Altered serotonin regulation in genetic and post-stroke models of anxiety and depression

Faranak Vahid-Ansari

This thesis is submitted as a partial fulfillment of the Ph.D. program in Neuroscience

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

AUTHORIZATION

All previously published content presented herein was obtained from articles where Faranak Vahid-Ansari was the primary author with the exception of figures 1, 2, 3, 4 in chapter 1: general introduction, obtained from the cited publications with the permission from correspondence authors.

ABSTRACT

Major depression is a complex disease involving genetic and environmental factors. Previous studies suggest that functional genetic polymorphisms that alter the serotonin (5-HT) system in combination with psychosocial stress can synergistically increase the strength of these associations. In addition, depression is associated with several neurological disorders involving neuronal injury, including stroke (i.e. post-stroke depression, PSD). Both forms of depression are treated with 5-HT-selective antidepressants like fluoxetine, but remission rates do not exceed 50%. Evidence showed that alterations affecting the 5-HT system, directly or indirectly, lead to anxiety or depression phenotypes and may elucidate determinants of response to antidepressants. To better understand common and unique alterations in both genetic- and injury-related depression, I have generated and investigated two novel mouse models that exemplify a serotonin-related genetic risk (Flx-Freud-1 mice) and an injury model (ischemic lesion), to identify similarities and differences in their behavioral phenotypes, and in response to fluoxetine treatment. In the Flx-Freud-1 mouse model, 5-HT neuron-specific adult knockout of Freud-1, a key repressor of the 5-HT1A receptor gene, led to overexpression of 5-HT1A autoreceptors thought to negatively regulate the 5-HT system. These mice showed increased 5-HT1A autoreceptor responses, reduced 5-HT levels and a robust anxiety and depression phenotype that was resistant to chronic fluoxetine treatment. These behaviors were dependent on increased 5-HT1A autoreceptors since they were not seen in mice lacking 5-HT1A autoreceptors in adult Freud-1 knockout background. Instead an opposite anti-depressed phenotype emerged, suggesting that Freud-1 might have additional functions in 5-HT cells. In the PSD model, the vasoconstrictor endothelin-1 was injected to induce transient ischemia in the left medial prefrontal cortex,

part of the circuitry thought to be damaged in PSD in humans. This stroke resulted in a persistent anxiety, depression and cognitive impairment. Chronic fluoxetine treatment alone or combined with voluntary exercise was effective to reverse the behavioral and cognitive phenotypes in this PSD mouse model The results of genetic and SSRI treated stroke models show that changes in 5-HT system contribute to widespread dysregulation of the neuronal circuitry implicated in depression, anxiety. Genetic alteration of the 5-HT system conferred fluoxetine-resistance, while cortical stroke which indirectly altered the 5-HT system remained responsive to fluoxetine. Following unilateral stroke, there was increased activity of the contralateral hemisphere, including the prefrontal cortex and limbic areas involved in anxiety and depression, and activation of the 5-HT system. Effective treatment with chronic fluoxetine alone or combined with exercise significantly reduced and balanced the contralesional neuronal activation in affected regions that correlated with improvements in phenotypes.

In conclusion, this work implicates genetic changes that directly alter the 5-HT system in resistance to chronic fluoxetine treatment. Therefore, the Flx-Freud-1-induced 5-HT1A autoreceptor overexpression mouse model may provide a useful pre-clinical model of antidepressant resistance. In contrast, in the PSD model, in which expression of 5-HT1A autoreceptors remained intact, chronic fluoxetine treatment reversed depression and anxiety phenotypes. This model may provide insight into changes in neuronal activity that allows antidepressants to mediate behavioral and cognitive improvement.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1: General Introduction

Chapter 3: Persistent post-stroke depression in mice following unilateral medial prefrontal cortical stroke

Chapter 4: Chronic fluoxetine but not exercise, induces behavioural recovery and balanced corticolimbic activity following unilateral ischemic lesion

LIST OF FIGURES

CHAPTER 1: General Introduction

CHAPTER 2: Abrogated Freud-1/CC2D1A repression of 5-HT1A autoreceptors induces antidepressant-resistant anxiety- and depression-like behavior

CHAPTER 3: Persistent post-stroke depression in mice following unilateral medial prefrontal cortical stroke

Chapter 4: Chronic fluoxetine but not exercise, induces behavioural recovery and balanced corticolimbic activity following unilateral ischemic lesion

LIST OF ABBREVIATIONS

+/+	containing no copies of the floxed gene
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin; 5-hydroxytryptamine
5-HT1A	serotonin 1A receptor
5-HTP	5-hydroxytryptophan
5-HTT	serotonin transporter
8-OH-DPAT	8-hydroxy-N,N-dipropyl-2-aminotetralin
^{125}I-MPPI	4-(2'-Methoxy-phenyl)-1-[2'-(n-2''-pyridinyl)-p-iodobenzamido]-ethyl-piperazine
Amyg	amygdala
ANOVA	analysis of variance
AP	anterior-posterior
BBK	beam break
BLA	basolateral amygdala
BM	Barnes maze
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CC2D1A	coiled-coil and C2 domain containing 1A
cF1ko	Freud-1conditional knockout
cF1/1A dko	Freud-/5HT1A conditional knockout
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CE	central amygdala
CG	cingulate cortex
CGPR	calcitonin gene-related peptide
CMS	chronic mild stress
CUMS	chronic unpredictable mild stress
CV	cresyl violet
CP	cortical plate
Cyl	cylinder test
DA	dopamine
DAT	dopamine transporter
DBS	deep brain stimulation
DRD	dorsal raphe dorsal
DG	dentate gyrus
Deaf1	deformed epidermal autoregulatory factor 1
DM14	*Drosophila melanogaster* 14
DRE	dual repressor element
DRN	dorsal raphe nucleus
DV	dorsoventral
E12	embryonic day 12
EPM	elevated plus maze
ER	estrogen receptor

ET-1	endothelin-1
F1wt	Freud-1 wild type
FLX	fluoxetine
flx	floxed; flanked by loxP sites
flx/flx	containing two copies of the floxed gene
Freud-1	five-prime repressor element under dual repression 1
FST	forced swim test
G	protein guanosine nucleotide-binding protein
GABA	gamma-Aminobutyric acid
GAD 67	Anti-Glutamic Acid Decarboxylase 67
GFAP	Glial fibrillary acidic protein
GFP	green fluorescent protein
Gi	G protein inhibitory
Go	G protein other
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GR/MR	glucocorticoid receptors
Gs	G protein stimulatory
Hes5	hairy/enhancer of split 5
HLH	helix-loop-helix
HPLC	high performance liquid chromatography
IBa1	ionized calcium binding adaptor molecule 1
IL	infra-limbic
i.p.	intraperitoneal
K^+	potassium
KO	knockout
LD	light dark test
LDR	lateral dorsal raphe
LFL	left forelimb
LHL	left hind limb
LHbl	lateral habenular nucleus
LSN	lateral septal nucleus
MAOI	monoamine oxidase inhibitor
MCAO	medial cerebral artery occlusion
mPFC	medial prefrontal cortex
MDD	major depressive disorder
M	mice
ML	medial–lateral
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
MnR	median raphe
mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
NE	norepinephrine
NeuN	Neuronal Nuclei
NSF	novelty suppressed feeding

ns	not significant
MWM	Morris water maze test
nGRE	negative glucocorticoid response element
NRE	NFkB response element
NSID	non-syndromic intellectual disability
NSMR	non-syndromic mental retardation
NUDR	nuclear Deaf1 related
OE	over-expression
OF	open field
OR	object recognition
P3	postnatal day 3
PET	positron emission tomography
PL	pre-limbic
PN	post-natal
p.o.	per oral
PSD	post stroke depression
PVN	paraventricular nucleus of hypothalamus
R	Rat
RE-1	REST element binding site
REST	repressor element 1-silencing transcription factor
RFL	right forelimb
RHL	right hind limb
RW	running wheel
s.c.	subcutaneous
S.E.M.	standard error of the mean
SERT	serotonin transporter
sham ctrl	sham control
SPT	sucrose preference test
siRNA	small interfering ribonucleic acid
SSRI	selective serotonin reuptake inhibitor
SVZ	subventricular zone
TAM	tamoxifen
TCA	tricyclic antidepressant
TPH	tryptophan hydroxylase
TRD	treatment resistant depression
TS	tail suspension
Veh	vehicle
VDR	ventral dorsal raphe
VGLuT	vesicular glutamate transporter
VLDR	ventral lateral dorsal raphe
WHO	World Health Organization
WT	wildtype

ACKNOWLEDGMENTS

"*You need to be aware of what others are doing, applaud their efforts, acknowledge them in their pursuits. When we all help one another, everybody wins" –Jim Stovall*

I would like firstly to thank Dr. Paul R. Albert, who has been the primary contributor to my evolution as a researcher. Dr. Albert has taught me to rationalize my thoughts unbiased and be persistent in science. Additionally, Dr. Albert's mentorship attitude has built up an environment where people mutually trust and enjoy while following their research interest. I am greatly thankful for that because these positive impacts revolutionized my life and my personality. Just must say "Thank you, Dr. Albert!"

I've been pleased of sharing my thoughts and results with several individuals from the past and current in the Albert laboratory and indeed I extend my gratitude for the help, advice from our own lab manager, Ms. Mireille Daigle.

I thank my thesis advisory committee members Dr. Ruth Slack, Dr. Diane Lagace and Dr. Richard Bergeron for their guidance through several educative discussion sessions in the direction of my project.

To achieve my goals during past years, the friendship and support of neuroscience members of Ottawa University were invaluable. I would like to express many thanks to Dr. Anthony Hakim and Dr. Diane Lagace who kindly provided me great references. Their supports inspired and motivated me more to be my best.

I am also grateful for the scholarship provided by the U of Ottawa (for 4 years) and trainee award offered by Canadian Partnership for Stroke Recovery (CPSR; for 2 years).

Lastly but foremost, I recall my appreciation and gratitude to my loved family members who were long-standing with me and honored my time mostly devoted to my studies instead to them. Just must say "Thank you to all".

Chapter 1

General Introduction

Major depression is predicted to have one of the highest global burdens of disease by 2030 (Kessler, 2003; Kessler and Bromet, 2013; McGrath et al. 2016). It currently has a lifetime prevalence of approximately 10–15% and is more frequent in women than in men (Branchi et al., 2013; Celada et al., 2013; Lam et al., 2012). Depression is a chronic disorder that is often comorbid with anxiety (Hyman, 2000). It is diagnosed by psychiatric criteria with diverse and sometimes opposite symptoms, leading to imprecise diagnostic categories and ineffective medication. Since 15% of depressed patients are suicidal, this lack of treatment is associated with an increased likelihood of suicide attempt or completion (Krishnan and Nestler, 2008). Unraveling the pathophysiology of depression is a complex challenge. Not only are syndromes heterogeneous and their etiologies diverse, but important symptoms such as guilt and suicidality cannot be reproduced in animal models. Nevertheless, other symptoms like anxiety or behavioral despair have been modeled in animals, and these, together with clinical data, are providing insight into the neurobiology of mood disorders. Recent studies combining behavioral, molecular and electrophysiological techniques in transgenic mice have revealed that activation or inactivation of specific subpopulations of neurons to alter neural circuit function results in anxiety- or depression-like behaviors. Understanding the underlying causes of these neuronal changes might offer a crucial new direction for the development of novel treatments for mood disorders in humans.

It is generally recognized that both genetic and environmental factors can synergistically contribute to predisposition of individuals to mental illness (Caspi and Moffitt, 2006). For example, both genetic polymorphisms of serotonin transporter (e.g., 5-HTT LPR) and adverse environment (early life stress) have been shown to associate

with major depression (Caspi et al., 2003, 2010), and this association is greatly strengthened if both risk factors are present.

In addition, several neurological conditions like stroke, Parkinson's disease, Alzheimer's disease and epilepsy are associated with increased prevalence of major depression by 20-50% (Evans et al., 2005) and are thought to result from changes in neuronal function. However, some of these disorders like stroke display acute neuronal loss, while major depression is a more chronic condition. In some cases, such as in Parkinsonian patients, depression can even start before motor symptoms arise (Evans et al., 2005). Conditions like stroke can contribute to predisposition and trigger episodes of depression (Hyman, 2000). Accordingly, because several lines of evidence discussed below implicate the serotonin (5-HT) system in mood disorders, I hypothesized that genetic alterations to suppress raphe 5-HT activity would lead to depression/anxiety resistant to chronic SSRI treatment; while brain injury associated with depression/anxiety will respond to chronic SSRI treatment. Thus, I characterized and compared two mouse models of anxiety and depression: a genetic depression model (TPH2-Cre/Flx-CC2D1A mice) and an injury model of post-stroke depression, to determine whether similar changes in the 5-HT system are occurring and affect the response to antidepressant treatment.

I. Monoamine hypothesis of depression

Original clinical observations on the effects of imipramine and related tricyclic compounds that were tested as neuroleptics showed that this medication reduces depressive symptoms. Imipramine, the first antidepressant (Lehmann et al., 1958), is a

tricyclic antidepressant (TCA) that inhibits the serotonin transporter (SERT) and norepinephrine transporter (NET) resulting in a longer half-life of the transmitter in the synaptic cleft (Pletscher, 1991). These and other observations led to hypothesize that reduced activity of monoamine systems, like NE and/or 5-HT, is associated with depression (Schildkraut, 1965; Coppen, 1967).

Acute tryptophan, a precursor of 5-HT, depletion studies also support a role for decreased 5-HT in depression, or at least in relapse of recovered depressed patients (Barnes and Sharp, 1999; Young and Leyton, 2002; Jans et al., 2007; Lanfumey et al., 2008). 5-HT and its metabolites are reduced in CSF of depressed patients and suicide victims. The reductions in 5-HT metabolism (Brown et al., 1982) and changes in 5-HT receptor levels are also seen by PET in living depressed patients and in post-mortem studies. These results suggest a reduced activity of the 5-HT system in major depression (Hesselgrave and Parsey, 2013; Savitz and Drevets, 2009). In agreement with the role of 5-HT, current first-line therapy for major depression uses antidepressant compounds that target the monoamine system, particularly selective 5-HT reuptake inhibitors (SSRIs), such as fluoxetine or citalopram that increase 5-HT neurotransmission. However, the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study showed that only one-third of the patients given citalopram achieved remission and that about 10–15% more responded to combination therapy (Trivedi et al., 2006). These data emphasize the need for improved depression models to optimize the pharmacological treatments and/or augmentation strategies for depression.

Several lines of evidence also implicate decreased 5-HT activity in anxiety but its importance in the etiology and severity of this disorder remains unclear (Millan, 2004;

Wong et al., 2005; Jans et al., 2007). Anxiety and depression are often co-morbid in humans and share several symptoms including disturbances in sleep, feelings of fatigue, difficulties with concentration, and differences in arousal. Like depression, anxiety is associated with environmental stress (aan het Rot et al., 2009; Booij et al., 2010). While anxiety is the immediate defensive response to an acute threat, depression could be a mechanism in response to a chronic threat- or sustained stress-induced fear.

As a further indication of the potential role of the serotonin system in these disorders, genetic polymorphisms in 5-HT related genes (e.g., 5-HTT, 5-HT1A receptor) have been associated with depression and anxiety, but these associations are weak and not always reproducible, suggesting that 5-HT may be a predisposing factor rather than a cause of depression or anxiety (Karg et al., 2011; Kishi et al., 2013).

While SSRIs are currently the first-line of treatment for depression and anxiety disorders, some anxiety disorders respond relatively well to acute antidepressant treatment while others remain only partially responsive to chronic treatment (Blanco et al., 2003; Walsh and McDougle, 2004). Although the pharmacological effect of these drugs to block 5-HT reuptake in the brain is rapid, there is a therapeutic delay of weeks to months before their anti-depressant/anxiety activity is manifest. This delay might reflect neuro-adaptive changes in pre- and postsynaptic cells, including long-term changes in gene expression and protein translation (Krishnan and Nestler, 2008). The role of 5-HT1A autoreceptors in controlling the serotonergic tone has led to the hypothesis that these receptors delay the effects of SSRIs and other drugs, which cause increases in 5-HT levels. Specifically, 5-HT1A autoreceptors exert negative feedback inhibition in response to increased levels of 5-HT in the raphe nuclei. Blier et al (1998) suggested that

the time required for 5-HT1A autoreceptor desensitization might be responsible for the delayed onset of therapeutic efficacy of these drugs.

1. The brain serotonin system: role of 5-HT1A receptor signaling

The brain serotonin system consists of a small group of neurons located in the raphe nuclei of the midbrain that are unique because they express the rate-limiting enzyme for serotonin synthesis, tryptophan hydroxylase-2 (TPH2), the brain specific isoform (Walther et al., 2003; Lenicov et al., 2007). The differentiation of neuronal progenitors to express serotonergic markers like TPH2 is driven by the transcription factor Pet-1, which is expressed only in 5-HT neurons of the brain and directly activates the TPH2 gene (Hendricks et al., 2003). 5-HT neurons of the rostral raphe nuclei, including the dorsal and median raphe nuclei, project widely throughout the brain to innervate key brain regions involved in anxiety and depression. Activation of serotonin neurons induces the release of serotonin at target neurons and within the raphe; via collateral branches (Kocsis et al., 2006; Bang et al., 2012), and is rapidly removed by the 5-HT transporter, the target of SSRI antidepressants.

In addition to its role in the regulation of mood and social behaviors, the 5-HT system also plays important roles in complex physiological functions including temperature regulation, appetite, and libido. The actions of 5-HT on these diverse functions are mediated by a large family of at least 15 distinct receptor subtypes (Hoyer et al., 2002). Although the relative contributions of individual receptor subtypes to the serotonergic regulation of mood are incompletely understood, attention has focused on the 5-HT1A receptor ~~subtype~~, one of the most abundant and widely expressed 5-HT receptors in the brain (Barnes and Sharp, 1999). 5-HT1A receptors are seven-

transmembrane-domain G-protein-coupled receptors (GPCR) that signal through Giα and Goα proteins to inhibit adenylyl cyclase, activate inward rectifying potassium channels, and inhibit voltage-gated calcium channels to reduce neuronal firing (Barnes and Sharp, 1999; Albert and Tiberi, 2001) and neurotransmitter secretion (Albert and Lemonde, 2004; Lanfumey and Hamon, 2004). Unlike most 5-HT receptors, the 5-HT1A receptor is expressed both as a presynaptic autoreceptor on the cell body and dendrites of raphe 5-HT neurons, and as a post-synaptic heteroreceptor on non-serotonergic neurons targeted by the 5-HT system. Serotonin released in the raphe activates 5-HT1A autoreceptors, which negatively regulates the firing of the serotonin system. Release of serotonin at target neurons activates 5-HT heteroreceptors including the 5-HT1A heteroreceptor which is abundantly expressed in the hippocampus, septum, amygdala, and PFC (Albert et al., 1990) where it mediates serotonin actions on fear, anxiety, stress, and cognitive function (Barnes and Sharp, 1999; Gross and Hen, 2004; Savitz et al., 2009; Meltzer et al., 2012; Donaldson et al., 2013; Garcia-Garcia et al., 2014). Thus, 5-HT1A receptors have the dual ability to modulate ~~both~~ global serotonin levels and to mediate local responses to released serotonin. The relatively selective 5-HT1A autoreceptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and antagonist WAY 100635 (Heisler et al., 1998) have been used as pharmacological probes of 5-HT1A receptor function. While 8-OH-DPAT is selective for 5-HT1A receptors, it can also activate 5-HT7 receptors with 10-fold lower affinity; while WAY100635 is highly specific to block 5-HT1A receptors. Systemic administration of 8-OH-DPAT produces acute hyperphagia, hypothermia, and an anxiolytic-like effect in rodents (Lo Iacono et al., 1998). The behavioral and physiological effects of 8-OH-DPAT are blocked by pretreatment with 5-

HT1A antagonist, WAY 100635. In clinical trials, 5-HT1A partial agonists, such as buspirone, are currently used as anxiolytics (Vinkers et al., 2010), and 5-HT1A receptor partial agonists and antagonists are reported to enhance the therapeutic effects of antidepressants (Blier et al., 2003).

In the serotonin system, 5-HT1A receptor functions as a major inhibitory receptor, while there are complementary roles for other stimulatory 5-HT receptors, such as 5-HT2, 5-HT3, 5-HT4, and 5-HT7 receptors (Beique et al., 2004). For example, the other major 5-HT receptor (particularly in cortex) is the Gq-coupled 5-HT2A receptor, which mediates excitatory actions of serotonin on target neurons (Celada et al., 2004; Puig and Gulledge, 2011; Llado-Pelfort et al., 2012). Thus, in terms of neural circuitry, the 5-HT1A receptor is inhibitory, while the 5-HT2A receptor is stimulatory. However, these receptors can also couple to protein kinase pathways in a cell-specific manner that may stimulate gene transcription and indirectly enhance neuronal function in the long term (Kushwaha and Albert, 2005; Mogha et al., 2012).

2. Functional subsets of 5-HT neurons: Afferent and Efferent connections

The midbrain raphe complex includes serotonergic systems located within the DR, the median raphe nucleus, caudal linear nucleus, pontomesencephalic reticular formation, supralemniscal cell group, and interpeduncular nucleus (Hale et al., 2012). Here I will summarize the organization of the DR. The DR is topographically organized and divided into sub-regions including the rostral, dorsal, ventral, ventrolateral, interfascicular, and caudal portions (Lowry, 2002; Lowry et al., 2005, 2008; Hale and Lowry, 2011; Hale et al., 2012). The major subdivisions related to this study will be described here.

2-1. Projections of the dorsal raphe nucleus (DRN)

The DR is located at -4.24 to -4.96 from Bregma in the mouse brain (Franklin and Paxinos, 2008). The dorsal part of DR (DRD) projects to forebrain regions associated with control of emotional behaviors like the central amygdala (CE), Basolateral amygdaloid (BLA), bed nucleus of stria terminalis (BNST), accumbennucleuss (NAc), medial prefrontal cortex (mPFC) and dorsal hypothalamus (Van Bockstaele et al., 1993; Commons et al., 2003; Hale et al., 2008). The DRD also receives the projections from several regions including the infralimbic cortices, BNST and different parts of hypothalamic nuclei (Peyron et al., 2008; Luo et al., 2015). The ventral part of the DR projects to sensorimotor, ventrolateral orbital, frontal, motor, and visual cortices and the caudate putamen (Steinbusch et al., 1980; Steinbusch, 1981; Waterhouse et al., 1986; Kazakov et al., 1993); while it receives projections from the cingulate and lateral orbital cortices, CE, and dorsomedial hypothalamic nucleus, with less dense projections from other amygdaloid nuclei and cortex (Peyron et al., 1998). The ventrolateral DR gives rise to projections involved in visual function including the superior colliculus and lateral geniculate nucleus (O'Hearn and Molliver, 1984; Waterhouse et al., 1993) while receive projections from the amygdala, with heavy innervation by the CE and moderate innervation by the dorsolateral medial amygdala with additional projections from the ventromedial prefrontal cortex, hypothalamus, and the retina (Hurley et al., 1991; Shen and Semba, 1994; Lee et al., 2003, 2007). In fact, these are limbic brain areas involved in the circuitry underlying mood and emotion and these massive afferent and efferent connections between DR and these forebrain regions suggests that the activity of DR is

important to modulate their functions (Dorocic et al., 2014; Weissbourd et al., 2014; Ogawa et al., 2014).

2-2. Neuron types in the DRN

The DR is a highly-conserved brain structure in vertebrates located ventral to the aqueduct. The largest population of 5-HT neurons (~9000 in mouse brain) in DR provides up to two-thirds of the 5-HT in the forebrain. The 5-HT neurons in DR are heterogeneous in cell morphology, neurochemical markers, projection sites, functional preference and electrophysiological properties (Abrams et al. 2004; Calizo et al. 2011; Bang et al. 2012). In addition to 5-HT, glutamate is released by many DRN projection neurons. Many DRN neurons express the vesicular glutamate transporter 3 (VGluT3) but not VGluT1 and VGluT2 (Gras et al., 2002). Approximately two-thirds of 5-HT neurons, especially those located along the midline express VGluT3. A small subset of VGluT3 positive neurons in dorsal DRN is not co-expressed in 5-HT cells while at the lateral sides of DR, the 5-HT cells lack VGluT3 expression. VGluT3/5-HTergic fibers mainly project to the cerebral cortex, lateral septum, hippocampus, and olfactory bulb while VGluT3 fibers alone are present in VTA/substantial nigra compacta (SNc), multiple hypothalamic and thalamic areas, preoptic area, ventral palladium, hippocampus, and medial septum. The inputs toVGluT3/5-HT cells have not been fully mapped yet (Luo et al, 2015).

Recent trans-synaptic tracing studies reveal that DR GABA neurons also share a largely similar input pattern with 5-HT neurons (Dorocic et al. 2014; Weissbourd et al. 2014). GABAergic neurons are mainly located in the lateral DRN while co-expressing only in 5-HT cells. GABA cells mediate feedback inhibition in the local 5-HT network in

DR and also project to the forebrain (Bang et al., 2012). The GABA neurons in DR receive projections from the central amygdala and BNST.

A small subset of DRN neurons (~1000 in mice) expresses the dopamine markers like tyrosine hydroxylase and the dopamine transporter (DAT) in the rostral-dorsal part of DR (Flores et al. 2004). These neurons are non-5-HTergic and form a separate neuron population (Hioki et al. 2010). They are an extension of A10 dopamine system and resemble VTA dopamine neurons.

Figure 1 schematically illustrates the different neuron types in DRN and connectivity of 5-HT neurons obtained from Luo et al, 2015.

