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Estrogen regulation of the human 5-HT1A receptor gene

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Abstract

Altered regulation of the 5-HT1A receptor is implicated in depression. Since depression is twice as prevalent in women versus men and major hormonal events in women trigger depression, I evaluated estrogen regulation of the 5-HT1A receptor in rat RN46A raphe cells. Estrogen (100 nM) induced no change in transcriptional activity of the 5-HT1A promoter (-1515bp/ATG). A putative estrogen response element (1AERE, –433bp) was identified in human and mouse but not rat 5-HT1A promoter. At least one nuclear protein complex from RN46A cells expressing estrogen receptor (ERβ) bound specifically to the 1AERE, which mediated estrogen repression that was blocked upon mutational inactivation of 1AERE. Mutation of 1AERE derepressed the human 5-HT1A promoter but, paradoxically, estrogen induced rat 5-HT1A RNA in RN46A cells. Thus estrogen, via ERβ action at 1AERE, represses human 5-HT1A promoter activity, but has an opposite effect on rat 5-HT1A expression that could reflect species-specific or post-transcriptional regulation.

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List of Abbreviations

1AERE	serotonin-1A Estrogen Response Element
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
8-OH-DPAT	8-hydroxy-2-(di-n-propyl-amino)-tetralin
AF-1 or 2	Activation Function 1 or 2
Ca2+	Calcium
cAMP	cyclic Adenosine MonoPhosphate
DBD	DNA Binding Domain
DRE	Dual Repressor Element
DRN	Dorsal Raphe Nucleus
E	Estrogen
ECT	ElectroConvulsive Therapy
EMSA	ElectroMobility Shift Assay
ER α/β	Estrogen Receptor α/β
ERE	Estrogen Response Element
ERKO	Estrogen Receptor Knock Out
ERT	Estrogen Replacement Therapy
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPCR	G Protein Coupled Receptor
GRK	G protein Receptor Kinase
HRT	Hormone Replacement Therapy

LBD	Ligand Binding Domain
LH	Luteal Hormone
MAO-A or B	MonoAmine Oxidase A or B
MAOi	MonoAmine Oxidase inhibitor
MAPK	Mitogen-Activated Protein Kinase
MDD	Major Depressive Disorder
MnR	Median Raphe nucleus
NF-kB	Nuclear Factor kappa B
NRSE	Neural Restrictive Silencing Element
P	Progesterone
PET	Positron Emission Tomography
PKA/C	Protein Kinase A/C
PLC	PhosphoLipase C
PMD	PeriMenopausal Depression
PMDD	PreMenstrual Dysphoria
PMS	PreMenstrual Syndrome
PPD	PostPartum Depression
PR	Progesterone Receptor
RNERb	RN46A cells stably expressing ERβ
SERM	Selective Estrogen Receptor Modulator
SERT/5-HTT	SERotonin Transporter
Sp1	Stimulating protein 1
SSRI	Serotonin Specific Reuptake Inhibitor

TCA	TriCyclic Antidepressant
TPH	Tryptophan Hydroxylase
vgERE	vitellogenin Estrogen Response Element
WAY-100625	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2pyridinyl)cyclohexanecarboxamide

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Introduction

Affective disorders are becoming an increasing concern in today's society. These illnesses include unipolar depression, or major depressive disorder (MDD), bipolar disorder and anxiety. It is estimated that approximately 19 million American adults are affected by depression and anxiety disorders annually, and approximately 12 million of those affected are women, roughly twice the rate of men (Association 2001).

Depression is an affective disorder characterised by an unpleasant mood that is present for most of the day. Other features include a general loss of pleasure (anhedonia) and interest as well as intense mental pain (anguish). For unipolar depression to be diagnosed, other symptoms must also be present including disturbed sleep, diminished appetite and weight loss, loss of energy, decreased sex drive, psychomotor agitation or retardation, difficulty concentrating, indecisiveness, feelings of worthlessness, guilt, pessimistic thoughts and thoughts about dying or suicide (suicidal ideation). Exclusion criteria also exist to rule out other potential diseases, and allowances should also be made for recent trauma, such as a family death, which generally precludes some symptoms of depression during the normal course of mourning (Kandel, Schwartz et al. 1991).

Serotonin neurotransmission

Serotonin (5-Hydroxytryptophan or 5-HT) is an important neurotransmitter in the brain. It is synthesised from tryptophan, a process which depends on two main enzymes: (1) tryptophan hydroxylase (TPH) which adds a hydroxyl group to the 5' position of the indole ring to generate 5-hydroxytryptophan (5-HTP) and (2) Aromatic amino acid

decarboxylase (AADC or DDC) that generates serotonin (Kandel, Schwartz et al. 1991). Serotonin is mostly synthesised peripherally by tryptophan hydroxylase 1 (TPH1) in enterochromaffin cells in the gut, when tryptophan is taken up from dietary sources into these cells (Yu, Fujimura et al. 1999). However, recently, a second isoform of TPH (TPH2) has been identified, and is believed to be primarily responsible for synthesis of 5-HT in the brain (Walther, Peter et al. 2003). Serotonin is synthesized in the cytosol, and is then packaged into secretory vesicles by vesicular monoamine transporter (VMAT2) (Kandel, Schwartz et al. 1991), and transported to nerve terminals. Invasion of the nerve terminal by action potentials depolarizes the terminal opening voltage-dependent calcium channels to increase $[Ca^{2+}]_i$ and induces calcium-dependent release of 5-HT. Upon release into the extracellular space, reuptake of 5-HT from the extracellular space is controlled by the serotonin reuptake transporter (5-HTT or SERT), which is the main method of termination of signaling to postsynaptic targets (Kandel, Schwartz et al. 1991). Serotonin in the cytosolic space is degraded mainly by monoamine oxidase A (MAO-A) although MAO-B seems to be the predominant isoform of this enzyme expressed in dorsal raphe neuron (DRN) 5-HT cells. Alternately, 5-HT can be recycled into vesicles by the vesicular monoamine transporter VMAT2.

Serotonin neurotransmission originates in the midline or rostral raphe nuclei (Törk 1990; Jacobs and Azmitia 1992). These presynaptic neurons extend to postsynaptic targets in the brain such as the hippocampus, hypothalamus, thalamus, striatum, cortex, amygdala, spinal chord and a variety of other regions in the nervous system (Kandel, Schwartz et al. 1991). The raphe nuclei themselves consist of a cluster of serotonergic neurons and/or

dendrites and non-monoaminergic neurons. The majority of 5-HT neurons originate from the dorsal raphe nucleus (DRN), while a smaller proportion project from the median raphe nucleus (MnR) (Adell, Celada et al. 2002). The dendrites of 5-HT neurons are described as having spines, but being poorly branched and staying within the neuropil of the raphe nuclei. The axons tend to be small and unmyelinated. It should be noted that the proximal portion of the axonal processes along with axonal varicosities and terminals extend within the raphe nuclei. These varicosities can be described as an area of the axon that is swollen and contains clusters of synaptic vesicles, but no specialised synaptic junctions. Transmitters can therefore be released from these axon collaterals, and exert an effect on the proximal soma and dendrites of the neuron. Interestingly, it appears that axons are the only portion of 5-HT neurons that actually extend beyond the physiological limits of the raphe nuclei and into other regions such as the periacqueductal gray and reticular formation. As well, studies of 5-HT release indicate that this neurotransmitter is released to a greater extent within the raphe nuclei than other regions in the forebrain (Adell, Celada et al. 2002).

There are fourteen known subtypes of serotonin receptors, divided into 7 families and 14 subfamilies, all of which are structurally and pharmacologically distinct (Hoyer, Clarke et al. 1994). With the exception of the 5-HT3 receptor, which is a ligand-gated ion channel, all 5-HT receptors are 7-transmembrane spanning, G protein-coupled metabotropic receptors or GPCRs. The 5-HT1 receptor family has 5 subtypes of 5-HT receptors, designated 5-HT1A-F. These receptors couple negatively to adenylyl cyclase via G proteins and share high sequence homology. The 5-HT1A, 5-HT1B and 5-HT1D

receptors are the only subtypes which have been identified on the presynaptic, or raphe neurons (Barnes and Sharp 1999). The 5-HT1A receptor will be described in greater detail below but the presynaptic receptor appears to be most important in modulating the rate of firing of serotonin neurons (Albert, Lembo et al. 1996; Pineyro and Blier 1999). It has been proposed that the 5-HT1B receptor functions as both an autoreceptor and a heteroreceptor to control transmitter release. Electrophysiological studies confirm that these receptors do not play a role in the regulation of rat 5-HT DRN firing (Sprouse and Aghajanian 1987). While it is unlikely that the 5-HT1B receptor plays a significant role in depression, studies of the 5-HT1B null mouse reveal a role in aggressive behaviour (Ramboz, Saudou et al. 1996). Studies of this mouse also demonstrate that the 5-HT1B receptor does exhibit the potential to compensate for deficits in the postsynaptic 5-HT1A receptor expression, as the sensitivity of this receptor in the striatum is increased in 5-HT1A receptor null mice (Knobelman, Hen et al. 2001). It has been proposed that 5-HT1D acts as an autoreceptor both pre- and postsynaptically, as well as a heteroreceptor, in a parallel manner to 5-HT1B, but with different pharmacology and expression patterns (Barnes and Sharp 1999). Recent studies have indicated a potential role for 5-HT1B and 5-HT1D modulation of serotonin release from DRN neurons. Although it has been shown that they do not modulate the actual firing of these neurons, the 5-HT1B/1D receptors appear to inhibit 5-HT efflux to postsynaptic targets (Stamford, Davidson et al. 2000). The remaining 5-HT1 receptor subtypes, 5-HT1E and F, are located postsynaptically and have only undergone limited study. Their physiological role in serotonin neurotransmission is as of yet undetermined (Barnes and Sharp 1999).

The 5-HT2 receptor family is composed of three subtypes, all of which are coupled positively to phospholipase C and lead to release of intracellular calcium (Barnes and Sharp 1999). This family has been implicated in a variety of mental disorders, including schizophrenia, anxiety, sleep and eating disorders and migraine headaches (Baxter, Kennett et al. 1995). The 5-HT3 receptor is a ligand-gated ion channel that has been implicated in anxiety, cognition, and dopamine function (Barnes and Sharp 1999). The 5-HT4 receptor family is positively coupled to adenylyl cyclase, and the efficiency of this coupling may depend on the splice variant or subtype of the 5-HT4 receptor. The increase in cyclic adenosine monophosphate (cAMP) modulated by this family of receptors has been linked to phosphorylation of a number of downstream targets and leads to increased excitability of the cell (Barnes and Sharp 1999). The 5-ht5 class of receptor is currently poorly understood. It has been cloned from a mouse cDNA library, but as of the publication of the Barnes review (1999), no evidence of a functional, native 5-ht5 receptor exists. Based on mRNA expression patterns, this receptor might play an early postnatal developmental role and is likely expressed mainly on astrocytes (Barnes and Sharp 1999). The 5-ht6 family of serotonin receptors is also poorly studied, and the identification of a native, functional receptor has not been achieved. Based on the predicted structure and recombinant expression of the receptor in artificial expression systems, it is thought that this family couples positively to adenylyl cyclase, however the physiological role of this receptor is still unclear, although it may be implicated in anxiety and depression (Barnes and Sharp 1999). The most recently identified 5-HT receptor family is the 5-HT7 receptors, of which there are at least four splice variants. These receptors are positively coupled to adenylyl cyclase through $G\alpha_s$, and their

activation generates an increase in intracellular calcium. It has been proposed that the 5-HT7 receptor plays a role in the regulation of circadian rhythm, modulation of neuronal activity and seizures (Barnes and Sharp 1999).

The 5-HT1A receptor

This metabotropic receptor couples to G_i/G_o proteins, which generally leads to inhibition of neuron firing. The $G\alpha_i$ protein is negatively coupled to adenylyl cyclase, which upon activation of the receptor, inhibits the formation of cAMP in the cell. The $G\beta\gamma$ subunits will, upon release from the activated receptor, induce the opening of potassium channels and close calcium channels in neuroendocrine and raphe cells (Penington, Kelly et al. 1991; Penington, Kelly et al. 1993; Albert 1994; Bayliss, Li et al. 1997; Bayliss, Li et al. 1997). This results in hyperpolarisation of the neuron, and consequently, inhibition of firing (Albert and Tiberi 2001). In different cell lines, such as mesenchymal cells, activation of the 5-HT1A receptor has also been shown to activate phospholipase Cβ (PLCβ), which is a stimulatory pathway leading to the activation of mitogen-activated protein kinase (MAPK) (Raymond, Mukhin et al. 1999). This has a variety of downstream effects on gene transcription and cell survival (Albert and Tiberi 2001). Modulation of MAPK phosphorylation via 5-HT1A receptor stimulation with the specific agonist 8-OH-DPAT has also been shown *in vivo*, however the effect opposed that seen in cell culture, where receptor stimulation induced a decrease in MAPK activation in the rat hippocampus (Chen, Shen et al. 2002).

The 5-HT1A receptor is coupled to L-type calcium channels via the G_o but not G_i G proteins (Liu, Jakobs et al. 1994; Liu, Ghahremani et al. 1999). Specifically, it is thought that the Gβγ subunit associates with the α1 subunit of the channels, and inhibits influx of Ca^{2+} (Clapham and Neer 1997; Albert and Tiberi 2001). This translates to a decrease in $[Ca^{2+}]_i$. However, experiments in different cell lines demonstrate coupling of the 5-HT1A receptor to PLCβ, which leads to increased turnover of phosphoinositol, increased levels of IP3 and release of cellular calcium stores to increase $[Ca^{2+}]_i$. This is another example of the signaling variability that is conferred simply by the environment in which a particular receptor is expressed (Albert, Lembo et al. 1996). In fact, the 5-HT1A receptor couples to different G proteins in different brain regions. In the hippocampus, the receptor is coupled to G_o, in the frontal cortex to G_o and G_{i3}, in the DRN to G_{i3} and to G_{i1}, G_{i3} and G_z in the hypothalamus (Hensler 2003).

Presynaptically, the 5-HT1A receptors are located on the soma and dendrites of 5-HT neurons (Riad, Garcia et al. 2000). Postsynaptically, this receptor is expressed on cholinergic neurons of the septum and glutamatergic neurons of the cortex and hippocampus including CA1 pyramidal neurons. Agonist stimulation of the postsynaptic receptor can influence release of both acetylcholine in the septum and noradrenaline in a variety of regions, including the hypothalamus, hippocampus, ventral tegmental area and frontal cortex (Barnes and Sharp 1999).

It should be emphasized that the downstream signaling of the 5-HT1A receptor is strongly dependent on its localisation within the brain, this also applies to the modulation

of serotonin neurotransmission by the 5-HT1A receptor. Electrophysiological studies suggest that the 5-HT1A receptor in the DRN may be more sensitive to agonist stimulation than in other areas, for example the MnR. Microdialysis studies also indicate regional differences for agonist-induced release of 5-HT, supporting the possibility that 5-HT1A autoreceptor control may be different between individual serotonin pathways (Barnes and Sharp 1999). A number of studies conducted with single cell recordings have demonstrated that presynaptic and postsynaptic 5-HT1A receptors have a differential response to drug treatments (Blier and de Montigny 1990; Blier, Lista et al. 1993; Blier, Lista et al. 1993). In fact, evidence has concluded that overall, long term administration of 5-HT1A agonists leads to desensitization of the presynaptic 5-HT1A receptor, but not of the 1A receptor in the CA3 region of the hippocampus (Blier and de Montigny 1994). A number of mechanisms have been proposed to address this including: the presence of a reserve of receptors in the raphe, but not the hippocampus; differential activation states of the receptor depending on its localisation; coupling to different G proteins and extracellular availability of 5-HT at the cell body (Pineyro and Blier 1999).

Stimulation of the 5-HT1A receptor induces a variety of behavioural effects, which are generally specific to presynaptic or postsynaptic activation of the receptor, although which of these is implicated is often unclear and specific to the experimental model examined. For example, the administration of the 5-HT1A agonist, 8-OH-DPAT, directly into the raphe evokes hypothermia in rats, but this is not blocked with 5-HT lesions or inhibited 5-HT synthesis (Bill, Knight et al. 1991; O'Connell, Sarna et al.

1992). In mice, the same experimental paradigm shows a loss of the agonist-induced hypothermic response when presynaptic 5-HT1A activation is blocked by 5-HT lesions (Goodwin, De Souza et al. 1985). This is suggestive of a species difference in the mechanism of the hypothermic response. It appears that 8-OH-DPAT-induced hypothermia is presynaptic in the mouse, but involves both pre- and postsynaptic mechanisms in the rat (Bill, Knight et al. 1991).

The importance of the obvious regional difference in 5-HT1A receptor function becomes apparent when you focus on the presynaptic autoreceptor. This is believed to be the primary 5-HT receptor involved in modulating firing of serotonergic neurons as well as modulating the release of 5-HT at the synapse. Sprouse and Aghajanian (Sprouse and Aghajanian 1987) were among the first to demonstrate that 5-HT1A agonists, but not 5-HT1B agonists, could attenuate the firing of DRN neurons in rats. Since that study, a number of groups have confirmed this in various experimental models (Albert, Lembo et al. 1996; Stamford, Davidson et al. 2000). It is also known that stimulation of the 5-HT1A autoreceptor will lead to a decreased basal release of 5-HT *in vivo* as well as an inhibition of stimulated release in cell culture (Stamford, Davidson et al. 2000).

Essentially, the activity of the 5-HT1A receptor strongly influences serotonin neurotransmission, and as such, changes in receptor expression and density is expected to have a variety of functional consequences. This can occur in two ways: receptor desensitisation and transcriptional regulation.

Receptor desensitization

When a GPCR is subjected to sustained agonist stimulation, it must eventually turn off the signal so as not to overload the cell. A general mechanism for this exists where the activated receptor is phosphorylated by receptor kinases (GRKs) or by protein kinases A or C (PKA/C) (Albert, Lembo et al. 1996). In the case of 5-HT1A, phosphorylation of the receptor by PKC leads to uncoupling of the G proteins from induction of PLCβ, but does not affect inhibition of adenylyl cyclase (Lembo and Albert 1995). If PKA also phosphorylates the receptor, uncoupling from PLC seems to be enhanced (Albert and Tiberi 2001). GRKs also function as a general mechanism for desensitization of GPCRs. After phosphorylation of the receptor, β-arrestin is recruited and binds to the receptor, preventing reassociation of the G protein subunits. This leads to internalisation of the receptor into clathrin-coated pits at which point, the receptor is either recycled to the membrane or degraded (Pitcher, Freedman et al. 1998). Thus far, actual characterisation of GRK-mediated inhibition of 5-HT1A has been limited, due to the rapidity of receptor resensitization. However, GRK2 has been implicated in desensitization of 5HT1B (Lembo, Ghahremani et al. 1999), and therefore may be important for 5-HT1A regulation (Albert and Tiberi 2001).

Agonist treatment has the ability to desensitize the 5-HT1A autoreceptor, but not the postsynaptic receptor in the hippocampus. It is also interesting to note that this effect does not require chronic agonist treatment, as is the case with antidepressant administration (Hensler 2003). In fact, it seems to be a trend that hippocampal 5-HT1A receptors are intrinsically less prone to desensitization, supporting the hypothesis that the

autoreceptor and its postsynaptic version are differentially regulated. The desensitization of the autoreceptor has been well-supported by electrophysiological studies and microdialysis studies of animals treated with 5-HT1A agonists (Hensler 2003).

Transcriptional Regulation

The promoter region that regulates 5-HT1A expression was identified in 1994, and encompasses the 5' flanking region from –1 to –6139 upstream of the ATG start site for the gene (Charest, Wainer et al. 1993; Albert, Lembo et al. 1996; Storring, Charest et al. 1999). A number of putative consensus sequences for a variety of transcription factors have since been identified by sequence analysis, and several of them have been shown to be functional, however most of these elements have been identified in the rat promoter.

The human and murine 5-HT1A promoter regions do not contain a TATA box, and their activity appears to be modulated by the transcription factors MAZ and Sp1, which bind to a GC-rich portion of the promoter region. This region was also shown to serve as a transcription initiation sequence, however whether MAZ and Sp1 were involved in this activation was not clear (Parks and Shenk 1996). By contrast, transcription of the rat 5-HT1A gene is initiated at a site 58-bp downstream from a consensus TATA element. Furthermore, several enhancer regions were identified, some of which were selective, i.e. were only responsive in 5-HT1A positive cell lines, while others were non-selective (Storring, Charest et al. 1999). A 31-bp repressor region was identified upstream of the enhancer regions, and it has since been shown to be functional in both 5-HT1A receptor expressing cell lines and to a greater degree in non-receptor expressing cells. It appears

that in the receptor positive cell line, a single protein complex bound to a 14-bp sequence within the repressor element. In receptor-negative cells, an additional protein complex would bind to an adjacent 12-bp element to repress expression of the gene to an even greater extent (Ou, Jafar-Nejad et al. 2000).

Modulation of the rat 5-HT1A promoter by corticosteroids has been mapped to a direct repeat of the glucocorticoid response element, separated by 6 base pairs. This element is conserved between the mouse, rat and human promoter region (Ou, Storring et al. 2001). This modulation has been demonstrated to be dependent on the location of the 5-HT1A receptor, whereby corticosterone will repress expression of the receptor in the hippocampus, but not in the raphe neurons (Meijer, Williamson et al. 2000). A second mechanism of repression by corticosteroids has been linked to the transcription factor NF-κB. This study showed that 2 functional NF-κB responsive elements exist in the rat promoter, and that this protein induces the 5-HT1A promoter. However, the NF-κB induction is repressed by corticosteroids, likely through the glucocorticoid receptor (GR) (Wissink, Meijer et al. 2000). The same transcription factor (NF-κB) is also modulated by 17-β-estradiol (Wissink, van der Burg et al. 2001) which is explained in further detail below, and does exert a transcriptional enhancement upon the 5-HT1A receptor.

The 5-HT1A receptor and its connection to anxiety and depression

It is now well known that serotonergic function is likely impaired in depressed patients. Preliminary evidence implicating serotonin as a major player in depression began with

the observation that depressed patients had lower levels of tryptophan, which is a precursor of serotonin. It was also observed that tryptophan depletion through diet resulted in negative mood changes and that administration of tryptophan could lead to an antidepressant effect (Middlemiss, Price et al. 2002).

Some studies attempted to link levels of the breakdown product of serotonin, 5-HIAA, to depression, theorizing that this measure was a good indicator of neuronal transmission. Several groups, but not all, show a reduction in levels of this metabolite in the cerebrospinal fluid (CSF) of depressed patients. However, there was no correlation of levels of 5-HIAA to the severity of depression (Mann 1999).

Another approach to teasing out the neurobiological mechanism of depression used neuroendocrine challenges with compounds such as fenfluramine, which causes the release of serotonin and blocks its reuptake. 5-HT causes the release of corticotrophin-releasing hormone, which then promotes the release of adrenocorticotrophic hormone (ACTH) and prolactin into the bloodstream. ACTH can then cause the release of cortisol from the adrenal cortex to the bloodstream, therefore producing two indices of increases in 5-HT measurable in a simple blood sample: prolactin and cortisol. The consensus within these studies is that depressed patients, even those who have undergone treatment and were considered to be in remission, had a blunted prolactin response. Extrapolating from this, it can therefore be concluded that depressed patients have impaired serotonergic function. Measurements with administration of tryptophan or 5-HT agonists show similar results with few exceptions (Mann 1999).

As research progressed, however, it became evident that the relationship between serotonin and depression could be based in any of the 14 5-HT receptors, or within serotonin transport, synthesis or degradation, or perhaps within several of these components. Currently, a significant amount of research has implicated the presynaptic 5-HT1A receptor as a dominant factor in major depression as explained later on in this review.

Antidepressant and agonist treatments

Among the most convincing evidence for the involvement of serotonin neurotransmission, and in particular the presynaptic 5-HT1A autoreceptor, is the pharmacological action of specific serotonin reuptake inhibitors (SSRIs). These drugs block the 5-HT reuptake transporter located at the synapse. This prevents 5-HT, released from the presynaptic neuron, from being taken back up into the presynaptic bouton. With acute SSRI treatment, there is no real increase in serotonin neurotransmission, as the serotonin feeds back via collateral axonal branches to the 5-HT1A autoreceptors on the soma and dendrites of the presynaptic neuron and activates them. This results in a compensatory reduction of neuron firing, and hence a lowering of 5-HT in the synapse. With chronic treatment, however, these autoreceptors become desensitized, and the serotonin in the synapse can build up to a level where it can increase firing in the postsynaptic neurons (Albert, Lembo et al. 1996). Recently, it has been shown that 5-HT1A receptor activation of G proteins in the brain was decreased in the DRN and lateral

septum, but increased at postsynaptic sites such as the hippocampus with SSRI treatment. This is suggestive of desensitization of the 5-HT1A autoreceptor, and sensitization of the postsynaptic receptor (Shen, Li et al. 2002). A second study confirms the decreased interaction of the autoreceptor with its G protein in the raphe, with no change in receptor number, with SSRI treatment, but shows no effect on postsynaptic receptors (Hensler 2002). A third study examining G protein coupling in the same manner supports the results of Shen *et al*, whereby a decrease in presynaptic receptor coupling was observed, concurrent with an increase in the hippocampal receptor coupling following chronic fluoxetine treatment, which is an SSRI (Elena Castro, Diaz et al. 2003).

