-HT1A gene promoter and antidepressant response in mood disorders: preliminary findings. Int J Neuropsychopharmacol 7:453-460.

Sestan N, Artavanis-Tsakonas S, Rakic P (1999) Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science 286:741-746.

Shammah-Lagnado SJ, Alheid GF, Heimer L (1999) Afferent connections of the interstitial nucleus of the posterior limb of the anterior commissure and adjacent amygdalostriatal transition area in the rat. Neuroscience 94:1097-1123.

Shammah-Lagnado SJ, Alheid GF, Heimer L (2001) Striatal and central extended amygdala parts of the interstitial nucleus of the posterior limb of the anterior commissure: evidence from tract-tracing techniques in the rat. J Comp Neurol 439:104-126.

Shammah-Lagnado SJ, Beltramino CA, McDonald AJ, Miselis RR, Yang M, de Olmos J, Heimer L, Alheid GF (2000) Supracapsular bed nucleus of the stria terminalis contains central and medial extended amygdala elements: evidence from anterograde and retrograde tracing experiments in the rat. J Comp Neurol 422:533-555.

Sharma SK, Nirenberg M, Klee WA (1975) Morphine receptors as regulators of adenylate cyclase activity. Proc Natl Acad Sci U S A 72:590-594.

Shekhar A, Truitt W, Rainnie D, Sajdyk T (2005) Role of stress, corticotrophin releasing factor (CRF) and amygdala plasticity in chronic anxiety. Stress 8:209-219.

Shemer A, Whitaker-Azmitia PM, Azmitia EC (1988) Effects of prenatal 5-methoxytryptamine and parachlorophenylalanine on serotonergic uptake and behavior in the neonatal rat. Pharmacol Biochem Behav 30:847-851.

Shemer AV, Azmitia EC, Whitaker-Azmitia PM (1991) Dose-related effects of prenatal 5-methoxytryptamine (5-MT) on development of serotonin terminal density and behavior. Brain Res Dev Brain Res 59:59-63.

Shi F, Visser WH, de Jong NM, Liem RS, Ronken E, Hoekstra D (2003) Antisense oligonucleotides reach mRNA targets via the RNA matrix: downregulation of the 5-HT1A receptor. Exp Cell Res 291:313-325.

Shilatifard A (2006) Chromatin Modifications by Methylation and Ubiquitination: Implications in the Regulation of Gene Expression. Annu Rev Biochem.

Shinonaga Y, Takada M, Mizuno N (1992) Direct projections from the central amygdaloid nucleus to the globus pallidus and substantia nigra in the cat. Neuroscience 51:691-703.

Sibley DR (1999) New insights into dopaminergic receptor function using antisense and genetically altered animals. Annu Rev Pharmacol Toxicol 39:313-341.

Sijbesma H, Schipper J, Cornelissen JC, de Kloet ER (1991) Species differences in the distribution of central 5-HT1 binding sites: a comparative autoradiographic study between rat and guinea pig. Brain Res 555:295-304.

Silva RC, Cruz AP, Avanzi V, Landeira-Fernandez J, Brandao ML (2002) Distinct contributions of median raphe nucleus to contextual fear conditioning and fear-potentiated startle. Neural Plast 9:233-247.

Sim LJ, Childers SR (1997) Anatomical distribution of mu, delta, and kappa opioid- and nociceptin/orphanin FQ-stimulated [35S]guanylyl-5'-O-(gamma-thio)-triphosphate binding in guinea pig brain. J Comp Neurol 386:562-572.

Simonin F, Valverde O, Smadja C, Slowe S, Kitchen I, Dierich A, Le Meur M, Roques BP, Maldonado R, Kieffer BL (1998) Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. Embo J 17:886-897.

Sims RJ, 3rd, Belotserkovskaya R, Reinberg D (2004) Elongation by RNA polymerase II: the short and long of it. Genes Dev 18:2437-2468.

Skoog DA, West DM, Holler FJ (1994) Evaluation of Analytical Data. In: Analytical Chemistry: An Introduction, Sixth Edition, pp 78-95. Philadelphia: Saunders College Publishing.

Slagsvold HH, Ostvold AC, Fallgren AB, Paulsen RE (2002) Nuclear receptor and apoptosis initiator NGFI-B is a substrate for kinase ERK2. Biochem Biophys Res Commun 291:1146-1150.

Smart D, Smith G, Lambert DG (1994) mu-Opioid receptor stimulation of inositol (1,4,5)trisphosphate formation via a pertussis toxin-sensitive G protein. J Neurochem 62:1009-1014.