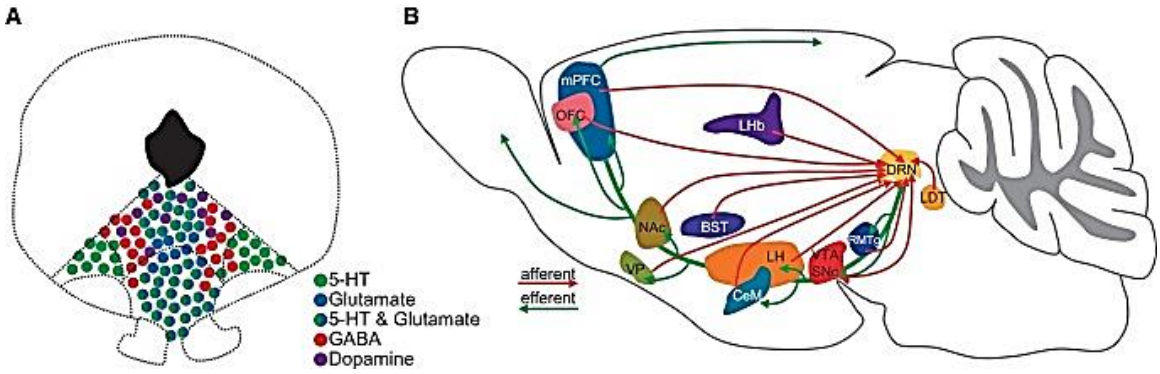


Figure 1. Neuron types in the DRN and the connectivity of 5-HT neurons.

(A) A cartoon shows the distribution of 5-HT, glutamate, GABA and dopamine neurons in the DRN. Note that some neurons express markers for both 5-HT and glutamate.

(B) Input and output patterns of DRN 5-HT neurons. (mPFC) medial prefrontal cortex, (OFC) orbitofrontal cortex, (VP) ventral pallidum, (NAc) nucleus Accumbens, (LHb) lateral Habenula, (BST) bed nucleus of the stria terminalis, (CeM) central amygdala, (LH) lateral hypothalamus, (DRN) dorsal raphe nucleus, (VTA) ventral tegmental area, (SNc) substantia nigra pars compacta, (RMTg) rostromedial tegmental nucleus, and (LDT) laterodorsal tegmental nucleus (Luo et al., 2015).

Although 5-HT plays a major role in raphe function, glutamate, GABA, DA and other subtypes also communicate with other brain regions to modify behavior, and form an intra-raphe network to regulate 5-HT neuron firing.

2-3. Distribution of 5-HT synaptic contacts and triads in the mouse brain

Recently, a quantitative method using the combination of confocal microscopy with high resolution and 3D reconstruction of 5-HTT-positive axons was used to map the 5-HT boutons located to excitatory/inhibitory synapses specifically in limbic brain regions.

Post-synaptic components of excitatory/inhibitory synapses form "triads". Briefly, asymmetrical synapses/excitatory triads were found in hippocampus, cortex, mPFC while symmetrical synapse/inhibitory triads were mainly located in DR, VTA, CeA, BLA. The combination of both (excitatory-inhibitory) was observed in areas like thalamic regions, BNST and NAc (Belmer et al., 2016, 2017). The preferential proximity of 5-HT boutons to neurochemical excitatory/inhibitory synapses could therefore suggest that serotonergic axons projecting to one area may preferentially target local glutamatergic, interneurons or both to modulate their activity. For example, the preferential proximity of 5-HT-positive axon terminals to GABA terminals engaged in symmetrical synapses in DR and amygdala sub-regions (CeA, BLA) suggests that 5-HT mainly modulates the activity of interneurons in DR and amygdala. In contrast, 5-HT axon terminals are mainly engaged in asymmetrical synapses in mPFC to modulate the activity of excitatory neurons. Therefore, alterations in 5-HT system activity which preferentially change the activity of different cell types in target brain regions could differentially impact behavioral output.

Figure 2, obtained from a review published by Belmer et al. (2016) illustrates the distribution of 5-HT synaptic contacts and triads in the mouse brain.

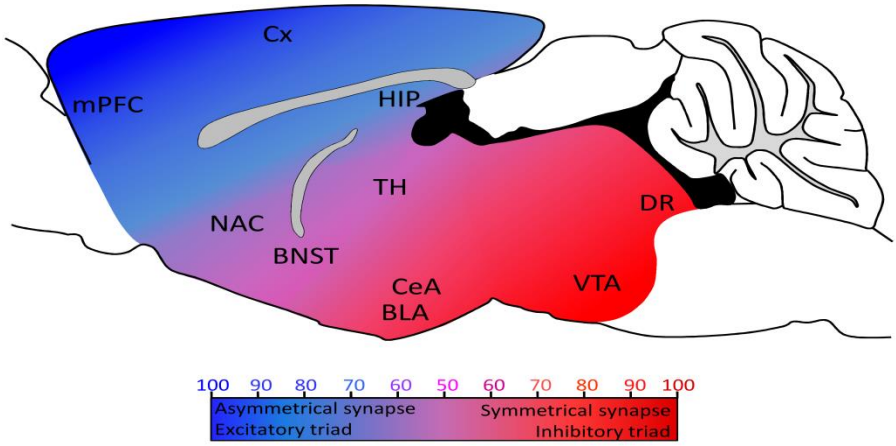


Figure 2. Distribution of 5-HT synaptic contacts and triads in the rodent brain.

Cx: cortex; mPFC: medial prefrontal cortex; NAC: nucleus accumbens; BNST: bed nucleus of the stria terminalis; TH: thalamus; CeA: central nucleus of the amygdala; BLA: the basolateral nucleus of the amygdala; VTA: ventral tegmental area; DR: dorsal raphe.

Color coded from blue to red represents the heterogeneous distribution of 5-HT axons connectivity (blue: asymmetrical synapses/excitatory triads; red: symmetrical synapse/inhibitory triads (Belmer et al., 2016).

3. Serotonin-PFC circuits in depression and anxiety

3-1. The role of the 5-HT1A receptor in depression and anxiety

Serotonergic dorsal raphe nucleus has been long linked to depression on the basis of both serotonin depletion studies and the efficacy of SSRIs, as discussed above. In addition, other limbic brain regions, like NAc, amygdala, hippocampus, hypothalamus and mPFC show changes in their structure and function in major depressive disorder patients (Duman and Aghajanian, 2012). Together, these brain areas comprise a limbic circuit controlling the mood, emotion, memory and cognition as well as sleep and feeding patterns (Warner-Schmidt, 2013). The anatomical connections of this circuit have been known for many years. However, the functional connectivity of this circuit or how the changes in the activity of one area affect the others is still under investigation.

As mentioned above, a key component of serotonin circuitry is the 5-HT1A autoreceptor, which functions as the major somatodendritic autoreceptor to negatively regulate the "gain" of the serotonin system. In addition, 5-HT1A heteroreceptors are abundantly expressed post-synaptically to mediate serotonin actions on mood and emotion. Importantly, in the PFC, 5-HT1A heteroreceptors are expressed on two antagonist neuronal populations: excitatory pyramidal neurons and inhibitory interneurons. Rodent models implicate the 5-HT1A receptor in anxiety- and depression-like phenotypes with distinct roles for pre- and post-synaptic 5-HT1A receptors. For example, global knockout of the 5-HT1A receptor results in an anxiety phenotype, which is rescued by expression of the post-synaptic forebrain 5-HT1A receptor in pyramidal neurons in the global 5-HT1A -/- mice (Gross et al., 2002). Similarly, early postnatal knockdown of the pre-synaptic 5-HT1A autoreceptor also results in an anxiety phenotype

(Richardson-Jones et al., 2011). On the other hand, a 30% knockdown of presynaptic 5-HT1A autoreceptors in the adult mice results in resilience to stress and depression (Richardson-Jones et al., 2010). Conversely, an increase in 5-HT1A autoreceptors would be predicted to lead to increased susceptibility to depression by reducing serotonergic activity (Albert 2012; Popova and Naumenko, 2013; Donaldson et al., 2013; Garcia-Garcia et al., 2014). Thus, alterations in the expression of presynaptic 5-HT1A receptors at different stages of life can significantly influence behavior phenotypes.

In a review (Albert et al., 2014), we presented a model of serotonin-PFC circuitry that integrates evidence from mouse genetic models of anxiety and depression involving knockout, suppression, over-expression, or mutation of genes of the serotonin system including 5-HT1A receptors. The model postulates that behavioral phenotype shifts as serotonin activity increases from none (anxious/aggressive) to low (anxious/depressed) to high (anxious, not depressed). Thus, the role of the 5-HT system and various changes in 5-HT1A models in depression/anxiety varies with the extent of 5-HT activity in the PFC.

3-2. The role of the PFC in depression

Several studies have examined brain stimulation approaches for targeting of the PFC for treatment of treatment-resistant MDD patients (Ressler and Mayberg, 2007, Lozano et al., 2012, Dunlop et al., 2017). Pathak et al. (2016) reported that Repetitive transcranial magnetic stimulation (rTMS) increases in GABA power in left dorsal PFC associated with improvements in symptoms of depression in major depressive disorder (MDD) patients. They also found that increase in delta band connectivity between L-DLPFC/amygdala or anterior cingulate cortex promotes recovery from depression. A recent report by Chau et al. (2017) presents a meta-analysis to assess and compare the

effects of three distinct types of intervention for MDD including SSRIs, electroconvulsive therapy (ECT), and rTMS on regional resting-state brain activity. Except for distinct areas affected by individual types of treatment, successful monotherapy of each commonly results in significant neuronal changes in anterior cingulate cortex, an area that receives massive 5-HT projections from DR. In addition, in a pre-clinical mouse model study, Wscieklica et al. (2017) reported that using deep brain stimulation (DBS) to stimulate the DRD is associated with an anxiolytic-like effect assessed in open field (OF) test. DRD stimulation also led to increases in Fos-immunoreactivity in the medial amygdala, lateral septum, and cingulate cortex. Therefore, DBS applied to the DRD appears to change the neuronal activity in this neurocircuitry implicated in the anxiolytic-like effects.

Taken together, the biological cause of depression may reside in part within the mPFC-DR circuitry, and targeting specific area or subpopulations of neurons in this circuitry may provide faster relief from depression symptoms.

3-3. Targeted genetic modification of the serotonin system and behavior: Transgenic and knockout approaches

Several mouse models have targeted the 5-HT1A receptor gene or genes involved in determining 5-HT levels or 5-HT neurotransmission in the brain (Table 1). These models have been examined for their anxiety and depression-like behavioral phenotypes and taken together have shown that:

- Alteration in serotonin genes (5-HT1A, 5-HTT, TPH2) produces an anxiety and/or depression phenotype.

- Early post-natal modulation of the 5-HT system can produce lifelong changes in anxiety/stress reactivity.
- Both pre- and post-synaptic 5-HT1A receptors contribute to anxiety and depression phenotypes, often in opposite ways.
- High (5-HT1A autoreceptor or 5-HTT knockout) or low (TPH2 or Pet-1 knockout) 5-HT neurotransmission can result in anxiety phenotype; very low 5-HT results in anxiety and aggressive behavior.
- Partial downregulation of 5-HT1A autoreceptor accelerates and enhances SSRI action
- 5-HT1A receptors are required for neurogenic and anti-anxiety actions of chronic SSRI treatment (from review by Albert, Vahid-Ansari, Luckhart, 2014)

Table 1. 5-HT genetic models summary.

Knockout/in	Target Gene Expression			Adult Behavior	REF
5-HT1A	**Embryo**	**Early PN**	**Adult**		
5-HT1A-/-	-	-	-	Anxiety, antidepressant	Ramboz et al.1998 Heisler et al. 1998
5-HT1A auto-	+	+	**-30%**	Antidepressed, SSRI+	Richardson-Jones et al., 2010
5-HT1A hetero-supp (CamKII)	**-95%**	**-95%**	**-95%**	Depression	Richardson-Jones et al., 2011
5-HT1A auto-supp (Pet1)	**-80%**	**-80%**	**-80%**	Anxiety	Richardson-Jones et al., 2011
5-HT1A auto-siRNA suppr.	+	+	**-80%**	Anti-depressed, SSRI+	Bortolozzi et al. 2012; Ferres et al. 2013
5-HT1A hetero-rescue (CaMKII)	-	+	-	Anti-anxiety	Gross et al., 2002
5-HT1A-heteroOE tg	+	++	+	Anti-anxiety Anti-depressed	Kusserow et al., 2004
5-HT1A-autoOE (Tph2)	**+300%**	**+300%**	**+300%**	Aggression, anxiety	Audero et al., 2013; Piszczek et al., 2013
5-HT1A-auto rescue	**+300%**	**+300%**	**+300%**	Anxiety, anti-depressed	Audero et al., 2013; Piszczek et al., 2013
5-HT					
TPH2-/-	-	-	-	**Anti-**Anxiety, depression, aggression	Mosienko et al., 2012
TPH2-R439H*	**-80%**	**-80%**	**-80%**	Anxiety, depression, 5-HT20%	Jacobsen et al., 2012; Sachs et al., 2013
PET1-/-	**-80%**	**-80%**	**-80%**	Aggression, anxiety	Hendricks et al., 2003
Pet1-adultKO	+	+	**-80%**	Anxiety	Liu et al., 2012
En1/Pet1- TetTX	**-***	**-***	**-***	Anti-Anxiety, cognitive+	Kim et al., 2009
5-HTT					
5-HTT-/-	-	-	-	Anxiety, anti-aggression	Holmes et al., 2002, 2003; Kalueff et al., 2006
5-HTT-FLX	+	-	+	Anxiety, depression	Ansorge et al., 2004
5-HTT+-G56A*	**+***	**+***	**+***	Subordinate, social-, SSRI+	Veenstra-Vanderweele et al., 2012

Genetic models are as defined in **Box1** with promoter used indicated in parentheses; rescue is an expression in the knockout background, over-expression (OE) is on wild-type background; auto, autoreceptor-specific; hetero, heteroreceptor-specific. The effect of the indicated genetic model on target gene expression (−, none; +, normal; ++, increased; +X, increased by X%) at indicated developmental time [embryo, early post-natal (PN) or adult] and adult behavior phenotype or response to SSRI are indicated. *loss of 5-HT release; enhanced activity 5-HTT mutant (from Albert et al., 2014).

4. Transcriptional modifiers of 5-HT1A receptor expression in anxiety and depression

An imbalance in the transcriptional regulation of neuronal genes involved in serotonergic neurotransmission, such as HTR1A (5-HT1A gene), may contribute to mood disorders. Thus, it is important to understand how key genes of the 5-HT system are regulated in health and disease. Alteration in the expression level of the 5-HT1A gene has been already implicated in depression/anxiety behavior (Abbas et al., 2007). Since depression follows a chronic time course, it is thus critical to identify the transcriptional mechanisms underlying the long-term regulation of critical regulators of the 5-HT system, such as the 5-HT1A receptor gene. The transcriptional regulation of the 5-HT1A receptor gene has been characterized by the genetic dissection of 50 regulatory DNA sequences of the gene (Albert and Fiori, 2014). The 5-HT1A promoter contains GC-rich sequences typical of a "house-keeping" promoter that directs ubiquitous expression in all tissues (Storring et al., 1999). Expression of this gene is regulated by several different transcription factors, and its expression is restricted to neurons by several repressors including REST, Deaf1 and Freud-1/Freud-2 repressor proteins (Fig. 3) (Albert, 2012). REST is mainly expressed in non-neuronal cells to restrict 5-HT1A gene expression to appropriate neurons. Similarly, repressors Hes1 and Hes5 are expressed in neural progenitors and are down-regulated during differentiation to allow expression of neuronal genes including the 5-HT1A receptor. Deaf1 (Deformed epidermal autoregulatory factor 1), also known as NUDR, is a regulator that is partly responsible for repressing transcription of 5-HT1A receptors in non-neuronal cells and 5-HT neurons (Czesak et al., 2006). Freud1 (five-prime repressor element under dual repression), also known as CC2D1A, is another repressor located of

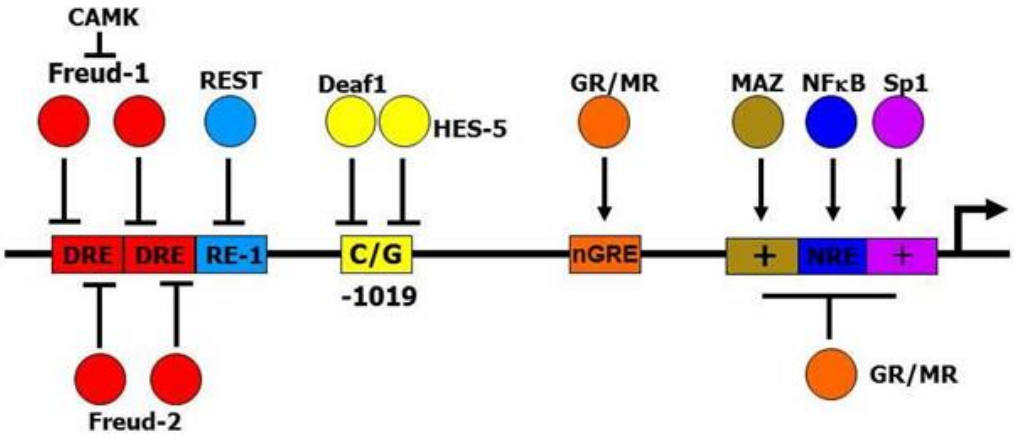


Figure 3. Human 5-HT1A receptor gene (HTR1A) promoter elements.

The location of identified DNA elements on the 5-HT1A 5' regions flanking the start of translation (bold arrow) is shown figuratively. Identified activators (arrows) or repressors (bars) of transcription are also shown. Within the minimal promoter (box) there are GC-rich Sp1 and MAZ elements (+); NFkB response element (NRE); and glucocorticoid receptors (GR/MR) that inhibit transcription by blocking Sp1. Further upstream in a repressor/enhancer regions, a negative glucocorticoid response element (nGRE) also mediates direct GR/MR-induced repression. Hes and Deaf1 proteins repress the 5-HT1A promoter at the C(-1019) allele, while in serotonin neurons Pet-1 exerts strong enhancer activity. A strong repressor region that silences expression in 5-HT1A-negative non-neuronal cells, but also represses in 5-HT1A-positive neuronal cells is located upstream that includes elements for REST (repressor element -1; RE-1), Freud-1 and Freud-2 (dual repressor element; DRE), (from Albert et al, 2011).

upstream of Deaf1 that negatively regulates the transcription of 5-HT1A receptors. Under certain conditions, such as increased stress, glucocorticoid and mineralocorticoid receptors can also bind to a negative GRE (glucocorticoid response element) on the promoter and repress the 5-HT1A transcription. By contrast, the 5-HT1A promoter also contains several Pet-1 sites recognized by the raphe-specific enhancer, Pet-1, which primarily enhances 5-HT1A autoreceptor expression (Jacobsen et al., 2008). Thus both positive and negative regulators play a role in regulating 5-HT1A receptor expression.

The 5-HT1A promoter is most strongly repressed by an upstream repressor region that contains a conserved and novel dual repressor element (DRE) in different species including rat, mouse, and human (Fig. 4A) (Storring et al., 1999; Ou et al., 2000). Deletion or mutation of the entire DRE de-represses 5-HT1A receptor gene transcription in all cell types (neuronal and non-neuronal). Mutation of the 5' portion of this element (FRE) led to a complete de-repression of the 5-HT1A promoter exclusively in neuronal cells suggesting that the FRE is a major regulator of 5-HT1A expression in neurons. Furthermore, using the 5-HT1A DRE as the target DNA element, Freud-1 was cloned by yeast one-hybrid screening of a mouse brain cDNA library (Ou et al., 2003). Using bacterially purified and *in vitro* transcribed/translated Freud-1, the specific complex of Freud-1-FRE was demonstrated by electrophoretic mobility shift assay (Lemonde et al., 2004). Finally, treatment of raphe RN46A serotonergic cells, but not non-neuronal cells, with antisense to Freud-1 induced 5-HT1A receptor transcription and protein levels (Ou et al., 2003), demonstrating the importance of Freud-1 as a negative regulator of the 5-HT1A gene in neuronal cells. However, its importance to regulate 5-HT1A receptor levels and the 5-HT system *in vivo* was not addressed.

4-1. Freud-1 repression of the 5-HT1A promoter

The mechanism by which Freud-1 represses 5-HT1A receptor transcription is dependent on cell type. Using a Gal4-based mammalian one-hybrid approach, transcriptional repressor activity was shown to be intrinsic to Freud-1 (Lemonde et al., 2004). Based on transcriptional reporter assays, the mechanism by which Freud-1 represses 5-HT1A transcription in neuronal cells was shown to be HDAC-dependent, while in non-neuronal cells it is HDAC-independent (Lemonde et al., 2004). The HDAC-dependent mechanism involves the recruitment by Freud-1 of the chromatin remodeling protein Brg1 with a Sin3A-HDAC repressor complex, while the HDAC-independent repression involves recruitment of a different Brg1-BAF complex lacking Sin3A and HDAC (Souslova et al., 2016). However, the importance of HDAC in Freud-1 actions *in vivo* remains unclear.

The human Freud-1 gene, also known as coiled-coil and C2 domain containing 1A (CC2D1A), is highly conserved through evolution. The protein contains four *Drosophila melanogaster* 14 domains (DM14) unique to Freud-1 homologues and of unknown function, one helix-loop-helix domain (HLH), a protein kinase C-like C2 domain, a proline-rich domain thought to be involved in protein binding (Williamson, 1994; Ou et al., 2000, 2003; Lemonde et al., 2004), coiled-coil oligomerization motifs (Burkhard et al., 2001), putative phosphorylation sites for protein kinases A and C and two calcium calmodulin-dependent protein kinase (CaMK) II/IV sites (Fig. 4) (Ou et al., 2003). Importantly, Freud-1 is negatively regulated by CaMK-dependent phosphorylation (Ou et al., 2003; Rogaeva et al., 2007), and increases in calcium signaling lead to de-repression of the 5-HT1A gene via Freud-1 inhibition (Ou et al., 2003).

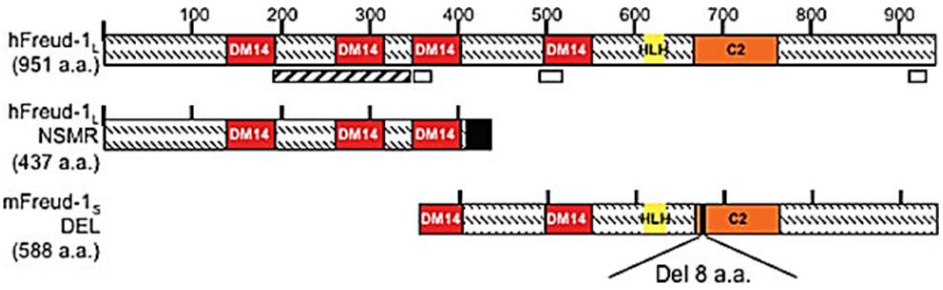


Figure 4. Schematic representation of Freud-1 and its isoforms. Long isoform of human Freud-1.

(hFreud-1L; NCBI accession No.Q6P1N0) with four DM14 domains, one helix-loop-helix (HLH), proline-rich region (diagonal line filled box), three coiled coil motifs (white boxes), and one C2 domain is demonstrated. hFreud-1 found in the patients with NSMR is also shown (hFreud-1L NSMR), lacking fourth DM14, HLH, and C2 domains but containing an additional 30 nonsense amino acids (AACPCQQGRLCPGPAAWPGSVSGGRPALW; black box) preceding a termination codon. Finally, the short isoform of mouse Freud-1 is demonstrated without two N-terminal DM14 domains (mFreud-1S; NCBI accession No. ABC54619). Furthermore, a location of the eight-amino-acid deletion that abolishes Freud-1 DNA binding abilities is shown. The amino acid scale is depicted at the top of hFreud-1L counted from the most upstream methionine codon. (from Rogaeva et al., 2007).

Freud-1 is broadly expressed throughout the rodent brain, particularly high levels in cortical and limbic regions and co-expressed with 5-HT1A receptors, suggesting an important function for Freud-1 in the central nervous system (Ou et al., 2003), and also in human cortex (Szewczyk et al., 2010). Immunostaining studies showed that Freud-1 protein is co-localized with 5-HT1A autoreceptors and 5-HT in raphe nuclei and also co-localized with postsynaptic 5-HT1A receptors in the hippocampus, cortex, and hypothalamus.

In addition to its role in regulating 5-HT1A receptors, Freud-1 may regulate other gene targets. Basel-Vanagaite and colleagues (2006) have documented the genetic linkage between mental retardation and Freud-1/CC2D1A, suggesting a neurodevelopmental role for Freud-1. In addition, our lab has identified an FRE in the human dopamine-D2 receptor gene that regulates its transcription in several cell lines (Rogaeva et al., 2007). While its importance in D2 regulation *in vivo* has not been shown, these examples highlight the multiple roles of Freud-1. Thus, a global knockout of Freud-1 could have multiple effects, and has been shown to result in immediate post-natal death (Zhao et al. 2011).

The developmental expression of Freud-1 has been assessed in the mouse brain (Basel-Vanagaite et al., 2006). By *in situ* hybridization, Freud-1 mRNA is detectable in cultured embryonic cortical neural stem cells and progenitors as early as E12 and persists through adulthood. Specifically, Freud-1 mRNA expression is found in the sub-ventricular zone (SVZ) pro-genitor cells, developing cortical plate (CP) in hippocampal neurons at E16. At P3, mRNA is broadly expressed in hippocampal pyramidal neurons, cerebral cortex, and several other brain regions and persists into adulthood (Basel-

Vanagaite et al., 2006). The expression in brain of Freud-1 from E12 to adulthood suggests that it participates in brain development (Ou et al., 2003; Albert and Lemonde, 2004; Basel-Vanagaite et al., 2006).