When comparing the physiological effects of other classes of antidepressants, a trend emerges: serotonin neurotransmission is facilitated. It should be noted that tricyclic antidepressants (TCA) are more potent inhibitors of norepinephrine reuptake, but several of them are also excellent inhibitors of 5-HT reuptake. It appears that though tricyclic antidepressant treatment does not lead to desensitisation of the 5-HT1A autoreceptor, effects on the hippocampal 5-HT1A receptors are fairly consistent (Hensler 2003). For example, the activation of presynaptic 5-HT1A-coupled G proteins is unaltered in tricyclic antidepressant-treated rats, however postsynaptic receptors are sensitised (Shen, Li et al. 2002). This is in contrast to another study of G protein activation that saw no change in hippocampal 5-HT1A G protein coupling (Hensler 2002). However, studies of behaviour and physiological responses to postsynaptic receptor activation have not shown any consistent effect (Hensler 2003). Electrophysiological studies of the postsynaptic 5-HT1A receptor, however, have consistently shown a sensitisation to 5-HT with TCA

treatment in regions of the brain such as the amygdala, the suprachiasmatic nucleus of the hypothalamus, the lateral geniculate body and the facial motor nucleus but not in the somatosensory cortex. In the hippocampus, studies have shown increased responsiveness of the 5-HT1A receptor with TCA treatment (Blier and de Montigny 1999).

Chronic treatment with monamine oxidase inhibitors (MAOi) shows decreased behavioural and physiological responses that are mediated by the postsynaptic 5-HT1A receptor. As well, it appears that this receptor has a decreased ability to inhibit adenylyl cyclase activation in the hippocampus (Hensler 2003). However, chronic MAOi treatment desensitized the presynaptic receptors, but had no effect in the hippocampus (Shen, Li et al. 2002).

Electroconvulsive therapy (ECT), a treatment used for patients that are resistant to traditional pharmacological therapies, can increase 5-HT1A receptor number in parts of the hippocampus, which may contribute to its antidepressant action (Mann 1999).

5-HT1A expression levels

PET studies examining the levels of the 5-HT1A receptor in the raphe nuclei have been somewhat inconclusive, with one study showing increased levels of 5-HT1A agonist binding in the raphe neurons of post-mortem brain from clinically depressed suicide victims (Stockmeier, Shapiro et al. 1998). However, a different group found that binding of WAY-100625, a 5-HT1A antagonist, in the midbrain of patients with primary,

recurrent, familial mood disorders was decreased, in opposition to expected results. However, this study examined unmedicated patients with both bipolar and unipolar depression, and therefore may not be as specific to major depressive disorder (Drevets, Frank et al. 1999).

Another study in depressed victims of suicide revealed that the concentration of 5-HT1A receptors in the DRN of suicide victims was unchanged, however the binding capacity of these receptors was significantly decreased compared to controls; a similar trend was observed in the MnR. Serotonin transporter (SERT) binding and mRNA were not different between the two groups, however there was a marked reduction in the number of neurons that expressed SERT in suicide victims (Arango, Underwood et al. 2001).

Behavioural studies

Behavioural studies in mice and rats have shown that 5-HT1A agonists will modify animal behaviour in two models of depression, the forced swim test and the learned helplessness paradigm. In the forced swim test, the animal is subjected to several pretests that induce a "behavioural despair" where the animal does not try to swim to escape, instead it remains immobile and floats. The time spent immobile is considered a measure of depression, and is decreased with antidepressant treatment.. In the learned helplessness paradigm, the animals are subjected to an inescapable stress, often electric shock, in a pre-trial. In the actual testing phase, the animals are then given means of escape from the stress, however their latency to take this escape is a measure of

helplessness. In these experiments, the effect was traced to presynaptic receptors based on the agonists being directly injected into the raphe nucleus, or by examining whether the effect was blocked by lesioning raphe neurons with 5,7-dihydroxytryptamine (Newman, Lerer et al. 1993). It has also been shown that giving antidepressants to mice reduces the hypothermic response in mice (described above) seen with administration of the selective 5-HT1A agonist 8-OH-DPAT (Newman, Lerer et al. 1993). This indicates that modulation of the 5-HT1A autoreceptor occurs with antidepressant treatment, suggesting that the activation of this receptor plays a role in the disorder itself.

SERT knockout Mice

The serotonin knockout mouse is considered to be a good model of chronic antidepressant treatment based on the functional consequences of this deletion on the 5-HT1A receptor. Studies have shown that within this animal model, application of SSRIs to brainstem slices inhibits neuronal firing in the DRN of the wild type mice, but not in the SERT-/- mice. 1A Agonist stimulation of the same preparations showed inhibition of neuron firing in the same region, however this effect was attenuated in SERT -/- mice. The same experiments done on hippocampal slices revealed no differences in these parameters between wild type and knock out mice. This suggests that deletion of the serotonin transporter, as with chronic SSRI treatment, leads to a functional desensitization of the 5-HT1A autoreceptor, without altering the postsynaptic receptor (Mannoury la Cour, Boni et al. 2001).

5-HT1A knockout mice

In 1998, Hen *et al* knocked out the 5-HT1A receptor in mice by insertion of a PGK-neo cassette into a region following the third transmembrane domain of the 5-HT1A gene (Ramboz, Oosting et al. 1998). Functionally, it was observed that the levels of 5-HT and 5-HIAA in the tissues of these mice were unchanged compared to wild type, as was the ratio of 5-HT/5-HIAA, suggesting that turnover of serotonin also remains consistent (Ramboz, Oosting et al. 1998). This is in contrast to a more recent study that found 5-HIAA concentrations were increased in specific brain regions, the substantia nigra, dorsal medial raphe nuclei and locus coeruleus, however the 5-HT content was unaffected, suggesting higher turnover in these regions (Ase, Reader et al. 2000; Ase, Reader et al. 2001). However, these experiments were performed on mice with a different genetic background, which may account for the divergence from the Ramboz (1998) study (Ase, Reader et al. 2000). Electrically-invoked release of 5-[^{3}H]-HT was unchanged in the knockout. There did appear to be some compensation for the lack of 5-HT1A receptors, as the inhibitory response to a 5-HT1B agonist was significantly greater in the -/- mouse (Ramboz, Oosting et al. 1998). Further study of the electrophysiology of the 5-HT1A knockout mouse revealed that the mean spontaneous firing activity of DRN neurons was nearly doubled as compared to wild type mice, but this did not lead to any significant change in 5-HT release (Richer, Hen et al. 2002).

The behaviour of these mice was examined in the open field and elevated plus maze test, and it was found that the knockout and heterozygote male mice were less active overall

and displayed increased anxiety-like behaviour. This was defined in the open field test by measuring the time spent in the centre of the field and the relative distance traveled in the centre. The female mice were also less active, although in fewer measures of behaviour than males, but their anxiety-like phenotype was unchanged. The authors postulate that this is due to a "floor" effect, as the anxious behaviour of female mice is already elevated. The elevated plus maze test, where the mice must choose between two open elevated arms and two closed arms, also point to an anxious phenotype. In this experimental paradigm, no effect of sex was observed, with the females showing elevated anxiety compared to wild type as well as males (Ramboz, Oosting et al. 1998). The mice were also tested in the forced swim test. There were no genotype effects in the pretest, however the knockout mice were significantly more tenacious in their efforts to get out of the water in the subsequent trials, while the wild type mice became progressively more immobile. The group confirmed that this behavioural data was directly related to a lack of 5-HT1A, and not developmental compensations, by administering pharmacological antagonists and measuring behaviour. The same trends were observed (Ramboz, Oosting et al. 1998).

More recently, a conditional and tissue-specific 5-HT1A knockout was designed to look more closely at whether these changes were due to loss of the receptor during a specific time in the mouse life cycle, and whether this is specific to loss of expression in a particular brain area. A transgenic mouse expressing the 5-HT1A receptor gene under control of the tetO promoter was crossed with another strain expressing tTA, the bacterial transcription factor which modulates tetO, under control of the α-calcium-calmodulin-

dependent protein kinase II (αCAMKII) promoter, which is functional in the hippocampus and cortex. As such, the progeny of this cross expressed the 5-HT1A receptor in the hippocampus and cortex, but not in the raphe nuclei, and expression of the receptor can be blocked by administration of doxycycline. This allows for inducible expression of postsynaptic 5-HT1A receptors without any expression of the presynaptic autoreceptor. This study found that forebrain expression of the serotonin-1A receptor was enough to rescue anxious behaviour of these mice. Furthermore, using the inducible feature of this knockout, the investigators showed that a lack of postsynaptic 5-HT1A receptors during the early postnatal period (P5-P21) was most important in the anxious phenotype (Gross, Zhuang et al. 2002). Thus far, nothing has been published examining inducible knockout of the presynaptic 5-HT1A receptor. However the current theory is that the anxiety phenotype is related primarily to the postsynaptic 5-HT1A receptor, while depression is mediated primarily by the autoreceptor. A raphe-specific knock-in of the receptor may further clarify the role of 5-HT1A in these two consequences of serotonergic dysfunction.

Estrogen and its physiological functions

Estrogens are members of the steroid hormones and are derived from cholesterol and released from the ovaries of the female. Cholesterol is taken up by the ovary, or synthesized within this structure from acetate, and converted into pregnenolone (Buxton 2003). Estrogen requires a carrier molecule, which is produced by the liver, in order to circulate within the body. This molecule functions to reduce the hydrophobicity of the steroid hormone, limit the availability of the hormone, and prolong the half-life of the

hormone. It also confers a certain amount of target specificity, as only certain cells have receptors to particular carrier-hormone complexes (Kapás 2001).

The major physiological roles of estrogen are numerous and varied. It affects a variety of physiological structures including uterus, vagina, fallopian tube and breast development, bone growth, metabolism, and the circulatory system (Buxton 2003). Estrogens have significant effects elsewhere than in reproduction and among them, is an involvement in the nervous system, influencing cognitive function, pain mechanisms, fine motor skills, mood and susceptibility to seizures. Other studies have also suggested that estrogen might have a neuroprotective effect in both stroke and Alzheimer's disease (McEwen 2001).

The estrous cycle

The estrous cycle in women has been divided into 4 main phases, the follicular or proliferative, ovulatory, luteal or secretory, and menstrual phases. The follicular phase occurs in days 1 to 13-14 of the cycle, and is the time when Follicle Stimulating Hormone (FSH) stimulates the ovary to prepare for release by generating a follicle and thickening the endometrium. FSH and Luteal hormone (LH) rise gently during this phase, in preparation for release of the ovum. The ovulatory phase is characterised by a peak in estrogen, FSH and LH, resulting in release of the ovum. Estrogen declines rapidly over days 10-15, while progesterone undergoes a sustained increase from this phase through to the end of the menstrual cycle. During the luteal phase, LH and FSH drop back to normal levels, while progesterone remains high and depletes estrogen

receptors. Estrogen shows another small peak in days 16-24, then returns to basal levels prior to menstruation. The menstrual phase is characterised by sloughing of the endometrium, and hence, menstrual bleeding, due to estrogen and progesterone withdrawal (Moses 2003).

The estrogen receptor

Among other things, estrogen is a powerful transcriptional regulator. It mediates this function with a specific nuclear receptor, called the estrogen receptor. There are two isoforms of the estrogen receptor, the estrogen receptor α (ERα), which was discovered first and the estrogen receptor β(ERβ). In general, nuclear receptors contain three functional domains, the ligand binding domain (LBD) at the C-terminus of the receptor, the DNA-binding domain, located centrally, and the activation domain (AF), of which there may be several and can be distributed throughout the molecule. The LBD confers specificity of a particular receptor for its steroid hormone, while the DBD targets the receptor to the genes that it is capable of regulating, based on a particular consensus sequence within the genome (DeMayo, Zhao et al. 2002). Alternatively, the receptor is divided into a C-terminal A/B domain, which contains AF1, the DBD, named region C, a hinge domain (region D, and the LBD (region E). Most interactions with co-activators and co-repressors occur through the AF-2 domain, which is contained within region E of the receptor (Safe 2001). The DNA binding domain contains two zinc fingers that are essential for binding of the receptor to its response element, and this region is 97% homologous between ERα and ERβ. The LBD shows 59% homology between the two isoforms (Safe 2001). Other regions of the ERβ show much less homology to ERα,

including the hinge domain, the E/F region, and the A/B region. The A/B region shows only 19% homology that may, along with ligand binding variation, be a source of functional variability for this receptor (Mosselman, Polman et al. 1996). The mechanism of action of AF-1 is still unclear, however it is known that it is required for the partial agonist activity of tamoxifen, which can act as both agonist and antagonist depending on the tissue type. AF-1 is also activated by MAPK-directed phosphorylation, suggesting a role in ligand-independent activation of the receptor (McDonnell and Norris 2002).

The differing roles of ERα and ERβ were discovered mainly by studying ER knockout animals, specifically mice (ERKO mice). The loss of ERα appears to cause a much more dramatic phenotype. While both knockouts show severe ovarian dysfunction, the αERKO also lacks sexual maturation of the gonadal ducts, and is infertile. The βERKO mouse, however have normal uteri and show normal cycling of the reproductive organs, however this mouse also shows reduced fertility because of infrequent and inefficient ovulation (Couse, Curtis Hewitt et al. 2000). Interestingly, knocking out the ERβ has revealed behavioural changes due to loss of this receptor. βERKO female mice show increased anxiety behaviour in the open field test and the elevated plus maze paradigm, which was associated with altered long term potentiation in the amygdala and increased 5-HT1A receptor expression in the amygdala of ERβ-deficient female mice (Krezel, Dupont et al. 2001).

The estrogen receptor β is encoded on human chromosome 14, while ERα is encoded on chromosome 6. The ERβ is expressed in the central nervous system, the cardiovascular

system, the immune system, the urogenital tract, the gastrointestinal tract, the kidneys and the lungs. The biological role of ERβ is becoming more obviously distinct in terms of function and modes of regulation. In the central nervous system, expression patterns of ERα and ERβ are also distinct. Both isoforms are expressed in the hypothalamus, whereas ERα is the dominant receptor in the hippocampus, olfactory lobe, cortex and cerebellum (Gustafsson 1999).

Transcriptional modulation by estrogen

The classical, ligand-dependent mechanism of transcriptional modulation by estrogen begins with the binding of the ligand to the receptor, which allows it to dissociate from chaperone proteins that keep it transcriptionally inactive. The activated form of the receptor changes conformation and dimerises with a second active receptor, conferring the ability to bind to its target response element (DeMayo, Zhao et al. 2002). Once activated by ligand binding, the hetero- or homodimerised receptor translocates from the cytoplasm to the nucleus and binds to its cognate hormone response element. However, in the case of the estrogen receptor, it is already localized in the nucleus, therefore this step does not occur (Beato, Truss et al. 1996). At this point, co-activators or co-repressors are recruited to the bound steroid receptor, and influence the activity of the basal transcriptional machinery (DeMayo, Zhao et al. 2002). Coactivators that have been identified include the Steroid Receptor Coactivator family (SRC), transcriptional intermediary factor-2 (TIF2 or GRIP1), receptor-associated coactivator 3 (RAC 3 or AIB1) and activator of thyroid and retinoic acid receptors (ACTR). These last three are of the p160 family of proteins which are capable of then recruiting other coactivators

such as CREB binding protein and the related p300 protein, High Mobility Group proteins (HMGs), E3 ubiquitin-protein ligases (E6-AP and RPF-1). p160 coactivators also recruit histone acetylases, which assist in the unwinding of chromatin to make the gene more accessible for transcription (DeMayo, Zhao et al. 2002). It has been shown these co-activators are recruited in a ligand and AF-dependent manner by the "NR-box" which is comprised of the motif LxxLL (Pike, Brzozowski et al. 2000). Other proteins recruited by ERα may allow for direct contact with the general transcriptional machinery, as is the case for thyroid hormone receptor-associated protein (TRAP220 or DRIP205) (McDonnell and Norris 2002). The activated receptor can also function as a repressor, recruiting histone deacetylases to inhibit the basal transcriptional machinery. It can also recruit other co-repressors including nuclear receptor corepressor (NcoR, silent mediator of retinoid and thyroid hormone receptors (SMRT), repressor of estrogen action (REA), short heterodimer partner (SHP), receptor-interacting protein 140 (RIP140), dosage-sensitive sex-reversal, Adrenal hypoplasia congenital, X chromosome (DAX-1), and repressor of tamoxifen activity (RTA). The activity of ERα can be negatively regulated by ERβ. In general, ERβ is a less efficient transcriptional activator, however it can function as a dominant inhibitor of ERα-mediated gene activity if both isoforms are expressed in the same cell line. This is physiologically relevant if you note that the sensitivity to E2 is greatly increased in ERβ knockout mice, supporting a role as a repressor for this receptor. The function of estrogen receptors is also repressed by ligand-activated progesterone receptors (McDonnell and Norris 2002). It is now evident that estrogen receptors have a cell and context-specific manner of interaction with a variety of transcription factors. However the role and method of recruitment and discrimination

between these proteins is still in the early stages of understanding (McDonnell and Norris 2002).

The core consensus sequence that binds the estrogen receptor consists of a 13-bp element containing 10 nucleotides that form an inverted repeat, separated by three base pairs, 5'GGTCAnnnTGACC3'(Driscoll, Sathya et al. 1998). Significant variation of this element has been found among many estrogen responsive sequences. Examination of ER binding by gel shift assays have shown that a single T to G mutation, at position –4 of the inverted repeat abolishes binding of the receptor completely (Driscoll, Sathya et al. 1998). Another study also showed that a single base pair change, a G to A mutation at position –5 of the perfect palindrome is not recognized by ERs, although this motif is contained within glucocorticoid and retinoic acid response elements (Sanchez, Nguyen et al. 2002). However, the sequences flanking the ERE are also important for binding, and are capable of restoring binding to a non-functional element. This is attributed to a flanking purine residue at each end of the sequence. Further examination indicated that the second flanking residue was also important, and that these sequences can rescue binding in a consensus sequence with up to two changes within the palindrome (Driscoll, Sathya et al. 1998).

Phosphorylation may play an important role in the function of hormone receptors. Several reports show that Ser/Thr phosphorylation is required for binding of the receptor to its response element. It appears that in general, the receptor is phosphorylated whether or not the ligand is present, however the level of phosphorylation increases once the

ligand is bound. However, it is unclear at what step, dimerisation, nuclear translocation, or DNA binding, this phosphorylation is important. Specifically, the estrogen receptor is phosphorylated at a tyrosine residue in the ligand binding domain, and it has been reported that this is important for optimal estrogen binding to the receptor (Beato, Truss et al. 1996). The ERα is also phosphorylated at ser106 and ser124 by MAPK, and studies indicate that this leads to the recruitment of SRC-1 to AF-1. Phosphorylation of Ser118 in human ERα activates both AF-1 and AF-2 functions (Govind and Thampan 2001).

Indirect mechanisms of transcriptional modulation

The estrogen receptor can interact with other transcription factors to modulate transcription. An example of this is the interaction of ER with Sp1 through Sp1$(N)_x$ERE or Sp1$(N)_x$ERE half sites. Both factors must bind to their consensus sequence for this interaction to occur, and other factors are also involved in the activation of transcription. Interestingly, the interaction of ERα with Sp1 is associated with gene activation, while ERβ-Sp1 interactions are more likely to cause repression of transcription. A number of estrogen responsive genes, which do not contain consensus EREs, are modulated in this fashion, including cathepsin D, heat shock protein 27, transforming growth factor-α. Studies of the uteroglobin gene has revealed that genes containing putative EREs might require interactions with Sp1 to fully induce a hormonal response (Safe 2001).

Estrogen responses also occur via interaction with G-C rich sequences, independent of the presence of a full or partial ERE, perfect or imperfect. This mechanism appears to

require the binding of Sp1, but not ERα to this region, however the Sp1 binding is greatly enhanced by its presence. It has been shown that ERα and ERβ can interact with the C-terminal zinc finger of Sp1, and given the number of transcription factors that the ERs are capable of recruiting, this allows for a great degree of variability in the mechanism of hormone-induced transcriptional regulation (Safe 2001).

The AP-1 response element, which normally binds members of the Jun/Fos family of transcription factors, can also be a site for estrogen receptor action. Two mechanisms, one AF-dependant, and one not requiring the ER AF domain, have been proposed for transcriptional modulation through these consensus sites. The AF-dependent proposal suggests that estrogen-liganded ER interacts with p160 coactivators that are recruited to the complex of proteins at the AP-1 site. ER is known to recruit these proteins when acting at an ERE, therefore it is logical that the receptor is also capable of the same interactions at other sites. This is then believed to trigger a higher activation state of the p160s, therefore inducing greater transcriptional activity. The proposed mechanism of AF-independent activation occurs by activated ERβ sequestering repressors away from the protein complex at the AP-1 transcription site (Kushner, Agard et al. 2000). Estrogen receptors are also capable of protein-protein interactions with GATA-1, IL-6, NF-kB, NF-IL6 and C/EBP transcription factors to modulate their activity in a cell-specific manner. However repression of NF-kB is reciprocal, given that this TF is also capable of repressing estrogen activation (Sanchez, Nguyen et al. 2002).

Ligand-independent mechanisms of hormone activation often involve membrane-signaling pathways. The ER can be located at the membrane, and its activation has been shown to activate kinase pathways that lead to the phosphorylation of the receptor. Homodimerisation takes place and the receptor can translocate to the nucleus and bind to its response element (DeMayo, Zhao et al. 2002).

Nongenomic mechanisms of estrogen activity

Genomic actions are generally long term changes that occur over an extended period of time. This does not account for some of the actions of estrogen, which can occur very rapidly. There is a subpopulation of ERα and ERβ located at the cell membrane, which may be involved in cell signaling through G proteins. Both ERα and ERβ have been shown to regulate MAPK activity in through an interaction with the SH2 domain of SRCwhich is believed to lead to cell proliferation in both cases. Furthermore, it has been shown that estrogen receptors can interact with the p85α regulatory subunit of PI3 kinase, leading to increased activation of this kinase which may be involved in some of the cardioprotective effects of estrogen (Sanchez, Nguyen et al. 2002). In the endothelial cell, estrogen can activate MAPK, which leads to phosphorylation and hence modification of the function of heat shock protein 27 (HSP27). E2 is also capable of inhibiting chemotherapy or radiation-induced JNK activation, which prevents formation of the apoptosome and hence promotes cell survival. It is also believed that the G protein activity of E2 leads to signaling-induced synthesis or activation of transcription factors. For example, the hormone can activate c-fos through extracellular signal-regulated

protein kinase (ERK) or PI3K-dependent pathways, and can stimulate the prolactin gene through ERK. Estrogen can rapidly induce activation of PI3K, within about 15 minutes of treatment. This leads to activation of a number of different transcription factors, downstream signaling molecules and genes coding for structural proteins, cytokines or enzymes, many of which have implications in the protective features of estrogen (Pedram, Razandi et al. 2002).

Estrogen can have rapid effects on neuronal excitability, which involve direct interactions of estrogen receptors and second messenger systems. These include activation of cAMP, MAPK, as well as modulation of G protein coupling. Effects on Ca^{2+}, an important second messenger, have also been observed. Estrogen can affect Ca^{2+} current into the cell and gonadotropin-releasing hormone release, as well as exert effects on calcium channels and entry of the ion into to the cell. Estrogen can also stabilise mitochondrial membrane potentials, prevent ATP depletion in the mitochondria and reduce the generation of oxygen free radicals, all of which reduce cell damage and promote cell survival (McEwen 2001).

Estrogen and progesterone play direct roles in the modulation of ligand-gated ion channels. For example, estrogen can act as a direct functional antagonist at the 5-HT3 receptor (Wetzel, Hermann et al. 1998). The currently favoured mechanism for this is that the steroid enters the membrane at the receptor-membrane interface and allosterically modulates the receptor function. Functional modification in this manner may have implications in the development and course of nausea during pregnancy and in

psychiatric disorders. Neuroactive steroid derivatives of progesterone have been shown to interact with mammalian $GABA_A$ receptors, and increase the frequency and duration of openings of the GABA-gated fluoride channel. Other ligand-gated channels are targets for steroids, including the NMDA receptor, the AMPA receptor and kainate receptors, actions at which involve potentiation of Ca^{2+} current which leads to neuroprotective effects, along with improvements in learning and memory. Furthermore, steroids activate the G protein-coupled oxytocin receptor, and it is believed that the sigma receptors also bind steroids, and can be modulated by steroid activity (Kelly, Lagrange et al. 1999; Brinton 2001; Kelly and Levin 2001; DeFazio and Moenter 2002)There is some evidence that exists to support a role for estrogen receptors in post-transcriptional control, although these studies are very limited (Govind and Thampan 2001).

Estrogen and its connection to anxiety/depression

As mentioned earlier, the global incidence of depression in women vs. men is 2:1 following puberty, while the incidence of childhood depression is equivalent between the sexes. Additionally, women are more vulnerable to depressive episodes at specific times in their lives, all of which involve major hormonal change: the premenstrual period, the postpartum period and the perimenopausal period (Epperson, Wisner et al. 1999).

Premenstrual Dysphoria (PMDD)

About 5% of all menstruating women experience PMDD (Epperson, Wisner et al. 1999). The DSM-IV requirements for a diagnosis of PMDD are that women report a minimum

of 5 of the 11 total symptoms (Steiner and Born 2000). These include anger/irritability, depressed mood, mood swings, anxiety/tension, low interest, decreased concentration, poor energy and changes in sleep and appetite, along with physical symptoms of PMS, serious enough to interfere with lifestyle and social functioning (Halbreich and Kahn 2001). The symptoms must worsen premenstrually, and lessen within a few days of the onset of menstruation, and this must have occurred with most menstrual cycles for the past year (Steiner and Born 2000). The symptoms of PMDD cease during pregnancy and postmenopause, and if cycling hormones are suppressed or prevented. It has also been noted that hormone replacement therapy can trigger cyclical mood changes in women with a history of PMDD. There is some divergence in the clinical features of PMDD as compared to major depression. For example, the hypothalamic-pituitary-adrenal axis shows normal function in PMDD, inconsistent with studies of MDD. PMDD tends to respond best to serotonin-selective antidepressants such as SSRI's, and symptom improvement generally occurs within the first cycle of treatment, and conversely, when treatment is stopped, symptoms return rapidly (Steiner and Born 2000). Women with PMS have shown a tendency in published studies to have increases in progesterone levels, however no firm, well-controlled study has consistently shown this. Although no differences in plasma levels of reproductive hormones have been noted, it is more likely that the etiology of PMS is based in differences in sensitivity to the cyclicity or rate of fluctuations in hormone levels (Halbreich and Kahn 2001).