Smidt MP, Burbach JP (2007) How to make a mesodiencephalic dopaminergic neuron. Nat Rev Neurosci 8:21-32.

Smidt MP, Smits SM, Burbach JP (2003) Molecular mechanisms underlying midbrain dopamine neuron development and function. Eur J Pharmacol 480:75-88.

Smidt MP, Asbreuk CH, Cox JJ, Chen H, Johnson RL, Burbach JP (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3:337-341.

Smiley JF, Levey AI, Ciliax BJ, Goldman-Rakic PS (1994) D1 dopamine receptor immunoreactivity in human and monkey cerebral cortex: predominant and extrasynaptic localization in dendritic spines. Proc Natl Acad Sci U S A 91:5720-5724.

Smith Y, Parent A, Seguela P, Descarries L (1987) Distribution of GABA-immunoreactive neurons in the basal ganglia of the squirrel monkey (Saimiri sciureus). J Comp Neurol 259:50-64.

Smits SM, Ponnio T, Conneely OM, Burbach JP, Smidt MP (2003) Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. Eur J Neurosci 18:1731-1738.

Sokoloff P, Giros B, Martres MP, Andrieux M, Besancon R, Pilon C, Bouthenet ML, Souil E, Schwartz JC (1992) Localization and function of the D3 dopamine receptor. Arzneimittelforschung 42:224-230.

Solinas M, Ferre S, You ZB, Karcz-Kubicha M, Popoli P, Goldberg SR (2002) Caffeine induces dopamine and glutamate release in the shell of the nucleus accumbens. J Neurosci 22:6321-6324.

Somers JM, Goldner EM, Waraich P, Hsu L (2006) Prevalence and incidence studies of anxiety disorders: a systematic review of the literature. Can J Psychiatry 51:100-113.

Song J, Ugai H, Nakata-Tsutsui H, Kishikawa S, Suzuki E, Murata T, Yokoyama KK (2003) Transcriptional regulation by zinc-finger proteins Sp1 and MAZ involves interactions with the same cis-elements. Int J Mol Med 11:547-553.

Song J, Ugai H, Ogawa K, Wang Y, Sarai A, Obata Y, Kanazawa I, Sun K, Itakura K, Yokoyama KK (2001) Two consecutive zinc fingers in Sp1 and in MAZ are essential for interactions with cis-elements. J Biol Chem 276:30429-30434.

Sotelo C, Cholley B, El Mestikawy S, Gozlan H, Hamon M (1990) Direct Immunohistochemical Evidence of the Existence of 5-HT1A Autoreceptors on Serotoninergic Neurons in the Midbrain Raphe Nuclei. Eur J Neurosci 2:1144-1154.

Stargell LA, Struhl K (1996) Mechanisms of transcriptional activation in vivo: two steps forward. Trends Genet 12:311-315.

Stenkamp RE, Teller DC, Palczewski K (2002) Crystal structure of rhodopsin: a G-protein-coupled receptor. Chembiochem 3:963-967.

Stevenson CW, Gratton A (2003) Basolateral amygdala modulation of the nucleus accumbens dopamine response to stress: role of the medial prefrontal cortex. Eur J Neurosci 17:1287-1295.

Steventon B, Carmona-Fontaine C, Mayor R (2005) Genetic network during neural crest induction: from cell specification to cell survival. Semin Cell Dev Biol 16:647-654.

Stewart WF, Ricci JA, Chee E, Hahn SR, Morganstein D (2003) Cost of lost productive work time among US workers with depression. Jama 289:3135-3144.

Stockmeier CA (1997) Neurobiology of serotonin in depression and suicide. Ann N Y Acad Sci 836:220-232.

Stockmeier CA, DiCarlo JJ, Zhang Y, Thompson P, Meltzer HY (1993) Characterization of typical and atypical antipsychotic drugs based on in vivo occupancy of serotonin2 and dopamine2 receptors. J Pharmacol Exp Ther 266:1374-1384.

Stone JM, Morrison PD, Pilowsky LS (2007) Glutamate and dopamine dysregulation in schizophrenia--a synthesis and selective review. J Psychopharmacol 21:440-452.

Strobel A, Gutknecht L, Rothe C, Reif A, Mossner R, Zeng Y, Brocke B, Lesch KP (2003) Allelic variation in 5-HT1A receptor expression is associated with anxiety- and depression-related personality traits. J Neural Transm 110:1445-1453.