4-2. Roles of Freud-1 *in vivo*

As described above, in cell lines over-expression of Freud-1 reduces 5-HT1A protein levels, whereas depletion of Freud-1 up-regulates 5-HT1A expression specifically in neuronal cells (Ou et al., 2003). Although, these results strongly suggest that Freud-1 functions as a key transcriptional repressor of the 5-HT1A receptor gene, there is a lack of data on the implication of Freud-1 in the mechanisms underlying genetically determined and experimentally altered 5-HT1A receptor system, *in vivo*, and possibly implicated in depression and anxiety. Some studies have begun to address the role of Freud-1 *in vivo*, and in regulating *in vivo* 5-HT1A receptor levels. In 2010, Naumenko's group showed a significant reduction of 5-HT1A receptor gene expression with a considerable increase of Freud-1 expression in a model of 5-HT1A receptor desensitization produced by chronic 5-HT1A agonist administration in mice (Naumenko et al., 2010). Basel-Vanagaite, et al, (2006) and Manzini, et al. (2014) showed that disruption of the CC2D1A/Freud-1 gene in humans is linked to NSID and autism, conditions often associated with reduced social interaction. The global knockout of Freud-1 is associated with impairments in cortical neuron differentiation and poor neonatal breathing, which result in high lethality (Zhao et al., 2011). Conditional knockout of Freud-1 in the postnatal mouse forebrain results in impaired cortical neuronal development, reduced cognitive function and social interaction, as well as anxiety-like behavior in the OF test (Oaks et al., 2016). This suggests that post-synaptic

Freud-1 is also important for normal behavioral development. Consistent with this, Freud-1 levels are reduced in the PFC following chronic stress in rats (Iyo, et al 2009), while both Freud-1 and 5-HT1A protein levels were reduced in PFC of young depressed subjects (Szewczyk et al., 2010), suggesting that early impairments in cortical Freud-1 levels may predispose to depression.

Taken together, the Freud-1 protein appears to be one of the factors that could determine the expression level of 5-HT1A receptors, mediate the actions of serotonin neurotransmission and regulate its level and activity.

5. Genetic model: Conditional Freud-1 knockout

Multiple genetic and environmental factors contribute to risk of major depression in humans. However, the focus of etiology and treatment has been the major monoamine systems and the serotonin system in particular. To address the role of genetics in depression, I have crossed TPH2CreERT2 with floxed Freud-1 to generate the conditional Freud-1 knockout mouse model, which should alter the expression of a key regulator of the 5-HT system, the 5-HT1A autoreceptor.

5-1. The Cre-Recombinase System

The Cre-loxP system is well known as an applicable irreversible gene expression/deletion system (Tsien et al., 1996). It consists of two identical loxP sites that are placed on the 5' and 3' position of an interest gene or transcriptional stop sequence (flanking the region of interest, floxed region), and expression of the Cre-recombinase molecule. Cre-recombinase recognizes LoxP sites and recombines the floxed locus, excises the DNA sequence which contains the gene of interest. Tissue specific expression of Cre by using transgenic targeting yields a spatial specificity of recombination. Temporal specificity is

obtained by modification of the system. An inducible Cre-recombinase has been generated by fusing the Cre protein with a modified estrogen receptor (ER) to create the Cre-ERT2 molecule (Metzger et al., 1995). The Cre-ERT2 protein remains in the cytoplasm. Following treatment with the ER ligand, tamoxifen, Cre-ERT2 translocates to the nucleus and recombination occurs. Thus, the gene of interest is only deleted in the target area after tamoxifen treatment. Notably, although this system is being commonly used, it can often present problems with penetrance, particularly in the brain. Typical recombination in the brain is up to 80 percent efficiency reported (Erdmann et al., 2007).

5-2. The floxed Freud-1 mice

The floxed Freud-1 mice used in this study include loxP sites that flank exons 14-16, the same region that is deleted in the human Freud-1 gene resulting in NSMR (non-syndromic mental retardation) (Oaks et al., 2016). Cre-induced recombination results in the truncation of the helix-loop-helix DNA binding and the C2 domains, crucial for Freud-1 repressor activity, and hence is predicted to generate an inactive or weakly dominant negative protein. The Flx-Freud-1 mice line was obtained from Dr. Walsh.

Objectives and Hypothesis:

Reduction in 5-HT1A autoreceptor expression should result in an enhancement of serotonergic transmission (figure 5). Among the transcriptional negative regulators of the 5-HT1A promoter gene, Freud-1 is my particular interest since it has been shown to have predominant repressor activity in 5-HT1A-positive 5-HT neuronal cells in culture (Ou et al., 2000). Based on these findings, I hypothesized that Freud-1 is a key regulator of the

5-HT1A gene *in vivo* and that deletion of Freud-1 will result in increased 5-HT1A autoreceptor expression-induced depression and /or anxiety like behavior.

To address this question, I have crossed Freud-1flx/flx with TPH2CreERT2 to generate inducible and conditional mouse lines for specific knockout of Freud-1 in 5-HT neurons of the raphe in adulthood, in order to avoid developmental consequences of Freud-1 deletion. To address the role of 5-HT1A receptors in the process, I have also crossed Freud-1flx/flx-TPH2CreERT2 mice with 5-HT1Aflx/flx to generate a double knockout in adult 5-HT neurons.

The aims of studies using the genetic model in this thesis are:

Aim 1. To address whether the behavioral phenotype induced by loss of Freud-1 results in 5-HT1A/autoreceptor overexpression-induced depression/anxiety (figure 5).

Aim 2. To address whether the behavioral phenotype induced by loss of Freud-1 is dependent on 5-HT1A/autoreceptor overexpression (figure 6).

Aim 1: Conditional Freud-1 KO in 5-HT neurons

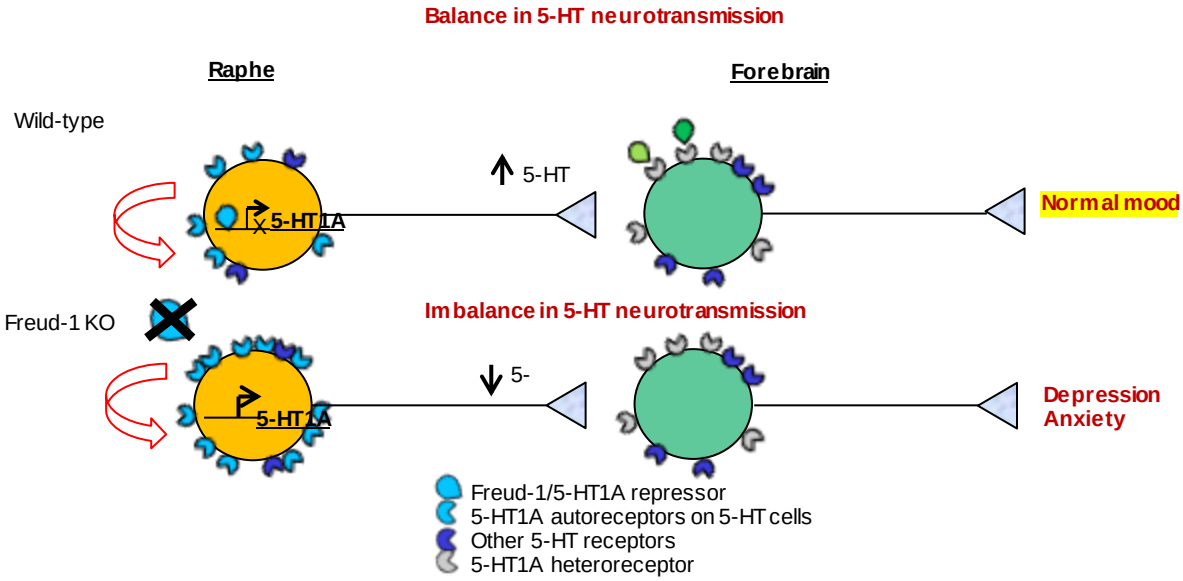


Figure 5. Freud-1 as a major repressor of the 5-HT1A receptor gene is conditionally knocked out in 5-HT cells in adulthood.

An inducible conditional Freud-1 KO mouse was generated by crossing TPH2-CRE-ERT2 x FLX-Freud-1. Freud-1 (about 90%) was specifically deleted from 5-HT cells located in dorsal raphe resulting in over-expression of 5-HT1A autoreceptors (about 2 folds). The behavioural studies test whether deletion of Freud-1 in the adult induces a depression/anxiety-like phenotype.

Aim 2: Freud-1/5-HT1A double KO in 5-HT neurons

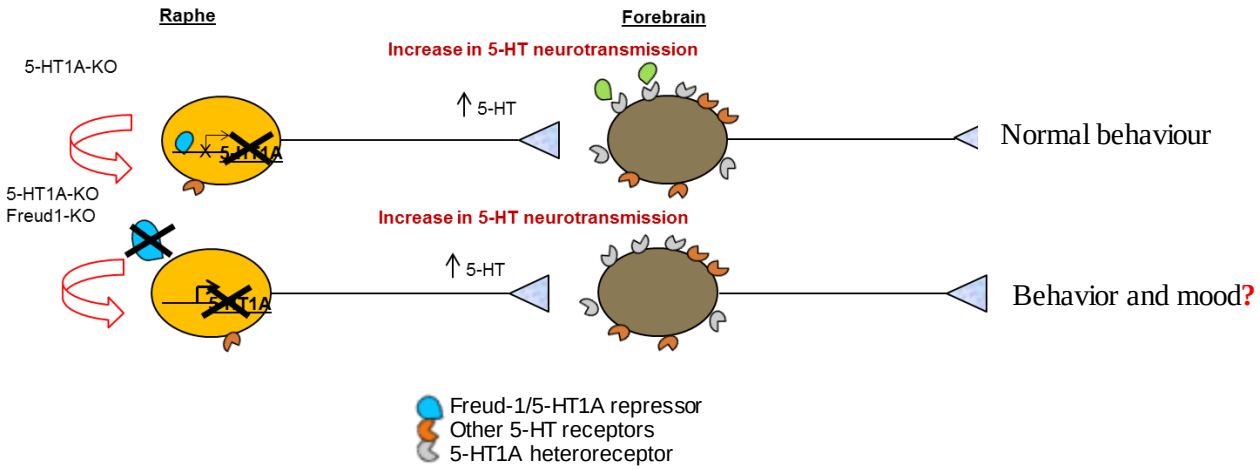


Figure 6. The 5-HT1A receptor gene is conditionally knocked out in 5-HT cells of conditional Freud-1 KO in adulthood.

An inducible double conditional 5-HT1A/Freud-1 KO mouse was generated by crossing FLX-5-HT1A x FLX-Freud-1 x TPH2-CRE-ERT2. About 90% of 5-HT1A autoreceptors was specifically deleted from 5-HT cells in conditional Freud-1 KO. The behavioural studies test whether conditional Freud-1-induced depression/anxiety-like phenotype is dependent on 5-HT1A autoreceptors.

To examine the role of serotonin in an injury model of depression, I chose to model post-stroke depression, in which a small stroke was induced in left mPFC. mPFC mainly projects to the limbic areas and midbrain DR and regulate the activity of these sites. The mPFC in rodents (homologue to Brodmann's area 25 in human) (Deverts, 2000) is involved in goal-directed behaviors and motivation, and regulates the serotonin system. I aimed to address whether unilateral lesion at left mPFC induces depression and/or anxiety involving dysregulation in mPFC-DR circuitry.

II. Post Stroke Depression model: Unilateral ischemia in mPFC

Post-stroke depression (PSD) is a common outcome following stroke, with 30% of patients developing major depression post stroke, and 80% of patients having milder forms of depression (Kotila et al., 1998; Paolucci et al., 2006). Stroke is the second leading cause of death worldwide (WHO, 2012) and when silent strokes (silent brain infarcts or micro-bleeds) are included the prevalence rises 10-fold, with one stroke in Canada every 10 minutes and 1 silent stroke each minute (Leary and Saver, 2003; Vermeer et al., 2007). Recently, it has been shown that late onset depression is associated with the presence of silent strokes, thus greatly increasing the prevalence of PSD (Brookes et al., 2014; Wu et al., 2014). On the other hand, the incidence of silent strokes in depressed patients is 46% (Vermeer et al., 2007), consistent with a bidirectional connection between the two (Hakim, 2011). Thus, there is a strong link between stroke and depression.

PSD appears to be directly/indirectly triggered by or linked to the ischemic event, as depression occurs within 3 months of a stroke (Paolucci et al., 2006), and is often co-

morbid with anxiety (Campbell Burton et al., 2011; Galligan et al., 2015). Patients that suffer PSD have a poor outcome, with reduced autonomy, decreased quality of life, a greater risk of suicide and increased overall mortality within 10 years (Whyte et al., 2006; Almeida and Xiao, 2007; Wolfe et al., 2011). PSD is also strongly associated with cognitive decline, impaired cognitive ability (Nys et al., 2006; Thomas and Lincoln, 2006; Barker-Collo, 2007; Hakim, 2011) and impaired motor recovery (Mayo et al., 1991; Goodwin and Devanand, 2008; Wulsin et al., 2012) which leads to increased hospitalization and health care costs (Ghose et al., 2005; Husaini et al., 2013; Atteih et al., 2015).

1. Pathogenesis and predisposing factors of PSD

How stroke triggers depression is unclear but appears to involve the size, number of ischemic lesions, and whether the lesions disrupt the midbrain, limbic and medial prefrontal cortical circuitry implicated in depression (Price and Drevets, 2012; Flaster et al., 2013). Particularly white matter lesions are associated with metabolic alterations in this circuitry (Capizzano et al., 2010) and are correlated with major depression (de Groot et al., 2000; de Leeuw et al., 2001; Terroni et al., 2011; Hoogenboom et al., 2014). In particular, a common type of ischemic stroke involving the anterior cerebral artery has been indirectly associated with altered activity in the anterior cingulate and medial prefrontal cortex implicated in depression (Paradiso et al., 2011). Several independent studies indicate that in ischemic stroke patients, lesions in the left frontal cortex, left basal ganglia or brainstem are correlated with depression, affective, or apathetic symptoms (Terroni et al., 2011; Murakami et al., 2013; Rajashekaran et al., 2013; Wu et al., 2014). Thus, an emerging concept that remains to be tested is that strokes or silent strokes may

disrupt the anxiety or depression circuitry (Albert et al., 2014) at several locations, resulting in PSD (Terroni et al., 2011; Zhang et al., 2012).

2. Modeling post stroke depression

Although post-stroke depression is prevalent clinically, preclinical research has mainly focused on motor deficits caused by stroke. However, the manifestation of PSD in humans has been associated with lesions located in the mPFC–midbrain–limbic circuitry that is implicated in depression and anxiety (as described above).

To validate the PSD phenotype in sub-chronic and chronic phases following stroke, the best practice guidelines for testing mouse behavior should be followed, which includes using: (i) more than one behavioral test; (ii) using tests that have construct and face validity, as well as outcomes that are objective, reproducible and sensitive enough to detect a long-term deficit (Crawley et al., 2008). To date, preclinical models of PSD have been used intraluminal suture, middle cerebral artery occlusion (MCAO) model that induces sensorimotor impairments. This model results in large stroke and is often combined with chronic stress to induce a depression-like phenotype. Kronenberg et al., 2015 reviewed the behavioral consequences of stroke models (induced by MCAO/chronic stress), which show that PSD can be modeled in rodents to extend our understanding in the neurobiology of PSD. However, because some MCAO models are often associated with massive and variable lesions affecting large regions of cortex and striatum, they induce pronounced motor impairments that can interfere with assessments of depression- and anxiety-like behavior. To augment the behavioral phenotype, chronic stress such as social isolation or chronic restraint stress has been used. But chronic stress itself induces depression like behavior, suggesting that these models are in part stress-

induced rather than post-stroke depression models. Therefore, the question of whether stroke in rodents leads to depression and/or anxiety remained to be addressed.

PSD is also strongly associated with cognitive decline and impaired cognitive ability (Nys et al., 2006). Although it is not clear whether cognitive deficits is a PSD outcome or stroke the recovery from PSD is associated with improvements in cognitive decline (Kimura et al., 2000).

Table 2 briefly classifies the different methodologies applied to induce stroke associated with changes in mood and emotion like depression/anxiety and also cognitive impairments. One of my goals was to develop a reliable model of PSD (Vahid-Ansari et al., 2016) that was not associated with motor impairments and that persists over time, to test response to relevant treatments (Vahid-Ansari and Albert, 2017).

Table 2. Behavioral consequences in some PSD models

PSD Model	Post -Stroke Behavior			Biological measurements	REF
	Depression	Anxiety	Lerning-Memory		
MCAO (M)	---	---	---	----	Gauer et al, 2010; Deplauque et al, 2011; Winter et al, 2005
MCAO/CMS (R)	---	---	---	↓ 5-HT/DA/NE in frontal lobe-hippocampus	Ji et al, 2014
MCAO/CUMS (R)	SPT-FST	OF	---	↑ CGPR in CSF-hippocampus	Shao et al, 2015
MCAO (suture-60 min)/social isolation (M)	FST	EZM-OF	---	↓ BDNF in cortex-striatum	O'Keefe et al, 2014
MCAO (suture30, 60, 70)/spatial restraint (M)	TST-FST-SPT	---	---	↓ 5-HT/DA/BDNF in hypothalamus, hippocampus, cortex, brainstem ↑ Serum cortisol	Zhang et al, 2015
Left MCAO/reperfusion (60 min) (M)	FST, SPT	EPM, NSF	---	↓ DA/DAT in striatum ↑ BDNF in striatum	Kronenberg et al, 2012
MCAO/reperfusion (60 min) (M)	---	---	MWM, PA	---	Bouët et al, 2007
ET1-mPFC (R)	---	OF, LD	OR, AS shifting, BM	---	Livingston-Thomas et al, 2015
PT-PFC (M)	---	---	OR	---	Zhou et al, 2016
ET1-L mPFC (M)	FST, TST	EPM, OF, LD, NSF	MWM	---	Vahid-Ansari et al, 2016

M, mice; R, rat; MCAO, medial cerebral artery occlusion; ET-1, endothelin-1; mPFC, medial prefrontal cortex; CMS, conditional mild stress; CUMS, chronic unpredictable mild stress; 5-HT, serotonin; DA, dopamine; NE, norepinephrine; DAT, dopamine transporter; CGPR, calcitonin gene-related peptide; SPT, sucrose preference test; FST, forced swim test; TST, tail suspension test; OF, open field; EM, elevated plus maze; LD, light dark test; MWM, Morris Water maze; OR, object recognition; BM, Barnes maze; NSF, novelty suppressed feeding.

3. Recovery from PSD

3-1. Antidepressants: SSRIs

Effective treatment to promote recovery from PSD has relied on psychopharmacotherapy developed to treat major depression (Hackett et al., 2008). The therapeutic effects of a treatment (like SSRIs) in PSD may exert beneficial effects on different aspects of stroke recovery (Mead et al., 2012). The FLAME study conducted by Chollet et al., 2011 showed that early treatment with fluoxetine, starting 5 to 10 days after the onset of stroke, significantly enhanced motor recovery after 3 months in non-depressed patients with ischemic stroke. Furthermore, 12-week treatment with antidepressants increases the survival of both depressed and non-depressed stroke patients (Jorge et al., 2003). In agreement, there is clinical evidence that in addition to improving anxiety and depression symptoms (Flaster et al., 2013; Mikami et al., 2014), SSRI antidepressants (including fluoxetine) can also enhance post-stroke cognitive ability (Sato et al., 2006; Flaster et al., 2013).

In the context of lesion size in animal models of ischemic stroke, Lim et al., 2009 showed that administration of fluoxetine within 30 min, 3 h or 6 h and as late as 9 h of MCAO in rats significantly decreased the lesion size. Interestingly, treatment with SSRI, citalopram, starting 7-day post stroke (induced by MCAO/reperfusion in rat) prevents post-stroke depression, also attenuates secondary extra focal neurodegeneration in dopaminergic midbrain cells (Kronenberg et al., 2012). Taken together, these findings elaborate the beneficial effects of SSRIs on various aspects in stroke and stroke outcomes. Furthermore, it highlights the contribution of 5-HT system activity in recovery from stroke and post stroke-behavior. However, as mentioned before, about 50% of

depressed patients respond to SSRIs and remission is seen in only 30%. Furthermore, Juang et al., 2015 found the use of antidepressants associated with an increased risk of stroke recurrence, especially for ischemic stroke, in a cohort study followed 16770 patients. The higher risk associated with antidepressants was observed in patients with diabetes. Mortensen et al., 2014 also showed SSRI-induced hemorrhage, which is a major concern in stroke since it could lead to secondary strokes. Thus, chronic SSRI therapy requires close monitoring for SSRI side effects in stroke survivors. Hence, there is a need to devise new strategies to treat PSD. For this purpose I have developed a new rodent model of PSD to elucidate the changes in neuronal activity that lead to depression and how effective treatment can modify these changes.

3-2. Rehabilitation therapy: exercise

Stroke patients often also receive rehabilitation therapy involving exercise (Selim and Molina, 2012). Exercise has several potentially beneficial effects including enhancing hippocampal neurogenesis and synaptic plasticity (Luo et al., 2007). Voluntary exercise has been shown to induce BDNF and hippocampal neurogenesis and has antidepressant and antianxiety effects in rodents (Duman et al., 2008; Snyder et al., 2009) and humans (Southwick et al., 2005). Exercise can be a promising treatment to complement pharmacological treatments in several different neurological diseases (Svensson et al., 2014). A potential antidepressant effect of exercise could also be important for recovery from PSD (Vaynman et al., 2004; Luo et al., 2007; Duman et al., 2008; Li et al., 2009). In a clinical study, Ohmatsu et al., 2014 showed that pedaling exercise activates the 5-HT system resulting in changes in cingulate cortex activity and improvement in negative emotion. In agreement, in a pre-clinical rat study, Otsuka et al., (2016) found that acute

treadmill running at a low speed significantly increases c-Fos expression in 5-HT neurons in the DRN whereas high-speed running significantly enhances c-Fos expression in CRF neurons in the PVN. Low-speed running also results in decreased anxiety- and depressive-like behaviors in rats. These studies have shown the beneficial effects of exercise in different depression/anxiety models. In fact, therapeutic effects of exercise can be enhanced further by antidepressants, such as SSRIs as shown in animal studies (Santarelli et al., 2003; Maya Vetencourt et al., 2008) that may also be of benefit in clinical PSD.

Objectives and Hypothesis:

Chronic treatment with SSRIs has beneficial effects to reverse the anxiety-depression phenotype as well as the sensorimotor deficit (Chollet et al., 2011; Mead et al., 2013). I hypothesize that recovery from PSD can be improved by combined chronic SSRI and voluntary exercise in a valid PSD model. Further, chronic SSRI treatment and exercise activate different brain regions that may be crucial for the behavioral recovery in PSD.

Aim 1: Persistent post-stroke depression in mice following unilateral medial prefrontal cortex stroke

Aim 2: Assessment of alterations in corticolimbic activity associated with post-stroke de pression and its treatment by SSRI/exercise.

Based on the evidence presented above, the overall hypotheses and objectives of this study are to compare the two models of depression and anxiety induced by different etiology: genetic and stroke. To address this I have generated 2 depression models: a genetic model using knockout of Freud-1 to genetically dysregulate 5-HT system; and a post-stroke model, in which a small stroke is induced in mPFC. Both models result in a robust anxiety and depression phenotypes. I predict that these models result in dys-regulation of the serotonin system along with the perturbations in mPFC-DR circuitry. In addition, successful SSRI treatment is associated with the alterations in pre- and post-synaptic neuronal activity to promote recovery from depression and anxiety.

Chapter 2

Abrogated Freud-1/CC2D1A repression of 5-HT1A autoreceptors induces antidepressant-resistant anxiety- and depression-like behavior

Abrogated Freud-1/CC2D1A repression of 5-HT1A autoreceptors induces fluoxetine-resistant anxiety/depression-like behavior

Faranak Vahid-Ansari MSc[1], Mireille Daigle[1], M. Chiara Manzini PhD[2], Kenji F. Tanaka PhD[3], René Hen PhD[4], Sean D. Geddes MSc[5], Jean-Claude Béïque PhD[5], Jonathan James[6], Zul Merali PhD[6], Paul R. Albert PhD[1]*

[1]Ottawa Hospital Research Institute (Neuroscience), UOttawa Brain and Mind Research Institute, Ottawa ON K1H-8M5 Canada

[2]Department of Pharmacology and Physiology, The George Washington University School of Medicine and Health Sciences, Washington, DC 20037, USA.

[3]Department of Neuropsychiatry, School of Medicine, Keio University, Tokyo, 160-8582, Japan

[4]Department of Psychiatry, Columbia University Medical Center and Research Foundation for Mental Hygiene, New York State Psychiatric Institute, New York, NY, 10032, USA

[5]Department of Cellular and Molecular Medicine, UOttawa Brain and Mind Research Institute, Ottawa ON K1H-8M5 Canada

[6]The Royal's Institute of Mental Health, affiliated with the University of Ottawa, Ottawa ON

Running Title: Freud-1 in fluoxetine-resistant anxiety-depression

*To whom correspondence should be addressed: OHRI (Neuroscience), UOttawa Brain and Mind Research Institute, 451 Smyth Road, Ottawa ON K1H-8M5 Canada; phone, 613-562-5800 8307; fax, 613-562-5403; email, palbert@uottawa.ca

- Accepted in Journal of Neuroscience (October 2017)

Author Contributions

FV-A, and PRA designed the study; FV-A generated and confirmed the mouse models, acquired the behavioral, immunofluorescence, 5-HT1A autoradiography, and DPAT-induced hypothermia data. FV-A and PRA analyzed the data; FV-A wrote the article, and PRA revised it and approved the article for publication.

ABSTRACT

Freud-1/CC2D1A represses the gene transcription of serotonin-1A (5-HT1A) autoreceptors, which negatively regulate 5-HT tone. To test the role of Freud-1 in vivo, we generated mice with adulthood conditional knockout of Freud-1 in 5-HT neurons (cF1ko). In cF1ko mice, 5-HT1A autoreceptor protein, binding and hypothermia response were increased, with reduced 5-HT content and neuronal activity in the dorsal raphe. The cF1ko mice displayed increased anxiety- and depression-like behavior that was resistant to chronic antidepressant (fluoxetine) treatment. Using conditional Freud-1/5-HT1A double knockout (cF1/1A dko) to disrupt both Freud-1 and 5-HT1A genes in 5-HT neurons, no increase in anxiety- or depression-like behaviour was seen upon knockout of Freud-1 on the 5-HT1A autoreceptor-negative background, rather a reduction in depression-like behaviour emerged. These studies implicate transcriptional dys-regulation of 5-HT1A autoreceptors by the repressor Freud-1 in anxiety and depression and provide a clinically relevant genetic model of antidepressant resistance. Targeting specific transcription factors like Freud-1 to restore transcriptional balance may augment response to antidepressant treatment.