Postpartum Depression (PPD)

The incidence of PPD is about 10%, with the major risk of depression occurring within the first 3 months postpartum (Epperson, Wisner et al. 1999). At this time, major hormonal shifts are taking place. Within 48 hours of birth, estrogen levels drop to prefollicular levels from a level which is about 100 times that of a non-gestating woman, while progesterone drops dramatically as well, with its levels during pregnancy being about 300 times the normal. Within 24 hours of delivery, the estrogen levels are about $1/25^{th}$ of what they were prior to birth (Fredericks 2003). Postpartum blues are experienced by most new mothers and are considered normal. The symptoms, including dysphoria, mood lability, irritability, crying, anxiety, insomnia, and poor appetite, generally appear during the first week postpartum, peak on the fifth day, and are gone by the end of the second week. In PPD, the symptoms last beyond the second week postpartum and are severe enough to resemble an episode of major depression (Halbreich and Kahn 2001).

The current hypothesis is that the abrupt withdrawal of hormones, particularly estrogen, is among the major factors leading to PPD, however this has not been conclusively proven. A study done in 2000 simulated the supraphysiologic gonadal steroid levels of pregnancy by administering the gonadotropin-releasing hormone agonist leuprolide acetate along with supraphysiologic doses of estrogen and progesterone. Withdrawal from these high levels to a hypogonadal state in women with or without a history of PPD, simulated the postpartum period. Using daily symptom self-ratings and standardized subjective and objective cross- sectional mood rating scales, it was found that five of the

eight women with a history of PPD, and none of the control women, developed significant mood symptoms after hormone withdrawal. The authors concluded that there is direct involvement of estrogen and progesterone in the etiology of PPD, and that women show a differential sensitivity to changes in these steroids (Bloch, Schmidt et al. 2000).

Perimenopausal Depression

Depression during perimenopause is an important phenomenon that likely contributes to the conclusions of several community-based studies where the incidence of depression between the ages of 45-55 was 3-4 times more elevated in women than men (Epperson, Wisner et al. 1999). The perimenopause is defined by the World Health Organization as the period before menopause until one year following the cessation of menstruation, and is associated with loss of ovarian follicular activity. This process begins at the median age of 45.5-47.5 years and lasts 4 years on average. Symptoms of perimenopause include hot flushes, experienced by 85% of women, night sweats, dyspareunia, urinary frequency, sleep disturbance, tiredness, depression and anxiety. During menopause, the circulating levels of sex steroids fluctuate, estrogen generally decreases over time, but serum levels can be quite variable from cycle to cycle (Soares and Cohen 2001).

A number of cross-sectional studies report more negative affect associated with the onset of perimenopause, with more perimenopausal women reporting depressive symptoms than premenopausal or postmenopausal women. There is a significant risk for major depression in women who have undergone surgical menopause. It is important to

remember though that psychosocial factors may play a large part in these symptoms, as this is often a time when women face events such as children leaving home, and a loss of what to them may be their primary function, child bearing (Soares and Cohen 2001).

Treatment options and estrogen replacement

A few epidemiological studies have examined the link between sex and treatment response. These have found that women aged below 40 had a decreased response to imipramine, a tricyclic antidepressant, than men or women over 40. A similar result was observed in women with PPD who responded better to TCAs than non-postpartum women, suggesting a hormonal factor. As well, women with the hormonal syndromes described above tend to respond very well to modulators of the serotonergic system, such as SSRIs (Epperson, Wisner et al. 1999). In fact, women with PMDD show improvement with SSRI treatment even if administered only during the luteal phase of the menstrual cycle (Halbreich and Kahn 2001). One report suggested that administration of estrogen could improve the response to antidepressant treatment, and another trial indicated that women on oral contraceptives responded better to sertraline, an SSRI, than premenopausal women (Young and Korszun 2002). Similarly, it was found that older depressed women taking estrogen replacement therapy (ERT) reported significantly greater improvement and quality of life with sertraline treatment than women not receiving estrogen (Schneider, Small et al. 2001). Comparable studies examining response to fluoxetine treatment show that ERT does improve the clinical outcome of administration of this SSRI in women over 60 (Schneider, Small et al. 1997). However a study in women aged 45 or over showed no significant improvement in treatment

response with ERT (Amsterdam, Garcia-Espana et al. 1999). Another study that is important to highlight in this review is one that examined the gender-based response to two different classes of antidepressants, TCAs (imipramine) and SSRIs (sertraline). The study found that women had a more favourable response to sertraline, while men responded better to imipramine. Women also responded significantly more slowly than men to imipramine, while men showed a trend towards a slower response to sertraline, although the data wasn't significant. The same study also suggested that premenopausal women responded better to sertraline than imipramine while postmenopausal women showed no difference. This data suggests that gonadal hormones likely contribute to the pathology of depression, and hence influence the treatment response (Kornstein, Schatzberg et al. 2000). These studies indicate that the presence of estrogen is beneficial for treatment response to antidepressants, in particular serotonin specific reuptake inhibitors. This strongly suggests that estrogens regulate the serotonergic system, and perhaps maintain its balance. When dysregulation of the serotonergic system occurs, estrogens likely minimise or modulate this dysregulation, which allows for a more robust response to treatments.

Estrogen as a treatment option

Women with severe premenstrual syndrome (PMS) have often been treated successfully with estrogen. One study found that a high dose of estrogen was more effective than placebo in treatment-resistant women with severe major depression, however although improved, the women remained symptomatic (Klaiber, Broverman et al. 1979). This result has not been replicated in women with major depression not related to a hormonal

event (Epperson, Wisner et al. 1999). Recently, two randomized, double-blind studies have suggested that transdermal administration of estrogen can act as an antidepressant in menopausal women (Schmidt, Nieman et al. 2000; Soares, Almeida et al. 2001). When administered during the follicular and luteal phases, estrogen treatment improved both the physical and psychological symptoms of severe PMS (Epperson, Wisner et al. 1999; Almeida and Barclay 2001). Several reports indicate that cyclical transdermal estrogen in dosages high enough to suppress ovulation is an effective treatment for the mood fluctuation seen in PMS (Halbreich and Kahn 2001). A study conducted in women with PPD who had low serum estradiol concentration found that depressive symptoms were drastically reduced after two weeks of estrogen treatment. This treatment was effective in increasing Montgomery-Asburg Depression Rating Scale (MADRS) scores to levels defined as "clinical recovery" in 19 of 23 patients. However, this was not a placebo-controlled trial, which would be necessary to definitively attribute the clinical benefit to estrogen replacement (Ahokas, Kaukoranta et al. 2001). Estrogen can also be used as a preventative strategy in women with a history of postpartum mood disorders. When these women are given high-dose estrogen immediately after birth, followed by decreasing dosage over the course of a month, the relapse rate fell from 35-60% to 9% (Epperson, Wisner et al. 1999). Similarly, rats dosed with estrogen and progesterone at levels that mimic the 23-day gestational period, defined as "pregnant", who continued to receive estradiol benzoate following the "pregnancy" showed prevention of "PPD". Behavioural testing indicated that hormone replacement following pregnancy is an important factor in preventing PPD, as measured by the forced swim test, a model of depression (Galea, Wide et al. 2001). A more recent study of female rats indicated that

estrogen and the synthetic estrogen ethinyl-estradiol reduce immobility time and increase swimming time in the forced swim test. This effect is consistent with the behavioural changes seen when these mice are given antidepressants, including the SSRI fluoxetine, and desipramine, a catecholamine reuptake inhibitor (Estrada-Camarena, Fernandez-Guasti et al. 2003).

There are often distinct differences in responses to synthetic and natural steroids, as well as different modes of administration. Interestingly, women treated with estrone sulfate in one particular study showed an improvement in mood that was correlated with increased levels of free tryptophan levels, perhaps implicating the serotonergic system in this observation (Aylward, Holly et al. 1974). Although estrogen may not be potent enough to be administered as a treatment for MDD, except in very few cases, it can be used effectively for depressive episodes related to major hormonal events. It can also be used in conjunction with traditional treatments to improve affect, making it important to understand the role of this hormone in depression and in the dysregulation of the serotonergic system.

Interactions between serotonin and estrogen

There are many levels of control within the serotonergic neural system that are potentially modulated by steroid hormones. This includes serotonin synthesis, reuptake, neural firing or activity that drives 5-HT release, degradation and receptor activation. An extensive array of studies has looked at all of these components, leading to what appears

to be a complex network of regulation by estrogen and progesterone. This section will attempt to put all of this data into perspective and perhaps explain the physiological reasons why women exhibit a much higher risk of depression than men do.

Expression patterns of steroid receptors in serotonin neurons

In nonhuman primates, the β isoform of the estrogen receptor is expressed in neurons in the raphe nucleus, including those that are positive for the serotonin transporter, however the ERα is not present. The expression of ERβ within the DRN is not induced by hormone treatment, however the progesterone receptor (PR), also located in the 5-HT neurons of the DRN, is induced with estrogen treatment. ERα is expressed in postsynaptic targets such as the hypothalamus (Bethea, Lu et al. 2002).

In mice, both ERα and ERβ co-localise in serotonin neurons of the raphe, however the β isoform is predominant (Bethea, Lu et al. 2002). In the rat raphe nuclei, ERβ is also the predominant receptor isoform in the DRN, however ERα is also expressed in this region, albeit not in 5-HT neurons. Further study has suggested the ERα is localized in the smaller interneurons of the dorsal raphe (Bethea, Lu et al. 2002). The guinea pig shows a similar expression pattern to the rat, with ERβ being the dominant isoform in the DRN, and ERα being expressed in nonserotonergic neurons of the periaqueductal gray region, which is adjacent to the raphe (Bethea, Lu et al. 2002). Overall, the trend in all species seems to be that ERβ is the predominant isoform of the estrogen receptor in 5-HT neurons of the raphe nucleus, suggesting that it is this particular receptor that plays a role in regulating serotonergic neurons in response to the presence of gonadal hormones.

Serotonin synthesis

Measurements of 5-HT in particular brain regions can be difficult to interpret. However some information can be gleaned from looking at the net serotonin within a specific region. Two studies have shown that female rats have higher levels of 5-HT in the central nervous system overall (Renner, Biegon et al. 1985; Carlsson and Carlsson 1988). Examination of the 5-HT content and turnover, based on measurement of 5-HIAA in the dorsal raphe of ovariectomised rats indicates a general trend towards an increase with estrogen, progesterone or a combination of the two (Ladisich 1974; Cone, Davis et al. 1981; Di Paolo, Diagle et al. 1983; Renner and Luine 1986). However, this can be difficult to interpret, as when using the ratio of 5-HT/5-HIAA as a measure of serotonin turnover, as both an increase in 5-HT or a decrease in 5-HIAA would lead to an increase in the ratio. However, both scenarios are a result of opposing effects on serotonin activity. Increasing 5-HT suggests more synthesis or less release, while more metabolite suggests more release, as it is not made unless 5-HT is available (Bethea, Lu et al. 2002). Both increases and decreases in the levels of the neurotransmitter have been observed with estrogen treatment in various brain regions. However, most recent studies confirm that 5-HT release is decreased in the ventromedial nucleus, and that the releasable pool of 5-HT is smaller in the female hypothalamus (Bethea, Lu et al. 2002).

Studies of 5-HT levels in humans are more limited, but some data does exist using more easily measured peripheral markers of serotonin activity, such as prolactin, which is elevated in response to increased 5-HT levels as described previously in this paper. It

was found that the increase in prolactin after fenfluramine challenge, which causes the release of serotonin and blocks its reuptake, was maximal during the follicular phase, suggesting an increase in 5-HT synthesis at that time in the cycle (O'Keane and Dinan 1991). Tryptophan depletion studies show greater effects on several parameters of mood in women than in men, suggesting a female vulnerability to precursor availability (Ellenbogen, Young et al. 1996). Furthermore, women given tryptophan during the late luteal phase, when estrogen and progesterone are high, showed a higher prolactin response than women loaded with tryptophan several days after menstruation, when levels of E and P have dropped (Bancroft, Cook et al. 1991). Researchers have shown that serum estradiol levels correlate positively with serotonin measured in blood samples, and that postmenopausal women show lower 5-HT levels in their blood, which is increased to premenopausal levels with estrogen replacement therapy (Gonzales and Carrillo 1993). Measurements of plasma 5-HT levels reveal an increase during the follicular phase of the menstrual cycle as compared to post-menopausal women. Estrogen replacement therapy in these women also led to an increase in plasma 5-HT (Blum, Vered et al. 1996). All of these studies come to a single conclusion: the presence of estrogen seems to promote higher levels of serotonin, and that a lack of this hormone is associated with decreased serotonin levels.

The effects of steroid hormones on tryptophan hydroxylase (TPH), the rate-limiting enzyme in the synthesis of serotonin have also been examined. In nonhuman primates, a ninefold increase in TPH expression as measured by *in situ* hybridization was observed with estrogen replacement. Supplementation with progesterone blunted this response

somewhat, and only a 5-fold increase was seen in these animals compared to control. However, in rats, no difference was seen in TPH mRNA levels in estrogen-treated rats compared to control. Western blot confirmed that the increase in TPH expression with estrogen treatment was reflected in the protein product in nonhuman primates, however progesterone did not affect estrogen-induced TPH protein levels, in contrast to inhibition of TPH mRNA induction (Bethea, Lu et al. 2002). However, recently, a second TPH isoform has been identified in the brain, which appears to be the main enzyme responsible for 5-HT synthesis (Walther, Peter et al. 2003). It is therefore unclear whether the changes seen with estrogen treatment are noteworthy, as modification of TPH2 is likely to be more physiologically relevant for modification of serotonin neurotransmission, and these studies do not discriminate between the two isoforms.

Serotonin reuptake and degradation

As was previously described, the serotonin transporter (SERT) is responsible for the vast majority of serotonin reuptake into the cell from the extracellular space.

A recent report of SERT sites in the amygdala, lateral septum and hypothalamus of the rat brain showed an increase in [^{3}H]-paroxetine binding, which is a more specific substrate, with acute estrogen treatment in all postsynaptic sites. Acute estrogen treatment also increased SERT mRNA in the dorsal raphe but not the median raphe (McQueen, Wilson et al. 1999). An earlier study showed a decrease in [^{3}H]-paroxetine binding in the female rat hippocampus with estrogen treatment and no effect in the cortex (Mendelson, McKittrick et al. 1993). This is a good indicator of the regional differences in regulation of SERT that likely exist within the brain.

Studies of nonhuman primates support regulation of SERT by estrogen, however the effect appears to be opposite than in rats. Estrogen and estrogen and progesterone-treated ovariectomised monkeys show a significant decrease in SERT mRNA expression in the dorsal and median raphe. A significant reduction in the number of cells that expressed the transporter was also observed in the dorsal raphe of hormone-treated animals (Pecins-Thompson, Brown et al. 1998). This decrease in SERT expression would suggest that, in the presence of ovarian hormones, serotonin remains in the extracellular space for a longer period of time, as is the case with SSRI treatment. Meanwhile, studies of [^{3}H]-citalopram binding in nonhuman primates reveal no changes in SERT in the DRN and MnR, with increases in the preoptic area, ventromedial nuclei, and dorsal and lateral hypothalamus, suggesting that changes in SERT mRNA are not necessarily reflected in levels of protein. As well, hormone treated animals showed a significant increase in the number of immunofluorescent SERT-expressing fibres in the hypothalamus but not the DRN (Lu, Eshleman et al. 2003). In humans, single-photon emission computed tomography (SPECT) studies (Malison, Price et al. 1998) and studies of postmortem brain tissue of individuals diagnosed with depression suggest that the number of SERT sites are lower (Gross-Isseroff, Biegon et al. 1998). It is becoming increasingly obvious that changes in SERT mRNA are not necessarily reflected in the expression of the transporter protein, and that there are many facets of regulation of SERT that require further study.

The degradation of serotonin is attributed mainly to the function of the enzyme monoamine oxidase (MAO). Two isoforms of this enzyme exist, and the mRNA for both MAO-A and MAO-B were detected in the monkey DRN (Luque, Bleuel et al. 1996). It seems that MAO-A selectively degrades serotonin and norepinephrine in rats, humans and monkeys, while MAO-B is mostly responsible for the degradation of dopamine. However, the distribution and function of this enzyme may not be so cut and dried, as reports show that MAO-B can degrade serotonin in the rat, bovine and pig brain and it is predominantly located in 5-HT neurons (Bethea, Lu et al. 2002).

Studies of the expression and function of MAO in response to ovarian hormone treatment in nonhuman primates suggest that MAO-A mRNA is significantly decreased in the DRN and several hypothalamic nuclei, including paraventricular nucleus (PVN) lateral hypothalamus (LH) and ventromedial nucleus (VMN)(Gundlah, Lu et al. 2002). Studies in rats show that both chronic and acute estrogen treatment is capable of downregulating MAO-A in the hypothalamus and chronic treatment also downregulated MAO-A expression in the amygdala (Bethea, Lu et al. 2002). Studies of MAO-B remain consistent between rats and monkeys, showing no hormone regulation in the DRN, but a decrease in function in the hypothalamus with estrogen and progesterone treatment. Examination of MAO-B mRNA showed no change in the DRN, but did decrease in the hypothalamic pre-optic area (POA), LH and VMN with E, P or E+P treatment (Gundlah, Lu et al. 2002). What is interesting is the trend that modulation of MAO-A by estrogen occurs in regions of the brain where mainly the ERβ receptor is expressed, while regulation of MAO-B takes place in regions where ERα is the predominant isoform.

Regions such as the VMN, where both isoforms of the estrogen receptor are expressed show regulation of both MAO-A and –B. There are exceptions to this generalisation, however, indicating that things are not as simple as a promoter preference for one estrogen receptor over another (Bethea, Lu et al. 2002). However, a change in MAO activity or expression would be a interesting point of control for serotonin neurotransmission by ovarian hormones, as decreases in this enzyme would lead to increased synaptic serotonin concentrations, and thus greater transmission. This would in turn lead to elevated mood, consistent with the general observations that estrogen has a positive influence on mood, and appears to be a negative regulator of MAO, while progesterone is a negative mood regulator, and increases MAO levels.

Postsynaptic serotonin receptors

Examinations of other families of 5-HT receptors have also yielded some other points of control of serotonergic neurotransmission by ovarian steroids. Binding studies of brain samples of postmenopausal women receiving hormone replacement therapy suggest that estrogen and estrogen in combination with progesterone can significantly increase 5-HT2A agonist binding in the cortex (Moses, Drevets et al. 2000). In rats, several studies have shown increases in 5-HT2A receptor labeling in the dorsal raphe nucleus, anterior frontal, anterior cingulate and primary olfactory cortex, with a single injection of estrogen (Bethea, Lu et al. 2002). Ovariectomy in rats has been shown to reduce levels of 5-HT2A mRNA and protein in the frontal cortex, while chronic estrogen replacement reverses this effect (Biegon, Reches et al. 1983; Cyr, Bosse et al. 1998). Altogether, research suggests that estrogen increases 5-HT2A binding capacity in higher forebrain

regions (Bethea, Lu et al. 2002). In humans, PET studies of the brain reveal that 5-HT2 receptor binding is significantly higher in men than women in 22 healthy, age-matched men and women, particularly in the frontal and cingulate cortices. However, this study did not distinguish between the 3 isoforms of the 5-HT2 receptor (Biver, Lotstra et al. 1996) although 5-HT2A is the predominant form in these regions. The 5-HT2A receptor has been implicated in suicide and depression, although it is unclear as to whether modulation of this receptor by steroid hormones is an important factor in mood, further investigation may reveal significant links between the two.

The 5-HT1A receptor

Given the previously described importance of the 5-HT1A receptor in serotonergic neurotransmission, and the importance of steroid hormones in the pathology of depression, it seems likely that there is a relationship between the two. Several studies have revealed that expression of 5-HT1A autoreceptor may be regulated by estrogen which would help explain the increased prevalence of depression in women, as well as the hormone-change associated depressive syndromes such as pre-menstrual dysphoric disorder, postpartum depression and perimenopausal depression.

A PET study using the 5-HT1A antagonist [^{11}C-carbonyl] WAY 100635 conducted in humans aimed to establish a link between age, sex, and aggressive traits and 5-HT1A binding potential in several brain regions. This group found that females had a significantly higher binding potential than men in the DRN, amygdala, anterior cingulate, cingulate body, medial prefrontal cortex and orbital prefrontal cortex. No difference was

observed in the hippocampus. There was no age correlation for 5-HT1A levels (Parsey, Oquendo et al. 2002). An earlier study examined the binding of labeled 8-OH-DPAT in postmortem brain, and found that there was no difference in the binding potential between males and females in the prefrontal cortex, temporal cortex, parietal cortex, occipital cortex and hippocampus. However, some aging differences were seen between males and females, with women experiencing a decrease in 5-HT1A receptor density in the hippocampus and parietal cortex, but men showing a decrease in the occipital cortex. Receptor density in the female occipital cortex showed an increase with age. The authors also postulate that in women, alterations in agonist binding are due to changes in receptor density, while in men, it is more due to changes in receptor affinity. This suggests that regulation of the 5-HT1A receptor in humans is likely quite different between the sexes (Palego, Marazziti et al. 1997). Another PET study using [^{11}C-carbonyl] WAY 100635 found decreases in 5-HT1A binding in healthy men with age, in all regions examined, but no change in healthy women, in contrast to what would be expected after a drop in estrogen levels after menopause (Meltzer, Drevets et al. 2001). A second group confirmed the decline in 5-HT1A receptor binding potential with age, but did not discriminate between gender, leaving it unclear as to whether the decline in 5-HT1A receptor levels was limited to men (Tauscher, Verhoeff et al. 2001). These PET studies do not reveal a definitive mechanism for differential regulation of the 5-HT1A receptor in men and women. It does suggest that differences do exist, and as such it may be that dysregulation of the 5-HT1A receptor in depressed subjects may exacerbate effects of steroid hormones upon serotonin neurotransmission, thus leading to an increased sensitivity of women to depressive episodes.

A number of laboratories have looked at the 5-HT1A mRNA in different areas of the brains of animal models in response to estrogen treatment, however the results have been variable. One study looked specifically at the raphe nuclei in nonhuman primates, where it was found that estrogen treatment in spayed monkeys markedly decreased 5-HT1A mRNA in the DRN. Simultaneous treatment with progesterone, which stimulates expression of the estrogen receptor, decreased levels of 5-HT1A mRNA to an even greater extent (Pecins-Thompson and Bethea 1999). However, a later study, which aimed to examine whether SERMs were capable of inducing similar changes found no change in 5-HT1A receptor mRNA in the midbrain of ovariectomised macaques treated orally with estrogen, raloxifene, or arzoxifene. The authors attribute the discrepancy to oral dosing being more variable than a silastic implant, which was the method employed in the first 5-HT1A mRNA study (Bethea, Mirkes et al. 2002). To follow up on the observed regulation of the 5-HT1A autoreceptor, the same group examined whether binding of [3H]-8-OH-DPAT in the DRN was altered with hormone treatment. They found that the reductions seen in mRNA levels were reflected in the ligand binding, suggesting a functional downregulation of this receptor in response to progesterone and/or estrogen treatment. The affinity of the ligand for the receptor was unaffected, however a reduction of approximately 20% in the number of binding sites was observed in the DRN, while a more modest difference was seen in the MnR and the periaqueductal grey. Further examination of the effects of hormone replacement on the DRN 5-HT1A in this study revealed that while the affinity of the receptor for its G protein was unaffected, the basal and stimulated binding of [^{35}S]-GTP-γ-S was significantly decreased in treated

animals. Furthermore, it appears that conjugated equine estrogen treatment in ovariectomised monkeys can significantly decrease the expression levels of the $G\alpha_{i3}$ protein in the DRN, while leaving the expression of $G\alpha_{i1}$, $G\alpha_o$ and $G\alpha_z$ proteins unaffected (Lu and Bethea 2002). This is consistent with the observation that chronic estrogen treatment in ovariectomised rats reduces hypothalamic levels of $G\alpha_z$, $G\alpha_{i1}$ and $G\alpha_{i3}$ proteins. It is likely that regulation of 5-HT1A function by ovarian steroids might be due, in part, to downregulation of its G protein subunits (Raap, DonCarlos et al. 2000).

Studies of the 5-HT1A autoreceptor in rats add to the evidence that progesterone and/or estrogen modulate it. An early study by Lakoski *et al* in 1988 (reviewed by Bethea, 2002) demonstrated that estrogen treatment reduced the ability of 8-OH-DPAT to decrease firing of DRN neurons. This is an indirect measure of reduced 5-HT1A binding sites in estrogen-treated rat DRN. Extracellular recordings of the firing activity of dorsal raphe neurons in female rats treated with 17-β-estradiol for seven days show a statistically significant increase compared to control, however the same treatment in males had no effect (Robichaud and Debonnel 2002).