Stromberg I, Kehr J, Fuxe K (2000) Restoration of dopamine transmission in graft reinnervated striatum. Evidence for regulation of dopamine D2 receptor function in regions lacking dopamine. Prog Brain Res 125:309-315.

Sugita S, North RA (1993) Opioid actions on neurons of rat lateral amygdala in vitro. Brain Res 612:151-155.

Sulek K (1969) [Nobel prize for Joshua Lederberg in 1958 for discovery of genetic recombination and organization of the hereditary material in bacteria. Prize for George W. Beadle and Edward L. Tatum for discovery of the regulatory effect of genes on biochemical processes]. Wiad Lek 22:418-420.

Sullivan PF, Neale MC, Kendler KS (2000) Genetic epidemiology of major depression: review and meta-analysis. Am J Psychiatry 157:1552-1562.

Suzuki M, Matsuda T, Asano S, Somboonthum P, Takuma K, Baba A (1995) Increase of noradrenaline release in the hypothalamus of freely moving rat by postsynaptic 5-hydroxytryptamine1A receptor activation. Br J Pharmacol 115:703-711.

Suzuki Y, Tsunoda T, Sese J, Taira H, Mizushima-Sugano J, Hata H, Ota T, Isogai T, Tanaka T, Nakamura Y, Suyama A, Sakaki Y, Morishita S, Okubo K, Sugano S (2001) Identification and characterization of the potential promoter regions of 1031 kinds of human genes. Genome Res 11:677-684.

Svenningsson P, Tzavara ET, Liu F, Fienberg AA, Nomikos GG, Greengard P (2002) DARPP-32 mediates serotonergic neurotransmission in the forebrain. Proc Natl Acad Sci U S A 99:3188-3193.

Svenningsson P, Lindskog M, Ledent C, Parmentier M, Greengard P, Fredholm BB, Fisone G (2000) Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa in vivo by dopamine D1, dopamine D2, and adenosine A2A receptors. Proc Natl Acad Sci U S A 97:1856-1860.

Swanson LW, Petrovich GD (1998) What is the amygdala? Trends Neurosci 21:323-331.

Takada M (1990) The A11 catecholamine cell group: another origin of the dopaminergic innervation of the amygdala. Neurosci Lett 118:132-135.

Takebayashi K, Akazawa C, Nakanishi S, Kageyama R (1995) Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. Identification of the neural precursor cell-specific promoter element. J Biol Chem 270:1342-1349.

Takeuchi Y, Fukunaga K (2004) Dopamine D2 receptor activates extracellular signal-regulated kinase through the specific region in the third cytoplasmic loop. J Neurochem 89:1498-1507.

Talkowski ME, Bamne M, Mansour H, Nimgaonkar VL (2007) Dopamine genes and schizophrenia: case closed or evidence pending? Schizophr Bull 33:1071-1081.

Tanaka M, Yoshida M, Emoto H, Ishii H (2000) Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. Eur J Pharmacol 405:397-406.

Tatum EL, Beadle GW (1942) Genetic Control of Biochemical Reactions in Neurospora: An "Aminobenzoicless" Mutant. Proc Natl Acad Sci U S A 28:234-243.

Terranova JP, Chabot C, Barnouin MC, Perrault G, Depoortere R, Griebel G, Scatton B (2005) SSR181507, a dopamine D(2) receptor antagonist and 5-HT(1A) receptor agonist, alleviates disturbances of novelty discrimination in a social context in rats, a putative model of selective attention deficit. Psychopharmacology (Berl) 181:134-144.

Tershner SA, Helmstetter FJ (2000) Antinociception produced by mu opioid receptor activation in the amygdala is partly dependent on activation of mu opioid and neurotensin receptors in the ventral periaqueductal gray. Brain Res 865:17-26.

Thompson MR, Li KM, Clemens KJ, Gurtman CG, Hunt GE, Cornish JL, McGregor IS (2004) Chronic fluoxetine treatment partly attenuates the long-term anxiety and depressive symptoms induced by MDMA ('Ecstasy') in rats. Neuropsychopharmacology 29:694-704.

Thompson PR, Fast W (2006) Histone citrullination by protein arginine deiminase: is arginine methylation a green light or a roadblock? ACS Chem Biol 1:433-441.

Tordjman S, Carlier M, Cohen D, Cesselin F, Bourgoin S, Colas-Linhart N, Petiet A, Perez-Diaz F, Hamon M, Roubertoux PL (2003) Aggression and the three opioid families (endorphins, enkephalins, and dynorphins) in mice. Behav Genet 33:529-536.