SIGNIFICANCE STATEMENT

Altered regulation of the 5-HT1A autoreceptor has been implicated in human anxiety, major depression, suicide and resistance to antidepressants. This study uniquely identifies a single transcription factor, Freud-1, as crucial for 5-HT1A autoreceptor expression in vivo. Disruption of Freud-1 in serotonin neurons in mice links up-regulation of 5-HT1A autoreceptors to anxiety/depression-like behavior and provides a new model of

antidepressant resistance. Treatment strategies to re-establish transcriptional regulation of 5-HT1A autoreceptors could provide more robust and sustained antidepressant response.

INTRODUCTION

Major depression and anxiety disorders are highly prevalent and often comorbid lifelong diseases (Kessler and Bromet, 2013; Whiteford et al., 2013) that involve reductions in the activity of monoaminergic systems, particularly the serotonin (5-HT) system (Jans et al., 2007; Krishnan and Nestler, 2008; Booij et al., 2015). The chronic course of depression and antidepressant action implicates long-term dys-regulation of 5-HT system. A critical node regulating 5-HT activity is the 5-HT1A receptor gene (HTR1A), that is expressed as a somatodendritic autoreceptor to inhibit the firing of 5-HT neurons, and as a heteroreceptor at targets of the 5-HT system implicated in mood, emotion, stress response and antidepressant action (Albert and Lemonde, 2004; Albert et al., 2014; Garcia-Garcia et al., 2014). Increases in 5-HT1A autoreceptors, which would tend to reduce 5-HT neurotransmission, are found in depressed and attempted suicide subjects and in depressed suicide post-mortem tissue (Albert et al., 2011; Hesselgrave and Parsey, 2013; Sullivan et al., 2015), and the latency to respond to antidepressant treatment has been attributed in part to the time required to desensitize 5-HT1A autoreceptors (Albert and Lemonde, 2004; Blier and El Mansari, 2013). However, the transcriptional mechanisms that dictate long-term receptor expression and their roles in anxiety, depression and response to antidepressants remain unclear.

The HTR1A gene contains a series of repressor elements upstream of the promoter that suppress its expression (Albert and Fiori, 2014). Within the repressor region, a strong dual repressor element (DRE) binds to Freud-1/Cc2d1a, which represses HTR1A transcription in neuronal and non-neuronal cells (Ou et al., 2000; Ou et al., 2003; Lemonde et al., 2004a; Rogaeva and Albert, 2007). In adult mice, Freud-1 is co-

expressed with 5-HT1A receptors throughout the brain (Ou et al., 2003). In the raphe, Freud-1 is co-expressed with 5-HT1A autoreceptors, unlike its homologue Freud-2/Cc2d1b, which is weakly detected in the raphe (Hadjighassem et al., 2009; Szewczyk et al., 2010). Knockdown of Freud-1 results in de-repression of 5-HT1A transcription in raphe cells (Ou et al., 2003), and thus we hypothesized that Freud-1 functions as a key repressor of 5-HT1A autoreceptor expression in vivo, that might affect 5-HT regulation, and depression and anxiety behavior, and have generated mice with knockout of Freud-1 in adult 5-HT neurons to address this hypothesis.

Materials and Methods

Experimental Design

Mouse Models: All animal studies were done in accordance with the University of Ottawa Animal Care Committee guidelines. Animals were maintained on a 12-hour light/dark cycle (7:00 AM to 7:00 PM) with ad libitum access to food and water. Both sexes were used and the proportion of male/female did not differ among groups; since no differences between male and female were observed in the tests conducted the data were pooled. The Cc2d1a (Freud-1)$^{flx/flx}$ mice (Oaks, 2016) were crossed with TPH2-CreERT2 mice (stock#016584, C57BL/6N background, Jackson Labs, https://www.jax.org/strain/016584) to generate heterozygous TPH2-CreERT2-Freud-1wt/flx mice, which were interbred to generate homozygous TPH2-CreERT2-Freud-1flx/flx (cF1ko) and TPH2-CreERT2-Freud-1wt/wt (WT) littermates (Fig. 5). At 8 weeks of age, mice were administered tamoxifen (Sigma, cat#T5648, 180 mg/day, approximately 3 mg/kg, i.p.) once/day for 5 consecutive days to activate CreERT2-induced recombination. To detect Cre-induced recombination, cF1ko mice were crossed into a ROSA26-flxSTOP-GFP C57BL/6J background (obtained from Dr. Diane Lagace, Univ. of Ottawa). The TPH2-Cre-ERT2/Freud-1wt/flx were crossed with fl1A mice (C57BL/6 x 129Sv x ROSA-Flpe background) (Samuels, 2015; Szewczyk, 2014) and bred to obtain TPH2-CreERT2-Freud-1flx/flx/1Aflx/flx mice (cF1/1Adko) (Fig. 5). To obtain Freud-1 WT mice on the 5-HT1AautoKO background, cFreud-1wt/wt:1Awt/flx were crossed with cFreud1wt/wt:1Awt/flx. To obtain mice on the corresponding 5-HT1A wild-type background TPH2-CreERT2-Freud-1wt/flx/1Awt/wt mice were bred to generate F1WT-1AWT mice.

Genotyping

Ear tissue samples were taken at 3 weeks of age and DNA extracted using the REDExtract-N-Amp Tissue PCR kit (Sigma). PCR was done using the following primers and conditions:

Cc2d1A$^{flx/flx}$: 5'-TAG AAA CAC TTA CCC TCC ACA TTG-3' and 5'-TAG GAA GTG CCC ACC CAG A-3'. The PCR conditions were: 94°C for 4min; 15 cycles at 94°C for 30s, 70°C for 30s, 0.5°C/cycle, 72°C for 30s; 20 cycles at 94°C for 30s, 62°C for 30s, 72°C for 30s; 62°C for 30s; 72°C for 10min, 10°C. This protocol results in 202-bp (wild-type) and 382-bp (floxed) products.

TPH2-CreERT2: TPH2-11679 5'-GCT GAG AAA GAA AAT TAC ATC G-3', CRE-125235'-TGG CTT GCA GGT ACA GGA GG-3', OIMR8744 5'-CAA ATG TTG CTT GTC TGG TG-3' and OIMR8745 5'-GCT AGT CGA GTG CAC AGT TT-3'. The PCR conditions were: 94°C for 1min; 35 cycles at 94°C for 15s, 57°C for 20s, 72°C for 10s; 94°C for 15s, 72°C for 2min, 10°C. This protocol results in 200-bp (wild-type) and 300-bp (transgenic) products.

ROSA-YFP: OIMR4982 5'-AAG ACC GCG AAG AGT TTG TC-3', OIMR8545 5'-AAA GTC GCT CTG AGT TCT TAT-3', OIMR8546 5'-GGA GCG GGA GAA ATG GAT ATG-3'. The PCR conditions were: 94°C for 3min, 94°C for 30s, 58°C for 1min, 72°C for 1min, 35 cycles: 94°C for 30s, 72°C for 10min, 10°C. This protocol results in 600-bp (wild-type) and 320-bp (transgenic) products.

1Aflx/flx: 5'-GGG CGT CCT CTT CAC GTA G-3' and 5'-CAG GGA CGT TGT GGT GTT GT-3'. The PCR conditions were: 94°C for 2min, 15 cycles at 94°C for 30s, 68°C for 30s -0.5°C/cycle, 68°C for 20s; 20 cycles at 94°C for 30s, 60°C for 30s, 68°C for 20s;

60°C for 30s, 68°C for 5 min, 10°C. This protocol results in 254-bp (wild-type) and 292-bp (floxed) products.

Immunofluorescence

Mice were anesthetized by lethal injection (0.01 ml/g, i.p.) of sodium pentobarbital (Somnitol; MTC Pharmaceuticals, Cambridge, ON, Canada) and perfused by cardiac infusion of 30 ml PBS, then 25 ml 4% paraformaldehyde. Whole brains were isolated, cryo-protected overnight in 20% sucrose and frozen at -80°C. Coronal brain slices (20 µm) were prepared using the following coordinates: Dorsal raphe: Bregma 4.36 to 4.72 mm (Paxinos and Franklin, 2001). Slices were thaw-mounted on Superfrost slides (Thermo-Fisher) and kept at -80°C. The sections were washed 3 × in PBS, blocked 1 h in PBS with 1% BSA, 10% NDS, 0.1% Triton X-100 (or 0.3% Tween 20 for 5-HT1A antibody), followed by overnight incubation at 22°C with chicken anti-GFP, (Abcam, ab13970, 1:500; RRID:AB_300798), sheep anti-TPH, (Millipore, ab1541 1:100; RRID:AB_90754), rabbit anti-5-HT1A receptor (custom made primary antibody raised to the i2 loop of the 5-HT1A receptor sequence; Cedarlane, Hornby, ON, Canada, 1:50, (Czesak et al., 2012)); rabbit anti-Freud-1, 1:1000 (Rogaeva and Albert, 2007); goat anti-5-HT (Abcam, ab66047, 1:500; RRID:AB_1142794) and rabbit anti-FosB (Santa Cruz, sc-48, 1:500; RRID:AB_631515). The sections were then washed three times in PBS and incubated for 1h in secondary antibody at 22°C. The secondary antibodies were as follows: Alexa Fluor 488 anti-chicken (Jackson, 103-545-155, 1:250; RRID:AB_2337390), anti-sheep Cy3 (Jackson, 713-165-003, 1:200; RRID:AB_2340727), Alexa Fluor 488 anti-rabbit (ThermoFisher, A-21206, 1:1000; RRID:AB_2535792), Alexa Fluor 594 anti-rabbit (ThermoFisher, A-21207, 1:200;

RRID:AB_141637), Alexa Fluor 647 anti-goat (ThermoFisher, A-21447, 1:200; RRID: AB_2535864) in blocking solution. Images of dorsal raphe were acquired with the Axiovision imaging software (RRID:SCR_002677) on a Zeiss Axio Observer D1 microscope under 10X and 20X magnification (n=4/group). Positive-stained cells were manually counted within a standardized template using ImageJ 1.48v software; RRID:SCR_003070.

5-HT1A autoradiography

For autoradiography, mice were sacrificed by cervical dislocation and decapitation. Extracted brains were frozen immediately on dry ice (-75°C) and maintained at -80°C until sectioning. Brains were cryo-sectioned at a thickness of 25 µm and mounted sections were maintained at -80°C until processing. Mounted sections were processed for ^{125}I-MPPI (Perkin Elmer, Boston, MA) autoradiography as described (Donaldson, 2014; Luckhart, 2016). Sections were exposed to Kodak BioMax MR film (VWR) for 24 h. Films were digitized at 1200-dpi resolution using an Epson Perfection V500 Photo Scanner, and signal density was measured using the mean luminosity function in ImageJ (1.49). Levels of 5-HT1A binding (µCi) were quantified by analyzing a standardized template outlining the region-of-interest, and adjacent background lacking specific binding subtracted. For raphe, data from sections at Bregma -4.36, -4.48, -4.60, -4.72 cm were averaged; for hippocampus, Bregma; -1.82 cm was used. Signals were within the linear range of the film and quantified based on standard curve using ARC146-F 14C standard (American Radiochemicals Inc, St. Louis, MO).

High Performance Liquid Chromatography (HPLC) Analysis

Levels of 5-HT and 5-HIAA were quantified in extracts of dissected tissues by HPLC (Czesak et al., 2012). For HPLC, cF1ko and matched WT littermate mice (n=4, 11 wks old) were sacrificed by cervical dislocation and decapitation. The entire dorsal raphe, hippocampus and prefrontal cortex were dissected, pooled, frozen immediately on dry ice and maintained at -80°C until homogenization and analysis (Czesak et al., 2012). In brief, 300 µL of homogenization solution (0.3 M monochloroacetic acid, 0.1 mM EDTA, 10% methanol and internal standard) was added to each sample followed by sonication. Following sonication, 100 µL was aliquoted and frozen for protein concentration determination (Pierce™ Coomassie (Bradford) Protein Assay). The remaining 200 µL was centrifuged and the supernatant analyzed for 5-HT and 5-HIAA content using HPLC (Agilent Technologies, Walbronn, Germany). A 10-µL volume of supernatant was injected via an autoinjector (1100 series Autosampler; Agilent, Walbronn, Germany) into the HPLC system equipped with an electrochemical detector (VT-03 flow cell, Intro detector; Antec Leyden, Montreal, Quebec, Canada) with an applied potential of 500 mV, a filter of 1 s, and a range of 100 nA/V. Separation of these analytes was achieved by their passage through a reverse phase analytical column (Phenomenex Kinetex® 2.6 µm C-18, 100 x 4.6 mm). The column was equilibrated at a flow rate of 0.5 ml/min with mobile phase consisting of the following (in mM): 90 NaH_2PO_4, 1.7 1-octane sulfonic acid (sodium salt), 50 citric acid (monohydrate), 5 KCl, 50 EDTA, and 14% acetonitrile, final pH 3.0. The quantification of the analytes was performed by comparing their area under the curve to those of known external standards using computerized Agilent ChemStation chromatography data acquisition system (Agilent).

DPAT-Induced Hypothermia

The hypothermia procedure was performed from 9-11 AM. Mice were weighed and internal temperature was taken using a rectal thermometer every 10 minutes for 40 minutes (4 baseline measurements). Animals were administered 8OH-DPAT (0.75 mg/kg, i.p., Sigma) followed by 3 measurements of basal body temperature at 10-min intervals (Martin et al, 1992). For analysis purposes, the first baseline temperature was discarded. The remaining three baseline values were averaged and the difference between the average baseline and recorded temperature was plotted across time.

Whole-cell Electrophysiology

Brainstem slices (300-µm) containing the DRN were prepared from 10-11 week old mice as previously described (Geddes et al., 2016). In brief, mice were anesthetized and sacrificed by decapitation. Once the brain was removed, coronal slices were made from a block of brain tissue while immersed in ice-cold cutting solution (in mM): 119 choline-Cl, 2.5 KCl, 1 CaCl2, 4.3 $MgSO_4$-$7H_2O$, 1 NaH_2PO_4, 1.30 sodium L-ascorbate, 26.20 $NaHCO_3$, 11 glucose at 37°C and equilibrated with 95% O_2, 5% CO_2. Slices were then transferred to a recovery chamber containing standard Ringer's solution (in mM): 119 NaCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$-$7H_2O$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose at 37°C, bubbled with 95/5% O_2/CO_2 and left to recover for > 1 h and equilibrated to room temperature (~25°C) prior to recordings. DRN neurons were visualized using an upright microscope (Examiner D1; Zeiss, Oberkochen, Germany) equipped with Dodt-gradient-contrast (40x/0.75NA objective). 5-HT neurons were identified by morphological and biophysical characteristics as previously established (Geddes et al., 2015). Whole-cell recordings carried out at room temperature in standard Ringer's solution using borosilicate glass patch electrodes (3-6 MΩ; World Precision Instruments). 5-HT1A

receptor-mediated currents were elicited by bath applying the 5-HT1A receptor agonist 5-carboxamidotryptamine (5-CT; 10 nM; Tocris) and holding current was monitored at 0.1 Hz (Vm = -55 mV. These recordings were carried out using an internal solution of the following composition (in mM): 115 potassium gluconate, 20 KCl, 10 sodium phosphocreatine, 10 HEPES, 4 Mg^{2+}-ATP, and 0.5 GTP (pH 7.25 adjusted with KOH; osmolarity, 280-290 mOsmol/L). Access resistance was continuously monitored by applying a 125 ms, 2 mV hyperpolarizing pulse every 10 s, and recordings were discarded if the access resistance changed by >30%.

Behavioral assays

Behavioral tests were conducted in littermates starting 2 weeks after the last tamoxifen injection, at 11 weeks of age. Mice were housed under normal light conditions and tests were performed beginning at 10:00 AM, after at least 1 h of habituation to the testing room. Testing was performed under white light illumination with the exception of the forced swim test (FST), which was performed under red light. All animals were of the same age at the start of testing and all tests were done in the order below and completed within 10 days. Each cohort included 10-32 mice/group. Throughout testing and behavioral analyses, experimenter was blind to the mouse genotype.

Elevated plus maze (EPM) test

The mice were placed in the center of an elevated two-arm plus maze, measuring ~ 20-cm high, ~ 6-cm wide and ~ 75-cm long (Noldus, The Netherlands). The arms of the maze are crossed with one arm having an open platform, the other arm having a closed platform with walls that are ~ 20-cm tall with overhead illumination (100-110 Lux) and camera. Mice were placed in the centre of the maze with the head toward the closed arm

of maze and allowed to explore the maze for 10 min. The mouse movements were videotaped and the time spent in closed and open arms was determined (Ethovision 10, Noldus IT, RRID:SCR_000441).

Open field (OF) test

The mice were placed in a corner of the arena (45-cm long in each side and 45-cm high) and allowed to explore the new environment for a total of 10 min at light levels of 300 Lux. Mouse movements were videotaped and the time spent in the outside of a center (24 × 24 cm) of the OF arena was analyzed (Ethovision 10, Noldus IT).

Tail suspension (TS) test

The tail of the mouse was secured with tape to a horizontal bar and the animals were suspended for 6 min in mouse TS boxes (Med Associates). An automated detection device (ENV-505TS Load-Cell Amplifier) was used to determine mobility and immobility time through Med Associates software (Ethovision XT, Noldus IT).

Forced Swim Test (FST)

Each mouse was placed into clear plastic cylinder 22-cm in diameter and 37-cm deep filled with 4 l of water (24°C). The mouse was videotaped from the side of the cylinder for 6 min under red light illumination and the duration of immobility time was quantified using an automated video-tracking software from Med Associates (Ethovision XT).

Novelty-suppressed feeding (NSF) test

The NSF test was used to assess anxiety-related behaviors (Santarelli, 2003). Briefly, animals were food deprived for 16 h. After 3 min of habituation they were placed in a new cage. Animals were individually placed in an arena (45-cm long in each side and 45-cm high; 300 lux) with a food pellet placed in the center. The latency of the mice to begin

eating food was recorded manually and immediately after mice approached the food or after 10 min had expired for the trial, the mice were removed from the arena and placed in their home cage and the latency to approach the food and the amount of food consumed in 5 min was measured.

Beam Break (BBK) test

Mice were placed into a novel home cage for 30 min. The home cage locomotor activity was measured by recording number of breaks of an invisible infrared light beams located on a frame surrounding the cage (Crawley, 2008) (Omnitech Electronics, Columbus, OH, USA).

Chronic SSRI treatment

For SSRI treatment, a separate cohort of mice, including conditional Freud-1 knockout and WT littermates (11 wk old, n=10), was single housed and received 18 mg/kg/day fluoxetine hydrochloride (Samuels et al., 2015; Santarelli et al., 2003) (Enzo life science, cat# 11181505, Farmingdale, NY, USA) in drinking water for 3-4 weeks using opaque bottles to protect the SSRI from light. Then, several behavioral tests were done as described above, with ongoing SSRI treatment, following the timeline shown in Figure 4. The consumption of fluoxetine was measured accurately by weighing to determine the amount of drinking water consumed every 3 days (~3 ml/day) and did not differ between groups.

Statistical Analyses

All analyses were done using the Statistical Package for the Social Sciences (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA; www.graphpad.com; RRID:SCR_002798). Data are expressed as mean ± SEM. $p \leq 0.05$

was used as the threshold for significance. Data comparing KO and WT littermates on one outcome measure were analyzed using unpaired t-test. One-way analysis of variance followed by Tukey's post-test was performed for multiple comparisons.

RESULTS

Adult knockout of Freud-1 in 5-HT neurons induces 5-HT1A autoreceptor upregulation

The cF1ko mice were generated for inducible knockout of Freud-1 in adult 5-HT neurons by crossing tamoxifen-inducible TPH2-CreERT2 and Cc2d1a (Freud-1)flx/flx mice (Oaks et al., 2016)(Figure 1A). Initially, to assess recombination specificity, cF1ko mice were bred on a ROSA26-flxSTOP-GFP background (cF1KO). Tamoxifen injection in adulthood revealed that recombination to produce GFP was not detected in non-serotonergic regions that express 5-HT1A receptors, including the PFC and hippocampus (Fig. 1B). However, GFP was present in 92% of TPH2-positive neurons in the raphe nuclei, and 100% of GFP-positive neurons were co-localized with TPH (Fig. 1C), indicating 5-HT neuron-specific recombination. Importantly, tamoxifen treatment of cF1KO compared to cF1wt mice strongly reduced the number of Freud-1-positive cells (114 WT vs. 22.5 cF1KO, t test, DF=6, t=11.566 ***p=0.0022), with ~90% of GFP- or TPH-positive raphe cells lacking Freud-1 (Fig. 1C-F). Freud-1 protein remained present in TPH-negative cells in the raphe (Fig. 1C, white arrowheads). In addition, there was no change in the number of TPH-positive cells, indicating that Freud-1 knockout did not alter the number of 5-HT neurons (Fig. 1E). Thus, tamoxifen efficiently induces Freud-1 knockout specifically in 5-HT neurons of the cF1ko mice.

To identify changes in 5-HT1A autoreceptors upon knockout of Freud-1 in 5-HT neurons, co-staining of 5-HT1A receptors and TPH in the dorsal raphe was examined (Fig. 1G). A significant increase in 5-HT1A-stained cells was observed in cF1ko compared to cF1wt sections (t test, DF=6, t=8.813 ***p=0.0001) with no change in total

TPH-positive cells (Figure 1F). There was a significant increase in 5-HT1A/TPH co-stained cells (WT, 49.50±6.752 vs cF1ko, 132.3±10.10 cells/template; t test, DF=6, t=6.810 ***p=0.0005), with no change in 5-HT1A/TPH-negative cell count (Fig. 1G, arrows), indicating a specific up-regulation of 5-HT1A autoreceptors upon Freud-1 knockout in 5-HT neurons. In the cF1ko raphe, we confirmed the reduction of Freud-1-positive cells (Fig. 1F, WT, 135.8±20.95; cFko, 27.50±6.538 cells/template; t test, DF=6, t=4.933 **p=0.0026). To further quantify the levels of 5-HT1A binding sites, autoradiography was performed using the selective 5-HT1A antagonist 125I-MPPI (Donaldson et al., 2014) and revealed a significant >1.5-fold increase in 5-HT1A binding in the dorsal and median raphe nuclei of cF1ko vs. cF1wt mice), but similar levels of 5-HT1A binding in hippocampus (Figure 1H). The 5-HT1A binding (uCi/region) was: DR: wt, 3.644±0.214 vs. cF1ko, 6.609±0.244, t test, DF=6, t=9.129, ***p=0.0001; MR: wt, 4.406±0.1895 vs. ko, 6.411±0.235, t test, DF=6, t=6.635, **p=0.0006; hippocampus, wt, 7.509±0.318 vs ko, 7.245±0.086, t test, DF=6, t=0.801, p=0.45. In summary, consistent with its repressor function, loss of Freud-1 in 5-HT neurons results in a significant up-regulation of 5-HT1A autoreceptors, while 5-HT1A heteroreceptor levels remain unchanged.

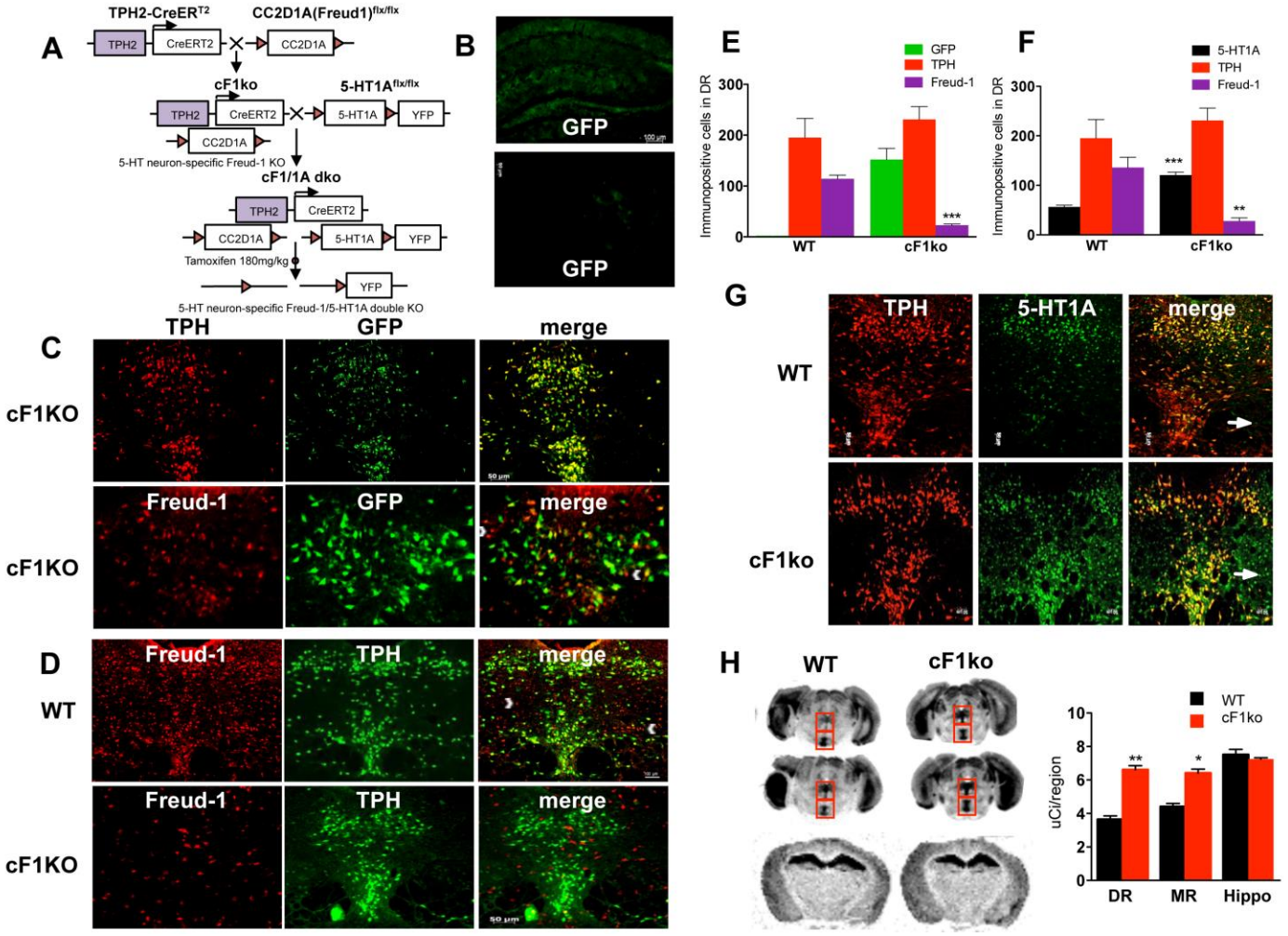

A
TPH2-CreER^{T2}
CC2D1A(Freud1)$^{flx/flx}$
TPH2
CreERT2
CC2D1A
cF1ko
5-HT1A$^{flx/flx}$
5-HT1A
YFP
5-HT neuron-specific Freud-1 KO
cF1/1A dko
Tamoxifen 180mg/kg
5-HT neuron-specific Freud-1/5-HT1A double KO
B
GFP
C
TPH
GFP
merge
cF1KO
Freud-1
D
WT
E
GFP
TPH
Freud-1
Immunopositive cells in DR
WT
cF1ko
F
5-HT1A
G
5-HT1A
H
uCi/region
DR
MR
Hippo

Figure 1. Loss of Freud-1 in 5-HT neurons increases 5-HT1A autoreceptors.