A study done in 1990 found that overall, female rats are more responsive to 5-HT1A receptor-mediated inhibition of serotonin synthesis in the hippocampus. In this study, it was found that basal serotonin synthesis was higher in females, but in the hippocampus, 8-OH-DPAT treatment brought female levels to match that of males (Haleem, Kennett et al. 1990). Similarly, treatment with 8-OH DPAT decreased the 5-HIAA/5-HT ratio in the hippocampus of adult male and female rats, but this decrease was least obvious in male

rats, and female rats in proestrous. Changes in this region are consistent with activation of the 5-HT1A autoreceptor, and the response was only prevented in ovariectomised rats with progesterone replacement or progesterone and estrogen, but not by estrogen alone (Maswood, Stewart et al. 1995). However, a change in this ratio could be due to activation of 5-HT1A, or to changes in the synthesis, release or degradation of 5-HT, making it unclear what is being modulated in this study. Another laboratory examined levels of this receptor in the dorsal raphe of ovariectomised female rats and found a 13% decrease in 5-HT1A mRNA with combined estrogen and progesterone treatment, but no effect of estrogen alone (Birzniece, Johansson et al. 2001). Studies of the transcriptional regulation of the rat 5-HT1A gene have shown that estrogen upregulates expression of this receptor via synergistic activation by NF-κB (Wissink, van der Burg et al. 2001). However, this effect was mediated through ERα, which is not present in rat serotonergic raphe neurons, but is expressed in smaller interneurons of the raphe. This may be a more important mechanism for regulation of the postsynaptic 5-HT1A receptor, where in areas such as the amygdala, and hypothalamus, ERα is more highly expressed (Sibille and Hen 2001).

It appears that estrogen has a robust effect on the 5-HT1A autoreceptor in primates, particularly at the level of its G proteins, and perhaps at a transcriptional level. Studies in rats, although indirectly suggesting regulation of 5-HT1A through electrophysiological studies, have not been as strong in demonstrating modulation of 5-HT1A mRNA. This could be due to a species difference in the sensitivity of the receptor to steroid hormones, or in a difference in the importance of changes in the presynaptic receptor. It is possible

that in rats, modulation of the postsynaptic receptor is more important for a depressive phenotype, while in primates, the presynaptic receptor is key.

Studies of the regulation by steroid hormones of postsynaptic 5-HT1A receptors have revealed many inconsistencies. In the nonhuman primate, no changes were observed in mRNA levels of 5-HT1A in the ventromedial hypothalamic nuclei with a month of E or E+P treatment (Gundlah, Pecins-Thompson et al. 1999), but up until now, no other postsynaptic sites in the monkey brain have been examined. The same group assayed specific binding of [^{3}H]-8-OH-DPAT in the hypothalamus of ovariectomised monkeys and found that 5-HT1A binding sites were reduced in several hypothalamic nuclei (Lu and Bethea 2002).

A study examining 5-HT1A mRNA and specific binding of radiolabeled ligand to the 5-HT1A receptor showed no effect of ovariectomy or chronic estrogen replacement in the hippocampus, prefrontal and cingulate cortex, or dorsal raphe of female rats. This may be due to the low concentration of ligand used in this study, which will preferentially bind to the G protein coupled form of the receptor than the uncoupled form (Landry and Di Paolo 2003). Another study, which looked at 5-HT1A mRNA levels in ovariectomised female rats treated with 17-β-Estradiol for 2 hours and 24 hours, found decreases in receptor expression in the medial amygdala, piriform cortex, and perirhinal cortex (24 hours only), but no change in the hippocampus or retrosplenial cortex (Osterlund and Hurd 1998). A study by the same group examining chronic (2 week) estrogen treatment under the same conditions found no change in mRNA levels. They

did observe reduced receptor binding in the amygdala, hippocampus, perirhinal cortex, and motor cortex but no change in the piriform or retrosplenial cortex. Yet another study looked at mRNA levels in response to acute (24 hours) estrogen treatment, however this study compared the effects in Flinders Sensitive Line (FSL) rats, and the control Flinders Resistant Line (FRL) rats. This study found that E treatment reduced 5-HT1A mRNA in the cingulate cortex, motor cortex, piriform cortex, and the medial anterodorsal amygdala nucleus, in a similar manner in both FSL and FRL rats (Osterlund, Overstreet et al. 1999).

Another study using receptor autoradiography to measure 5-HT1A levels at various stages of the menstrual cycle, as well as after ovariectomy in rats, found that 5-HT1A levels were upregulated in the ventromedial hypothalamic nucleus during estrous, compared to diestrous. They also noted that ovariectomy decreased levels of 5-HT1A in this region compared to estrous and proestrous. This observation was reversed by estrogen replacement. However, receptor levels were unaffected in other regions such as the medial preoptic area, bed nucleus of the striata terminalis, lateral septum, cingulate cortex, amygdala, hippocampal region CA1, and layers V and VI of the occipital cortex. This data suggests positive regulation of 5-HT1A receptor levels by estrogen in this brain region (Flugge, Pfender et al. 1999). It is known that administration of 8-OH-DPAT into the ventromedial nucleus of the hypothalamus inhibits the lordosis behaviour of female rats, providing a direct behavioural measure of postsynaptic 5-HT1A activation. It has been seen that two weeks of treatment with estradiol benzoate will reduce this behavioural response in ovariectomised rats, suggesting that the hormone replacement

desensitises the 5-HT1A receptors in this region (Jackson and Uphouse 1996; Uphouse, Andrade et al. 1996). Although there is no consistent change in 5-HT1A mRNA or receptor in any single brain region in these studies, there is a lack of consistency between experimental approaches. There is a large reserve of 5-HT1A receptors in the DRN which can significantly influence responses to 8-OH-DPAT (O'Connell and Curzon 1996) thus the length of treatment may not allow for depletion of this reserve, and thus an observable change. The length of time following ovariectomy before estrogen replacement, and the method of estrogen administration may also have an effect on the results. Overall, it appears that the function of the 5-HT1A receptor is attenuated at particular postsynaptic sites with estrogen treatment, however, this may not be primarily mediated at the level of transcription, there are likely many post-transcriptional mechanisms involved. Several studies have addressed these possibilites:

An early study by Clarke and Maayani (Clarke and Maayani 1990) came to the conclusion that while 4 days of estrogen replacement in ovariectomised rats did not alter 5-HT1A binding sites labeled with [^{3}H]-8-OH-DPAT in the hippocampus, the concentration response curve for inhibition of adenylyl cyclase was shifted to the left. The maximal and basal responses to 5-HT were not affected. The same study saw no change in G protein coupling, measured by a decrease in [^{3}H]-8-OH-DPAT binding with administration of Gpp(NH)p, which promotes a low-affinity receptor state. The study also ruled out an increase in K^+ channel and adenylyl cyclase responsiveness. This suggests that estrogen may be playing a role in modulating postsynaptic intracellular responses mediated by 5-HT1A.

Some recent studies suggest that 5-HT1A coupling to G proteins is reduced in the rat cortex, hippocampus and amygdala and piriform cortex with acute estrogen treatment, as measured by the binding of the receptor to [^{35}S]-GTPγS. The response of the receptor was blunted by 25% in the hippocampus. Chronic estrogen treatment failed to produce these results (Mize and Alper 2000). This group went on to demonstrate in an *in vitro* system that estrogens act directly via the estrogen receptor to produce the desensitization of the 5-HT1A receptors in the frontal cortex and hippocampus (Mize, Poisner et al. 2001). Further investigation into this observation revealed that 17-β-estradiol was capable of inducing PKA and PKC in rat hippocampal preparations within 10 minutes of treatment; this induction resulted in the phosphorylation of 5-HT1A. This is similar to what was seen in the macaque DRN, and activation of PKA and PKC may be an important mechanism in estrogen-mediated modulation of the 5-HT1A receptor, and hence in the influence of estrogen and progesterone on mood.

There is also the potential for post-translational modulation of the 5-HT1A receptor mediated by estrogen. For example, recent research has shown that estrogen modulates the protein levels and activity of GRK2 in the frontal cortex of the rat brain (Ansonoff and Etgen 2001). Expanding on this mechanism may suggest that modulation of GRKs, which phosphorylate activated receptors and recruit β-arrestin, leading to internalisation of the receptor (Pitcher, Freedman et al. 1998), will thus affect the activity of the 5-HT1A receptor in the raphe nuclei.

Hypothesis

Based on the above studies, I hypothesise that the human 5-HT1A receptor is downregulated by estrogen, which results in the potentiation of serotonin neurotransmission and leads to elevated mood. I propose that regulation of the presynaptic 5-HT1A autoreceptor takes place mainly at the level of transcription, such that upon administration of estrogen, transcription of the 5-HT1A autoreceptor is repressed. This may occur either indirectly or directly though an estrogen response element, and as a result levels of this receptor decline in the raphe nuclei.

Approach

Several deletion constructs of the human 5-HT1A promoter region together with the luciferase reporter gene have already been created in our laboratory. The human 5-HT1A promoter was chosen for analysis because of its clinical relevance, and because primates appear to show greater transcriptional response to estrogen. The 5-HT1A promoter was cloned upstream of the gene which encodes the enzyme luciferase. This protein oxidises luciferin, with concomitant production of a photon (Promega, 1996). Thus, the amount of luciferase expressed is proportional to the light emitted from the reaction, and transcription of this gene depends on the activation or repression of the 5-HT1A promoter. To identify a region that responds to estrogen, the transfected cells will be treated with estrogen and luciferase activity will be measured to evaluate whether the hormone modulates a particular region of the promoter. Should a region of the promoter respond to estrogen, the transcription factor responsible will be identified using sequence homology to identify potential DNA elements of interest. An online transcription factor

search through TRANSFAC (Bioinformatics 2001) or an equivalent program can determine potential binding proteins in the region of interest.

Experimental model

The experimental model should mimic *in vivo* conditions as closely as possible. This may be accomplished by using a cell line derived from rat embryonic medullary cells (White, Eaton et al. 1994). Infecting embryonic day 13 rat medullary raphe cells with a temperature-sensitive mutant of the SV40 large T-antigen (T-ag) produced such a cell line, named RN46A. At 33C, the cells divide with a doubling time of approximately 9h and appear similar to fibroblasts. However, at 39C, the mitotic action of the T-ag is inactivated and the cells will begin to differentiate. At this point, they take on a neuronal morphology, with long processes that can be multipolar. RN46A cells express a number of neuronal markers, such as neuron-specific enolase (NSE), whose expression is potentiated with differentiation, and neurofilament proteins. The cells do not express any glial markers, such as glial fibrillary acidic protein (GFAP) or galactocerebroside (Gal C). It was shown that these cells also express tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (AAAD), two key enzymes in the synthesis of 5-HT (Kandel, Schwartz et al. 1991). This suggests that these cells have the capability to secrete serotonin, a feature that is enhanced upon differentiation. The RN46A cell line has also been shown to express the 5-HT1A receptor as well as the sodium-dependent serotonin reuptake transporter (SERT) in both its undifferentiated and differentiated forms (Eaton, Staley et al. 1995). The data examining these features of this cell line

suggest that the 5-HT1A receptor is autoregulatory in this cell system, which parallels the role of this receptor in the raphe nucleus.

Another notable feature of the experimental paradigm is the pattern of expression of estrogen receptors within the RN46A cell line. Bethea *et al* showed via *in situ* hybridization and immunocytochemistry that the only estrogen receptor expressed in nonhuman primate raphe cells is the β isoform (Bethea, Lu et al. 2003). In rats, guinea pigs, and mice the predominant isoform of the estrogen receptor is also ERβ, although ERα is also present in serotonergic neurons of the mouse raphe nuclei (Bethea, Lu et al. 2002). It has also been demonstrated that RN46A cells express the mRNA corresponding to the beta isoform of the estrogen receptor, but mRNA of the alpha isoform was undetectable (Streicher, Earl et al. 2001; Bethea, Lu et al. 2003). This evidence supports the use of the RN46A cell line as a strong model for serotonergic neurons of the raphe nuclei.

Methods

Cell Culture

RN46A cells and the stable clones derived from this cell line were maintained in Neurobasal medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 0.5 mM L-glutamine (Wisent). 24 hours prior to estrogen treatment, in order to deplete endogenous estrogens, the medium was changed to phenol-red-free 50% Dulbecco's modification of Eagle's Medium/50% F-12, containing L-glutamine (DMEM/F12, Gibco) supplemented with 5% charcoal-treated fetal bovine serum (Gibco BRL). This approach to estrogen depletion was described in Wissink *et al* (2001). Cells were incubated at 33°C in 5%CO_2. Medium was changed every three days while the cells were being grown.

17-β-Estradiol (E2, Sigma) was added at a final concentration of 10^{-7} M for the indicated times from a 1 mM stock solution in ethanol, unless otherwise stated.

Plasmids

The 5-HT1A promoter constructs were designed and cloned by Sylvie Lemonde, a doctoral candidate in our laboratory. Briefly, deletion fragments of the promoter region up to 5 kB upstream of the 5-HT1A coding sequence were inserted 5' to the luciferase gene in a promoterless luciferase reporter gene expression vector, pGL3-Basic (pGL3B, Promega) (Figure 1A). The region of the promoter that was examined was from –1 to

–1515 (Figure 1B). The pGL3B vector was used as a negative control, to assay basal expression of the luciferase gene. The pGL3-Promoter (pGL3P, Promega) construct was used as a positive control, based on the expression of the luciferase gene under the control of the SV-40 promoter.

The human estrogen receptor α and β cDNA constructs were kind gifts from Dr. Pierre Chambon of the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Collège de France, Strasbourg, France.

The vitellogenin estrogen response element (vgERE) and the putative serotonin-1A estrogen response element (1AERE) were cloned upstream of the SV-40 promoter in the pGL3P. The oligonucleotides:

5'-CGGGGTACCCCGGGTCACAGTGACCTCCCCGGGAT-3' and

5'-ATCCCCGGGAGGTCAGTGTGACCCGGGGTACCCCG-3' or

5'-GGGGGTACCCCGAGGGTCACAGAGTGACCGTGTCCCCCGGGGGA-3' and

5'-TCCCCCGGGGGACACGGTCACTCTGTGACCCTCGGGGTACCCC-3' for vgERE and 1AERE respectively (Invitrogen) were annealed by heating the combined oligonucleotides to 95°C for 5 minutes and then allowing them to cool slowly to 37°C for 20 minutes. The EREs were then left at room temperature for a further 20 minutes.

The pGL3P vector and relevant ERE were digested with KpnI (New England Biolabs (NEB)) at 37°C for 2 hours and the enzyme inactivated by heating to 65°C for 20 minutes. A sequential digest was then performed with SmaI (NEB) overnight at 25°C.

The vector was gel purified on a 1% agarose gel and the elements were purified on a 2% agarose gel. The bands were excised under UV light and eluted from the gel using the DNA gel extraction kit (Millipore). Ligations were done with vector to element in a 1:8 ratio, using T4 DNA ligase (New England Biolabs) at 16°C overnight. The ligation products were then transformed into competent Escherichia coli DH5α cells using heat shock, and positive clones were identified by sequencing using the Sanger dideoxy termination method (Sanger, Nicklen et al, 1977).

The 1AERE construct was also generated by mutating the vgERE construct to incorporate 2 additional base pairs into the half site spacer, and by modifying the 3-bp flanking sequence to match that of the 5-HT1A promoter. The oligonucleotides used for this purpose were, with the mutations shown **in bold**. Forward: 5'-CATTTCTCTATCGATAGGTACC**GAG**GGTCACAG**AG**TGACC**GTG**CGGGGATGG GCTCGAGATCTG-3' and Reverse: 5'-CAGATCTCGAGCCCATCCCCG**CAC**GGTCA**CT**CTGTGACC**CTC**GGTACCTATC GATAGAGAAATG-3'. The mutations were incorporated using the Quikchange® XL Site-directed Mutagenesis Kit (Stratagene). Briefly, PCR reactions were assembled containing 300ng of each primer (forward and reverse), 500ng of DNA template, 2μL of dNTP mix, 10μL of 10x reaction buffer and ddH2O to a volume of 100μL. *PfuTurbo* DNA polymerase was added to the reaction mixture, and this was aliquoted in 4 tubes that were run at a T_m of 62°C in the Biometra T gradient thermal cycler.

Mutation of the 1AERE within the 5-HT1A promoter (located from –419 to –429 bp) was done using the Quikchange® XL Site-directed Mutagenesis Kit (Stratagene). The forward primer used for this reaction was: 5'-GGGAGAGGAGGATCACAGAGTGATCGTGGAGGATGGG-3' and the reverse primer was: 5'-CCCATCCTCCACGATCACTCTGTGATCCTCCTCTCCC-3'. The luciferase construct used for the mutation was the –724luc construct, which was the first fragment of the 5-HT1A promoter containing the 1AERE at about –433. The resultant construct was named –724mut.

Mutation of the 1AERE in front of SV-40 was done using the 1AERE construct described above, using the Quikchange® XL Site-directed Mutagenesis Kit (Stratagene). The forward primer used for this mutation was 5'-CGATAGGTACCGAGGATCACAGAGTGATCGTGCGGGGATGGG-3' and the reverse primer was 5'-CCCATCCCCGCACGATCACTCTGTGATCCTCGGTACCTATCG-3'.
The success of the mutations was evaluated using manual sequencing by the Sanger dideoxy termination method (Sanger, Nicklen et al 1977), and confirmed using automated sequencing. The construct was named 1Amut.

Plasmid Isolation

Plasmids were purified from 500mL overnight cultures grown in Luria-Bertani (LB) broth (1% bacto-tryptone, 0.5% yeast extract, 1%NaCL) containing 100µg/ml ampicillin (Amp). Plasmid DNA was isolated using the alkaline lysis method (Birnboim and Doly,

1979) in the presence of RNase A (50μg/mL). Plasmids were further purified by phenol-chloroform extraction and ultracentrifugation in a cesium chloride gradient at 50 000g overnight. The pure plasmid DNA was precipitated with 3 volumes of ethanol and resuspended in Tris-EDTA buffer (TE buffer, Ausubel *et al*, 1995). Some plasmids were purified using the GenElute High Performance (HP) Plasmid Maxiprep kit (Sigma). Purity of the plasmid DNA was determined by restriction digest analysis on an agarose gel.

Transient Transfections

RN46A cells were trypsinised and plated into 6-well Primaria plates (Stratagene) at 60-75% confluence. The following day, the cells were transfected using lipofectamine and lipofectamine-PLUS reagent (Invitrogen). To correct for transfection efficiency, 3 μg of pCMV-lacZII (pCMVβgal) plasmid were co-transfected with 7.5 μg luciferase reporter constructs and 1.5μg hERβ cDNA, in RN46A cells. In the stable hERβ-expressing clones (described in the following section), 9μg of the luciferase reporter construct, was transfected, along with 3μg of pCMVβgal. In parallel experiments, transfection efficiency in RN46A cells was approximately 10-30%.

The DNA was incubated with 16μL lipofectamine-PLUS reagent in 300μL Opti-MEM medium (Gibco) for 15 minutes. In a separate tube, the lipofectamine (2 x μg DNA) was incubated for 5 minutes in 300 μL Opti-MEM medium. The tubes were then combined and vortexed briefly to mix the lipid carrier with the PLUS-DNA complex. The medium on the cells was changed to 500 μL of serum-free Opti-MEM medium. After allowing

the lipid carrier to complex with the DNA for a minimum of 20 minutes, 100 µL of the transfection reaction was added to each well and the transfection was allowed to proceed at 33°C in 5% CO_2 for 4 to 6 hours. Once the transfection was complete, fresh medium was added and the cells were allowed to recover overnight. The following morning, the cells were rinsed with PBS and plated in estrogen-free medium (see Cell Culture).

HEK293 cells were transfected in 10cm plates using $CaPO_4$. Briefly, DNA was combined with 1mL of 0.25M $CaCl_2$, and 140uL of 0.1M $NaPO_4$ solution was diluted into 2X HBSS (50 mM Hepes; 10 mM KCl; 280 mM NaCl; 12 mM Glucose), to a final concentration of 1.5-2mM. While vortexing, the DNA-$CaCl_2$ solution was added to the $NaPO_4$, drop by drop. The mixture was incubated for 5 minutes, then vortexed and added to the cells. The mixture was left for 10 minutes, and medium was the added to the cells. The transfection was left to proceed overnight, and the cells were split into 6-well plates the following day. After allowing the cells to recover for 24 hours, the cells were changed to an estrogen-free environment and treated as described previously. The optimal concentration of $NaHPO_4$ was determined by examining the mixture of $CaCl_2$ and HPO_4 under the microscope and choosing the concentration that yielded the finest precipitate.

Reporter Assays

Cells were rinsed with PBS buffer and scraped into 150µL 1X Reporter Lysis buffer (Promega), then placed on ice. After a single freeze-thaw cycle, insoluble material in the sample was removed by centrifugation at 20 000xG for 2 minutes at 4°C.

For the luciferase assay, 30μL of extract was aliquoted into cuvettes, and allowed to equilibrate to room temperature for 2-3 minutes. In a dark room, the extract was mixed with 100 uL of 47 μM luciferin (Molecular Probes) in luciferase buffer (20 mM tricine, 2.67 mM $MgSO_4$; 1.07 mM $(MgCO_3)_4Mg(OH_2)5H_2O$; 0.1 mM EDTA; 33.3 mM DTT; 270 μM Co-enzyme A; 530μM ATP) and placed immediately into the BioOrbit 1250 luminometer. The luciferase activity, defined as the light produced by the reaction for a period of 10 seconds, was recorded. β-galactosidase activity was determined in the same samples using a MUG assay (Ausubel, 1995). 30 μL of extract and 30 μL of MUG substrate (4-methylumbelliferyl β-D-galactoside; Sigma) in 15 mM Tris-HCl, pH 8.8 were incubated at 37°C for 30 minutes. The reaction was stopped using a MUG stop solution (300 mM Glycine; 15 mM EDTA, pH 11.2) and mixed with 2 ml of Z-buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$). The conversion of the substrate to the highly fluorescent molecule methylumbelliferone quantitated using a Perkin-Elmer LS50 spectrofluorometer.

The ratio of luciferase to β-galactosidase activity was determined in triplicate samples and corrected for basal activity of the vector (pGL3B). Values were then normalised to SV-40 driven activity of the luciferase gene (pGL3P). All data are presented as the mean±SD of at least 3 independent experiments.

Generation of stable RN46A cells expressing human ERβ

RN46A cells were stably transfected as described above with an expression vector encoding the β isoform of the human estrogen receptor along with a PGK-promoter driven puromycin resistance gene (pGS5/puromycin) (Dr P Chambon, Strasbourg, France). Cells were allowed to recover for 48h, and then selected for puromycin resistance with 5 μg/mL puromycin for 2-3 weeks. After this time, very little cell death was evident, and cells began to grow in colonies. These were picked with a sterile pipette tip and individual colonies were grown in a multiwell plate. The colonies were expanded after reaching confluency, and assayed for the presence of the overexpressed estrogen receptor by Northern blot and electromobility shift assay (EMSA). Cells were also transfected transiently with the pGL3P-vitERE construct (described above) to determine whether an estrogen-mediated induction of promoter activity via stably-expressed hERβ was observed within the stable clones.

Isolation of mRNA and Northern analysis

Total cellular RNA was isolated from 3.5cm wells at 80-90% confluency. All solutions were prepared with DEPC-treated water, and autoclaved where possible to minimise RNase contamination. Cells were harvested by centrifugation at 2000 rpm (Eppendorf centrifuge 5471R) at 4°C. The medium was suctioned off to the last drop, and 100 μL of buffer A was added (10 mM Tris, pH 8; 1.5 mM $MgCl_2$; 5 mM KCl; 0.5 mM DTT; 0.5 mM PMSF; 0.5% NP-40) and the cells incubated on ice for 10 minutes. The preparation was the centrifuged (Eppendorf centrifuge 5471R) at 2000rpm for 15 minutes. The

supernatant was then transferred to an eppendorf containing 400μL of denaturing buffer (4 M guanidium thiocyanate; 25 mM sodium citrate, pH 7; 0.5% sarcosyl). 50 μL of 2 M sodium acetate, pH 4 was added, followed by 500μL of phenol and 100 μL chloroform. The solution was vortexed briefly, and incubated on ice for 10minutes, followed by a 5-minute incubation at room temperature. The samples were then spun in a centrifuge (Eppendorf centrifuge 5471R) at 4°C for 20 minutes and the aqueous phase transferred to a clean tube. 1 mL of ethanol was added, and preparations were stored at -80°C until ready for use. On the day of Northern analysis, RNA preparations were centrifuged (Eppendorf centrifuge 5471R) at 14000rpm for 20 minutes at 4°C. The mRNA pellet was resuspended in 50 μL DEPC water for analysis.

A 1.3% agarose gel in 1X MOPS buffer (20 mM 3-[N-morpholino]propanesulfonic acid (MOPS); 5 mM sodium acetate; 1 mM EDTA, pH 7) was poured containing 0.16% formaldehyde and allowed to solidify at room temperature. The formaldehyde was added when the gel mix had reached 55°C to prevent evaporation. The RNA sample was then mixed in a 1:2 ratio with denaturing buffer (25% formaldehyde; 50% formamide; 25% 10X MOPS) and incubated at 65°C for 10-15 minutes, then placed on ice. The samples were separated on the agarose gel in 1X MOPS buffer containing 0.16% formaldehyde at 75-80V.

The RNA was then transferred to a nitrocellulose membrane (Hybond-N; Amersham Pharmacia Biotech) using 10X SSC (1.5 M sodium chloride; 0.15 M sodium citrate, pH 7) Membranes were visualised by UV and the position of the 28S and 18S fragments

were indicated on the membrane. The RNA was then immobilised by UV crosslinking on the CL-1000 UV crosslinker (UVP) at 1200J for 1.2 minutes. Blots were prehybridised with ULTRAhyb™ hybridization buffer (Ambion) at 42°C for a minimum of 30 minutes.