Torres G, Bitran M, Huidobro-Toro JP (1992) Co-release of neuropeptide Y (NPY) and noradrenaline from the sympathetic nerve terminals supplying the rat vas deferens; influence of calcium and the stimulation intensity. Neurosci Lett 148:39-42.

Trudeau LE, Gutierrez R (2007) On cotransmission & neurotransmitter phenotype plasticity. Mol Interv 7:138-146.

Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865-871.

Ulfig N, Setzer M, Bohl J (2003) Ontogeny of the human amygdala. Ann N Y Acad Sci 985:22-33.

Usiello A, Baik JH, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, Piazza PV, Borrelli E (2000) Distinct functions of the two isoforms of dopamine D2 receptors. Nature 408:199-203.

Vallone D, Picetti R, Borrelli E (2000) Structure and function of dopamine receptors. Neurosci Biobehav Rev 24:125-132.

Van-Ham II, Banihashemi B, Wilson AM, Jacobsen KX, Czesak M, Albert PR (2007) Differential signaling of dopamine-D2S and -D2L receptors to inhibit ERK1/2 phosphorylation. J Neurochem In Press.

Van Ameringen M, Mancini C, Oakman JM (1998) The relationship of behavioral inhibition and shyness to anxiety disorder. J Nerv Ment Dis 186:425-431.

van Doorninck JH, van Der Wees J, Karis A, Goedknegt E, Engel JD, Coesmans M, Rutteman M, Grosveld F, De Zeeuw CI (1999) GATA-3 is involved in the development of serotonergic neurons in the caudal raphe nuclei. J Neurosci 19:RC12.

van Megen HJ, Westenberg HG, den Boer JA, Kahn RS (1996) Cholecystokinin in anxiety. Eur Neuropsychopharmacol 6:263-280.

van Riel E, van Gemert NG, Meijer OC, Joels M (2004) Effect of early life stress on serotonin responses in the hippocampus of young adult rats. Synapse 53:11-19.

Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA (2003) The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A 100:4903-4908.

Vazquez DM, Lopez JF, Van Hoers H, Watson SJ, Levine S (2000) Maternal deprivation regulates serotonin 1A and 2A receptors in the infant rat. Brain Res 855:76-82.

Veinante P, Freund-Mercier MJ (1997) Distribution of oxytocin- and vasopressin-binding sites in the rat extended amygdala: a histoautoradiographic study. J Comp Neurol 383:305-325.

Vicentic A, Francis D, Moffett M, Lakatos A, Rogge G, Hubert GW, Harley J, Kuhar MJ (2006) Maternal separation alters serotonergic transporter densities and serotonergic 1A receptors in rat brain. Neuroscience 140:355-365.

Villalba RM, Raju DV, Hall RA, Smith Y (2006) GABA(B) receptors in the centromedian/parafascicular thalamic nuclear complex: An ultrastructural analysis of GABA(B)R1 and GABA(B)R2 in the monkey thalamus. J Comp Neurol 496:269-287.

Vitalis T, Cases O, Passemard S, Callebert J, Parnavelas JG (2007) Embryonic depletion of serotonin affects cortical development. Eur J Neurosci 26:331-344.

Vizi ES (1982) Non-synaptic intercellular communication: presynaptic inhibition. Acta Biol Acad Sci Hung 33:331-351.

Vizi ES, Labos E (1991) Non-synaptic interactions at presynaptic level. Prog Neurobiol 37:145-163.

Volpicelli F, Caiazzo M, Greco D, Consales C, Leone L, Perrone-Capano C, D'Amato LC, Porzio U (2007) Bdnf gene is a downstream target of Nurr1 transcription factor in rat midbrain neurons in vitro. J Neurochem 102:441-453.

Voorn P, Jorritsma-Byham B, Van Dijk C, Buijs RM (1986) The dopaminergic innervation of the ventral striatum in the rat: a light- and electron-microscopical study with antibodies against dopamine. J Comp Neurol 251:84-99.

Walker DL, Davis M (2002) The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction. Pharmacol Biochem Behav 71:379-392.

Walther DJ, Peter JU, Bashammakh S, Hortnagl H, Voits M, Fink H, Bader M (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science 299:76.

Wang H, Moriwaki A, Wang JB, Uhl GR, Pickel VM (1996) Ultrastructural immunocytochemical localization of mu opioid receptors and Leu5-enkephalin in the patch compartment of the rat caudate-putamen nucleus. J Comp Neurol 375:659-674.