A. Conditional Knockout Strategy. To delete Freud-1 in 5-HT neurons, the cF1ko mouse was generated by crossing CC2D1A (Freud-1)flx/flx mice with TPH2-CreERT2 mice. At 8 weeks of age, mice were administered tamoxifen to activate CreERT2-induced recombination. To delete both Freud-1 and 5-HT1A autoreceptors in 5-HT neurons, the cF1ko mice were mated to the 5-HT1Aflx/flx mice, in which the 5-HT1A gene is flanked by LoxP sites and a YFP cassette to generate the Freud-1/5-HT1A double knockout mice following tamoxifen administration. B. Tamoxifen-induced recombination specificity. Hippocampal and prefrontal cortex sections from tamoxifen-treated conditional Freud-1 knockout/ROSA-GFP mice (cF1KO) show background GFP staining. C. Tamoxifen-induced recombination and loss of Freud-1 in dorsal raphe. DR sections from tamoxifen-treated cF1KO mice were stained for GFP and either TPH or Freud-1 (10x magnification, scale bar = 20 μm; inset, 20x). GFP was present in 92% of TPH+ cells, while 88% of Freud-1 was in GFP- cells in cF1KO sections (n=4). D. Loss of Freud-1 in 5-HT neurons. Freud-1/TPH-labeled cells in DR were almost absent in cF1ko compared to WT. By contrast, Freud-1+/TPH- cells remained (white arrowheads) (n=4). E. Quantification of GFP-, TPH- and Freud-1-stained cells in dorsal raphe of cF1KO and WT mice (n=4), shown as mean ± S.E. ($p < 0.001$). F. Quantification of 5-HT1A-, TPH- and Freud-1-stained cells in dorsal raphe of cF1ko (non-ROSA-GFP) and WT mice (n=4), shown as mean ± S.E. ($p < 0.001$). G. Loss of Freud-1 and increased 5-HT1A-positive cells in dorsal raphe of cF1ko mice. DR sections from tamoxifen-treated cF1ko vs. F1wt (WT) mice (scale=50 μm, n=4) were stained for TPH and 5-HT1A receptors (arrow, 5-HT1A in TPH- cells); 5-HT1A receptors were increased in TPH+ cells. H. Increased 5-HT1A binding in raphe of cF1ko mice. At left are representative images of 125I-MPPI autoradiography of sections from cF1ko and WT mice in dorsal and median raphe (boxes) at two levels (Bregma -4.60 and -4.72 mm) and hippocampus (Bregma -1.70). At right is quantification of 125I-MPPI binding. Data represent mean ± SEM (n=4/group), *$p < 0.05$; **$p < 0.01$. 5-HT1A binding was increased in raphe of cF1ko mice; DR (unpaired two-tailed Student's t-test, DF=6, t=9.129, **$p<0.001$), MR (unpaired two-tailed Student's t test, DF=6, t=6.635 *$p<0.01$).

Enhanced 5-HT1A function and reduced raphe 5-HT levels in cF1ko mice

In order to determine the effect of Freud-1 deficiency on 5-HT1A autoreceptor function in vivo, we measured hypothermia in response to acute administration of the 5-HT1A agonist 8OH-DPAT, which in mice is dependent on 5-HT1A autoreceptor levels (Albert et al., 2014). Within 30 min, 0.75 mg/kg 8OH-DPAT induced a maximal reduction in body temperature in the cF1ko mice that was significantly greater than that in the cF1wt mice and persisted for 70 min, indicating an enhanced 5-HT1A autoreceptor response to the 5-HT1A agonist (Figure 2A). To assess 5-HT1A autoreceptor function in single neurons, we performed whole-cell voltage-clamp recordings from dorsal raphe slices and bath applied the 5-HT1A receptor agonist 5-CT (10 nM). The magnitude of 5-HT1A-mediated outward currents was similar in cF1ko and F1wt mice (Figure 2B) and similar results were obtained using 100 nM 5-CT (data not shown). Limitations of the slice preparation, including a lack of 5-HT auto-inhibition or increased 5-CT induced internalization (Bouaziz et al., 2014; Andrade et al., 2015) could obscure the effect of 5-CT to produce increased response in the presence of excess 5-HT1A receptors.

Because 5-HT1A autoreceptors exert inhibitory tone on 5-HT neurons, we determined whether 5-HT levels are altered in cF1ko mice. In cF1ko compared to cF1wt dorsal raphe, there was a significant decrease (~50%) in 5-HT-positive cells from 68±10.5 to 35±8.2 cells/template (t test, DF=6 t=5.635, **p=0.0013), with no change in the number of TPH-positive cells (Figure 2C). Similarly, quantitative measurement of 5-HT and its major metabolite 5-HIAA revealed a ~50% reduction in raphe 5-HT content in Freud-1 knockout vs. wild-type mice (t test, DF=4 t=6.375, **p=0.0031), with no significant change in raphe 5-HIAA or in hippocampal or PFC 5-HT or 5-HIAA levels (Figure. 2D).

To address whether the activity of 5-HT neurons was changed, FosB staining was used as a marker for acute and chronic cellular activation. There was a ~50% reduction in FosB/TPH stained cells (49±11 vs. 25±7.9 cells/template, t test, DF=6 t=4.035, **p=0.0023) in the dorsal raphe of cF1ko compared to F1wt mice, suggesting a chronic reduction of 5-HT neuronal activity. Taken together, these data indicate an up-regulation of 5-HT1A autoreceptor expression and function upon loss of Freud-1 associated with increased auto-inhibition of 5-HT neuron activity and reduced raphe 5-HT levels.

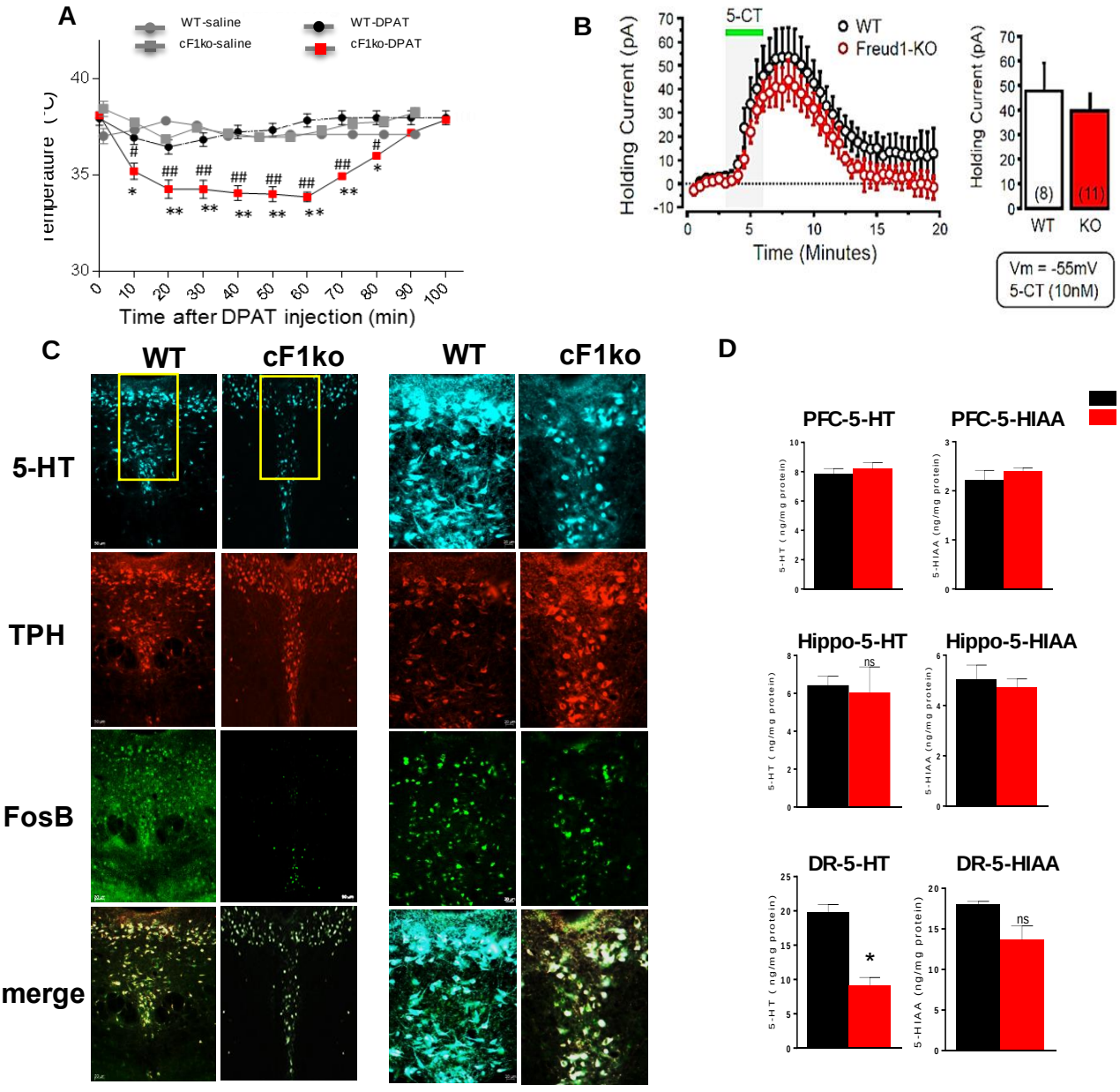

A
WT-saline
cF1ko-saline
WT-DPAT
cF1ko-DPAT
Temperature (°C)
Time after DPAT injection (min)
B
5-CT
WT
Freud1-KO
Holding Current (pA)
Time (Minutes)
(8)
(11)
WT
KO
Vm = -55mV
5-CT (10nM)
C
WT
cF1ko
WT
cF1ko
5-HT
TPH
FosB
merge
D
WT
cF1ko
PFC-5-HT
PFC-5-HIAA
Hippo-5-HT
Hippo-5-HIAA
DR-5-HT
DR-5-HIAA
5-HT (ng/mg protein)
5-HIAA (ng/mg protein)
ns
*

Figure 2. Loss of Freud-1 augments 5-HT1A autoreceptor function and reduces 5-HT neuron activity and 5-HT levels.

A. 5-HT1A-induced hypothermia. 8-OH-DPAT (0.75 mg/kg, i.p.) induced a greater body temperature reduction in cF1ko compared to F1wt (WT). Data represent mean±SEM (n=3/group); *p<0.05 vs. WT-saline, **p<0.001 vs. wild-type-DPAT, #p<0.05 vs. cF1ko-saline, ##p<0.001 vs. cF1ko-DPAT. B. Whole-cell voltage-clamp recordings (Vm = -55 mV) of 5-HT neurons in slices of dorsal raphe in vitro, from cF1ko or F1wt mice (n = 4) in response to 5-CT (10 nM). No significant difference in 5-HT1A receptor-induced outward current was observed. C. Reduced 5-HT- and FosB-stained cells in cF1ko raphe. DR sections of cF1ko or F1wt (WT) mice stained for 5-HT, TPH, and FosB shown at 10x (left, scale=50 μm) or 20x magnification of boxed region (right, scale=20 μm). D. Reduced raphe 5-HT content in cF1ko mice. Tissue 5-HT and 5-HIAA content was quantified by HPLC for dorsal raphe, hippocampus (Hippo) and prefrontal cortex (PFC) of cF1ko vs. WT mice. Data represent mean ± SEM, n=3/group; reduced raphe 5-HT content in cF1ko vs. WT mice (unpaired two-tailed Student's t test, DF=4, t=6.675 **p<0.01).

Fluoxetine-resistant anxiety- and depression-like behavior in cF1ko mice

The behavioural phenotype of adult cF1ko mice was assessed using multiple validated tests (Figure 3, timeline). In contrast, compared to F1wt littermates, cF1ko mice displayed robust anxiety-like behavior in the elevated plus maze (EPM), open field (OF) and novelty suppressed feeding (NSF) tests (Fig. 3A-C). In the EPM test, cF1ko mice had a significant 50% reduction in time spent in the open arms, with no difference in total distance or closed arm time (Fig. 3A). In the OF test, cF1ko mice spent significantly less time in the center of the arena, and more time in the corners (Fig. 3B). In the NSF test, the cF1ko mice displayed significantly greater latency to feed in the novel cage, while no difference was observed in latency for home cage food consumption (Fig. 3C). A depression-like phenotype was also detected in the cF1ko mice, with increased immobility in the forced swim (FST) (Figure 3D) and a trend ($p=0.07$) in the tail suspension (TS) test (Figure 3E). No difference in open field locomotor activity was detected between cF1ko and F1wt littermates in the beam break (BBK) test (Fig. 3F). These results were confirmed in a second independent cohort of mice (data not shown). These data indicate that adult knockout of Freud-1 in 5-HT neurons confers both anxiety- and depression-like phenotypes.

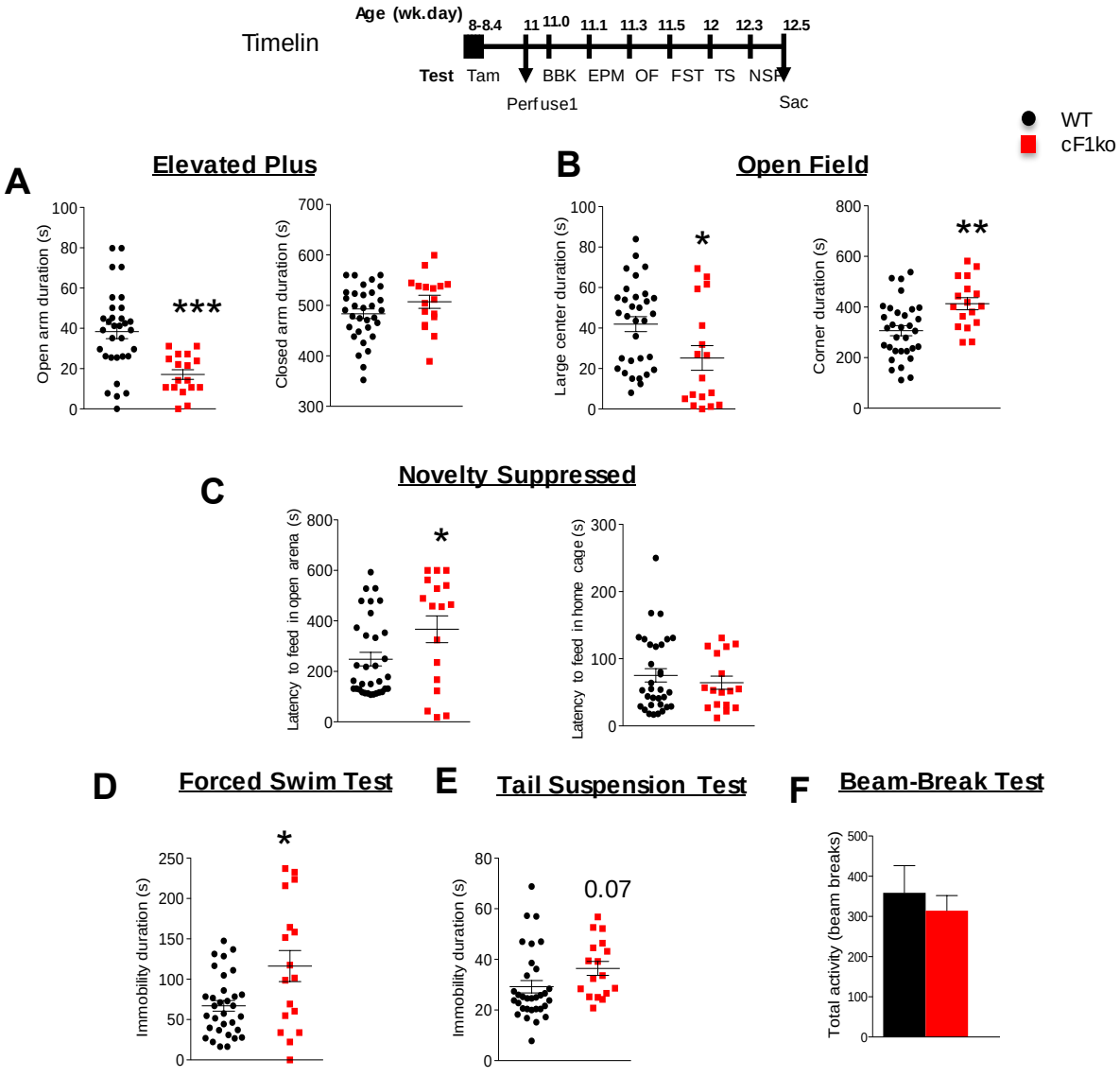
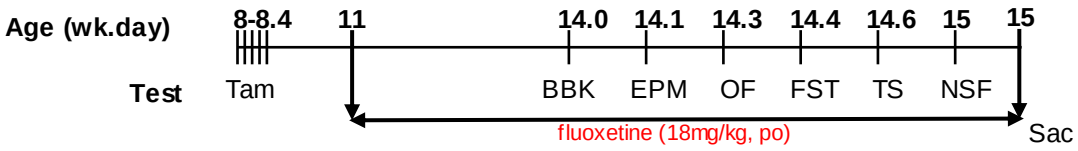

Timelin
Age (wk.day)
8-8.4
11
11.0
11.1
11.3
11.5
12
12.3
12.5
Test
Tam
BBK
EPM
OF
FST
TS
NSF
Perfuse1
Sac
WT
cF1ko
A
Elevated Plus
Open arm duration (s)
***
Closed arm duration (s)
B
Open Field
Large center duration (s)
*
Corner duration (s)
**
C
Novelty Suppressed
Latency to feed in open arena (s)
*
Latency to feed in home cage (s)
D
Forced Swim Test
Immobility duration (s)
*
E
Tail Suspension Test
Immobility duration (s)
0.07
F
Beam-Break Test
Total activity (beam breaks)

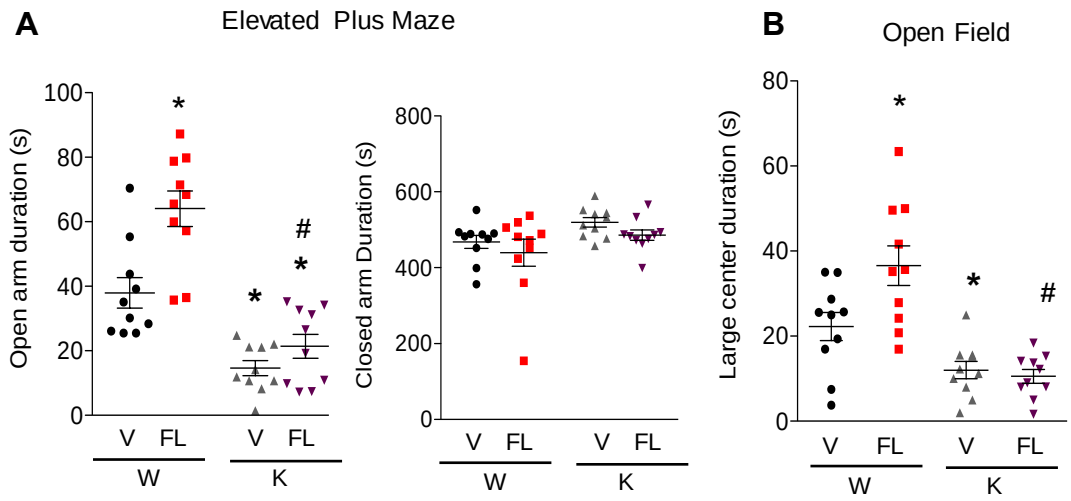
Figure 3. Increased anxiety- and depression-like behavior in cF1ko mice

The cF1ko and WT littermates underwent the indicated behavioural tests or assays according to the timeline shown, 2 weeks after the last tamoxifen injection (11-wk of age). A-C. Increased anxiety in cF1ko mice. A. EPM test. Compared to WT, cF1ko mice spent less time in open arms (unpaired two-tailed Student's t-test, DF=47, t=4.104**p<0.01), with no difference detected in closed arm time. B. OF. cF1ko mice displayed significantly reduced distance travelled in large center (unpaired two-tailed Student's t-test, DF=47, t=2.486 *p=0.0165), with no change in total distance (not shown). C. NSF test. cF1ko mice showed greater latency to approach food in the novel arena (unpaired two-tailed Student's t test, DF=48, t=2.221 *p=0.0311), but no difference in the home cage. D-E. Depression-like behaviour in cF1ko mice. D. FST. The cF1ko showed significant greater immobility duration in the FST compared to F1wt mice (unpaired two-tailed Student's t-test, DF=47, t=2.962 *p<0.05). E. TST. No difference in immobility duration between cF1ko and WT was seen. F. Locomotion test. Results from the beam break (BBK) test showed no difference in total 30-min activity comparing cF1ko and F1wt (WT) mice. Data represent mean ± SEM in cF1ko mice (n=17) vs. F1wt (WT) (n=32).

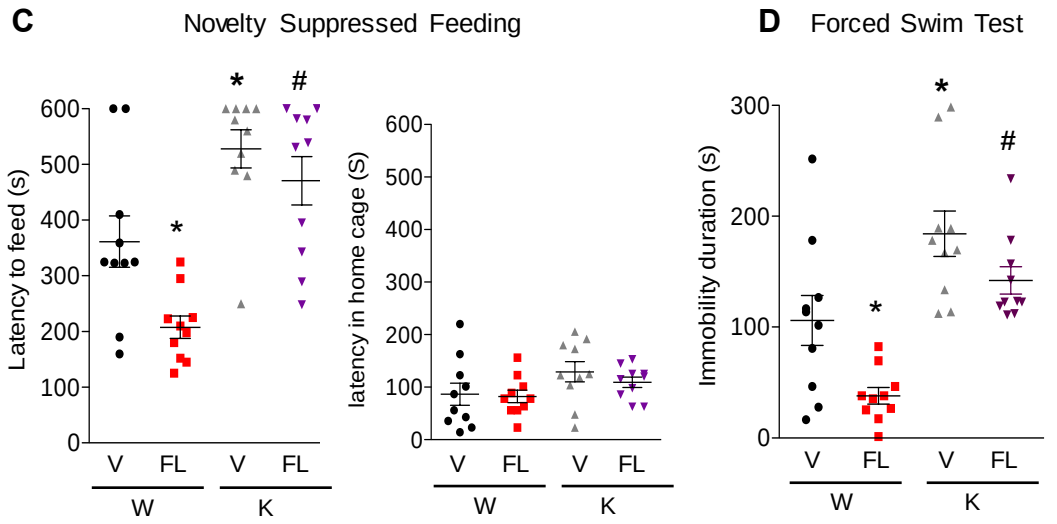
Increase in 5-HT1A autoreceptors is thought to reduce responsiveness to chronic SSRI treatment (Albert and Le Francois, 2010; Richardson-Jones et al., 2010). The behavioral response to fluoxetine (FLX) was tested in singly housed cF1ko and F1wt littermates (11 wk old, n=10/group) treated or not with FLX in drinking water for 3-4 weeks using the above tests. In each test, the effect of conditional Freud-1 knockout to increase anxiety/depression-like behavior was replicated. In the anxiety tests (EPM, OF and NSF), chronic fluoxetine treatment reduced anxiety-like behaviors in the cF1wt mice, but did not alter the anxiety phenotype in cF1ko mice, compared to the vehicle-treated group (Fig. 4A-C), with no changes in control measures.

Furthermore, the cF1ko mice had increased immobility but showed no significant response to FLX in the FST, while the F1wt showed reduced immobility in FLX-treated vs. vehicle (Fig. 4D). Neither cF1ko nor F1wt mice showed any differences or response to FLX in the TS (Fig. 4E), nor in the beam break test (Fig. 4F). These results indicate that the anxiety/depression-like phenotype seen in the cF1ko mice and associated with overexpression of 5-HT1A autoreceptors is resistant to chronic fluoxetine treatment.