Blots were incubated overnight with a random-primed cDNA probe labeled with [α-^{32}P]-dCTP using the *redi*prime™ II random prime labeling system (Amersham Pharmacia biotech). Briefly, 25 ng of the DNA to be labeled was denatured by heating to 95-100°C and snap cooled on ice. The denatured DNA was added to the reaction tube along with 50μCi of [α-^{32}P]-dCTP and mixed by pipetting up and down several times. The reaction mixture was then incubated at 37°C for at least 10 minutes, and subsequently purified on a G-50 sephadex column. The labeled DNA probe was denatured by heating to 95-100°C and snap cooled on ice for 5 minutes prior to hybridisation.

The following day, the blots were washed twice at low stringency (2X SSC; 0.1% Sodium dodecyl sarcosyl (SDS) at 42°C for 5 minutes and then washed at high stringency in 0.1XSSC; 0.1% SDS at 65°C for 1-10 minutes. Membranes were then exposed to a phospor-screen (Kodak) for a minimum of 24 hours. The image was then visualised using the Storm 860 Phosphoimager (Molecular Dynamics). RNA loading levels were evaluated using a probe for the human β-actin gene (Clonetech), whose levels do not change with estrogen treatment.

ElectroMobility Shift Assay (EMSA)

Nuclear extracts from five 15-cm plates at 85-95% confluency were obtained from cells that had been grown in estrogen-free medium (see Cell Culture) for 24h. The cells were trypsinised and rinsed in 1X HBBS (0.12 M NaCl; 4.6 mM KCl, 10 mM D-glucose; 20 mM HEPES). They were then rinsed in buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCL_2$; 10 mM KCl). Cells were incubated at 4°C for 10 minutes in Buffer A containing 0.5 mM DTT and 0.1% NP-40. The cell extract was centrifuged and the pellet resuspended in Buffer B (20 mM HEPES; 25% glycerol; 420 mM NaCl; 1.5 mM $MgCL_2$; 0.2 mM EDTA; 0.5 mM PMSF; 0.5 mM DTT; 0.5 mM Benzamidine; 20 mM Leupeptin). This was then centrifuged again, and the supernatant was combined with Buffer D (20 mM HEPES; 20% glycerol; 0.05M KCl; 0.2 mM EDTA; 0.5 mM PMSF; 0.5 mM DTT; 0.5 mM Benzamidine; 20 mM Leupeptin) for storage at -80°C until use in EMSA. *In vitro* transcribed/translated ERβ protein was generated using the TNT coupled Wheat Germ Extract system (Promega) as well as with the TNT T7 coupled Reticulocyte Lysate system (Promega). Briefly, the reaction components were assembled and incubated at 30°C for 2 hours. A simulataneous radioactive reaction using [^{35}S]methionine was assembled and separated on a 12% denaturing ployacrylamide gel. The gel was dried for 1 hour and exposed to film at -70°C for 24 hours to assess production of the protein.

Complementary 19-bp oligonucleotides containing the 1AERE or the vgERE were annealed and end-labelled with [γ-^{32}P]dCTP using DNA polymerase 1 large fragment (Klenow) (NEB). Nuclear extracts were pre-incubated, with or without E2, for 20

minutes at room temperature with or without competitor DNA or antibodies in a 20μL reaction containing EMSA buffer (20 mM HEPES; 0.2 mM EDTA; 0.2 mM EGTA; 100 mM KCl; 5% Glycerol and 2 mM DTT) and 5μg Poly d[I-C] (Roche). ^{32}P end-labelled probe (50 000 cpm) was added and incubated at room temperature for a further 20 minutes. The reaction was separated on a 5% polyacrylamide gel at 4°C, which was dried and exposed to film for 24-72 hours at –80°C with an intensifying screen.

Western Blot

Cultured cells were washed twice in PBS and scraped in NP-40 buffer containing 0.5 mM PMSF, 0.5 mM DTT, 0.5 mM Benzamidine and 20 mM Leupeptin. The cell extract was sonicated on a Fisher Scientific Sonic Dismembrator, model 60, for two 5-second pulses at 5-6 watts. Protein concentrations were determined using the BCA protein assay reagent kit (MJS Biolynx, Inc.). 25 μg of Protein was denatured in sample buffer by boiling for 5 minutes and resolved on 12% SDS-polyacrylamide gels by electrophoresis (SDS-PAGE). The protein was transferred to polyvinylidenefluoride (PVDF) polyscreen membranes (BioRad) at 4°C for 1hr at 250 mA. The membranes were blocked with 5% skim milk powder dissolved in PBS containing 0.1% Tween-20 (PBS-T) at room temperature for 1 hour. The blots were then incubated overnight at 4°C with the appropriate primary antibodies at a 1:1000 dilution in blocking solution. The membranes were rinsed 3 times with PBS-T prior to a 1-hour incubation at room temperature with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse at a 1:1000 dilution in blocking solution. The blot was rinsed 3 times for 10 minutes with PBS-T with a final

wash in PBS for 5 minutes and detected using the BM chemiluminescence blotting substrate (Roche).

Antibodies

A rabbit polyclonal antibody specific for GRK2 (Santa Cruz Biotechnology) and a crude serum custom antibody against 5-HT1A (Sigma) were used for Western Blot analysis. A mouse antibody against β-Actin (Sigma) was used as an internal control for protein loading. A goat polyclonal antibody (Santa Cruz Biotechnology) specific for the estrogen receptor β was used for supershift analysis in the EMSA examining nuclear protein binding to estrogen response elements. A monoclonal antibody specific to the FLAG-tag (Sigma) was used as a non-specific supershift reagent.

Real-Time RT-PCR

mRNA from cells treated with vehicle (ethanol) or 100nM estrogen for 8, 16 and 24 hours was isolated using Trizol (Invitrogen), according to the manufacturer's instructions.

Primers to 5-HT1A were designed to amplify a 400kb fragment in the I3 loop of the rat 5-HT1A gene. The sequence of the upper primer was 5'-GCCATCGCGCTAGACAGGTA-3' and the lower primer was 5'-GCGGTGCCGACGAAGTT-3'. GAPD RNA levels were measured to normalise the amount of RNA in each sample and were designed to amplify a 200kb fragment. The

upper primer sequence was 5'-CATGGCCTTCCGTGTTCCTACCC-3' and the lower primer was 5'-CCTCGGCCGCCTGCTTCA-3'.

5-HT1A mRNA levels in treated versus untreated RNERb20 cells were quantified using real-time RT-PCR. GAPDH mRNA levels were used for normalization. The relative concentration of mRNA or GAPDH was calculated in relation a standard dilution series of the Dbx plasmid, which contains the rat 5-HT1A gene. The standard curve was constructed with serial dilutions of Dbx of 10, 5, 1 and 0.01 ng/μL. Briefly, 1μL of the appropriate dilution was mixed with 0.25pM of the 5-HT1A primers, described above, 1 μL SYBR Green (SYBR Green I, Molecular Probes; diluted 1:1000) along with 22.5 μL of Platinum® *Taq* ploymerase supermix (Invitrogen; contains $MgCL_2$, dNTPs, and PCR buffer). Each sample was used in each reaction in triplicate, and amplified using the SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen). In a total volume of 25μL, 1 μL SYBR Green was mixed with 5 pM of each primer, 2X reaction buffer, and RT/Platinum Taq, according to manufacturer's instructions. The following PCR conditions in the Rotor-Gene 3000 (Montreal Biotech, Inc) were used: denaturation for 2 mins at 94°C, followed by 40 cycles with denaturation for 15 s at 94°C, annealing for 30 s at 60°C for both 5-HT1A and GAPDH and elongation for 30 s at 72°C. Data was acquired during elongation, and a melt analysis over a temperature gradient of 72-99°C was performed to correct for the possibility of primer dimers.

Results

Estrogen does not change the level of luciferase expressed in wild type RN46A cells

Four deletion constructs encoding the region from –1 to –1515 upstream of the human 5-HT1A ATG translational start site were cloned upstream of the luciferase reporter gene (Figure 1). Within the human 5-HT1A promoter, several regulatory elements exist. The NF-kB and Sp1 sites were identified using TRANSFAC transcription factor search (Bioinformatics 2001). The DRE (Dual Repressor Element) and NRSE (Neural Restrictive Silencing Element) were identified upstream of –1515bp in the human 5-HT1A promoter in our laboratory, and exert a significant repression on 5-HT1A expression (Lemonde, Rogaeva et al. 2003). Reporter constructs containing these elements did not show much expression, and thus too little activity to identify an inhibitory estrogen response. The constructs encompassed the region upstream of the translational initiation site from –1 to –391 bp (-391luc), from –1 to –724 bp (-724luc), from –1 to –1133 bp (-1133luc) and from –1 to –1515 bp (-1515luc) (Figure 1). Basal expression of luciferase was measured using the pGL3B construct, which contains the luciferase gene, but no promoter region to drive expression. Expression of luciferase driven by the viral SV-40 promoter (pGL3P) was used as a positive control of luciferase expression. The plasmids were transfected into wild-type RN46A cells and after a recovery period, changed to an estrogen-free medium as described and starved of hormones in phenol red-free media with charcoal-treated serum for 24 hours prior to estrogen treatment.

In multiple independent experiments, each construct displayed significant activity in the absence of estrogen, that was highest for the -391 and -724luc constructs, consistent with the presence of multiple Sp1/MAZ enhancer sites in these fragments (Parks et al., 1996). In the presence of 100 nM estrogen, no significant change in the activity of these fragments was observed, suggesting that none of these promoter fragments are significantly modulated by estrogen. Each individual experiment showed no significant difference between the control and treated group, although there was a slight increase in the activity of the -391luc construct. In further experiments, RN46A cells transfected with the luciferase reporter constructs and treated with 100 nM progesterone also did not generate any activation or repression of the 5-HT1A promoter constructs (data not shown). Thus I obtained no evidence for a strong transcriptional action of estrogen on transcriptional activity of the 5-HT1A receptor promoter in wild-type RN46A cells.

Estrogen does not induce the vitellogenin estrogen response element (vgERE) in wild type RN46A cells

Initially, the concentration and time of estrogen and progesterone treatment were based on conditions employed by Wissink et al (2001) for positive regulation of the rat 5-HT1A receptor promoter in HEK 293 cells cotransfected with ER cDNA constructs. To confirm that there were no transcriptional effects of estrogen on the 5-HT1A promoter region, a series of control experiments was conducted to establish that the lack of effect was not due to an inappropriate estrogen concentration or treatment time. To do this, it was necessary to generate a plasmid construct that is known to respond to estrogen. The

approach was to clone the vitellogenin estrogen response element (vgERE) upstream of the SV-40 promoter contained within the pGL3P luciferase reporter construct. This element is strongly activated upon binding both ERα and ERβ (Hall *et al*, 2002). Upon transfecting this construct into RN46A cells, however it became evident, as shown in Figure 3, that 17-β-estradiol treatment did not transactivate the vgERE-SV-40 construct. This indicates that perhaps the estrogen receptor, if present in RN46A cells as suggested by previous studies , may be desensitized, non-functional, or not abundant.

Optimal induction of luciferase is achieved with 100nM estrogen for 48 hours, with co-transfection of ERβ in a 5:1 construct to receptor ratio

To address whether RN46A cells could mediate an estrogen response, the experimental approach was changed to include co-transfection of the ERβ, under the control of the SV-40 promoter, into the RN46A cells to determine if there was an induction or repression of the luciferase reporter gene. The ERβ was chosen because this is the predominant subtype expressed in raphe neurons *in vivo* (Bethea, Lu et al. 2003). Initially, it was necessary to establish the proper concentration and time course of 17-β-estradiol treatment, as well as the optimal ratio of ERβ to be transfected along with the luciferase reporter construct. As illustrated in figure 4A, a concentration of 10 nM to 1 μM induced a maximal 8-fold induction of the SV-40 promoter at 48 hrs in the presence of the vgERE. Maximal induction appeared to occur at 48 hours for both 100 nM and 1 μM concentrations. Figure 4B illustrates that there was a steady increase of luciferase activity from about 8 hours, at each estrogen concentration tested, using a ratio of luciferase to receptor construct of 3:1. This demonstrates that the conditions under which

the previous transcriptional assays in wild-type RN46A cells have been performed were appropriate, and that these cells can mediate transcriptional responses to estrogen at a consensus ERE, if an exogenous estrogen receptor (ERβ) is cotransfected.

Because it became evident that it was necessary to provide exogenous functional estrogen receptor in the RN46A cells, a control experiment was conducted to establish the ratio of ERβ to reporter construct that yields maximal estrogen-mediated transcriptional effects. Figure 4C shows that in the absence of the estrogen receptor, there was no induction of the vgERE-pGL3P construct (ratio 1:0). However, when any amount of the ERβ receptor was transfected into the cells along with the reporter construct there was a 3-fold induction of luciferase expression with 100nM 17-β-estradiol administration for 24 hours. Interestingly, there was a statistically significant increase in the basal luciferase activity of the vgERE-pGL3P construct to approximately 3-fold as ERβ was increased, suggesting some spontaneous activity of vgERE in the presence of ERβ. The possibility that this basal activity of vgERE/ERβ was due to estrogens in the medium was examined below. In order to obtain maximal luciferase values, it is desirable to transfect a maximal amount of construct, therefore a ratio of construct to receptor of 5:1 was chosen for experimental purposes.

Endogenous estrogens are not present within this experimental model

To eliminate the possibility of endogenous estrogens within the experimental system modulating the promoter construct, the antiestrogen ICI 182,780 was used to block any such activation which may exist (Figure 5). Treatment with ICI 182,780 blocked

induction of the vgERE construct, indicating that the compound was functioning as an antiestrogen. A comparison of vehicle-treated and ICI-treated cells showed no significant difference in the basal luciferase activity, indicating that there is no induction of the vgERE by endogenous estrogens.

Estrogen does not modulate the 5-HT1A promoter region in the presence of ERβ

Experiments were performed in RN46A cells cotransfected with hERβ and 5-HT1A reporter constructs. It became apparent that there was no significant effect of estrogen on the 5-HT1A promoter region from –1 to –1515 upstream of the ATG start site (Figure 6A). When comparing the expression of luciferase in estrogen treated versus untreated cells, no significant change was observed. Assays were also conducted in HEK 293 cells to further verify any results observed in the RN46A cell line. In the latter, the estrogen receptor β was co-transfected with the luciferase reporter construct using the calcium phosphate precipitation method. The results of these experiments are depicted in figure 6B. Interestingly, compared to RN46A cells, the expression of the luciferase protein appears to be very low in the HEK 293 cells, which unlike RN46A cells do not express 5-HT1A receptors. Overall, there was no significant effect of estrogen upon the promoter in either cell line in the presence of the β isoform of the estrogen receptor.

Overexpression of ERβ in RN46A cells

In order to examine transcriptional effects of estrogen on endogenous 5-HT1A receptor levels, as well as to evaluate 5-HT1A reporter constructs in a cell line which endogenously expresses a functional estrogen receptor β, a line of RN46A cells stably transfected with hERβ was generated. After selecting the clones with puromycin, whose resistance gene was contained within the plasmid containing hERβ cDNA, colonies were picked and tested the expression level of hERβ RNA by Northern blot analysis (Figure 7A). Several clones (#7, 9, 20) expressed ERβ RNA, and were chosen for closer examination. In order to test for functional expression of hERβ, the response of the vgERE construct to estrogen treatment in these cell lines was studied. It was found that all of the hERβ-positive clones induced the activity of SV-40 with estrogen treatment, through the vgERE (Figure 7B, and data not shown). However, clone 9 (RNERb9), which had the highest expression levels tended to lose this response rapidly with passaging (about three passages). Some work was done with this clone, but it was limited due to the rapid loss of hERβ expression in these cells. Clone 7 did not show a large difference between basal expression (pGL3B) and SV-40 driven luciferase activity (pGL3P). Clone 20 (RNERb20) showed a good separation of activity between the basal and positive control (pGL3P), as well as a nice induction of vgERE with estrogen treatment, therefore most work was done using this clone.

Estrogen does not modulate the 5-HT1A promoter in RNERb20 cells

It was important to examine whether the lack of estrogen response in RN46A cells exogenously transfected with ERβ was due to a limited amount of the receptor entering the cells within the lipid complex. It was possible that not enough ERβ was being expressed transiently to induce changes in luciferase expression. Therefore, use of a cell line that stably expresses endogenous ERβ would reveal subtle changes in luciferase expression that are dependent on having adequate amounts of the receptor present. However transfection of luciferase reporter constructs into RNERb20 confirmed that there was no significant response to estrogen (100 nM) of the 5-HT1A promoter region up to –1515 (Figure 8), although the –391luc construct showed a small induction. By contrast, the vgERE construct was induced by about 2-fold in these experiments.

A putative estrogen response element (1AERE) exists in the 5-HT1A promoter region and binds a nuclear complex

An examination of the sequence of the human 5-HT1A promoter revealed that an ERE-like element was present at position –419/-433 bp. This sequence contained the ideal estrogen palindrome with a 5-base pair spacer between the half sites. The perfect ERE sequence is 5'-GGTCAnnnTGACC-3', while the putative ERE within the 5-HT1A promoter, named 1AERE, has the sequence 5'-GGTCAcagagTGACC-3'. Interestingly, the first half-site is lacking the initial G nucleotide (changed to A) in the rat 5-HT1A sequence, but in the mouse 5-HT1A sequence, both ERE half-sites are conserved and located in approximately the same position in their respective promoters (Figure 9).

To determine if a protein complex can bind to the 1AERE, nuclear extracts were harvested from wild-type RN46A cells, which did not appear to express a functional estrogen receptor (see above), and from the stable clones expressing ERβ. Two specific complexes were bound to the 1AERE in both wild-type RN46A cells (Figure 10A) and ERβ-expressing clone RNERb9 (Figure 10B), although complex 1 was more abundant in the ERβ clone. The complexes were competed specifically by an excess of unlabelled 1AERE probe (lanes 8-10) and to a lesser extent by unlabelled vgERE (lanes 11-13). A mutant form of the ERE (named 1AEREmut) with the mutated base pairs shown **in bold** (5'-GGTCAcagagTGACC3' to 5'G**A**TCAcagagTGA**T**C-3'), which changes the element to resemble a glucocorticoid response element half site (GRE), did not compete for binding of the complex (lanes 14-15). These observations suggest that two protein complexes bind specifically at the 1AERE. Similar results were seen in RNERb20 cells. Incubation of the reaction in the presence of estrogen did increase the intensity of the complex in some experiments (lane 5, Figure 9A), however this was not consistent (lane 5, Figure 9B). Addition of a monoclonal anti-ERβ antibody (lane 6) did not supershift or compete the complex, however it was determined later by western blot that the antibody was not effective for detection of the receptor. Since complex 1 was more abundant in the ERβ clone it may represent the ERβ-1AERE complex. However it was not supershifted by anti-ERβ, but this may reflect weak binding of the antibody under incubation conditions. Complex 1 appears to represent a different complex of unknown composition. The presence of protein complexes that recognize the 1AERE may result in transcription regulation of the 5-HT1A promoter in RN46A cells that was not detectable using the large promoter-luciferase constructs in previous studies.

In vitro transcribed/translated ERβ was also tested to determine if the receptor was able to bind to the 1AERE, but background in the wheat germ system prevented meaningful conclusions, and I was unable to obtain detectable ERβ expression using the rabbit reticulocyte system (data not shown).

Mutation of the 1AERE within the context of the 5-HT1A promoter did not change the promoter activity

Although protein complexes bind to the 1AERE, the functionality of the element remained to be evaluated. The initial approach to address its functionality was to mutate the 1AERE within the 5-HT1A promoter to the same sequence that did not displace the nuclear complex in RN46A and RNERb cells. The luciferase activity of the wild-type 5-HT1A reporter construct and the mutated form (Figure 11) were then compared. Mutation of the element did not appear to have a substantial effect on the promoter activity in RN46A cells co-transfected with ERβ in a 5:1 ratio. The transcriptional activity of these constructs was comparable both with and without the presence of estrogen (Figure 11A). Transfection studies in RNERb20 cells showed no induction or repression of the –724bp region of the promoter (-724luc) with estrogen treatment (Figure 11B), consistent with previous experiments (Figure 8B). There is also no induction or repression of the same promoter region containing the mutated form of the 1AERE (-724mutluc, Figure 11B). However, the basal transcriptional activity was significantly greater for the mutated versus non-mutated 1AERE promoter region. This result suggests that in the presence of ERβ at the 1AERE may exert a basal repressive

effect on the 5-HT1A promoter, which is somewhat independent of estrogen. However, this construct includes a large portion of the 5-HT1A promoter, and therefore the small effect of the mutation on estrogen responsiveness may not be obvious. The basal repression observed may be attributable to interference of the mutation with interaction of a different protein at an adjacent site, thus examination of the isolated 1AERE element is necessary.

The 1AERE mediates a repressive response to estrogen that is abolished with mutation of the element

The 1AERE was cloned upstream of the SV-40 promoter in pGL3P (1AERE) and transfected into wildtype RN46A cells with the ERβ (Figure 12A) as well as into RNERb20 cells (Figure 12B). In both cells lines, estrogen showed a trend towards repression, which was much more evident in RNERb20 cells, likely due to higher expression of the ERβ in these cells. This effect was not significant, however, due to high variability between experiments. This repression was significant in 2 of the 5 experiments ($p= 0.0026, 0.0118$), however it was only a trend in 3 of the 5 experiments. Mutation of the ERE (1Amut) abolished this trend in both cells lines. These results suggest that the nuclear complex that associates with the 1AERE might mediate a functional repression of the 5-HT1A promoter, which is eliminated by mutation of the element.

Endogenous 5-HT1A mRNA levels are increased with estrogen treatment

In order to address whether estrogen has any effect on endogenous (rat) 5-HT1A receptor expression, endogenous 5-HT1A mRNA levels in RNERb20 cells were determined by real-time quantitative RT-PCR. Estrogen treatment upregulated rat 5-HT1A RNA expression by 5-fold at 8 hr, an effect that declined at 16 and 24 hours (Figure 13A). Interestingly, the basal expression of 5-HT1A RNA also increased with time from 8-24 hrs of incubation in hormone- and serotonin-free medium, likely due to the effects of the 0.1% of ethanol added as a vehicle repressing the expression of 5-HT1A at the 8 hr time point. After 24hrs, however, these levels recovered. Because ethanol was added to both conditions (vehicle and estrogen), this overall repression was induced in both treatments, therefore differences between control and treated seen at the specific time points are likely due to estrogen. The reaction curve typically seen for amplification of 5-HT1A is depicted in figures 13B, and the threshold for quantification is indicated with a red line. This is the point at which the incorporation of Sybr Green was used to extract quantitative readings of the amount of mRNA present in the initial sample. The standard curve had an R-value of 0.992, indicating that the graph generated was linear, and results were reproducible (Figure 13C). A melt curve analysis shows melting temperature peaks for the PCR product of rat 5-HT1A amplified from mRNA samples (peak 1), for GAPDH (peak 2) and for Dbx 5-HT1A cDNA (peak 3) used to construct the standard curve (Figure 12D). By this analysis, we can be confident that the product generated was the complete 5-HT1A fragment and not primer dimers which would have a lower melting temperature.

The induction of rat 5-HT1A RNA by estrogen is in opposition to the data seen with human 5-HT1A promoter analysis, and suggests that there may be a species difference in regulation of the 5-HT1A receptor by steroid hormones. Comparison of the promoter fragments containing the 1AERE (Figure 9) indicates that the palindrome for the rat 1AERE is not complete, as it contains only 4 bases of the first half-site, making it an imperfect ERE. The murine promoter contains an element identical to the human promoter, suggesting that this may be a better animal model to conduct further study on. Alternately, estrogen may have post-transcriptional actions that lead increase in 5-HT1A RNA levels.

Figure 1: Schematic diagram of human 5-HT1A promoter reporter constructs.

Human 5-HT1A promoter fragments (depicted in purple, with known transcriptional elements indicated) were inserted upstream of the luciferase reporter gene within the pGL3-basic vector. Transfection efficiency was measured by the activity of pCMVβgal. Promoter activity was corrected for basal activity and normalised to the activity of the viral SV40 promoter inserted in pGL3B. Constructs were made and sequences confirmed by Sylvie Lemonde. NF-kB: nuclear factor kappa site B; Sp1: Stimulating protein 1; DRE: Dual Repressor element; NRSE: Neural Restrictive Silencing Element; ERE: Estrogen Response Element.