Wang J, Bannon MJ (2005) Sp1 and Sp3 activate transcription of the human dopamine transporter gene. J Neurochem 93:474-482.

Wang Q, Zhao J, Brady AE, Feng J, Allen PB, Lefkowitz RJ, Greengard P, Limbird LE (2004) Spinophilin blocks arrestin actions in vitro and in vivo at G protein-coupled receptors. Science 304:1940-1944.

Wang RY, White FJ (1985) Electrophysiological effects of cholecystokinin: central dopaminergic systems. Prog Clin Biol Res 192:95-103.

Wang S, Barres BA (2000) Up a notch: instructing gliogenesis. Neuron 27:197-200.

Wang Z, Benoit G, Liu J, Prasad S, Aarnisalo P, Liu X, Xu H, Walker NP, Perlmann T (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 423:555-560.

Waraich P, Goldner EM, Somers JM, Hsu L (2004) Prevalence and incidence studies of mood disorders: a systematic review of the literature. Can J Psychiatry 49:124-138.

Waselus M, Galvez JP, Valentino RJ, Van Bockstaele EJ (2006) Differential projections of dorsal raphe nucleus neurons to the lateral septum and striatum. J Chem Neuroanat 31:233-242. .

Wasserman D, Geijer T, Sokolowski M, Rozanov V, Wasserman J (2006) The serotonin 1A receptor C(-1019)G polymorphism in relation to suicide attempt. Behav Brain Funct 2:14.

Watson JD, Crick FH (1953a) The structure of DNA. Cold Spring Harb Symp Quant Biol 18:123-131.

Watson JD, Crick FH (1953b) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 171:737-738.

Wegel E, Shaw P (2005) Gene activation and deactivation related changes in the three-dimensional structure of chromatin. Chromosoma 114:331-337.

Weintraub D, Stern MB (2005) Psychiatric complications in Parkinson disease. Am J Geriatr Psychiatry 13:844-851.

Weisskopf MG, Zalutsky RA, Nicoll RA (1993) The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. Nature 362:423-427.

Weller A, Leguisamo AC, Towns L, Ramboz S, Bagiella E, Hofer M, Hen R, Brunner D (2003) Maternal effects in infant and adult phenotypes of 5HT1A and 5HT1B receptor knockout mice. Dev Psychobiol 42:194-205.

Welsh GI, Hall DA, Warnes A, Strange PG, Proud CG (1998) Activation of microtubule-associated protein kinase (Erk) and p70 S6 kinase by D2 dopamine receptors. J Neurochem 70:2139-2146.

Westlund KN, Coulter JD (1980) Descending projections of the locus coeruleus and subcoeruleus/medial parabrachial nuclei in monkey: axonal transport studies and dopamine-beta-hydroxylase immunocytochemistry. Brain Res 2:235-264.

Whitaker-Azmitia PM (1991) Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. Pharmacol Rev 43:553-561.

Whitaker-Azmitia PM, Lauder JM, Shemmer A, Azmitia EC (1987) Postnatal changes in serotonin receptors following prenatal alterations in serotonin levels: further evidence for functional fetal serotonin receptors. Brain Res 430:285-289.

Whitaker-Azmitia PM, Druse M, Walker P, Lauder JM (1996) Serotonin as a developmental signal. Behav Brain Res 73:19-29.

White LA, Eaton MJ, Castro MC, Klose KJ, Globus MY, Shaw G, Whittemore SR (1994) Distinct regulatory pathways control neurofilament expression and neurotransmitter synthesis in immortalized serotonergic neurons. J Neurosci 14:6744-6753.

Wilson MA, Mascagni F, McDonald AJ (2002) Sex differences in delta opioid receptor immunoreactivity in rat medial amygdala. Neurosci Lett 328:160-164.

Wilson MA, Burghardt PR, Lugo JN, Jr., Primeaux SD, Wilson SP (2003) Effect of amygdalar opioids on the anxiolytic properties of ethanol. Ann N Y Acad Sci 985:472-475.

Wisniewski JR, Zougman A, Kruger S, Mann M (2007) Mass spectrometric mapping of linker histone H1 variants reveals multiple acetylations, methylations, and phosphorylation as well as differences between cell culture and tissue. Mol Cell Proteomics 6:72-87.

Wissink S, Meijer O, Pearce D, van Der Burg B, van Der Saag PT (2000) Regulation of the rat serotonin-1A receptor gene by corticosteroids. J Biol Chem 275:1321-1326.