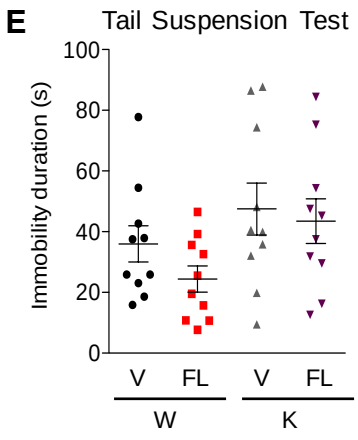

Age (wk.day)
8-8.4
11
14.0
14.1
14.3
14.4
14.6
15
15
Test
Tam
BBK
EPM
OF
FST
TS
NSF
fluoxetine (18mg/kg, po)
Sac


A
Elevated Plus Maze
Open arm duration (s)
Closed arm Duration (s)
V FL V FL
W K

B
Open Field
Large center duration (s)
V FL V FL
W K


C
Novelty Suppressed Feeding
Latency to feed (s)
latency in home cage (S)
V FL V FL
W K

D
Forced Swim Test
Immobility duration (s)
V FL V FL
W K


E
Tail Suspension Test
Immobility duration (s)
V FL V FL
W K

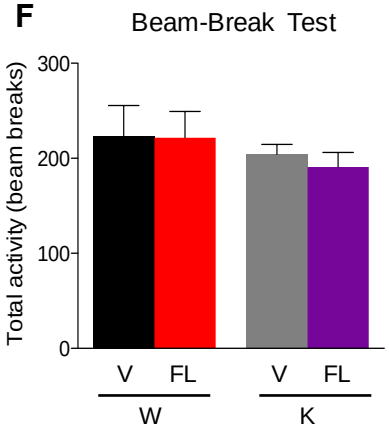

F
Beam-Break Test
Total activity (beam breaks)
V FL V FL
W K

Figure 4. Resistance to chronic SSRI treatment in cF1ko mice.

Wild-type (WT) or cF1ko (KO) mice were treated with fluoxetine (FLX) or vehicle (V) for 3 weeks and throughout the behavioural assays (timeline). A-C. Anxiety phenotype: chronic FLX reduced anxiety in WT mice, but did not affect the increased anxiety seen in cF1KO mice. A. EPM, significant changes in time spent in open arms in EPM test: one-way ANOVA treatment X genotype interaction, $F_{3.36}=27.01$, $p<0.01$; post-hoc Tukey **p < 0.01 vs WT-Veh , ##p < 0.01 vs WT-FLX. B. OF, changes in distance travelled in large center: one-way ANOVA treatment X genotype interaction, $F_{3.041}=14.53$, $p<0.01$; post-hoc Tukey **p < 0.01 vs WT-Veh , ##p < 0.01 vs WT-FLX. C. NSF, latency to approach food in novel arena: one-way ANOVA treatment X genotype interaction, $F_{3.36}=14.16$, $p<0.01$; post-hoc Tukey **p < 0.01 vs WT-Veh , ##p < 0.01 vs WT-FLX. D-E. Depression phenotype. D. FST. Depression-like behavior in cF1ko (veh) compared to F1wt (veh) was indicated by increased immobility in the FST. Chronic FLX significantly reduced immobility in F1wt, but not in cF1ko mice FST, one-way ANOVA treatment X genotype interaction, $F_{3.36}=13.62$, $p<0.01$; post-hoc Tukey **p < 0.01 vs WT-Veh , ##p < 0.01 vs WT-FLX. E. TST, no significant changes were observed. F. Beam break. No differences in 30-min activity in novel arena were observed. Data represent individual animals with mean ± SEM, n=10/group; **p < 0.01 vs. WT-Veh , ##p < 0.01 vs WT-FLX.

Requirement of 5-HT1A autoreceptors for Freud-1-dependent behavioral effects

To address whether the behavioral phenotype induced by loss of Freud-1 is dependent on the increased level of 5-HT1A autoreceptors, the cF1ko mice were mated to the flx-1A mice (Samuels et al., 2015), in which the 5-HT1A gene is flanked by LoxP sites and a YFP cassette (Fig. 1A). Tamoxifen-induced recombination in 5-HT neurons in cF1/1Adko mice was verified by co-staining for YFP and TPH (Fig.5A). Staining for 5-HT1A receptors revealed a strong reduction in the number of 5-HT1A/TPH co-labeled cells in cF1/1Adko compared to cF1ko/1Awt littermates (Fig. 5B, cF1ko/1Awt, 120.5±6.19; cF1ko/1Ako: 16± 2.48 cells; t test, DF=6, t=15.650 ***p=0.0001). The presence of weak 5-HT1A staining in TPH-negative cells (Fig. 5B, white arrows) may represent 5-HT1A heteroreceptors in non-5-HT neurons of the raphe (Calizo et al., 2011). The extent of loss of 5-HT1A receptors in the cF1/1Adko mice was determined by autoradiography using the selective 5-HT1A antagonist 125I-MPPI. The results show significant reduction of 5-HT1A binding in the dorsal (6.327±0.40 vs 1.339±0.1770 uCi; t test, DF=4, t=11.24 ***p=0.0004) and median (5.015±0.326 vs. 1.779±0.5248 uCi; t test, DF=4, t=5.235 **p=0.0064) raphe of cF1/1Adko (Figure 5C). No difference in post-synaptic 5-HT1A receptor levels was observed in hippocampus of cF1/1Adko compared to cF1ko/1Awt littermates. Thus, the cF1/1Adko mice show a significant reduction in the number of 5-HT1A autoreceptors.

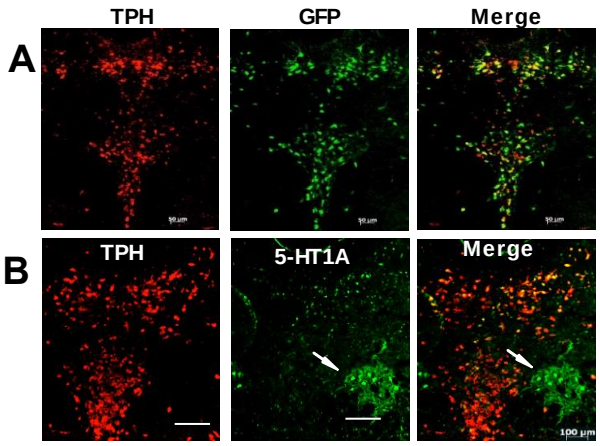
The effect of Freud-1 knockout on a 5-HT1A autoreceptor-negative background was examined using the cF1/1A dko mice. In Freud-1 wild-type mice, knockout of the 5-HT1A autoreceptor in adults (comparing cF1wt/1Ako to cF1wt/1Awt) did not alter anxiety or depression behavior (Fig. 5D-H), consistent with previous results (Richardson-

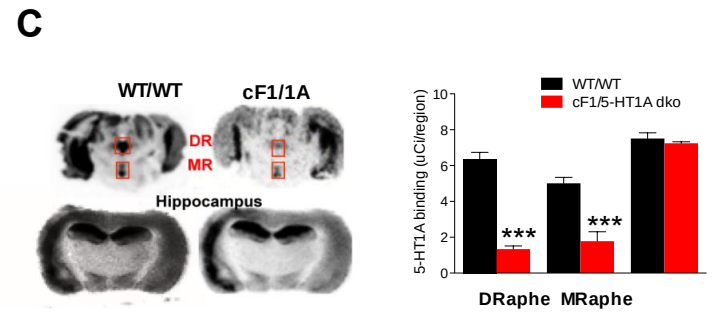
Jones et al., 2010). In mice lacking 5-HT1A autoreceptors, there was no difference in anxiety behaviors between Freud-1 knockout (cF1/1Adko) and wild-type (cF1wt/1Ako) mice in the EPM, OF or NSF tests, nor in control measures (Fig. 5D-F). Surprisingly, although the knockout of Freud-1 in the 5-HT1A wild-type background increased immobility in the FST, knockout of Freud-1 in the 5-HT1A-knockout background induced a stress resilient phenotype with reduced immobility in FST and TS compared to control groups (cF1wt/1Ako vs. cF1/1Adko, Fig. 5G-H). Thus, the absence of 5-HT1A autoreceptors not only blocked the pro-depressant effect of Freud-1 deletion but unmasked an antidepressant-like effect. No significant change in locomotor activity among genotypes was observed in the beam-break test (Fig. 5I). Taken together, these results indicate that the anxiety/depression phenotype observed upon conditional knockout of Freud-1 in 5-HT neurons depend on the presence of 5-HT1A autoreceptors.

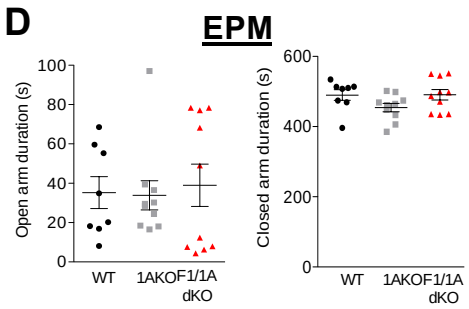
The effect of Freud-1 knockout on a 5-HT1A autoreceptor-negative background was examined using the cF1/1A dko mice. In Freud-1 wild-type mice, knockout of the 5-HT1A autoreceptor in adults (comparing cF1wt/1Ako to cF1wt/1Awt) did not alter anxiety or depression behavior (Fig. 6D-H), consistent with previous results (Richardson-Jones et al., 2010). In mice lacking 5-HT1A autoreceptors, there was no difference in anxiety behaviors between Freud-1 knockout (cF1/1Adko) and wild-type (cF1wt/1Ako) mice in the EPM, OF or NSF tests, nor in control measures (Figure 6D-F). Surprisingly, although the knockout of Freud-1 in the 5-HT1A wild-type background increased immobility in the FST, knockout of Freud-1 in the 5-HT1A-knockout background induced a stress resilient phenotype with reduced immobility in FST and TS compared to control groups (Fig. 6G-H). Thus, the absence of 5-HT1A autoreceptors not only

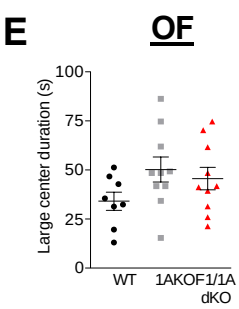
blocked the pro-depressant effect of Freud-1 deletion but unmasked an antidepressant-like effect. Taken together, these results indicate that the anxiety and depression phenotypes observed upon conditional knockout of Freud-1 in 5-HT neurons depend on the presence of 5-HT1A autoreceptors.

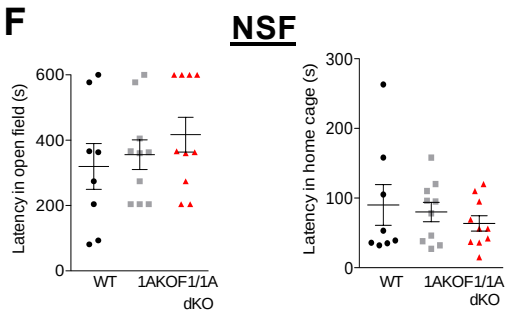
cF1/1A dKO

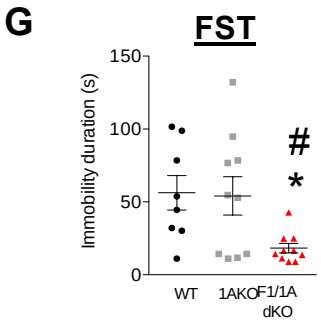

A
TPH
GFP
Merge
B
TPH
5-HT1A
Merge
100 µm

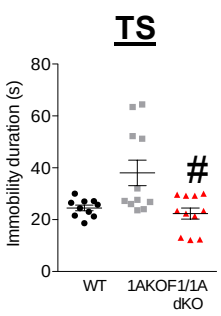

C
WT/WT
cF1/1A
DR
MR
Hippocampus
WT/WT
cF1/5-HT1A dko
5-HT1A binding (uCi/region)
***
***
DRaphe
MRaphe


D
EPM
Open arm duration (s)
Closed arm duration (s)
WT
1AKO
F1/1A dKO


E
OF
Large center duration (s)
WT
1AKO
F1/1A dKO


F
NSF
Latency in open field (s)
Latency in home cage (s)
WT
1AKO
F1/1A dKO


G
FST
Immobility duration (s)
#
*
WT
1AKO
F1/1A dKO


H
TS
Immobility duration (s)
#
WT
1AKO
F1/1A dKO

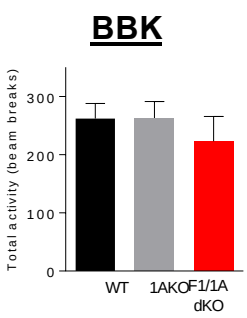

I
BBK
Total activity (beam breaks)
WT
1AKO
F1/1A dKO

Figure 5. Reversal of anxiety- and depression-like phenotypes upon loss of Freud-1 in the absence of 5-HT1A autoreceptors

Tissues from mice with conditional knockout of Freud-1 and 5-HT1A receptor in adult serotonin neurons (cF1/1A dKO) were compared to 5-HT1A conditional knockout (c1AKO) or wild-type littermates (WT/WT). A. Tamoxifen-induced recombination. Co-staining for YFP and TPH in the dorsal raphe. Recombination occurred exclusively in 5-HT cells; 92% of TPH-positive cells were YFP-positive (scale bar = 100 μm). B. Loss of 5-HT1A autoreceptors. Immunostaining for 5-HT1A and TPH showed significant loss of 5-HT1A immunostaining in TPH-positive cells of cF1/1A dKO mice. White arrows indicate the presence of 5-HT1A receptors in non-TPH positive cells in dorsal raphe (scale=100 μm). C. 5-HT1A autoradiography. 5-HT1A receptor autoradiography using 125I-MPPI of representative midbrain sections from WT/WT and cF1/1A dKO mice, including dorsal (DR) and median (MR) raphe (boxes) and hippocampal sections. DR and MR showed significant decreases in 5-HT1A autoreceptor binding: DR (unpaired two-tailed Student's t-test, DF=4, t=11.24, ***p<0.001), MR (unpaired two-tailed Student's t-test, DF=4, t=5.235, *p<0.01). D-H. Behavioral studies were done in either wild-type (WT) or conditional knockout (KO) for Freud-1 (F1) and/or 5-HT1A receptor (1A) in littermate mice. D. EPM. No change in anxiety-like behavior (open arm time) was observed. E. OF. No difference among groups was observed in time spent in the large centre. F. NSF. The latency to feed was not altered. G. FST. Immobility duration was reduced in F1/1AKO vs. WT and 1AKO mice (one-way ANOVA genotype X genotype interaction, $F_{2,25}=4.463$, $p=0.0067$; post-hoc Tukey *p<0.05 vs. WT, #p<0.05 vs. 1AKO). H. TS. Immobility time was reduced in F1/1AKO compared to WT or 1AKO (one-way ANOVA genotype X genotype interaction, $F_{2,29}=6.998$, $p=0.0033$; post-hoc Tukey *p<0.05 vs. WT, #p<0.05 vs. F1/1AKO). I. Beam break test. No difference in 30-min activity in novel arena was observed. Data points represent individual mice, with mean ± SEM: WT, n=10; F1WT/1AKO, n=11; F1/1AKO, n=11.

DISCUSSION

Freud-1 represses 5-HT1A autoreceptors to regulate 5-HT, anxiety and depression

A reduction in 5-HT neurotransmission implicated in anxiety and depression (Mann, 1999; Jans et al., 2007) has been associated with increased 5-HT1A autoreceptor expression in depressed subjects (Parsey et al., 2010) and depressed suicides (Stockmeier et al., 1998; Boldriniet al., 2008). Thus, the transcriptional "set point" of 5-HT1A autoreceptor expression may be elevated in depression, suggesting altered function of transcription factors (Albert et al., 2011). Here we show that loss of Freud-1 in adult 5-HT neurons leads to up-regulation of 5-HT1A autoreceptors (Figure. 1D-G), and is correlated with increased 5-HT1A response to DPAT-induced hypothermia (Figure 2A), reduced raphe 5-HT levels (Figure 2C-D), and anxiety/depression-like behaviors (Figure 3) that are resistant to chronic SSRI treatment (Figure 4). These findings implicate a key role for the endogenous repressor Freud-1 in 5-HT1A autoreceptor expression leading to reduced raphe 5-HT and antidepressant-resistant anxiety and depression. In contrast, global knockout of the repressor Deaf1 resulted in up-regulation of 5-HT1A autoreceptor expression, reduced raphe 5-HT, but only a mild anxiety phenotype (Czesak et al., 2012; Luckhart et al., 2016), consistent with a stronger effect of Freud-1 to repress the human 5-HT1A gene in raphe cells (Lemonde et al., 2003; Ou et al., 2003).

Previous studies have indirectly implicated transcriptional dys-regulation of the human HTR1A gene in psychopathology and treatment resistance (Albert et al., 2011). For example, within the human HTR1A repressor region, a C(-1019)G polymorphism that prevents binding and repression by Deaf1/NUDR (Lemonde et al., 2003) has been associated with major depression and bipolar depression (Kishi et al., 2013) and with

SSRI resistance (Le Francois et al., 2008; Kato et al., 2015; Takekita et al., 2015) and with increased 5-HT1A autoreceptors levels in depressed subjects (Hesselgrave and Parsey, 2013). Allele-specific RT-PCR analysis has revealed that this polymorphism leads to lifelong alterations in 5-HT1A RNA levels in human PFC, which is attenuated in depressed subjects (Donaldson et al., 2016). Our findings of a major role for Freud-1 in repressing 5-HT1A autoreceptor expression and in SSRI-resistantanxiety/depression phenotype in mice are consistent with a key role for Freud-1-mediated repression in human anxiety and depression.

Importantly, we demonstrate that the behavioral effect of Freud-1 deletion is dependent on the presence of 5-HT1A autoreceptors in adult mice (Fig. 5). Previously, a mild (30%) knock-down of the 5-HT1A autoreceptor in adulthood resulted in no change in anxiety or depression behaviors, but improved stress-coping (Richardson-Jones et al., 2010), a phenotype that we did not assess in these studies. The anti-depressed phenotype seen upon Freud-1 deletion suggests that Freud-1 has a pro-depressant effect that is revealed when 5-HT1A autoreceptors are absent. Loss of Freud-1 in 5-HT1A-negative 5-HT neurons could alter the transcription of other genes, including de-repressing dopamine-D2 receptors (Rogaeva et al., 2007), which have been shown to increase 5-HT neuron activity (Aman et al., 2007), which in turn could lead to an antidepressant effect. We did not address whether other gene targets of Freud-1 contribute to behavioral outcomes in cF1ko mice, apart from increased 5-HT1A autoreceptor expression. In addition to 5-HT1A and dopamine-D2 gene repression, Freud-1 induces NF-κB expression (Matsuda et al., 2003; Zhao et al., 2010). Gene deletion of Freud-1 reduces NF-κB signaling to synaptic plasticity in cortical development, leading to abnormal cortical dendrite

organization and reduced dendritic spine density (Manzini et al., 2014). However, these effects of Freud-1 deletion appear to be developmental, and deletion of Freud-1 in adulthood did not appear to affect neuronal organization (Oaks et al., 2016).

SSRI resistance in Freud-1 conditional knockout mice

The three-week latency for clinical efficacy of SSRI treatment is thought to involve desensitization of 5-HT1A autoreceptors to release 5-HT neurons from recurrent inhibition (Pineyro and Blier, 1999; Albert et al., 2011). Our results suggest that loss of Freud-1 may render 5-HT neurons resistant to desensitization by chronic SSRI treatment. In human depression, increased 5-HT1A autoreceptors are correlated with resistance to SSRI treatment (Parsey et al., 2010). Furthermore, the G(-1019) allele associates with resistance to SSRI treatment (Lemonde et al., 2004b; Le Francois et al., 2008; Parsey et al., 2010). The G-allele is also associated with resistance of negative symptoms to treatment with atypical antipsychotics that act in part by targeting 5-HT1A receptors (Reynolds et al., 2006; Newman-Tancredi and Albert, 2012). Taken together, these results implicate the increase in 5-HT1A autoreceptors upon loss of Freud-1 in resistance to chronic SSRI treatment. This resistance could suggest a role for activation of Freud-1 in desensitizing 5-HT1A autoreceptors in response to antidepressants. 5-HT1A receptors couple to inhibition of intracellular calcium levels (Albert et al., 1990; Penington et al., 1991), which could activate Freud-1 DNA binding and repression (Ou et al., 2003), leading to 5-HT1A autoreceptor desensitization. The loss of Freud-1 would prevent this process, leading to SSRI resistance. Alternately, the elevated levels of 5-HT1A autoreceptors may require high SSRI doses or longer times to fully desensitize. In this regard, 5-HT1A partial agonists or allosteric modulators that enhance Freud-1 signaling

to desensitize presynaptic 5-HT1A receptors may prove useful to augment SSRI response in treatment resistant subjects (Trivedi et al., 2006). The cF1KO mouse may provide a useful model to test novel antidepressants like ketamine or deep brain stimulation for their dependence on 5-HT auto-regulation, which has previously been examined using 5-HT depletion (Hamani et al., 2010; du Jardin et al., 2016).

Roles of Freud-1 *in vivo*

Our data suggest that susceptibility to anxiety and depression due to transcriptional dys-regulation of the 5-HT1A receptor by Freud-1 can be induced in adulthood and leads to reduced raphe 5-HT/5-HIAA level, suggesting a chronic reduction of 5-HT activity. Acute tryptophan depletion leads to relapse in recovered depressed but not control subjects, suggesting that depression confers increased susceptibility to 5-HT depletion that could be due to persistent transcriptional dys-regulation in recovered depressed subjects as exemplified by the cF1KO mice. Furthermore, patients that relapse often become more resistant to SSRI treatments, consistent with the SSRI-resistant phenotype of the cF1KO mice, and suggesting a role for loss of Freud-1 function in the development of this resistance.

Disruption of the CC2D1A/Freud-1 gene in humans is linked to NSID and autism (Basel-Vanagaite et al., 2006; Manzini et al., 2014), conditions often associated with reduced social interaction. Knockout of Freud-1 in the postnatal mouse forebrain results in impaired cortical neuronal development, reduced cognitive function and social interaction, as well as anxiety-like behavior in the OF test (Oaks et al., 2016). This suggests that post-synaptic Freud-1 is also important for normal behavioral development. Consistent with this, Freud-1 levels are reduced in PFC following chronic stress in rats

(Iyo et al., 2009), while both Freud-1 and 5-HT1A protein levels were reduced in PFC of young depressed subjects (Szewczyk et al., 2010), suggesting that early impairments in cortical Freud-1 levels may predispose to depression. The importance of presynaptic Freud-1 in 5-HT1A autoreceptor regulation and anxiety/depression behavior reported here, and its complementary role in the forebrain in anxiety and cognitive function suggest that enhancing Freud-1 expression or function may provide a useful target, particularly in treatment-resistant forms of these diseases.

In summary, this study provides evidence that loss of Freud-1 in 5-HT neurons causes over-expression of 5-HT1A autoreceptors, associated with SSRI-resistant and 5-HT1A autoreceptor-dependent anxiety/depression phenotypes and a 5-HT1A autoreceptor-independent anti-depressed phenotype, implicating Freud-1 as a key transcriptional regulator in 5-HT function and behaviour.

ACKNOWLEDGEMENTS

We thank Dr. C. A. Walsh (Harvard University) for providing the Cc2d1a (Freud-1)$^{flx/flx}$ mice, Drs. K. Nautiyal and Z. Donaldson, Columbia University for helpful discussions, and Dr. D. C. Lagace and the UOttawa Behavioral Core for helpful advice and technical assistance. This research was supported by grants from the Canadian Institutes for Health Research (MOP-115098 and MOP-123426 to P.R.A.) and trainee support to F.V.-A. from the Canadian Partnership for Stroke Recovery. These agencies had no role in the design of the study, collection and analysis of data or decision to publish.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

Chapter 3

Persistent post-stroke depression in mice following unilateral medial prefrontal cortical stroke

Persistent post-stroke depression in mice following unilateral medial prefrontal cortical stroke

F Vahid-Ansari,[1,2] D C Lagace,[2] and P R Albert[1,2]

[1]Ottawa Hospital Research Institute (Neuroscience), University of Ottawa Brain and Mind Research Institute, Ottawa, ON, Canada

[2]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada

Published in the Journal Translational Psychiatry, 2016 Aug; 6(8): e863

Published online 2016 Aug 2. doi: 10.1038/tp.2016.124

Author Contributions

FV-A, DCL and PRA designed the study; FV-A acquired the data; FV-A, DCL and PRA analyzed the data; FV-A wrote the article, and DCL and PRA revised it and approved the article for publication.

Abstract

Post-stroke depression (PSD) is a common outcome following stroke that is associated with poor recovery. To develop a preclinical model of PSD, we targeted a key node of the depression–anxiety circuitry by inducing a unilateral ischemic lesion to the medial prefrontal cortex (mPFC) stroke. Microinjection of male C57/BL6 mice with endothelin-1 (ET-1, 1600 pmol) induced a small (1 mm^3) stroke consistently localized within the left mPFC. Compared with sham control mice, the stroke mice displayed a robust behavioral phenotype in four validated tests of anxiety including the elevated plus maze, light–dark, open-field and novelty-suppressed feeding tests. In addition, the stroke mice displayed depression-like behaviors in both the forced swim and tail suspension test. In contrast, there was no effect on locomotor activity or sensorimotor function in the horizontal ladder, or cylinder and home cage activity tests, indicating a silent stroke due to the absence of motor abnormalities. When re-tested at 6 weeks post stroke, the stroke mice retained both anxiety and depression phenotypes. Surprisingly, at 6 weeks post stroke the lesion site was infiltrated by neurons, suggesting that the ET-1-induced neuronal loss in the mPFC was reversible over time, but was insufficient to promote behavioral recovery. In summary, unilateral ischemic lesion of the mPFC results in a pronounced and persistent anxiety and depression phenotype with no evident sensorimotor deficits. This precise lesion of the depression circuitry provides a reproducible model to study adaptive cellular changes and preclinical efficacy of novel interventions to alleviate PSD symptoms.