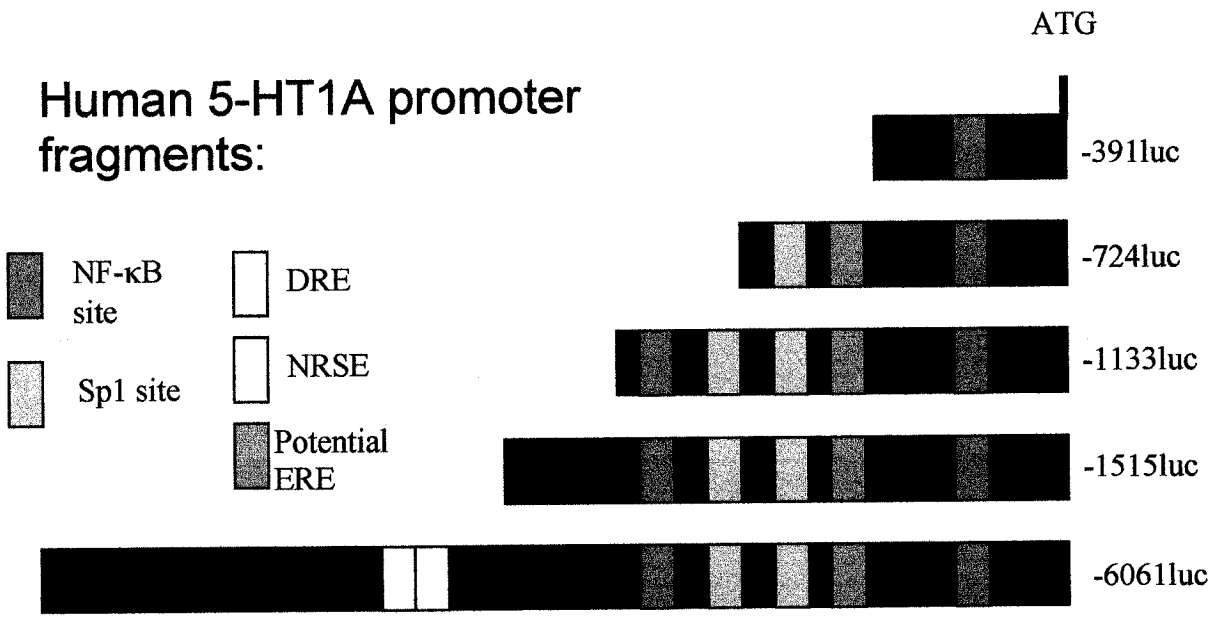

ATG
Human 5-HT1A promoter fragments:
NF-κB site
Sp1 site
DRE
NRSE
Potential ERE
-391luc
-724luc
-1133luc
-1515luc
-6061luc

Figure 2: Estrogen action on human 5-HT1A receptor promoter in RN46A cells

Summary of the results of transient transfection experiments in rat raphe RN46A cells. Constructs were transfected using a lipid carrier. Cells were estrogen-starved for 24 hours prior to treatment with 100 nM estrogen for 24h. Data for -391luc is mean ± standard deviation (SD) of triplicate samples (N=4); -724luc is mean ± standard deviation (SD) of triplicate samples (N=5); -1133luc is mean ± standard deviation (SD) of triplicate samples (N=4); -1515luc is mean ± standard deviation (SD) of triplicate samples (N=4). Basal expression of luciferase ranged from approximately 20 to 90 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 400-900 relative light units, on average.

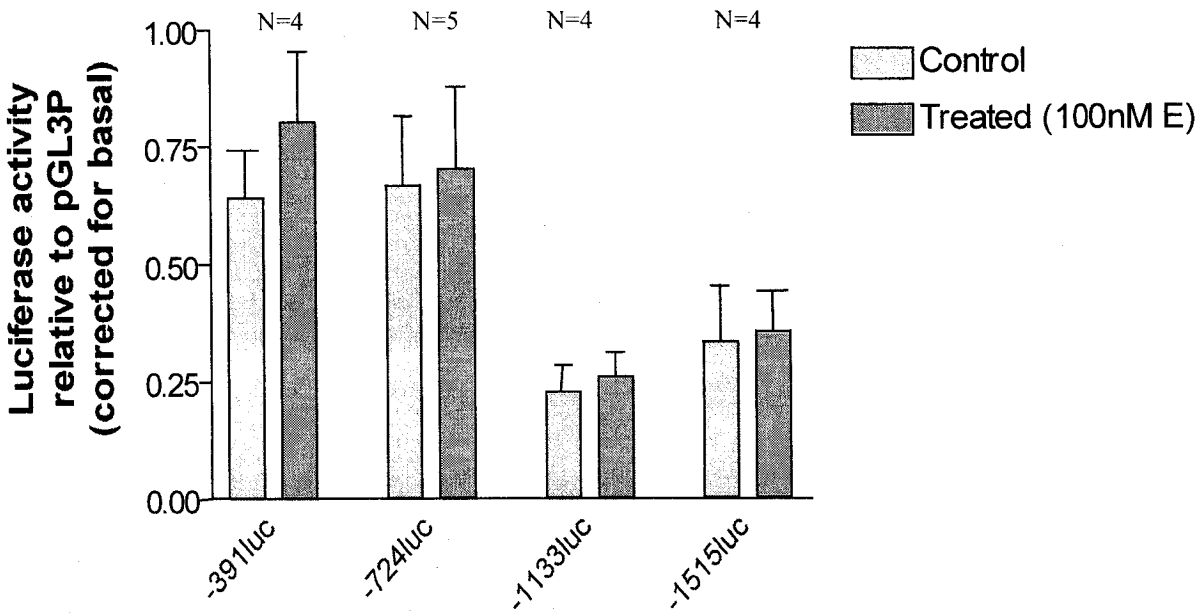

Luciferase activity relative to pGL3P (corrected for basal)
1.00
0.75
0.50
0.25
0.00
N=4
N=5
N=4
N=4
-391luc
-724luc
-1133luc
-1515luc
Control
Treated (100nM E)

Figure 3: Estrogen does not induce vitellogenin estrogen responsive element (vgERE) in wild-type RN46A cells

The vgERE was cloned upstream of the SV-40 promoter in pGL3P reporter assay construct. Cells were transfected using a lipid carrier and estrogen-starved for 24 hours prior to treatment with 100nM hormone for 24h; data is expressed as mean of triplicate samples ± standard deviation (SD). Basal expression of luciferase was approximately 90 relative light units, and SV-40 driven luciferase expression (pGL3P) was about 900 relative light units, on average

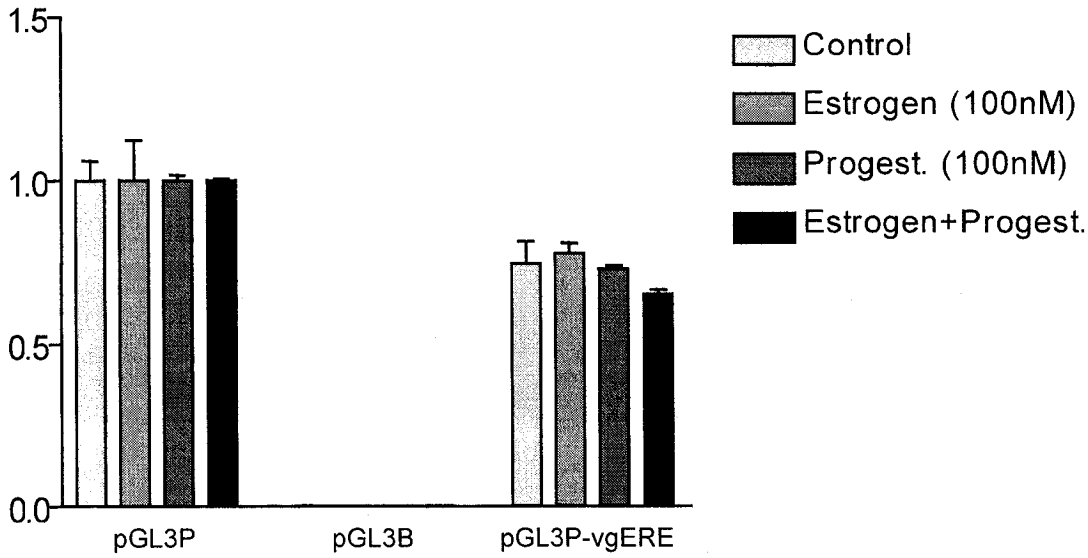
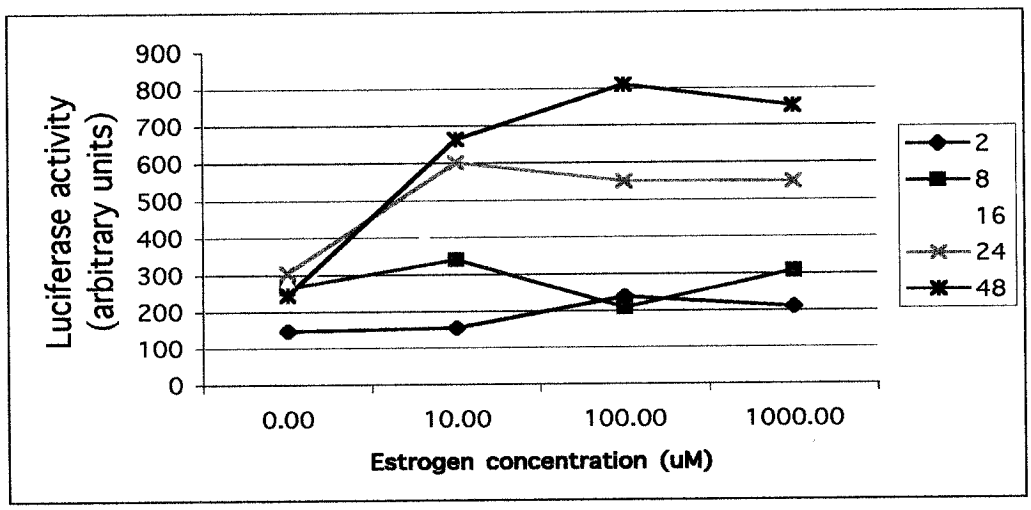

1.5
1.0
0.5
0.0
pGL3P
pGL3B
pGL3P-vgERE
Control
Estrogen (100nM)
Progest. (100nM)
Estrogen+Progest.

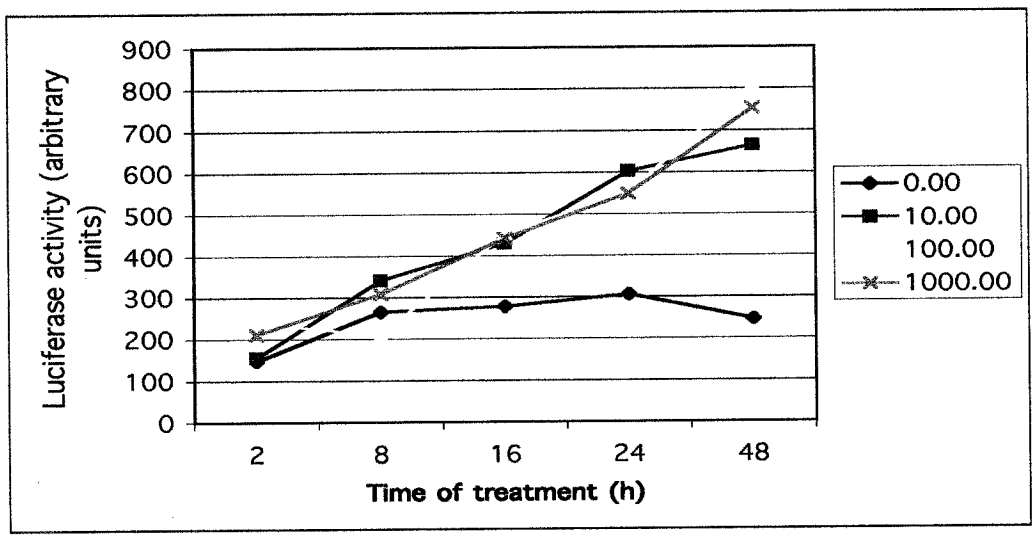
Figure 4: Optimal conditions for estrogen responsive gene transcription

Optimal experimental conditions were determined for estrogen response in estrogen-starved (24 h) RN46A cells transiently cotransfected using a lipid carrier with pGL3P-vitERE reporter and ERβ constructs. (A) Estrogen (10 nM) activates luciferase activity maximally after 48h of treatment in ERβ-transfected RN46A cells. The pGL3P-vitERE and ERβ constructs were transfected in a ratio of 3:1 and luciferase activity was measured and plotted against the concentration of 17-β-estradiol treatment (for 2-48 hr), from 0-1000 μM. Data represent mean ± SD of triplicate samples from a single experiment. (B) Estrogen activates luciferase activity maximally at a concentration of 10 nM in ERβ-transfected RN46A cells. Concentrations greater than 10 nM did not further enhance luciferase activity. Transfections were as in A, and luciferase activity upon treatment with indicated estrogen concentrations was plotted versus time, from 2 to 48 h; average of triplicate samples from a single experiment. (C) Optimal ratio of pGL3P-vitERE and ERβ. The pGL3P-vitERE and ERβ plasmids were cotransfected at indicated ratios and treated with 100nM 17-β-estradiol for 24h. Data are average of triplicate samples with standard deviation from a single experiment. Note that estrogen did not induce the SV-40 promoter in the absence of the ERβ (ratio 1:0), but did induce transcription at with ERβ present. Differences in basal expression of vgERE with or without receptor expression were measured with and unpaired, two-tailed Student's t-test, *: $p<0.05$ **: $p< 0.01$ ***$p<0.001$, n=3.

A


Luciferase activity (arbitrary units)
900
800
700
600
500
400
300
200
100
0
0.00
10.00
100.00
1000.00
Estrogen concentration (uM)
2
8
16
24
48

B


Luciferase activity (arbitrary units)
900
800
700
600
500
400
300
200
100
0
2
8
16
24
48
Time of treatment (h)
0.00
10.00
100.00
1000.00

C

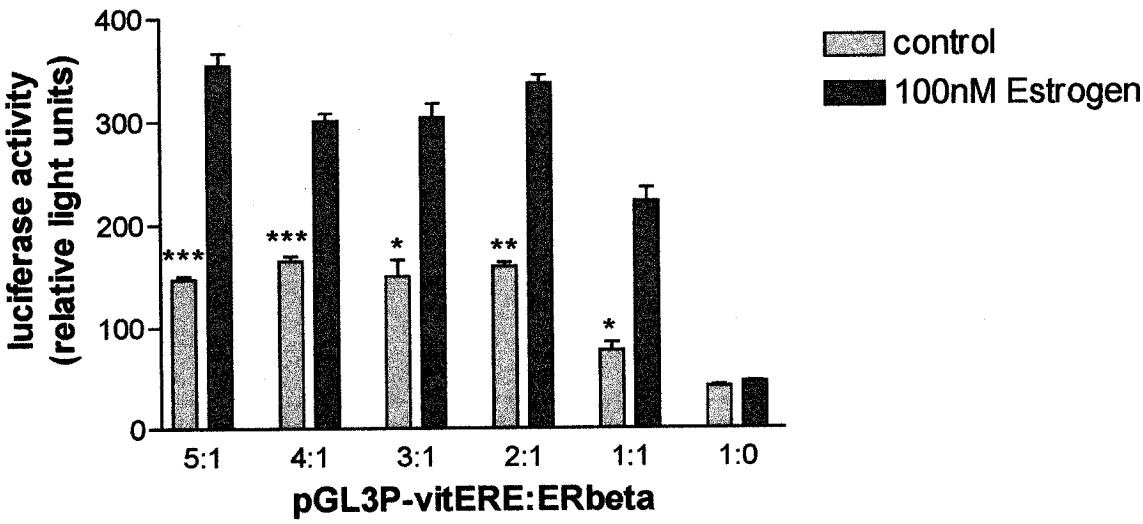

luciferase activity (relative light units)
400
300
200
100
0
***
***
*
**
*
5:1
4:1
3:1
2:1
1:1
1:0
pGL3P-vitERE:ERbeta
control
100nM Estrogen

Figure 5: Estrogen response requires ERβ and is blocked by antagonist

RN46A cells were transiently transfected with pGL3P (vector) or pGL3P-vitERE luciferase reporter constructs and ERβ (5:1), and were estrogen-starved for 24 hours prior to treatment with vehicle, estrogen (100 nM), the estrogen antagonist ICI 182,780 (100 μM), or both estrogen and ICI 182,780. ICI 182,780 blocked the activation of estrogen on the vgERE ($p=0.0133$; t-test), but showed no effect on the basal activity of the vgERE construct. Neither estrogen nor ICI 182,780 altered activity of pGL3P, which lacks vgERE. Results presented are typical of 2 independent experiments of triplicate samples. Mean and SD were determined from triplicate values obtained in a single experiment. Basal expression of luciferase ranged from approximately 20-200 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 900-8000 relative light units, on average, approximately a 40-fold increase over basal. Statistical analysis compared to control (1:0) was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$

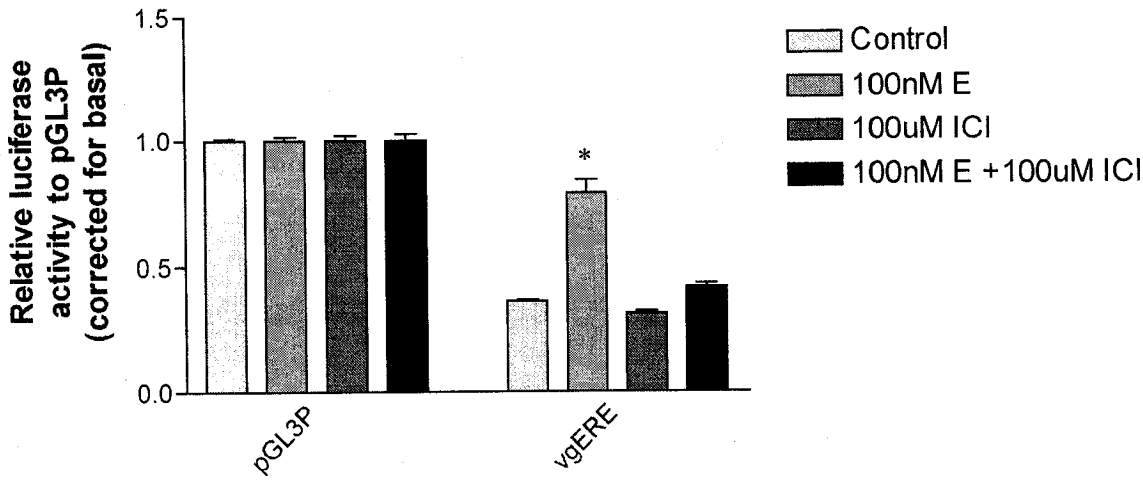
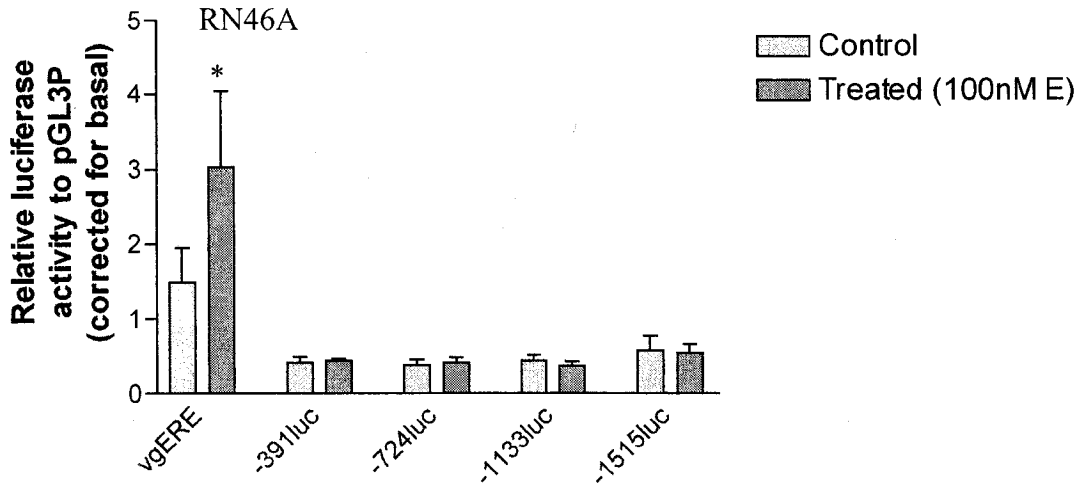

Relative luciferase activity to pGL3P (corrected for basal)
1.5
1.0
0.5
0.0
*
pGL3P
vgERE
Control
100nM E
100uM ICI
100nM E +100uM ICI

Figure 6: Estrogen fails to regulate the human 5-HT1A promoter in RN46A or HEK293 cells

(A) Summary of the results of transient transfection experiments in *RN46A* cells. The indicated 5-HT1A or pGL3P-vitERE (vgERE) luciferase reporter constructs and ERβ were cotransfected using a lipid carrier and cells were estrogen-starved for 24 hours prior to treatment with 100 nM 17-β-estradiol (E) for 48h. Results are mean ± SD from 4 independent experiments of triplicate samples (N=4). Basal expression of luciferase ranged from approximately 3 to 90 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 100 to 3000 relative light units, on average. Note that only for the vgERE did estrogen have a significant effect (P=0.0323, t-test). (B) Summary of the results (mean ± SD) of transient transfection experiments in *HEK 293 cells*. Constructs and ERβ were transfected using $CaPO_4$. Cells were estrogen-starved for 24 hours prior to treatment with 100nM 17-β-Estradiol for 24h. N values for independent experiments in triplicate samples were: -391luc (N=3); -724luc (N=5); -1133luc (N=5); -1515luc (N=5). Basal expression of luciferase ranged from approximately 2 to 4 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 50 to 60 relative light units, on average. There were no significant differences between control vs. estrogen treated samples. Statistical analysis was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$

A


RN46A
Relative luciferase activity to pGL3P (corrected for basal)
5
4
3
2
1
0
*
vgERE
-391luc
-724luc
-1133luc
-1515luc
Control
Treated (100nM E)

B

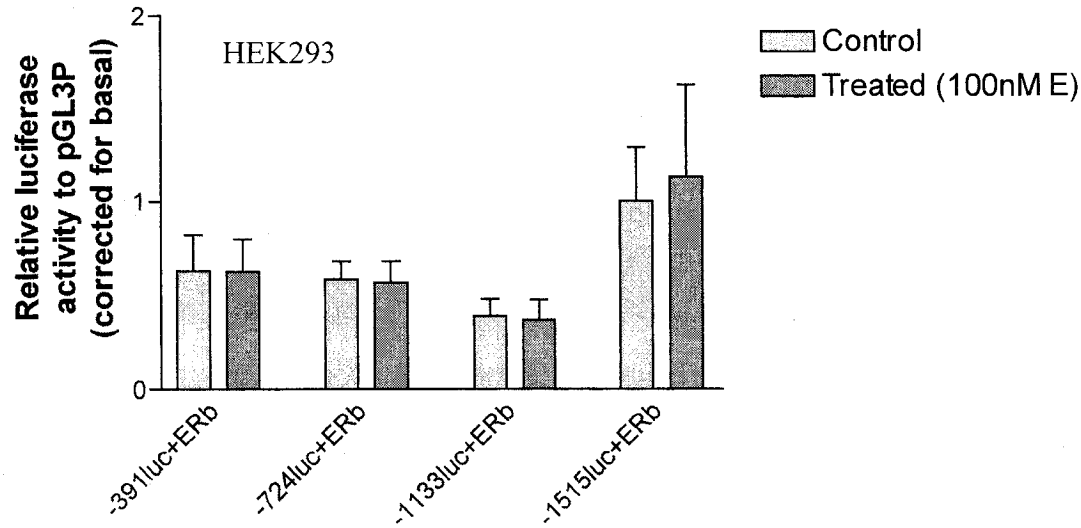
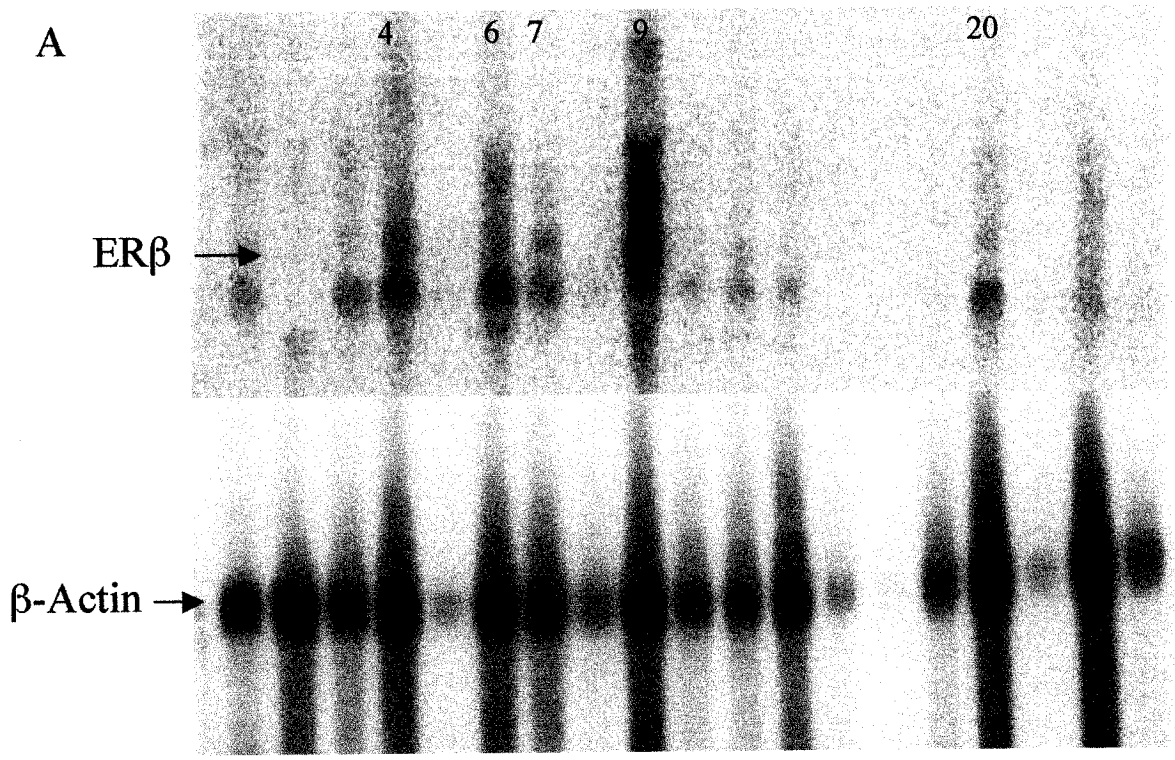

HEK293
Relative luciferase activity to pGL3P (corrected for basal)
2
1
0
-391luc+ERb
-724luc+ERb
-1133luc+ERb
-1515luc+ERb
Control
Treated (100nM E)