Witta J, Baffi JS, Palkovits M, Mezey E, Castillo SO, Nikodem VM (2000) Nigrostriatal innervation is preserved in Nurr1-null mice, although dopaminergic neuron precursors are arrested from terminal differentiation. Brain Res Mol Brain Res 84:67-78.

Wojciak JM, Clubb RT (2001) Finding the function buried in SAND. Nat Struct Biol 8:568-570.

Wong SK (2003) G protein selectivity is regulated by multiple intracellular regions of GPCRs. Neurosignals 12:1-12.

Xie Z, Lee SP, O'Dowd BF, George SR (1999) Serotonin 5-HT1B and 5-HT1D receptors form homodimers when expressed alone and heterodimers when co-expressed. FEBS Lett 456:63-67.

Xin JH, Cowie A, Lachance P, Hassell JA (1992) Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. Genes Dev 6:481-496.

Xu PY, Liang R, Jankovic J, Hunter C, Zeng YX, Ashizawa T, Lai D, Le WD (2002a) Association of homozygous 7048G7049 variant in the intron six of Nurr1 gene with Parkinson's disease. Neurology 58:881-884.

Xu R, Hranilovic D, Fetsko LA, Bucan M, Wang Y (2002b) Dopamine D2S and D2L receptors may differentially contribute to the actions of antipsychotic and psychotic agents in mice. Mol Psychiatry 7:1075-1082.

Yashiro K, Corlew R, Philpot BD (2005) Visual deprivation modifies both presynaptic glutamate release and the composition of perisynaptic/extrasynaptic NMDA receptors in adult visual cortex. J Neurosci 25:11684-11692.

Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell 93:755-766.

Yoon K, Gaiano N (2005) Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat Neurosci 8:709-715.

Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N (2004) Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 24:9497-9506.

Yuferov V, Zhou Y, Spangler R, Maggos CE, Ho A, Kreek MJ (1999) Acute "binge" cocaine increases mu-opioid receptor mRNA levels in areas of the rat mesolimbic mesocortical dopamine system. Brain Res Bull 48:109-112.

Yung KK, Bolam JP, Smith AD, Hersch SM, Ciliax BJ, Levey AI (1995) Immunocytochemical localization of D1 and D2 dopamine receptors in the basal ganglia of the rat: light and electron microscopy. Neuroscience 65:709-730.

Zaborszky L, Alheid GF, Beinfeld MC, Eiden LE, Heimer L, Palkovits M (1985) Cholecystokinin innervation of the ventral striatum: a morphological and radioimmunological study. Neuroscience 14:427-453.

Zetterstrom RH, Williams R, Perlmann T, Olson L (1996) Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Brain Res Mol Brain Res 41:111-120.

Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (1997) Dopamine neuron agenesis in Nurr1-deficient mice. Science 276:248-250.

Zhang YQ, Gao X, Huang YL, Wu GC (2000) Expression of 5-HT1A receptor mRNA in rat dorsal raphe nucleus and ventrolateral periaqueductal gray neurons after peripheral inflammation. Neuroreport 11:3361-3365.

Zhao ZQ, Scott M, Chiechio S, Wang JS, Renner KJ, Gereau RWt, Johnson RL, Deneris ES, Chen ZF (2006) Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. J Neurosci 26:12781-12788.

Zhong P, Ciaranello RD (1995) Transcriptional regulation of hippocampal 5-HT1a receptors by corticosteroid hormones. Brain Res Mol Brain Res 29:23-34.

Zhou FM, Liang Y, Salas R, Zhang L, De Biasi M, Dani JA (2005) Corelease of dopamine and serotonin from striatal dopamine terminals. Neuron 46:65-74.

Zhuang X, Gross C, Santarelli L, Compan V, Trillat AC, Hen R (1999) Altered emotional states in knockout mice lacking 5-HT1A or 5-HT1B receptors. Neuropsychopharmacology 21:52S-60S.

Ziabreva I, Poeggel G, Schnabel R, Braun K (2003) Separation-induced receptor changes in the hippocampus and amygdala of Octodon degus: influence of maternal vocalizations. J Neurosci 23:5329-5336.

Zigmond MJ (2000) When it comes to communications between neurons, synapses are over-rated: insights from an animal model of parkinsonism. Prog Brain Res 125:317-326.

Zoli M, Jansson A, Sykova E, Agnati LF, Fuxe K (1999) Volume transmission in the CNS and its relevance for neuropsychopharmacology. Trends Pharmacol Sci 20:142-150.