Introduction

Focal ischemic stroke can lead to both neurological symptoms, including impairments in sensorimotor function and cognition, and behavioral changes in anxiety, depression and emotionality (Esparrago et al., 2015; Williams et al., 2005; Carota et al., 2005). Within 3 months of a stroke, 20–50% of patients are diagnosed with post-stroke depression (PSD) following a focal stroke can persist for >1 year after diagnosis (Kotila et al., 1998; Paolucci et al., 2006). Patients with PSD often display both anxiety and depressive symptoms, (Campbell et al., 2011; Galligan et al., 2015) leading to decreased quality of life, increased mortality and heightened risk of recurrent stroke or suicide (Almeida and Xiao, 2007; Wolfe et al., 2011; Whyte et al., 2006). PSD is also associated with cognitive decline(Hakim 2011; Barker-Collo, 2007; Nys et al., 2006; Thomas and Lincoln, 2006) and interferes with stroke recovery (Mayo et al., 1991; Goodwin and Devanand, 2008; Wulsin et al., 2012) leading to increased hospitalization and health care costs (Atteih et al., 2015; Ghose et al., 2005; Husaini et al., 2008). In addition to PSD associated with focal ischemia, PSD due to silent strokes (vascular depression) is mainly associated with white matter lesions (Wu et al., 2014), with a 10-fold greater prevalence compared with overt strokes (Leary and saver, 2003; Vermeer et al., 2007) . When diagnosed, patients with PSD or vascular depression are treated with antidepressants, such as serotonin-selective reuptake inhibitors (Campbell et al., 2011; Mead et al., 2012). However, these drugs take at least 3–4 weeks to elicit a clinical response and they are only 50% effective in treating anxiety and depression, with only 30% of patients achieving remission (Trivedi et al., 2006). Thus, there is a need for preclinical models to develop improved treatment strategies.

There are several preclinical models of PSD in rodents and non-human primates that have induced large infarcts involving both cortical and subcortical gray and white matter and have reported ‘depressive-like syndromes' characterized by varying degrees of anxiety, despair and anhedonia (Kronenberg et al., 2014). For example, the middle cerebral artery occlusion (MCAO) model in combination with chronic mild stress evokes stress- and stroke-induced behavioral phenotypes that are amenable to antidepressant medications (McCann et al., 2014). However, the MCAO models are often associated with large and variable lesions affecting large regions of cortex and striatum and thus are confounded by pronounced motor impairments that can interfere with assessments of depression and anxiety. Therefore, one of our goals was to develop a reliable model of PSD that was not associated with motor impairments and that persists over time, to test relevant treatments.

Modeling PSD has been challenging due to the size and variability in the location of stroke in patients diagnosed with PSD (Robinson and Jorge, 2016). Specific focal lesions associated with PSD are rarely identified since PSD is difficult to diagnose without overt physical impairment. Although PSD is generally more frequent in subjects suffering from left hemispheric lesions (Barker-Collo, 2007; Astrom et al., 1993; Narushima et al., 2003) recent reports have found that right hemisphere lesions are also associated with PSD during acute post stroke period ($\leqslant$3 months) (Wei et al., 2015). Clinical studies have additionally demonstrated that PSD is associated with lesions involving cortico-limbic circuitry (de Groot et al., 2000; Gong and He, 2015; Liao et al., 2013; Brookes et al., 2014). In particular, recent studies support a positive relation between PSD and damage to some basal ganglion or frontal lobe structures including the medial prefrontal cortex

(mPFC) (Flaster et al., 2013). Furthermore, alterations in the activity of the mPFC have been strongly implicated in depression-like behavior (Klein et al., 2010; Covington et al., 2010; Vialou et al., 2014), as well as projections between mPFC and the basal ganglia and the nucleus accumbens (Warner-Schmidt, 2013; Belzung et al., 2014). Given that large infarcts that may impair mPFC function can induce depression and anxiety, we tested whether a small unilateral focal stroke targeted to left mPFC would be sufficient to disrupt affective circuitry and yield a potentially useful model of PSD.

To produce a focal stroke in the mPFC, we used the endothelin-1 (ET-1) model. ET-1 is a vasoconstrictor peptide that when injected results in a discrete ischemic lesion by causing blood vessels constrict acutely to reduce blood flow in the target area (Fuxe et al., 1989). The ET-1 model was initially characterized in rats as a simple and reproducible method of focal ischemia (Fuxe et al., 1989; Windle and Corbett, 2005). More recently, others have shown in mice that ET-1 can also be used to make a specific lesion of the mouse motor cortex that is associated with consistent locomotor impairments (Tennant and Jones, 2009; Roome et al., 2014). In this study, ET-1 microinjection in the left mPFC in male C57/BL6 mice was used to generate a unilateral and reproducible focal ischemic lesion, resulting in a robust anxiety and depression phenotype that persisted for at least 6 weeks without sensorimotor impairment. These findings suggest that the ET-1 lesion to the mPFC may be useful to examine changes in regional brain activity that lead to the PSD phenotype, as well as to develop, test and optimize treatments for PSD.

Materials and methods

Animals

Seventy one male C57/BL6, 10–11 weeks old (Charles River Laboratories, Montreal, QC, Canada) weighing 25–28 g at the time of surgery were used in this study. Mice were pair-housed in standard Plexiglass cages on a 12/12 h light/dark cycle with ad libitum access to food and water. Animals were allowed to acclimate to the housing facility for 2 weeks prior to surgery. Forty mice were tested in 2 cohorts of 20 mice for behavioral assays based on published literature for these tests (Belzung and Griebel, 2001; Crawley, 2007). For each cohort, the mice were randomly divided into either the sham control (n=10) or stroke (n=10) group and no blinding was done. For histology, 11 additional mice were used including n=3 at 48 h and n=4 at 1 week, 2 weeks, n=4 per group; for 6 weeks post surgery 4 mice from the behavioral cohort were used. A separate cohort of mice was used to test depression-like behavior at 6 weeks post stroke (sham=10; stroke=10). The University of Ottawa Animal Care Committee approved all experimental procedures in accordance with guidelines established by the Canadian Council of Animal Care.

ET-1 injection

Mice were anesthetized with isoflurane (5% induction, 1.5–2% maintenance with oxygen 1 l min−1) prior to stereotaxic surgery. During surgery, body temperature was maintained between 36.5 and 37.5 °C using a feedback homeothermic heating blanket. ET-1 (Calbiochem, San Diego, CA, USA) was suspended in sterile water for complete solubility (2 μg μl−1=800 pmol μl−1) by cold sonication at 4 °C for 15–20 min and 1.0 μl was injected using an infusion pump set at a rate of 0.20 μl min−1. ET-1 was injected at two sites within the left mPFC at the following coordinates (in mm) relative to Bregma (Franklin and Paxinos, 2007) (Figure 1a): Injection 1, (AP), 2.0; medial–lateral (ML),

+0.5; dorsoventral (DV), −2.4; Injection 2, AP 1.5; ML +0.5; DV −2.6. At the end of the infusion, the needle was left in place for 3 min before gradual withdrawal and closure of the incision that was treated with 0.1 ml of 2% transdermal bupivacaine (Chiron, Guelph, ON, Canada) as a topical anesthetic. Post surgery, the mice were placed in a 37 °C incubator to maintain body temperature until they regained mobility. At ~3 h post surgery, the mice were given a single injection of buprenorphine (0.03 mg kg−1 s.c., Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) for pain management and then returned to their housing room. Sham control mice underwent the same procedures except that the ET-1 was replaced with vehicle (sterile water).

Cresyl violet staining

At 2, 7, 14 or 42 days post surgery, mice were deeply anesthetized by administration of Euthanyl (149.5 mg kg−1 i.p., Bimeda-MTC Health, Cambridge, ON, Canada) and transcardially perfused with phosphate-buffered saline (PBS) 0.1 m, followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were removed and post fixed in 4% PFA overnight at 4 °C, then transferred to sucrose 30% (w/v) in 0.1 m phosphate buffer solution until saturated. Coronal sections (25 µm thickness) were cut through the mPFC and collected in 10 serial sections using a microtome. For each animal, every fourth section throughout the mPFC lesion was stained with cresyl violet (CV) (Tureyen et al., 2004).

Infarct volume assessment

Using ImageJ (NIH, Bethesda, MD, USA, http://imagej.nih.gov/ij/), the area of contralateral and intact ipsilateral tissue remaining was measured for every fourth serial coronal section (stained with CV) throughout the lesion in mPFC. Infarct volume in mm^3

was calculated as follows: Σ (area of contralateral lesion tissue−area of intact ipsilateral tissue) × thickness of the interval between sections (Windle et al., 2006).

Immunofluorescence

Coronal brain slices (25-µm thickness) of mPFC including cingulate cortex (CG), infra- and pre-limbic mPFC (coordinates; Bregma +1.70 to +1.54 mm) (Franklin and Paxinos, 2007) were obtained from brain tissue from PSD mice at 48 h (n=3) and 6 weeks (n=3) post stroke. The sections were washed 3 × in PBS, blocked 1 h in PBS with 1% BSA, 10% NDS, 0.1% Triton X-100 followed by 3-h incubation at RT with rabbit anti-IBa1 1:1000 (Wako, Richmond, VA, USA, 019–19741), and goat anti-GFAP 1:500 (Santa Cruz, Dallas, TX, USA, sc-6170) and mouse anti-NeuN 1:1000 (Millipore, Mississauga, ON, Canada, MAB377). Sections were washed three times in PBS followed by 1-h incubation at RT with Alexa Fluor 488 donkey anti-rabbit IgG, 1:1000 (Life Technology, Beverly, MA, USA, A-21206); donkey anti-goat Cy5, 1:250 (Life Technology, A-21447) and Alexa Fluor 594 donkey anti-mouse IgG, 1:200 (Thermofisher, Mississauga, ON, Canada, A-21203) in blocking solution. Images of left mPFC were acquired with the Axiovision imaging software (Carl Zeiss Canada, North York, ON, Canada) on a Zeiss Axio Observer D1 microscope (Carl Zeiss Canada).

Behavioral assays

Several validated behavioral tests measuring anxiety, depression and sensorimotor deficits were performed at the UOttawa Behavioral Core Facility in littermates following a unilateral PFC stroke. Mice were housed under normal light conditions and tests were performed beginning at 1000 hours, 2 h after lights were turned on and at least 1 h after the mice were habituated to the testing room. Testing was performed under white light

illumination with the exception of the forced swim test (FST) and cylinder test (Cyl), which were performed under red light. All animals were of the same age at the start of testing, and behavioral testing began at 1 week post surgery and lasted up to 6 weeks post surgery, two cohorts n=10 per group. Tests used included the elevated plus maze (EPM), open field (OF), FST, tail suspension (TS), novelty-suppressed feeding (NSF), beam break (BBK), horizontal ladder, Cyl and light–dark test (LD) that were performed according to the timeline presented (Figure 4a), although not all tests were done in each cohort as indicated by n-values in figure legends. One additional cohort (n=10 per group) was used to test for FST at 6 weeks post stroke. Throughout testing and behavioral analyses, experimenters were blind to the treatment groups.

Elevated plus maze

EPM is a test that assesses anxiety-like behaviors (Carobrez and Bertoglio, 2005). The mice were placed in the center of an elevated two-arm plus maze, measuring ~20-cm high, ~6-cm wide and ~75-cm long (Noldus, Wageningen, The Netherlands). The arms of the maze are crossed with one arm having an open platform, the other arm having a closed platform with walls that are ~20-cm tall. Mice were placed in the maze with the head toward the closed arm of maze and allowed to explore the maze for 10 min. The mouse movements were videotaped and the time spent in closed and open arms was analyzed (Ethovision 8, Noldus Information Technologies, Leesburg, VA, USA).

Open field test

The OFT measures exploration in response to a novel large open arena as a measure of anxiety (Belzung and Griebel, 2001). The mice were placed in a corner of the arena (45-cm long in each side and 45-cm high) and allowed to explore the new environment for a

total of 10 min at light levels of 300 lux. Mouse movements were videotaped and the time spent in the center (24 × 15 cm) and corners (squares with 10-cm sides) of the OF arena was analyzed (Ethovision 8).

Light–dark test

The light–dark paradigm is a measure of anxiety based on aversion to bright light and exploratory behavior in a novel environment in the presence of mild stressors (Hascoet and Bourin, 1998). Mice were placed into the light–dark chamber (Med Associates, St Albans, VT, USA), which is a box divided into two equal areas measuring 27 × 13.5 cm, with the dark area being completely black and covered, whereas the open area is transparent. The chamber is housed in a sound proof box that is additionally equipped with a fan and two bright light bulbs that produce 390 lux white illumination. The mouse is placed into the corner of the open area and allowed to move freely between the two areas for 10 min through an opening between the two chambers. The distance traveled in each zone, the total number of transitions, the time spent in each zone and the latency to enter the light compartment were recorded by using photo beam-based video-tracking software (Med Associates).

Novelty suppressed feeding test

The NSF test was used to assess anxiety-related behaviors (Santeralli et al., 2003). Briefly, animals were food deprived for 16 h. After 3 min of habituation they were placed in a new cage. Animals were individually placed in an arena (45-cm long in each side and 45-cm high; 100 lux) with a food pellet placed in the center. The latency of the mice to begin eating food was recorded manually and immediately after mice chewed the food or

after 10 min had expired for the trial, the mice were removed from the arena and placed in their home cage and the amount of food consumed in 5 min was measured.

TS test

The TS test measures stress coping response (Castagne et al., 2011). For this test, the mouse TS boxes from Med Associates were utilized and the tail of the mouse was secured with tape to a horizontal bar and the animals were suspended for 6 min. The automated detection device (ENV-505TS Load-Cell Amplifier, Med Associates, Fairfax, VT, USA) was used to determine mobility and immobility time through Med Associates software.

Forced swim test

The FST is used for assessing antidepressant-like activity (Castagne et al., 2011). The animals are subjected to an inescapable swim stress for a short period of time. Briefly, each mouse was placed into clear plastic cylinder 22 cm in diameter and 37-cm deep filled with 4 l of 24 °C water. The mouse was videotaped from the side of the cylinder for 6 min under red lighting and the duration of mobility and immobility time was quantified using Ethovision XT automated video-tracking software from Med Associates.

Home cage locomotor activity

Mice were placed into a novel home cage for 30 min and activity was measured by number of breaks of an invisible infrared light beams located on a frame surrounding the animal cage using the home cage locomotor activity system (Crawley, 2008) (Omnitech Electronics, Columbus, OH, USA).

Horizontal ladder test

Horizontal ladder is a locomotor test used to evaluate forelimb and hind limb stepping, placing and coordination (Metz and Whishaw, 2009). The animals were trained (1 trial) and tested (3 trials) to walk on a horizontal ladder (composed of two clear Plexiglas walls (69.5 × 15 cm) containing 121 metal rungs (0.15 cm in diameter, 2 cm from bottom of wall), spaced regularly (1 cm apart) or irregularly (0.5–2.5 cm apart)) while being video recorded. The number of successful and slips or missed steps were quantified.

Cylinder test

The cylinder test is used to assess sensorimotor function of the forelimb (Tennant and Jones, 2009). Mice were placed into a glass cylinder (10 cm diameter, 15-cm high) under red light condition and video recorded. Mice were required to perform a minimum of 20 rears at which time the test ended. The amount of time each limb was used to support the mouse throughout rearing was manually quantified.

Statistical analyses

All analyses were done using the Statistical Package for the Social Sciences (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Data are expressed as mean±s.e.m. Using the ROUT method in GraphPad Prism to identify outliers, one subject in TS and two subjects in EPM and FST, 6 weeks post stroke, were excluded from analyses. The behavioral data for the two cohorts of mice tested were not significantly different and therefore all data shown is the combined data from the two cohorts. Data comparing the sham and stroke mice on one outcome measure were analyzed using an unpaired t-test. One-way analysis of variance followed by Dunnett's multiple comparisons test was performed for comparing infarct volume at different time points.

Results

ET-1-induced infarct within the mPFC

ET-1 was microinjected sequentially in two locations within the left mPFC and at 48 h post ET-1 injection a focal lesion confined to the left mPFC was observed (Figure 1a). The lesion was significantly different from sham control mice that had only minimal needle track injury (Figure 1b). In the stroke mice, the lesion included all sub-regions of the mPFC including CG, pre-limbic and infra-limbic areas (Figure 1a). Detailed analysis of the location of the lesion through the longitudinal axis of the brain also showed very little variability between mice, and a peak in infarct volume between Bregma +1.54 and +1.78 (Figure 1c). In addition, MRI analysis was done on a subgroup of each cohort used for behavioral studies to visualize and confirm successful and accurate lesion of the left mPFC in vivo (Figure 1d). The MRI data showed consistent lesions, with similar average volume to that obtained by CV staining (Figure 1e). Thus, the ET-1-induced lesions were small, precise and reproducible, and no animals died or were excluded.

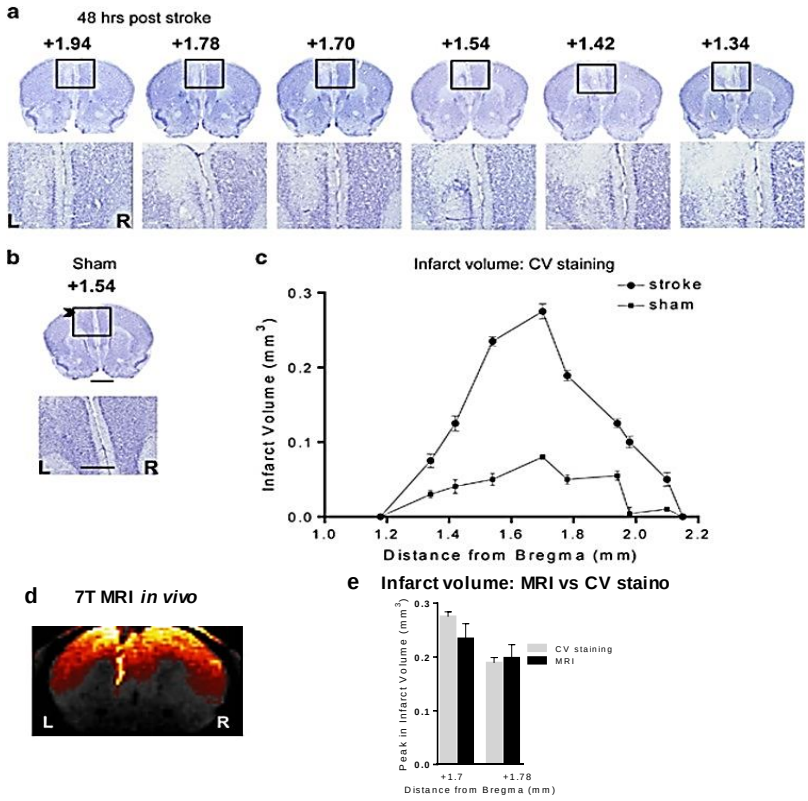


Figure 1. Lesion location following ET-1 injection into left mPFC.

(a) Representative microphotographs of CV-stained brain sections from a representative ET-1-injected mouse showing cell loss at 48 h post surgery shown at low (× 3.5) and high (× 20) magnification of the lesion site (box) with bregma locations indicated above. Scale bar, 400 and 50 µm. (b) A representative microphotograph of a CV-stained section of a sham control showing a trace of the needle track (arrowhead) detectable on left (L) side compared with the right (R) at 3.5 × and 20 × magnification. Scale bar, 400 and 50 µm. (c) Quantification of ET-1-induced lesion volumes at 48 h post surgery through the longitudinal axis of the lesion according to bregma locations (sham, n=3; stroke, n=3). (d) ET-1 lesion site visualized in vivo by MRI. Shown is a representative 7 T MRI image done in an anaesthetized living mouse at 4 days post stroke showing a 300-µm MRI section in which the lesion site is visualized and limited to the left mPFC (left, L; right, R); n=4–5 per experimental group. (e) Comparison of lesion size in MRI versus CV staining. Images obtained from in vivo MRI scanning or post-mortem CV staining of the same mice were quantified at two different distances from Bregma. Infarct volumes showed low variability and did not differ between the two measures. n=4. CV, cresyl violet; ET-1, endothelin-1; mPFC, medial prefrontal cortex; MRI, magnetic resonance imaging.

The size of the ischemic lesion at later times during which behavior was studied was determined. Examination of the stroke lesion site at 2 weeks post ET-1 injection (Figure 2a) revealed no significant change in lesion volume compared with either 48 h or 1 week post ET-1 injection (Figure 2c). In contrast, the lesion cavity was no longer evident at 6 weeks post stroke (Figures 2b and c). To determine the phenotype of the cells located within the lesion site, brain sections including both left CG (Figure 3a) and limbic cortex (Figure 3b) were analyzed by immunohistochemistry using antibodies for markers expressed in either microglia (IBa-1), astrocytes (GFAP) or neurons (NeuN). At 48 h post stroke, the lesion site had mainly IBa-1- and GFAP-positive cells, with few NeuN-positive cells at the lesion periphery, as shown in Figure 3 (left panels) and quantified in Table 1. By contrast, at 6 weeks post stroke, few IBa-1- or GFAP-positive cells were detected at the periphery of the lesion, while the lesion site was largely populated with NeuN-positive cells (Figure 3, Table 1). These results suggest that the ischemic lesion initially triggers a glial response with activation and invasion of microglia and astrocytes, followed by infiltration of neuronal cells expressing NeuN within the lesion by 6 weeks post stroke.

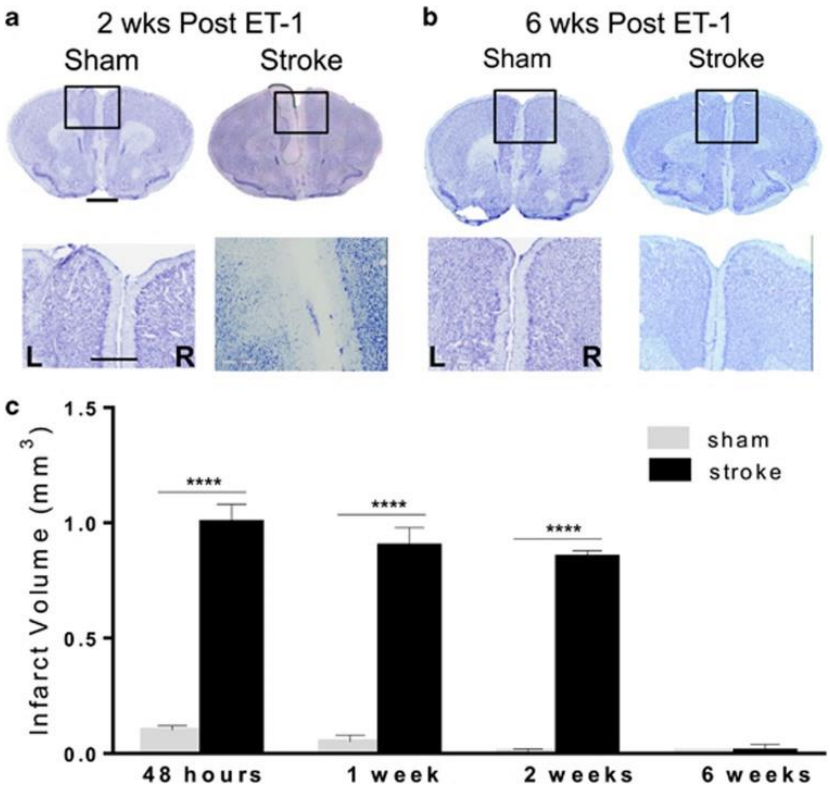


Figure 2. Quantification of infarct volume over time.

(a–c) Quantification of ET-1-induced lesion size by CV staining over time. Representative microphotographs of CV-stained brain sections of sham and ET-1 lesions in left (L) mPFC versus right (R) mPFC at 2 weeks (a) or 6 weeks (b) post stroke. High magnification (20x) images of the lesion site (box) are shown. At 2 weeks post stroke, an infarct with cell loss restricted to the left mPFC is visible, but is no longer observed at 6 weeks post stroke in any sub-regions of the left mPFC. Scale bar, 400 µm; 50 µm. (c) Infarct volume as quantified from CV-stained sections from sham and ET-1-injected mice at 48 h (n=3), 1 week (n=4), 2 weeks (n=4), and 6 weeks (n=4) post surgery (mean±s.e.m.) ****P<0.001 sham versus stroke; P<0.001 sham group at 6 weeks versus the other sham time points, P<0.001, stroke at 6 weeks versus all other stroke time points. CV, cresyl violet; ET-1, endothelin-1; mPFC, medial prefrontal cortex.

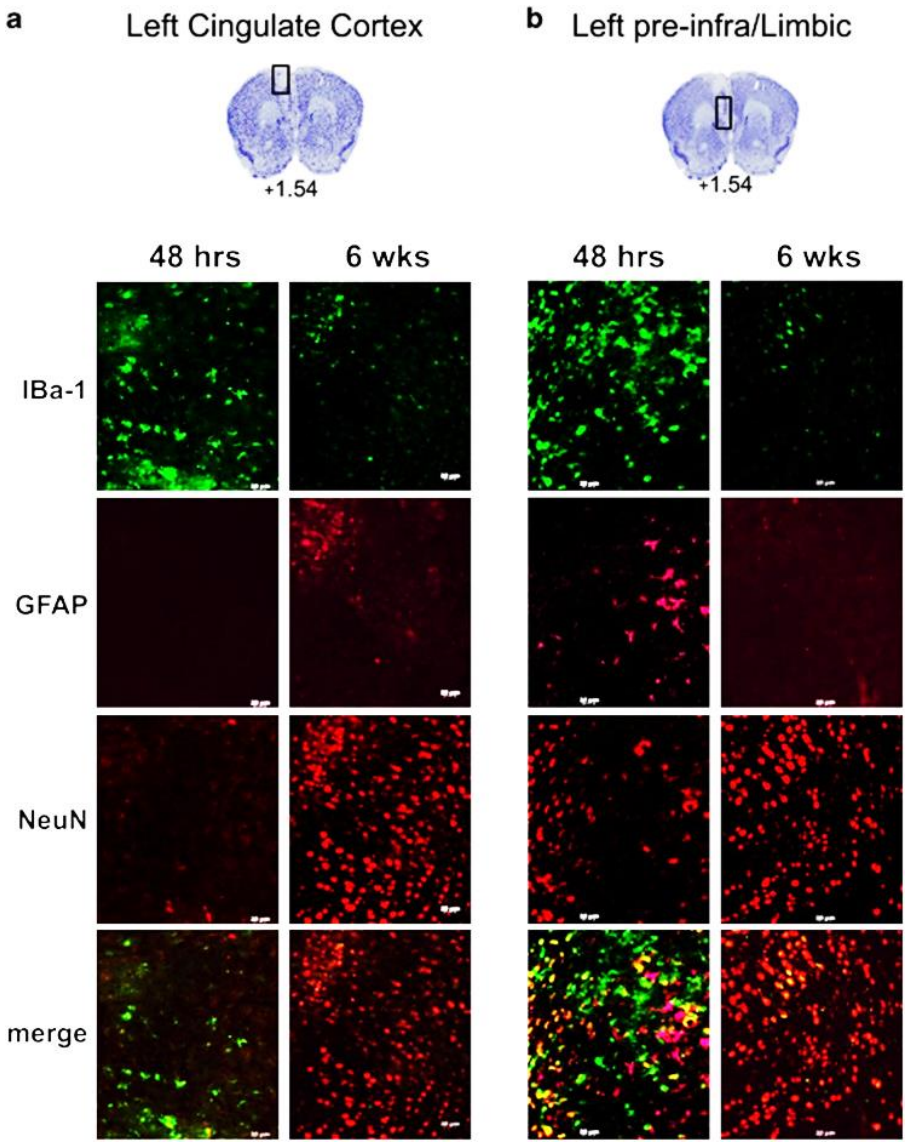


Figure 3. Cellular changes in the ischemic lesion site over time.