Figure 7: Analysis of RN46A clones stably-expressing functional ERβ

RN46A cells were transfected with hERβ expression plasmid and clones selected using puromycin as described in Methods. (A) Northern blot analysis of total RNA from stable ERb-transfected RN46A clones (positive clones indicated by numbers) using probe 1, recognising a 601bp fragment in the centre of hERβ cDNA. Whole cell RNA (from one 10-cm. Dish/lane) was electrophesed and transferred to nitrocellulose membrane and incubated with ^{32}P-labeled probe overnight. Strongly expressing clones are indicated with their assigned number. The appropriate band corresponding to hERβ RNA is indicated with arrow. Blots were also stripped and reprobed for β-Actin to correct for loading. (B) Transfection experiments in stable RN46A cell lines expressing hERβ. Luciferase reporter constructs and ERβ were transfected using a lipid carrier and estrogen-starved for 24 hours prior to treatment with 100nM hormone for 24 h, and data are mean ± S.D. of triplicate samples. Clones 7 ($p=0.010$; t-test) and 20 ($p=0.0007$; t-test) both displayed significant estrogen induction of pGL3P-vitERE but not vector controls. However clone 7 did not show a good separation of basal luciferase activity (pGL3B) and SV-40 driven luciferase activity. Assay was also done for clone 9, but estrogen response was lost after several passages of the cell line. Statistical analysis was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$, ***: $p<0.0005$


A
4
6
7
9
20
ERβ
β-Actin

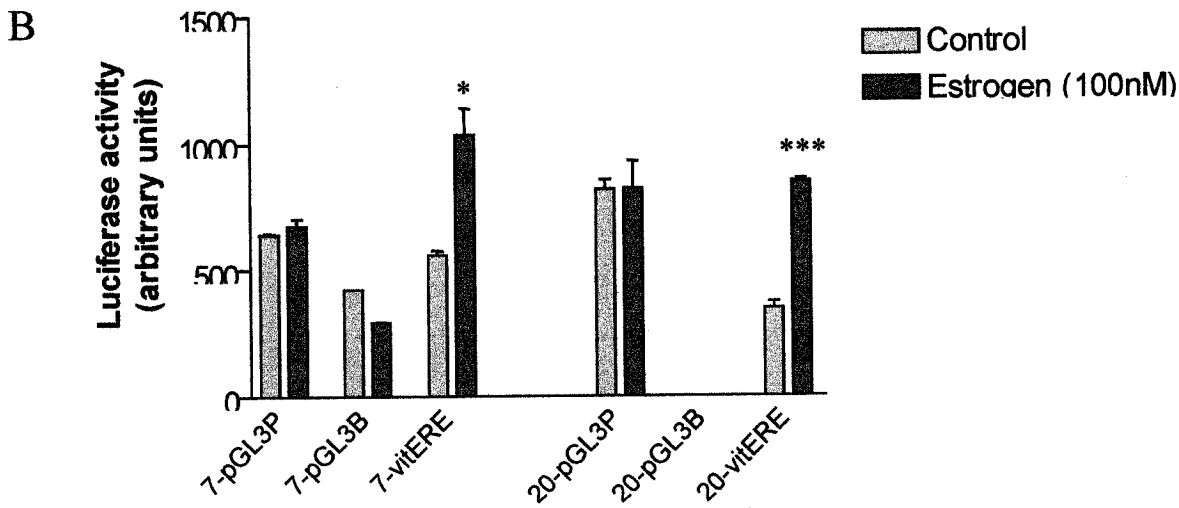

B
Luciferase activity (arbitrary units)
1500
1000
500
0
Control
Estrogen (100nM)
*
***
7-pGL3P
7-pGL3B
7-vitERE
20-pGL3P
20-pGL3B
20-vitERE

Figure 8: Estrogen does not regulate 5-HT1A promoter activity in RNERb20 cells

Human 5-HT1A and pGL3P-vitERE (vgERE) luciferase reporter constructs were transfected using a lipid carrier and estrogen-starved for 24 hours prior to treatment with 100 nM estrogen for 48h; Results are from 4 independent experiments of triplicate samples (N=4) and represent mean ± S.D. Only the vgERE showed an estrogen response, although statistical analysis suggests that it is not significant ($p=0.0816$); t-test). Basal expression of luciferase ranged from approximately 30 to 50 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 1000-3000 relative light units, on average. Statistical analysis was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$

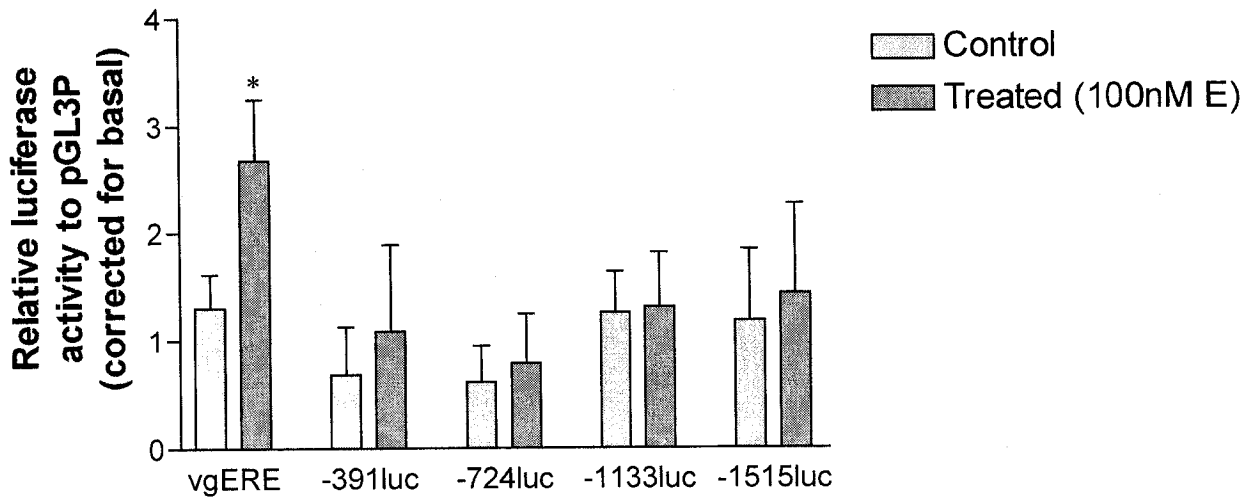

Relative luciferase activity to pGL3P (corrected for basal)
4
3
2
1
0
*
vgERE
-391luc
-724luc
-1133luc
-1515luc
Control
Treated (100nM E)

Figure 9: Sequence alignment of human, rat, and murine putative ERE sequences

Alignment of the 1AERE sequence in the human, rat, and murine promoter regions reveal that the sequence is conserved in the human and murine promoters, but the first half-site of the palindrome in the rat promoter is lacking a G, making it an imperfect ERE which may function differently than in the human and mouse.

Human: AGGAGGGTCAcagagTGACCGTGGA

Rat: AGATGAGTCAcagagTGACCCCGGA

Murine: ATGAGGGTCAtcgagTGACCCGGGAG

Figure 10: Protein-DNA complexes with 1AERE probe in RN46A and RNERb9 cells detected by EMSA.

Complementary 1A-ERE primers were end-labeled with ^{32}P (*1AERE*) and incubated (x cpm/sample) with nuclear extract (x μg/sample) from (A) RN46A and (B) RNERb9 cells. Nuclear extracts were pre-incubated with or without the indicated –fold excess (ng/ng labeled 1AERE or vgERE) of competitor DNA, which were double-stranded unlabeled 1A-ERE (*1AERE*), the positive control vitellogenin ERE *(vgERE)* or a mutated form of the 1AERE which is not expected to bind the estrogen receptor (*1AEREmut*). 1A-ERE specifically bound two protein complexes (1 and 2 as indicated) from both cell lines. No bands were detected in the absence of nuclear extracts (lanes 1-3).

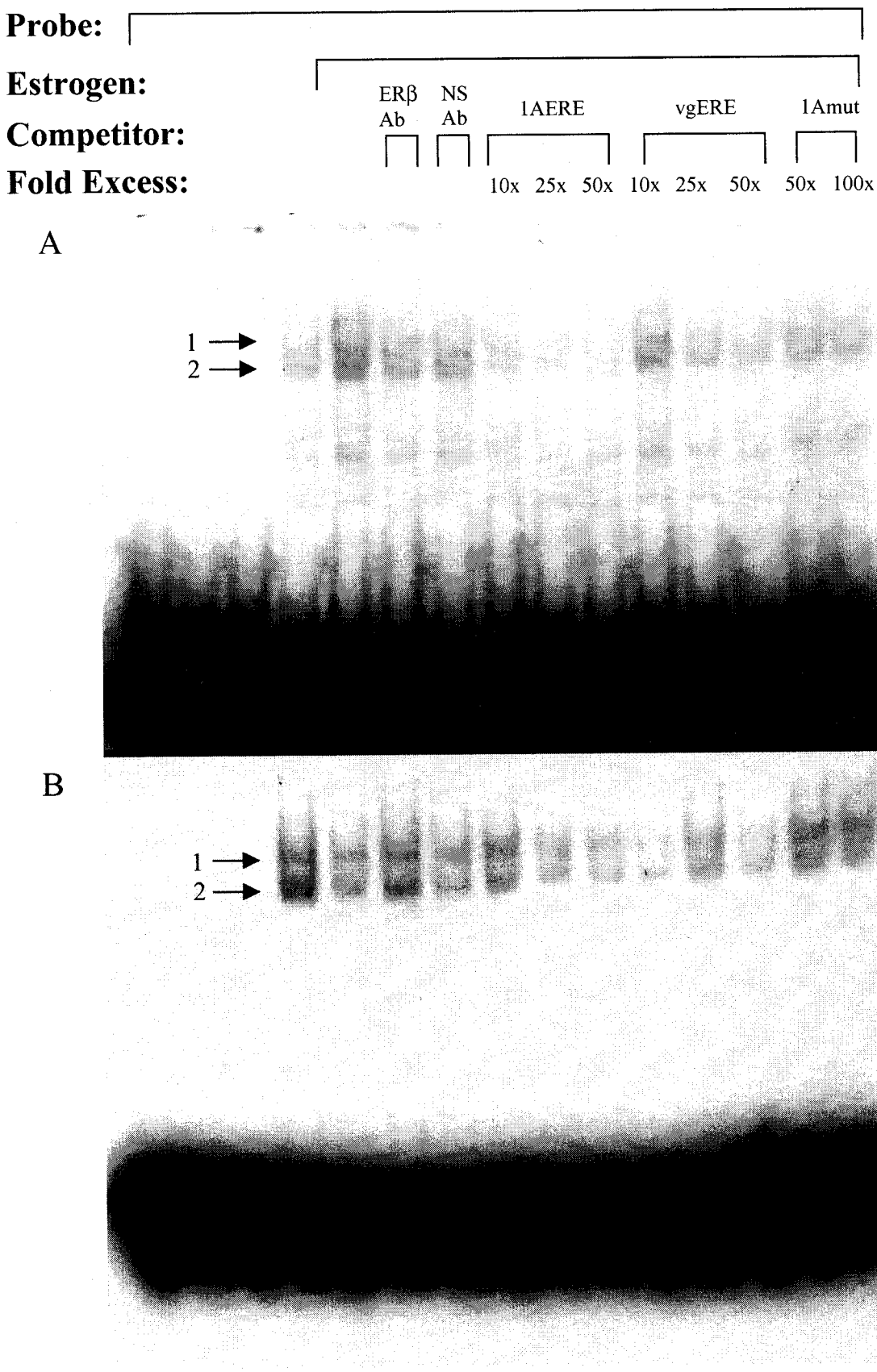
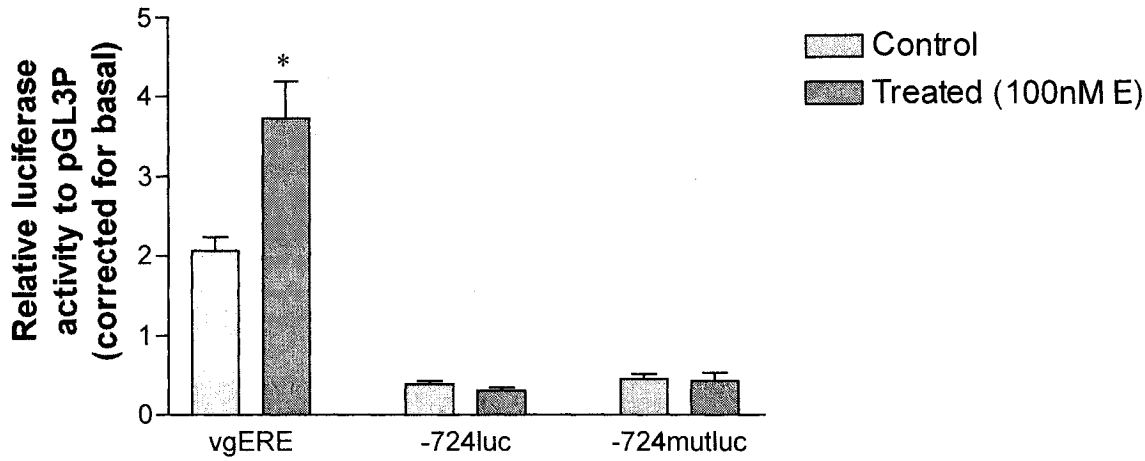

1AERE
Probe:
Estrogen:
Competitor:
Fold Excess:
ERβ Ab
NS Ab
1AERE
vgERE
1Amut
10x 25x 50x 10x 25x 50x 50x 100x
A
1
2
B
1
2

Figure 11: Mutation of the 1A-ERE derepresses 5-HT1A promoter activity in RNERb20 cells.

Human 5-HT1A (-724luc or ERE mutant -724mutluc) or pGL3P-vitERE (vgERE) luciferase reporter constructs and ERβ were transfected in a ratio of 5:1 using a lipid carrier and cell were estrogen-starved for 24 hours prior to treatment with 100 nM estrogen (E) for 48h. Data are expressed activity relative to pGL3P activity. Absolute basal luciferase activity (pGL3B) ranged from approximately 6 to 30 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 150-1000 relative light units, on average. Results are presented as mean ± S.D. from 4 independent experiments of triplicate samples (N=4). (A) Summary of results of transient transfections in *RN46A* cells. Significant estrogen induction was observed for only vgERE ($p=0.0143$; t-test). (B) Summary of results of transient transfections in *RNERb20* cells. Significant estrogen induction was observed for only vgERE ($p=0.0067$; t-test). Mutation of the ERE significantly derepressed basal activity of the 5-HT1A promoter ($p=0.0147$; t-test). Statistical analysis was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$, **: $p<0.001$, ns = not significant

A


Relative luciferase activity to pGL3P (corrected for basal)
5
4
3
2
1
0
*
vgERE
-724luc
-724mutluc
Control
Treated (100nM E)

B

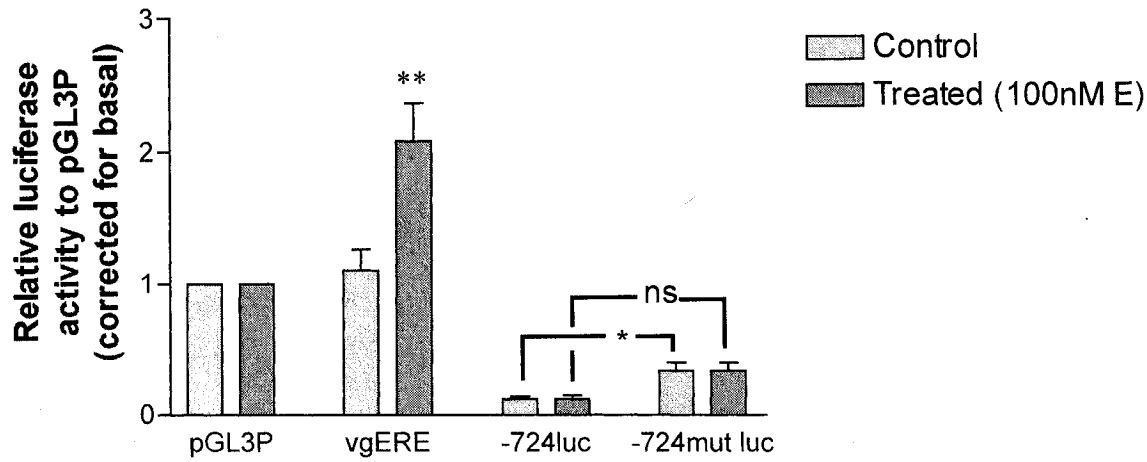
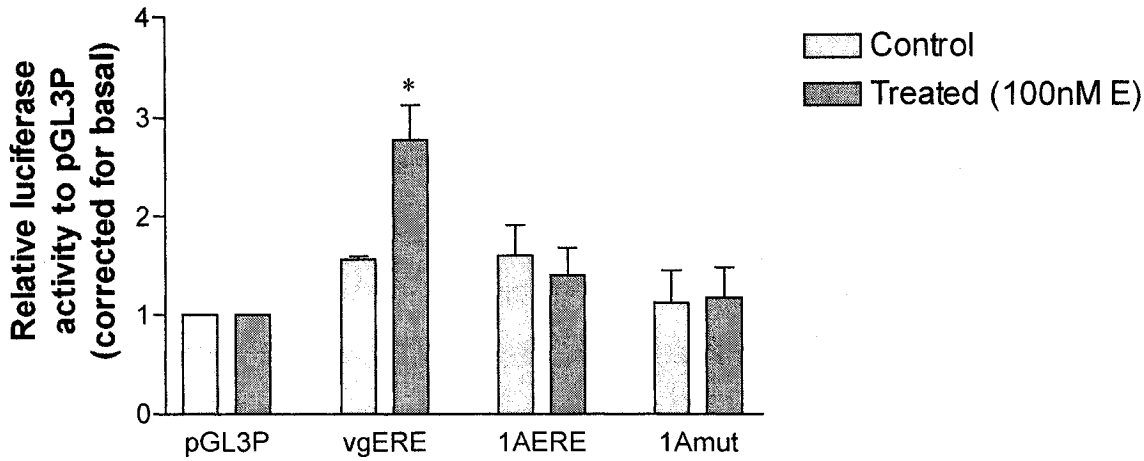

Relative luciferase activity to pGL3P (corrected for basal)
3
2
1
0
**
ns
*
pGL3P
vgERE
-724luc
-724mut luc
Control
Treated (100nM E)

Figure 12: Estrogen-induced repression by the 1A-ERE but not mutated 1AERE

SV40 promoter vector (pGL3P), or pGL3P with vitERE (vgERE), 1AERE-, or mutant 1AERE (1Amut) luciferase reporter constructs and ERβ were transfected in a mass ratio of 5:1 using a lipid carrier and cells were estrogen-starved for 24 hours prior to treatment with 100 nM estrogen (E) for 48h. Data are expressed activity relative to pGL3P activity. Results are presented as mean ± S.D. from 4 independent experiments of triplicate samples (N=4). (A) Summary of results of transient transfections in *RN46A* cells. A significant estrogen effect was observed for the vgERE construct only ($p=0.0144$; t-test). Absolute basal luciferase activity (pGL3B) ranged from approximately 6 to 30 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 150-1000 relative light units, on average. (B) Summary of results of transient transfections in *RNERb20* cells. Absolute basal luciferase activity (pGL3B) ranged from approximately 30 to 50 relative light units, and SV-40 driven luciferase expression (pGL3P) ranged from 1000-3000 relative light units, on average. Estrogen induced the vgERE construct ($p=0.1368$), although this was not statistically significant due to high S.D., and tended to repress the 1AERE ($p=0.2936$). Results are presented as mean ± S.D. from 5 independent experiments of triplicate samples (N=5). Statistical analysis was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$

A


Relative luciferase activity to pGL3P (corrected for basal)
4
3
2
1
0
*
pGL3P
vgERE
1AERE
1Amut
Control
Treated (100nM E)

B

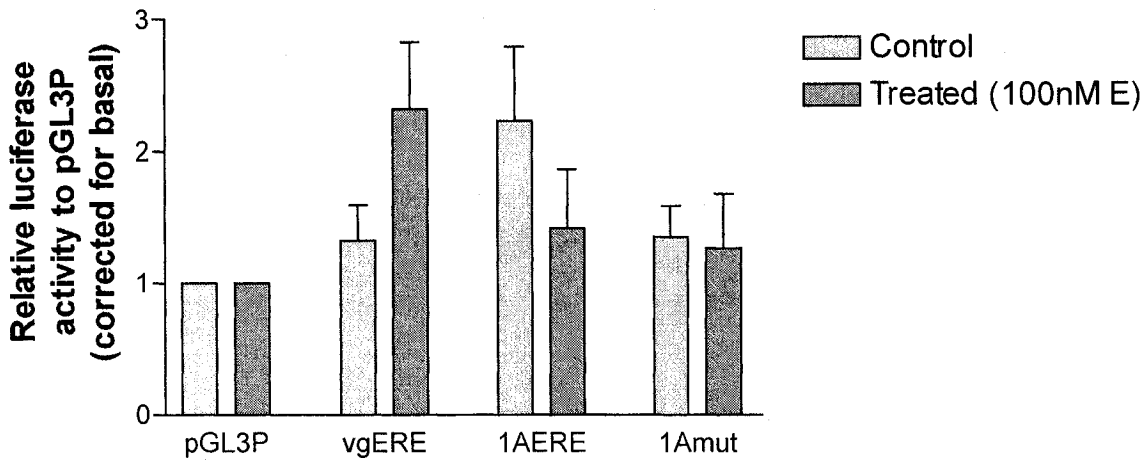
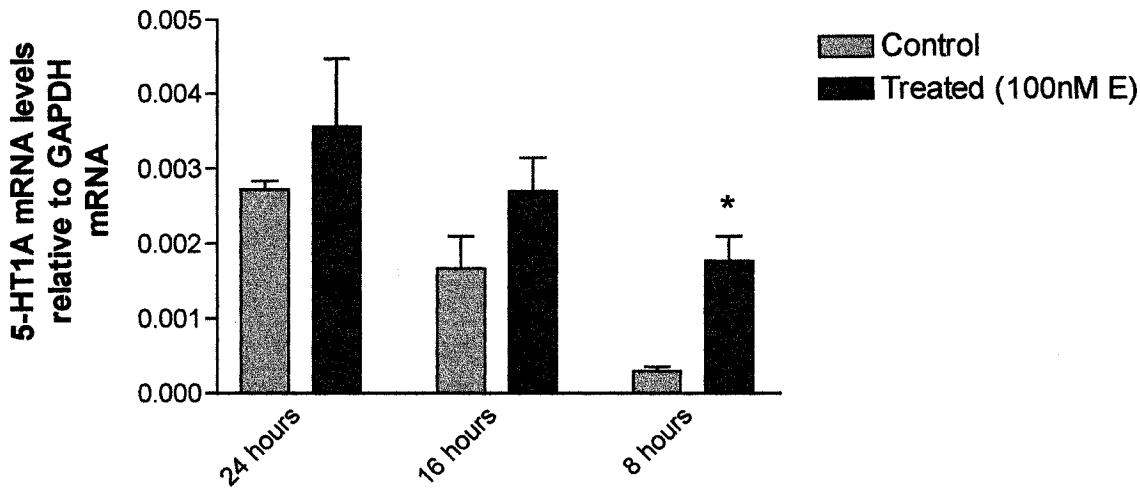

Relative luciferase activity to pGL3P (corrected for basal)
3
2
1
0
pGL3P
vgERE
1AERE
1Amut
Control
Treated (100nM E)

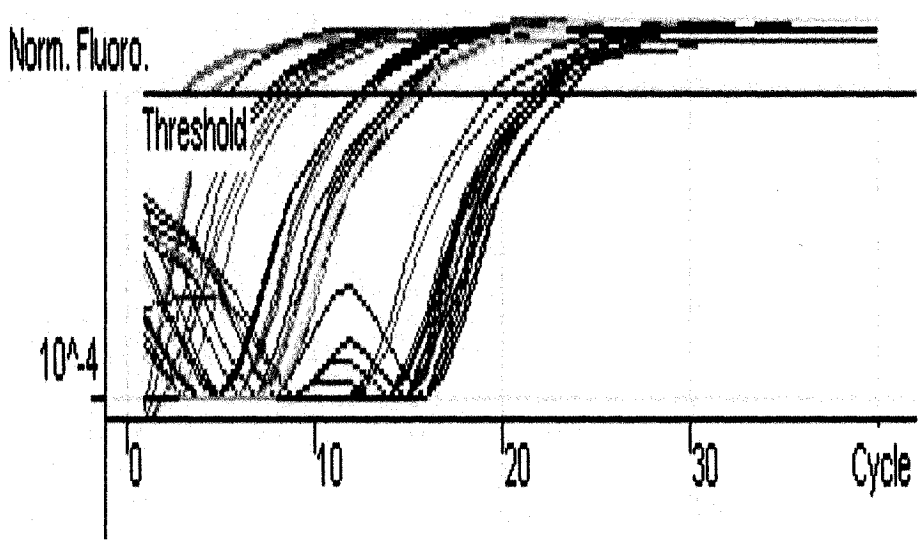
Figure 13: Estrogen increases 5-HT1A RNA in RNERb20 cells

(A) 5-HT1A RNA level is upregulated by 100nM estrogen treatment in RNERb20. Cells were estrogen-starved for 24 hours prior to treatment with vehicle or 100nM estrogen for the indicated times. Total RNA was isolated using Trizol (Invitrogen). 5-HT1A RNA was detected by real-time RT-PCR and quantified relative to GAPDH RNA in the samples. Data represent the mean ± S.D. of three independent experiments. Estrogen significantly induced 5-HT1A RNA levels at 8 hrs ($p=0.0109$), and showed a trend towards increase at 16 and 24hrs. (B) Standard curve used to quantify 5-HT1A RNA was constructed with known concentrations of the Dbx plasmid, containing rat 5-HT1A cDNA. (C) Typical amplification reaction indicating threshold point of reaction analysis. (D) Melt curve analysis of real-time PCR reactions. No primer dimers were formed during the reactions. Statistical analysis was done with an unpaired, two-tailed Student's t-test, *: $p<0.05$, $n=3$

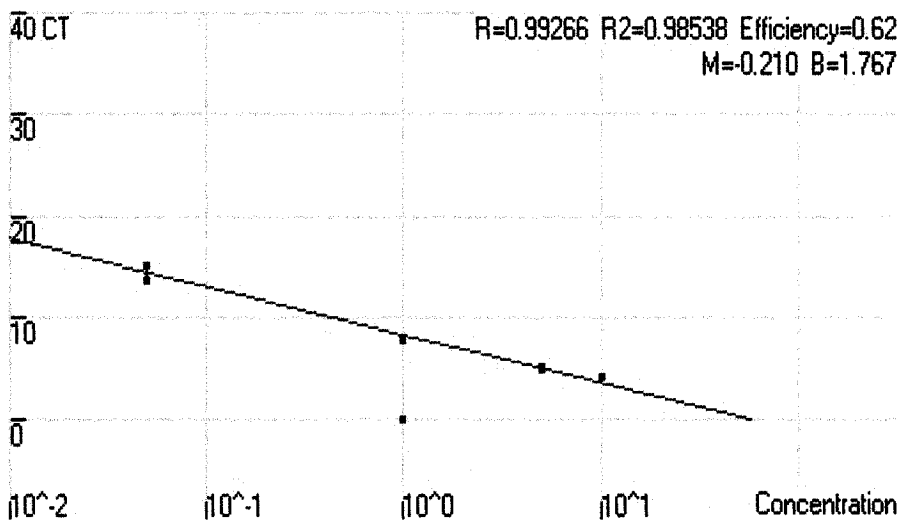
A


5-HT1A mRNA levels relative to GAPDH mRNA
0.005
0.004
0.003
0.002
0.001
0.000
24 hours
16 hours
8 hours
*
Control
Treated (100nM E)

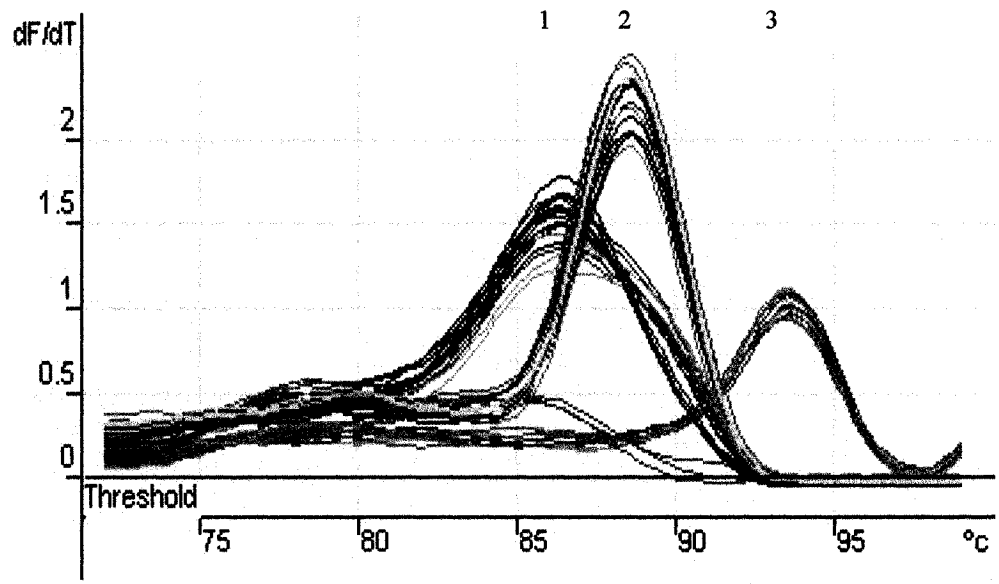
B


Norm. Fluoro.
Threshold
10^-4
0
10
20
30
Cycle

C


40 CT
R=0.99266 R2=0.98538 Efficiency=0.62
M=-0.210 B=1.767
30
20
10
0
10^-2
10^-1
10^0
10^1
Concentration

D


dF/dT
1
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°c

Discussion

Estrogen modulation of the 5-HT1A receptor

The main findings of this research project are that the human 5-HT1A promoter contains an estrogen-response element, 1AERE, that may negatively regulate gene transcription. This element resembles a perfect ERE, however there is a 5 base-pair spacer between the half sites, instead of the conventional 3 base pairs. The RN46A cells appear to express no or very little functional ER as assessed by the lack of estrogen induction of vgERE (Figure 3), thus transfection of additional receptor was necessary to see any effects of estrogen in these cells. Two nuclear complexes bound to the 1AERE, in both wild type RN46A cells, as well as in RN46A cells stably expressing ERβ RNA, although complex 1 was more abundant in RNERb cells. The binding of these complexes was not competed by a mutated form of the element, indicating that the mutation renders the ERE deficient in protein binding, and presumably incapable of mediating transcriptional modulation (Figure 10). The 1AERE on its own weakly repressed SV40 promoter activity only in RNERb cells (Figure 12). This trend towards repression was abolished with mutation of the element (1Amut), indicating that one or both of the protein complexes are responsible for mediating the repression. However since complex 1 is more abundant in RNERb cells, this complex may include the ERβ and mediate the estrogen-induced repression of 1A-ERE. In the context of the 5-HT1A promoter, when focusing on the –724luc construct, which contains the element, not much transcriptional effect was seen (Figure 11). The lack of estrogen repression in the 5-HT1A promoter construct likely reflects the complexity of the promoter and competing effects of multiple

elements that determine its activity. However, endogenous levels of 5-HT1A mRNA in RNERb cells show an increase in response to estrogen treatment (Figure 12), which may account for the small effect on the human 5-HT1A promoter. These experiments were conducted in rat cells, and it appears that the rat 5-HT1A receptor gene is regulated in a manner opposite to the human promoter in this model. This is somewhat consistent with the results of Wissink et al. (Wissink, van der Burg et al. 2001), who found that ERα mediated induction of short rat 5-HT1A receptor gene constructs via NF-κB sites, although ERβ was inactive. It is possible, then, that the rat RN46A cell model does not contain all of the factors required for estrogen-induced repression, or that they are not expressed to the required levels for a stronger effect of estrogen on the promoter. Furthermore, the presence of a consensus 1AERE in the human and mouse 5-HT1A sequences, but a mutated and probably inactive variant in the rat 5-HT1A gene (Fig. 9) suggests a lack of repression in the rat may be due to an inactive 1AERE.

It is likely that ERβ is involved in mediating the transcriptional effects of estrogen at the 1AERE, as the presence of the receptor was required for repression of SV-40 activity by estrogen. When this experiment was attempted in wild type RN46A cells not expressing ERβ, the repression was not observed (data not shown). However, because the nuclear complexes bound to the 1AERE in both wildtype and stable clones it suggests that ERβ may not be the primary factor binding to this element. Additionally, EMSA analysis of the 1AERE revealed two nuclear complexes (complex 1 and 2; Figure 9) could bind to the element, with complex 1 being less abundant in wild type than in stable cells. Neither complex is induced by estrogen, supporting the idea that these complexes are associated

with the element at all times, and may not require activation of the estrogen receptor. In this experimental paradigm, it is possible that enough of the ERβ in the nuclear extract was activated so that this effect was already maximal in the stable cells, thus addition of exogenous estrogen had no visible effect on the binding of complex 2. Further to this, in figure 4C, transfection of the ERβ seemed to increase basal activity of the vgERE, suggesting that the presence of ERβ may be promoting transcription. However, treatment with ICI 182,780 did not change the activity of the vgERE construct (Figure 5), suggesting that a ligand-independent mechanism of receptor activation may exist in this cell model.

Proposed mechanisms by which estrogen modulates 5-HT1A

ER can interact with Sp1 through Sp1$(N)_x$ERE or Sp1$(N)_x$ERE half sites, and both factors are required for transcriptional effects (Safe 2001). There are a number of Sp1 sites in the human 5-HT1A promoter, as found by a TRANSFAC transcription factor search (Bioinformatics 2001), one of which is proximal to the 1AERE, leading to the possibility that Sp1 remains constitutively bound to the element. Activation of ERβ, perhaps in a ligand-independent manner, may bring the receptor to the complex, thus allowing transcriptional repression to take place.

Alternatively, it is possible that activation of the ER functions to dislodge a gene regulatory complex that is constitutively bound to the 1AERE, suggesting a competitive mechanism for estrogen activity. If the associated complex is an activator, competition at the 1AERE site by ERβ would lead to a repression of gene expression. A good example

of this was identified with the orphan nuclear receptor Chicken ovalbumin upstream promoter-transcription factor (COUP-TF). This protein co-precipitated with the estrogen receptor in a ligand-independent manner, while retention of COUP-TF on an ERE separated the two. Further examination showed that COUP-TF bound as a dimer to ERE and ERE half-sites, but no nuclear receptors, including the ER, bound the element. Expression of COUP-TF lead to a concentration-dependent inhibition of estrogen-induced luciferase expression, suggesting that COUP-TF and ER were competing for direct DNA binding, resulting in regulation of transcription (Klinge, Silver et al. 1997). This would be consistent with the observation that in both non-ERβ expressing wild type RN46A cells, complex 2 is strongly bound to the 1AERE, while complex 1 is not very strong. In the RNERb9 cells, complex 1 and 2 are equally strong, suggesting that in these cells, perhaps ERβ is bound (complex 1) and competing away complex 2.

Studies have shown that ERα and ERβ are capable of mediating opposing effects on transcription, and can act as antagonistic transcriptional modulators. When the two receptors heterodimerise, this can result in the inhibition, and even reversal, of a transcriptional effect (Hall and McDonnell 1999). One example of this was seen in osteoblast cells, where co-expression of the two receptors diminished the capacity of each receptor alone to increase transcription. In the same study, it was shown that expression of SRC-1 preferentially enhanced the activation of ERβ, while SRC-2 had the same effect on ERα (Monroe, Johnsen et al. 2003). This demonstrates that the presence of either isoform of estrogen receptor, as well as the appropriate co-factors is important in determining what a specific response will be. This is likely to be pivotal in identifying an

estrogen response specific to the human 5-HT1A promoter, as the proper cellular context is obviously crucial. Furthermore, the interaction of the estrogen receptor with specific co-factors, such as SRC, can be ligand dependent or independent, and this also depends on the SRC isoform (Bai and Giguere 2003), yielding yet another mechanism by which estrogen function can be modulated depending on the species and cell type.

It has been shown that only the β isoform of the estrogen receptor is expressed in the raphe nuclei of the nonhuman primate and mouse (Alves, McEwen et al. 2000; Gundlah, Lu et al. 2001). In the rat brain, while ERβ is predominantly expressed in serotonergic neurons, some ERα is also present (Alves, Weiland et al. 1998; Bethea, Lu et al. 2002). Based on the knowledge that this differential distribution exists, and evidence that ERα and ERβ can modulate eachother, as well as have differential interactions with co-activators, it seems likely that transcriptional modulation of the rat promoter would be different from the human, and possibly murine 5-HT1A promoters.

One example of this may lie in the fact that the rat 5-HT1A gene is regulated by NF-κB and ERα to functionally activate transcription of the 5-HT1A receptor (Wissink, van der Burg et al. 2001). It is possible that in this rat cell model, this mechanism must compete with the repression of the 5-HT1A receptor mediated by ERβ. Although published reports show that ERα is not present in RN46A cells (Bethea, Lu et al. 2003), it is possible that over time, the cell line that has been propagated in this laboratory has different features to that which was maintained by Bethea *et al.* The RN46A cells in this laboratory lost expression of ERβ, and although it is doubtful that they regained ERα, it

is possible that enough NF-κB was present in the cells to activate transcription independent of the presence of ER.

Closer examination of the rat promoter sequence (Storring, Charest et al. 1999) reveals that the 1AERE is incomplete in the rat (Figure 9), suggesting that it may be nonfunctional, leaving no competition to the activation of the gene by NF-κB through ERα (Wissink, van der Burg et al. 2001). In the mouse, NF-κB has been shown to modulate the 5-HT1A receptor (Abdouh, Storring et al. 2001) and increased expression of the human 5-HT1A receptor occurs concurrently with upregulation of NF-κB, suggesting a role for this protein in transcriptional control (Cowen, Molinoff et al. 1997). However, no one has shown a direct interaction of NF-κB with the human or murine 5-HT1A promoter. Given the fact that some evidence exists to suggest that estrogens are negative modulators of NF-κB in the CNS of humans and mice (Evans, Eckert et al. 2001; Galea, Santizo et al. 2002), the 5-HT1A promoter may be regulated differently in the mouse and human. Specifically, in the DRN of nonhuman primates, it was found that the number of NF-κB-positive neurons was decreased in response to estrogen replacement, while progesterone, alone or in combination with estrogen, did not significantly affect levels of NF-κB (Bethea, Lu et al. 2002). Thus in primates, estrogen may reduce 5-HT1A transcription *in vivo* by decreasing NF-κB levels (Wissink, van der Burg et al. 2001). This repression is perhaps potentiated by negative regulation via the 1AERE, which is functional in the human 5-HT1A gene. Alternately the observed enhancement of rat 5-HT1A by ERα and NF-κB may be inhibited by ERβ-induced regulation via 1AERE in the mouse or human raphe, which express mainly ERβ but little

ERα. In summary, it seems likely that estrogen regulation of 5-HT1A expression has species-specificity that could depend on differences in ER subtype expression patterns, and ERE-like elements in the 5-HT1A promoter. In the rat, estrogen appears to induce or have little effect on 5-HT1A receptor expression, whereas in human and primate estrogen appear to repress the 5-HT1A receptor in raphe cells.

The physiological consequences of estrogen action on 5-HT1A

If estrogen represses the expression of 5-HT1A receptor, then this would indicate that the withdrawal of the hormone would result in more receptor being expressed in the raphe nuclei, and hence decrease neurotransmission to postsynaptic sites (Albert, Lembo et al. 1996; Pineyro and Blier 1999). In women, who are prone to depressive episodes during hormone fluctuation, this may be an important mechanism. Women with postpartum depression tend to suffer within the first three months after delivery. During pregnancy, estrogen levels are extremely high, particularly near the end of the pregnancy, which would lead to an increased repression of the 5-HT1A promoter, and would perhaps account for the euphoric state that many women experience while expecting. Postpartum estrogen levels drop within 5 days to prefollicular levels (Fredericks 2003), thus the repression of 5-HT1A would not be removed until this occurred. Once this repression is removed, the receptor levels should increase, likely within about 8 days, as the receptor half-life is 4 days. Thus, depressive symptoms, possibly due to overexpression of the 1A receptor would occur at this time. Women experiencing perimenopausal depression also experience a sustained loss of estrogen, likely leading to transcriptional changes in 5-HT1A receptor levels. However, mood tends to recover in these women following

menopause (perimenopause is defined as the onset of menopause, when estrogen levels are falling) (Soares, Almeida et al. 2001), suggesting that over time, some compensatory mechanisms exist to upregulate serotonin neurotransmission and elevate mood.

Depression can occur in the late luteal phase of the menstrual cycle, called PMDD, which is when the estrogen peak that triggered release of the ovum has subsided, and estrogen levels are low. However, it seems much more likely that in these women, a more rapid, nongenomic effect is taking place. PMDD responds rapidly to treatment, in fact, administration of SSRIs during the luteal phase of the menstrual cycle can alleviate symptoms (Halbreich and Kahn 2001), indicating that a chronic treatment, and thus genomic regulation of 5-HT1A, is not necessary.

Further study

A potential approach for determining the nature of estrogen-based transcriptional modulation that may exist for the 5-HT1A receptor would be to use an *in vivo* nonhuman primate and/or mouse model. Similar to the Bethea study, the animal could be ovariectomised and then treated with estrogen to determine effects on 5-HT1A mRNA levels by immunohistochemistry. Such a study should be complemented by evaluation of the functional protein levels as well. Additionally, the βERKO mouse may also be a useful model for study. Elimination of the ERβ, the predominant receptor in the DRN, may clarify whether the absence of estrogen modulation in the raphe nuclei exerts a significant effect on expression of the 5-HT1A receptor.

There is a variety of experimental evidence to implicate estrogen in depression, and the studies conducted here simply address one aspect of this. It seems evident that estrogen modulates the 5-HT1A receptor, however there may be several mechanisms over and above transcriptional regulation that take place *in vivo*. Based on the studies with PMDD, that are suggestive of a more rapid effect, there may be a non-genomic effect taking place in the human raphe which also modulates 5-HT1A receptor function. I described above how acute estrogen treatment appears to uncouple 5-HT1A from its G protein in particular regions of the rat brain, as measured by the binding of the receptor to [^{35}S]-GTPγS (Mize and Alper 2000). As a follow up to this, the same group found that 17-β-estradiol treatment induced PKA and PKC that resulted in the phosphorylation of 5-HT1A (Mize and Alper 2002). Concordantly, the basal and stimulated binding of [^{35}S]-GTP-γ-S was significantly decreased in the raphe of estrogen-treated macaques (Lu and Bethea 2002). Therefore it would be interesting to see if a similar mechanism exists to uncouple 5-HT1A rapidly in response to estrogen treatment in the primate. This could be done in a similar manner to the rat, with membrane preparations from the brain region of interest, in this case the DRN. PKC/PKA activation could also be examined *in vivo*, using western blotting to look for phosphorylation of the 5-HT1A receptor in response to acute estrogen treatment in ovariectomised animals, as well as looking for activated forms of the protein kinase.

There is also the potential for post-translational modulation of the 5-HT1A receptor mediated by estrogen. For example, recent research has shown that estrogen modulates the protein levels and activity of GRK2 in the frontal cortex of the rat brain (Ansonoff

and Etgen 2001). Expanding on this mechanism may suggest that modulation of GRKs, which phosphorylate activated receptors and recruit β-arrestin, leading to internalisation of the receptor (reviewed by Pitcher et al, 1998), will thus affect the activity of the 5-HT1A receptor in the raphe nuclei. Some study of GRK by Western blot was undertaken, however, this was done in wild type RN46A cells, which do not express a functional estrogen receptor. No change in GRK2, which is the predominant GRK isoform expressed in serotonergic neurons, and is known to modulate the function of 5-HT1B (Albert and Tiberi 2001), was observed (data not shown), however, it is possible that this mechanism could be important in RNERb clones.

Studies of the SERT-/- mouse show that there are specific regional and gender differences in the density and expression of the 5-HT1A autoreceptor, but no change in the G protein coupling. In the DRN, the density of receptors was significantly reduced, an effect that was potentiated in female mice. The hypothermic response to 8-OH-DPAT stimulation was greatly attenuated in male SERT-/- mice, and absent in females, while the 5-HT1A mRNA was significantly reduced in the raphe. This indirectly suggests that in mice, estrogen acts a repressor, consistent with the data extrapolated from the human 5-HT1A promoter (Li, Wichems et al. 2000). The firing activity of DRN in SERT -/- mice was examined, and it was noted that in both female and male knockout mice, the response of the neurons to 8-OH-DPAT stimulation was shifted to the right, indicating a desensitization of the 5-HT1A autoreceptors. This was significantly more pronounced in female knockout mice, consistent with the observations of Li et al (2000). There was no gender difference in response to 8-OH-DPAT in the wild type mice (Bouali, Evrard et al.

2002). This data supports the idea that dysregulation of the serotonergic system desensitises the 5-HT1A autoreceptor, perhaps augmenting the effects of estrogen on the 5-HT1A promoter, and explaining why women who are prone to depression are vulnerable to hormonal changes.

The positive effects of estrogen in the SERT knockout mouse model, which is a model of chronic SSRI treatment, would suggest that this hormone has the potential to augment the beneficial effects of antidepressant treatments, in particular, SSRI treatment. As explained previously, SSRIs work by desensitising the 5-HT1A receptor after chronic treatment with the drug. Knowing that estrogen has a genomic effect on the 5-HT1A receptor may enhance this effect, and perhaps accelerate the benefit. The studies in the SERT mouse show that female knockouts show a greater desensitisation of 5-HT1A, which is probably due to the presence of estrogen. Thus it is likely that co-administration of estrogen with SSRIs would be beneficial in some women. This is supported by a number of studies that have seen that estrogen in conjunction with antidepressants can have positive effect (Schneider, Small et al. 1997; Kornstein, Schatzberg et al. 2000; Schneider, Small et al. 2001; Young and Korszun 2002).

It would be interesting to conduct further examination of transcriptional regulation of 5-HT1A in mouse and human models, instead of in rats, which may give a clearer picture of what is taking place. The ERE identified in the human promoter is conserved in the mouse and thus, may be functional, unlike the 1AERE in the rat, whose first half-site is not conserved. It is also more likely that mouse cell lines, such as the SN-48 cells, which

express 5-HT1A (Charest, Wainer et al. 1993) would maintain the proper environment for regulation of the promoter region, in comparison to rat cells, which appear to modulate 5-HT1A differently via estrogen. Not many studies have focused on this receptor in mouse models, most work has been done in rats. However, it is interesting that in the ERβ knockout mouse, increases in 5-HT1A receptor in the amygdala were observed, suggesting that a repression of this receptor had been removed (Krezel, Dupont et al. 2001). There was a study conducted that shows opposite effects on the 5-HT1A receptor to what is being proposed in this thesis: the hypothermic effect mediated by 8-OH-DPAT was pronounced in female than in male mice. In addition, ovariectomy attenuated this observation, while estrogen replacement enhanced the hypothermic response and simultaneous testosterone blocked this increase (Matsuda, Nakano et al. 1991). Given that the hypothermic response is due to the presynaptic 5-HT1A receptor, and an increased hypothermia suggests increased receptor functioning, this is not what would be expected. This is also not what was seen in the SERT-/- mice, which further supports the idea that some dysregulation of the serotonergic system is involved in the interaction of estrogen and 5-HT1A. As mentioned, in wild type mice, no gender difference in DPAT-induced hypothermia was observed (Bouali, Evrard et al. 2002).

Some evidence exists to suggest testosterone acts as a regulator of 5-HT1A function. Differences exist in terms of vulnerability to depressive episodes between men and women, and these have traditionally been attributed to estrogen, as levels of this hormone are significantly elevated in women, and fluctuate in accordance with particular depressive syndromes. However, testosterone is also significantly elevated in men as

compared to women, and this may influence the function of the 5-HT1A receptor as well. A study conducted in tree shrews found that testosterone replacement in shrews subjected to chronic stress for 28 days kept 5-HT1A receptors in the hippocampus and occipital cortex at normal levels as compared to animals who were not given testosterone, and had increased 5-HT1A levels (Flugge, Kramer et al. 1998). This suggests that testosterone may also have a repressive effect on the receptor, which may help explain why men are less prone to depression that women.

A mouse model would be useful for examining how *in vivo* regulation of the 5-HT1A receptor takes place. As has been mentioned, study in the mouse is limited. To further characterize this, it would be desirable to examine both mRNA and protein levels of the 5-HT1A receptor in ovariectomised mice treated acutely and chronically with estrogen. The binding potential of the receptor could also be assessed, and it would be important to examine levels of the G protein subunits that couple to 5-HT1A, in a similar manner to what has been done in nonhuman primates. By doing this, it would provide the opportunity to establish the best animal model of steroid hormone effects on the 5-HT1A receptor so that the data may be extrapolated to humans.

In addition to this, much benefit would be derived from PET studies in the human brain designed to determine what effect gonadal hormones have on 5-HT1A receptor expression levels. PET studies have been somewhat inconclusive, with one study indicating that 5-HT1A levels were increased slightly in women, and another showing no difference in binding potential between gender, but a difference in age-related changes

between genders (Meltzer, Drevets et al. 2001; Parsey, Oquendo et al. 2002). PET studies across different stages of the menstrual cycle are an important undertaking and would determine if the binding potential of the 5-HT1A receptor changes with hormone fluctuation. It would also be interesting to compare 5-HT1A binding in women at different stages in their live, i.e. prepubertal, adult (pregnant, nonpregnant and postpartum), perimenopausal, and postmenopausal in order to evaluate the impact that these hormonal events have on 5-HT1A binding potential. Should such studies be conducted with comparison of depressed-and non-depressed patients, it would certainly clarify the importance of the 5-HT1A receptor in depression associated with major hormonal events. It may also provide insight into whether hormonal events trigger dysregulation of the serotonergic system, or whether the opposite is true, and it is fluctuation in serotonergic neurotransmission that amplifies emotional reaction to reproductive change.

Conclusion

Based on the above studies, it seems possible that estrogen represses expression of the human 5-HT1A receptor. Although the model system used in these experiments was not ideal (rat cells vs. human gene), the effect of the element alone appeared to be significant, however in the context of the entire 5-HT1A promoter, there was no significant repression of the 5-HT1A promoter. However, evidence suggests that regulation of this receptor by hormones may be quite different in rats as compared to humans, thus it would be much more beneficial to examine this response in a more physiologically-relevant model. This in turn may lead to a significant discovery about the regulation of the 5-HT1A receptor by estrogen, and in turn, the influence of this steroid on depression. Such knowledge would be instrumental in advancing the efficacy of antidepressant treatment in women, as well as in the control of hormone-shift induced episodes of depression, such as PPD, PMD and PMDD.

With further study and a focus on this novel element identified in the 5-HT1A promoter, it may be possible to identify polymorphisms in this area that affect binding of the transcriptional machinery, and in turn create a vulnerability to hormonal shifts. This could be used to identify risk, take preventative measures and design appropriate treatment paradigms. As a result of this research, there is the potential for benefit to 66% of the patients affected by depression, and to society as a whole.

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