Brain sections of the left mPFC (Bregma +1.54) were examined by immunofluorescence at the (a) cingulate gyrus (CG) and (b) pre- and infra-limbic cortex from mice 48 h (left) or 6 weeks (right) following ET-1 microinjection in left mPFC. Representative images of sections stained with anti-IBa1 (microglia), GFAP (astrocyte) and NeuN (neurons). At 48 h post stroke, the lesion site had IBa1-expressing cells in the absence of NeuN-expressing cells (left panels). In contrast at 6 weeks post stroke, the lesion site had NeuN-expressing cells in absence of IBa1 cells (right panels). Scale bar, 20 µm (48 h, n=3; 6 weeks, n=4). ET-1, endothelin-1; mPFC, medial prefrontal cortex.

Table 1. Quantification of cellular changes in the ischemic lesion site over time.

	Left cingulate gyrus		***Left pre/infra-limbic cortex***	
	48 h	***6 weeks***	***48 h***	***6 weeks***
IBa-1	63 ± 4.4	19.7 ± 2.7***	113 ± 10.0	23.3 ± 3.5***
GFAP	1.3 ± 0.3	19.0 ± 3.8**	46.3 ± 6.1	7.0 ± 1.5**
NeuN	5.0 ± 1.5	266 ± 22**	31.7 ± 4.5	311 ± 40**

Sections of the left medial prefrontal cortex from mice at 48 h (n=3) or 6 weeks (n=4) post stroke were stained with anti-IBa-1 (microglia), GFAP (astrocyte) and NeuN (neurons), as shown in Figure 3. The number of cells per section within the left CG or pre- and infralmbic cortex were quantified and presented as mean values±s.e.m. **P<0.01; ***P<0.001, compared with 48 h.

Anxiety and depression phenotype following infarct of the left mPFC

To determine whether unilateral stroke in the left mPFC was sufficient to elicit anxiety- or depression-like behavior or altered motor function, sham and stroke mice were compared using multiple behavioral tests (Crayen and Holmes, 2005). Beginning at 1 week after ET-1 injection, the mice were tested for anxiety- and depression-like behavior sequentially, with the least stressful anxiety tests first, then for locomotor function, at times shown in the timeline (Figure 4a). In each of the behavioral tests, the stroke mice displayed a clear phenotype compared with sham controls. In the EPM test, the stroke mice had a significant 80% reduction in open arm duration (Figure 4b). In the OF test, the stroke mice spent significantly less time in center of the arena and more time in corners of the arena compared with control mice (Figure 4c). In the light–dark test, the stroke mice also had a significant reduction time spent in light zone compared with control mice (Figure 4d). In the NSF test, the stroke mice displayed significantly greater latency to feed in novel cage while similarly consumed food in home cage (Figure 4e). On the TS test, the stroke mice had significantly greater immobility time compared with controls (Figure 4f). Stroke mice also showed significantly greater immobility in the FST compared with control mice (Figure 4g). Overall, these behavioral analyses show a very strong anxiety- and depression-like phenotype in the ET-1-lesioned mice in all tests compared with sham control mice.

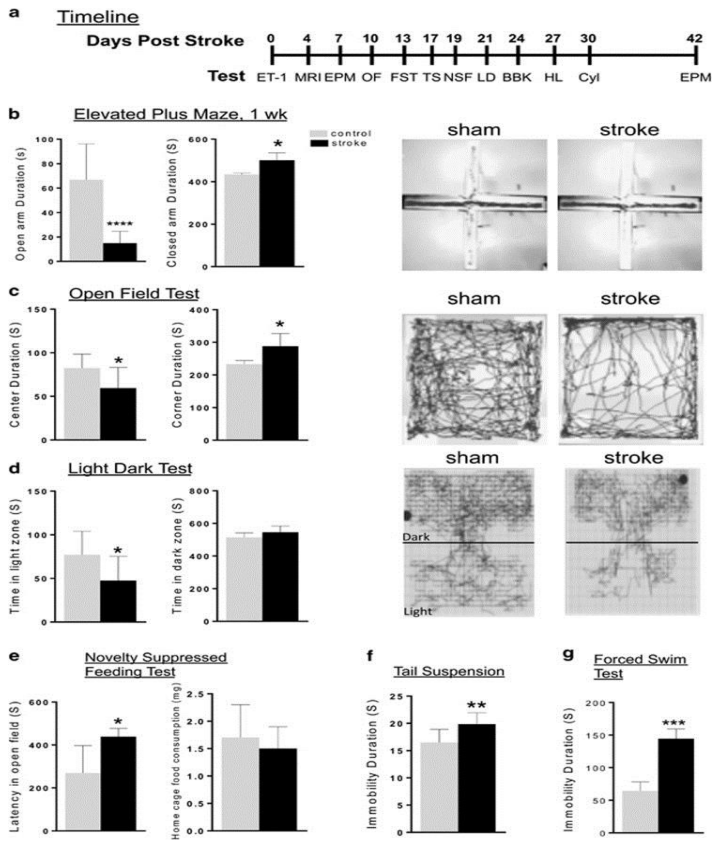


Figure 4. Anxiety and depression phenotype in stroke compared with sham mice.

(a) Timeline for behavioral studies post stroke. After ET-1-induced lesion of the left mPFC (day 0), a battery of tests was conducted from 1–6 weeks post lesion, including: BBK, beam break; Cyl, cylinder test; EPM, elevated plus maze; FST, forced swim test; HL, horizontal ladder test; LD, light dark; NSF, novelty-suppressed feeding test; OF, open field; TS, tail suspension. One or both cohorts were run on selected tests as indicated. (b) EPM: stroke mice spent significantly less time in the open arm and increased time in the closed arm duration compared with sham mice (single mouse tracking shown at right). Sham n=20; stroke n=20. (c) OF: the stroke mice spent significantly less time in center and more time in corner of the open field compared with sham mice (single mouse tracking shown at right). Sham n=10; stroke n=10. (d) LD: the stroke mice spent significantly less time in light zone of the light dark box compared with sham mice (single mouse tracking shown at right). Sham n=10; stroke n=10. (e) NSF: the stroke mice had a longer latency to feed compared with sham mice. The total food consumption in 5 min in the home cage was not different between stroke (n=10) and sham mice (n=10). (f) TS: the stroke mice had significant more immobility compared with sham mice on tail suspension test. (g) FST: the stroke mice showed significantly more immobility compared with sham mice on forced swim test. Data represent mean±s.e.m.; sham, n=20, stroke, n=20. *P<0.05; **P<0.01; ***P<0.001. ET-1, endothelin-1; mPFC, medial prefrontal cortex.

Intact locomotor activity and sensorimotor performance following lesion to the mPFC

Because the ET-1-induced lesion was restricted to the left mPFC and did not include the sensorimotor cortex, we hypothesized that locomotor activity and sensorimotor function would not be affected. In agreement with this hypothesis, locomotor activity monitored by total distance moved in both in the EPM (total activity, sham versus stroke; 476±35.8 cm versus 482±20.8 cm) and OF tests (total activity, sham versus stroke; 3789±127.2 cm versus 3579±168.5 cm) (Figures 4b and c) did not differ between stroke and control mice. Furthermore, there was no difference in number of BBKs between the stroke and sham mice up to 30 min after placement into a novel cage during the home cage activity test (Figure 5a). Together, these findings indicate the stroke mice show normal locomotor activity.

Persistent behavioral phenotype in stroke mice

Since histological assessment suggested that cellular remodeling of the lesion site had occurred at 6 weeks post stroke, we addressed whether the behavioral phenotype was still present at 6 weeks post stroke. When re-tested in the EPM, the stroke mice showed a significant 80% reduction in open arm time compared with sham mice (Figure 5d), similar to the phenotype observed in these mice at 1 week post stroke (Figure 4b). In a separate cohort of mice tested in the FST at 6 weeks post stroke, the stroke mice displayed about 50% increase in immobility compared with sham (Figure 5e). These results indicate that at 6 weeks post stroke, the stroke mice display both anxiety- and depression-like behavior. The retention of the anxiety and depression phenotype in the stroke mice indicates that the cellular remodeling of the infarct in the left mPFC at 6 weeks post stroke is not associated with functional recovery.

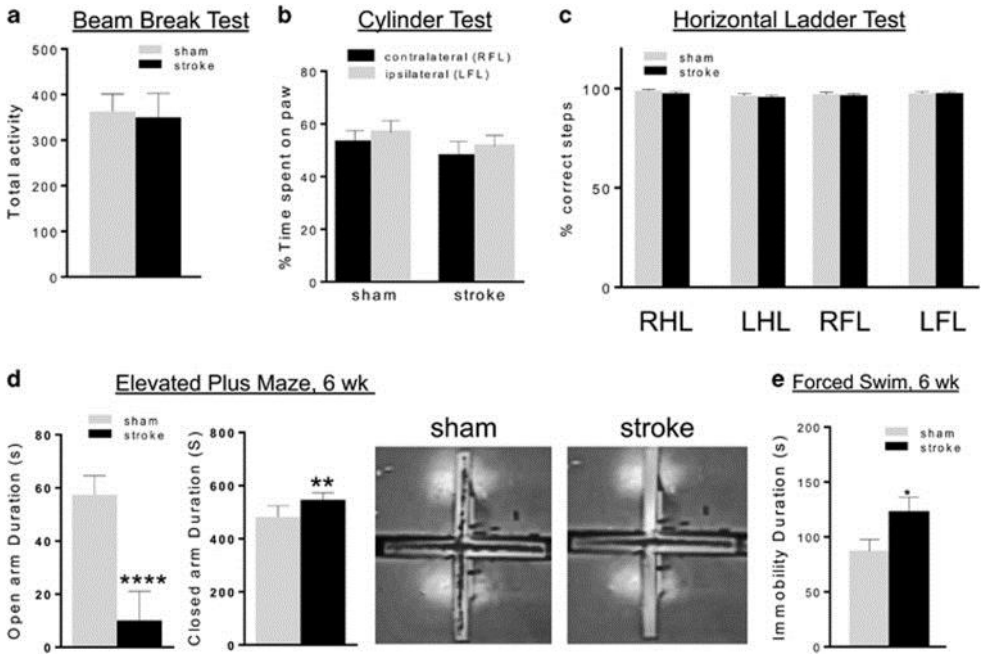


Figure 5. Persistent behavioral phenotype without locomotor impairment in stroke mice.

(a–c) Locomotor/sensorimotor tests. (a) Activity test: the total locomotor activity when placed in a novel cage was monitored for 30 min and was not different between sham and stroke mice. (b) Cylinder test: no differences in time spent on contralateral and/or ipsilateral paw were detected using the cylinder test between stroke and sham mice. (c) Horizontal ladder test: no differences in correct foot placement or foot faults were observed between stroke and sham mice (LFL, left forelimb; LHL, left hind limb; RFL, right forelimb; RHL, right hind limb). Data represent mean±s.e.m.; sham, n=10; stroke, n=10. (d, e) Anxiety and depression tests at 6 weeks post stroke. (d) EPM, week 6: after 6 weeks, EPM was repeated and stroke mice displayed reduced open arm and increased closed arm duration compared with sham mice, indicating a persistent anxiety phenotype that does not spontaneously recover (single mouse tracking shown at right). Sham, n=20; stroke, n=20. (e) FST, week 6: in a separate cohort at 6 week post stroke, the stroke mice displayed greater immobility duration compared with sham mice (sham, n=10; stroke, n=10). *P<0.05; **P<0.01; ****P<0.001. EPM, elevated plus maze; FST, forced swim test.

Discussion

Emotional disturbances associated with PSD, such as lack of motivation, depressed mood, agitation and anxiety, are common after stroke (Carota et al., 2005). Current antidepressant treatments result in remission in only 30% of depressed patients, hence improved preclinical ischemic models are needed to develop and optimize therapies for PSD (Kronenberg et al., 2014). Herein, we have developed a mouse model of PSD using unilateral microinjection of ET-1 into the left mPFC. This results in a very small stroke that is associated with a persistent depression- and anxiety-like phenotype in the absence of motor deficits.

The manifestation of PSD in humans has been associated with lesions located in the mPFC–midbrain–limbic circuitry that is implicated in depression and anxiety (Flaster et al., 2013; Price and Drevets, 2012; Singh et al., 2000; Terroni et al., 2011). The PSD mouse model developed here is relevant more generally to PSD following larger overt strokes that affect the PFC–subcortical circuitry. Although strokes restricted to the mPFC are rare in humans, we targeted this region to determine the outcome of an ischemic lesion in the mPFC, given that this region has been implicated in PSD (Singh et al., 2000; Terroni et al., 2011; Groenewegen et al., 1997; Riga et al., 2014; Yang et al., 2015). We also decided to use mice to generate a model that could exploit the large variety of conditional and inducible gene knockout mouse strains for future studies to further investigate the role of cellular remodeling and circuitry changes in PSD. Our results suggest that unilateral lesion of the PFC is sufficient to induce a robust anxiety and depression phenotype, in the absence of infarct in other cortical and subcortical gray and white matter.

Our model of unilateral mPFC lesion may also have implications in understanding why the prevalence of vascular depression increases with the number of white matter lesions, since these lesions can progressively disrupt connections between the mPFC and subcortical nuclei (De Groot et al., 2000; Gong and He, 2015; Liao et al., 2013; Brookes et al., 2014). By disrupting the axons, white matter strokes also may induce retrograde damage to projection neurons in the mPFC, including neurons and their axons that are targeted in our unilateral lesion model. However, animal models of white matter lesions leading to anxiety and depressive symptoms are yet to be reported. Although the etiology may differ, our model may be useful for understanding the progression and treatment of silent white matter strokes.

Unilateral ischemia in the mPFC as a model of PSD

The PSD mouse model we describe was generated by microinjection of ET-1 in the left mPFC. In addition, because a symmetric bilateral stroke would be extremely rare clinically, a unilateral rather than bilateral stroke has greater clinical relevance. The left side was targeted since left MCAO in rodents, and left hemisphere stroke in humans is more strongly associated with PSD, and is followed by other poorer stroke outcomes associated with PSD (Kronenberg et al., 2014). Since this ET-1 ischemia model specifically targets the left mPFC, it has several major advantages compared with the often utilized MCAO stroke model (Kronenberg et al., 2014). With the MCAO model, the stroke lesion is often large, and produces inconsistent infarct size unless occlusion durations are very long, due to inherent inter-animal variations in the collateral blood supply (Kronenberg et al., 2014; Carmichael, 2005). In our model, we have obtained very low variability in lesion size and location between mice (Figure 2) and there was no

mortality. Unlike some MCAO models, there was no need to combine mPFC lesion with chronic mild stress to observe depression-like behavior (McCann et al., 2014). In addition, the small ET-1-induced lesion was limited to the left mPFC and did not result in detectable locomotor or sensorimotor impairment. In contrast, the MCAO model is generally associated with sensorimotor impairments that can confound standard behavioral measures used to detect emotional function that rely on sensorimotor function, including assays used in this study (Carmichael, 2005). Electrolytic or excitotoxic lesions have previously been used to selectively ablate mPFC (Schiller and Weiner, 2004) but can have distinct consequences (Glenn et al., 2005). However, these lesions do not mimic the key characteristics of an ischemic lesion of a reversible loss of blood supply that may lead to long-term behavioral impairments that are potentially reversible. Thus, we believe that the ET-1 stroke model is superior to mimic the key aspects of stroke-induced injury.

Microinjection of ET-1 (400 pmol) is widely used and has been reported as a simple and reproducible method of focal ischemia that in rats produces lesions 14–20 mm^3 in size (Windle and Corbett, 2005; Cordova et al., 2014). In contrast, in mice the use of ET-1 has been more limited and the results are more variable (Tennant and Jones, 2009; Roome et al., 2014; Horie et al., 2008). In this study we used a total amount of 4 μg (1600 pmol) of ET-1, compared with the administration of 1 μg (400 pmol) that has been used in mice by others (Brookes et al., 2014; Vialou et al., 2014). Consistent with previous reports (Horie et al., 2008; Wang et al., 2007), our pilot studies confirmed that microinjection of 1 μg of ET-1 produced minimal lesions in C57/BL6 mice (data not shown). Our preliminary studies showed that to optimize consistent drug delivery and avoid denaturation and incomplete solubility that could account for inconsistent results, a high concentration of

ET-1 is completely solubilized in sterile water using sonication at 4 °C for 15–20 min, rather than in saline by vigorous vortexing (data not shown). Our results indicate that higher concentrations of ET-1 are necessary to obtain consistent and reproducible focal ischemic lesions in mice compared with rats.

To validate the PSD phenotype in sub-chronic and chronic phases following stroke, we have followed best practice guidelines for testing mouse behavior, which includes using: (i) more than one behavioral test; (ii) using tests that have construct and face validity, as well as outcomes that are objective, reproducible and sensitive enough to detect a long-term deficit (Crawley, 2008; Fisher et al., 2009). The ET-1-induced ischemic lesion of the left mPFC produced a significant anxiety phenotype detected in all four tests: EPM, OF, LD and NSF; and a behavioral despair phenotype in both the TST and FST. Thus this is a robust phenotype that can be detected in all measures used to detect anxiety and depressive-like behaviors.

Recently, large bilateral ischemic lesions to the PFC in the rat and mouse have been found to induce learning and memory deficits (Livigston-Thomas et al., 2015; Zhou et al., 2016). Given the role of the mPFC in cognitive function (Riga et al., 2014) and the association of impaired cognition with anxiety and depression in left hemisphere clinical stroke (Barker Collo, 2007), it would be interesting to test whether our unilateral lesion of the left mPFC is sufficient to elicit cognitive deficits. Results of MCAO ischemia in rodents have suggested a laterality with increased locomotor activity after right MCAO and anxiety after left MCAO, but others have not seen increased locomotion or anxiety (Kronenberg et al., 2014). This inconsistency may reflect the variability of MCAO strokes in different studies. A recent study in rats found that bilateral ET-1-induced lesion

of the rat mPFC showed no change or reduced anxiety in the light–dark or OF test, but did show cognitive impairment (Livingston-Thomas et al., 2015). The robust behavioral phenotype obtained with discrete unilateral lesion in the left mPFC compared with larger bilateral lesions, could suggest that anxiety and depression phenotypes may be more pronounced for unilateral lesions, but this remains to be further tested.

Cellular changes at PSD lesion site fail to rescue behavior

An additional goal of this study was to establish a stroke model with a persistent behavioral phenotype to study and optimize recovery from PSD. In many animal models, lesion size has been used as a measure of stroke severity without functional assessment (Kronenberg et al., 2014). On the other hand, clinical studies focus on functional recovery outcomes following stroke, which are not often correlated with infarct volume (Mark et al., 2008; Riley et al., 2011; Page et al., 2013). Our ET-1-induced stroke model displays a strong depression and anxiety phenotype that persists for at least 6 weeks post stroke, while at this time the lesion site in the left mPFC is barely detectable by CV staining. In addition, by 6 weeks post stroke the microglia that were observed at 48 h post stroke had largely vacated the lesion and a large number of neurons were found in the previous location of the lesion. Shrinkage of the lesion site could explain the repopulation, but tissue shrinkage in response to ischemia appears to involve brain atrophy (Kraemer et al., 2004). The initial glial reaction suggests that an active remodeling of the lesion may be occurring to allow neurons to migrate from adjacent tissue (penumbra) or from neurogenic sites, although mainly newborn glia reach the stroke site (Li et al., 2010). This neuronal repopulation is in contrast to what is found in ET-1 ischemic lesions that are 10- to 500-fold larger, in which lesion site forms a glial

scar populated by astrocytes and microglia that may prevent repopulation by neurons (Abeysinghe et al., 2014). Furthermore, most studies examine lesions at earlier times than 6 weeks post stroke that may not be sufficient to observe refilling of the lesion, as we observed no change in the lesion size at 2 weeks post stroke. Despite the presence of neurons within the lesion site, the stroke mice show persistent anxiety and depression behavior at 6 weeks post stroke.

The observed dissociation between lesion size and behavioral recovery in this PSD preclinical model indicates that the neurons within the lesion site are not correctly integrated in neural networks to restore behavior. This result highlights the importance of measuring functional outcomes of recovery rather than lesion size as an index of recovery in preclinical animal studies, similar to what is done in clinical stroke studies (Page et al., 2013; Chen et al., 2000). Because after 6 weeks the behavioral phenotype did not improve, the NeuN-expressing neurons that infiltrated the stroke lesion did not appear to be integrated into the behavioral circuitry, and this could be verified using electrophysiological analysis. Therapeutic strategies to enhance integration of these neurons and promote recovery could be assessed in future studies. In addition, altered activity through diaschisis of brain regions that are connected to the mPFC may contribute to the behavioral phenotype (Silasi and Murphy, 2014), which remains to be addressed in this stroke model. By identifying stroke-induced activity changes in the anxiety-depression circuitry over time, it may be possible to modify abnormal activity to normalize behavior. In this regard the impact chronic antidepressant treatment on the behavioral phenotype and brain activity may help elucidate whether this PSD model

displays a similar or different treatment response compared with models of stress-induced depression.

Conclusion

Taken together, we have shown that injection of ET-1 into the left mPFC of mice produces a robust model of PSD associated with persistent anxiety- and depression-like behavior suitable for preclinical work aimed to improve PSD recovery outcomes.

Acknowledgments

We thank Dr. Ania Serefko, Greg Cron (University of Ottawa Preclinical Imaging Core), Anthony Carter (Canadian Partnership for Stroke Recovery (CPSR), Mirela Barclay and Christine Luckhart (UOttawa Behavioral Core Facility) for technical assistance, and Drs Dale Corbett, Antoine Hakim and members of the CPSR for helpful discussions. We acknowledge grant support from the Canadian Institutes of Health Research and the Canadian Partnership for Stroke Recovery (CPSR) (PRA and DCL) and studentship support from CPSR to FV-A.

Notes

The authors declare no conflict of interest.

Chapter 4

Chronic fluoxetine-induced activity changes in recovery from post-stroke anxiety, depression and cognitive impairment

Chronic fluoxetine-induced activity changes in recovery from post-stroke anxiety, depression and cognitive impairment

Faranak Vahid-Ansari and Paul R. Albert*

Ottawa Hospital Research Institute (Neuroscience)

UOttawa Brain and Mind Research Institute

Ottawa ON K1H-8M5

Running Title: Fluoxetine-induced recovery from post-stroke depression

*To whom correspondence should be addressed: OHRI (Neuroscience), UOttawa Brain and Mind Research Institute, 451 Smyth Road, Ottawa ON K1H-8M5 Canada; phone, 613-562-5800 8307; fax, 613-562-5403; email, palbert@uottawa.ca

- Neurotherapeutics (July 2017), under-revision

Author Contributions

FV-A and PRA designed the study; FV-A acquired the data; FV-A and PRA analyzed the data; FV-A wrote the article, and PRA revised it and approved the article for publication.

ABSTRACT

Background: Post-stroke depression (PSD) is a common outcome of stroke that limits recovery and increases mortality and is thought to involve cortical-limbic alterations, but the changes involved are unclear. Using a mouse PSD model of left medial prefrontal cortex (mPFC) lesion, we addressed changes in cellular activity associated with PSD and its treatment.

Methods: Following ischemia, PSD mice were treated chronically (4-wk) with fluoxetine (SSRI) and/or voluntary exercise (free running wheel) and tested for behavior and spatial learning-memory. Then, we quantified brain-regional FosB-positive cells co-labeled for glutamate/pyramidal (VGLuT1-3/CaMKIIα), GABA (GAD67) and 5-HT (TPH) in PSD and following treatment.

Results: In addition to depression and anxiety behavior, PSD mice displayed cognitive impairment. PSD brains showed widespread activation with greater activation of the right vs. left brain in glutamate cells of the mPFC, nucleus accumbens, amygdala, hippocampus and in midbrain 5-HT neurons. Chronic treatment with SSRI alone or with exercise (but not exercise alone) reversed the behavioral phenotype and balanced bilateral neuronal activity, reducing glutamatergic (mPFC, NAc) or increasing GABAergic activity (septum, amygdala) or both (hippocampal CA3/DG, raphe).

Conclusion: These data show that chronic SSRI reverses the PSD phenotype, providing a novel model of serotonin-mediated recovery from stroke-induced anxiety, depression and cognitive impairment. Cellular activation analysis indicated that changes in pyramidal cell activity, especially of the contralateral cortical-limbic pathway, drive the PSD

phenotype, which is reversed by chronic SSRI treatment, in part through activation of GABAergic cells.

Learning objectives:

Understand which treatments (SSRI or exercise) are effective for post-stroke depression

Understand changes in brain activity in post-stroke depression

Understand changes in brain activity following treatment for post-stroke depression

INTRODUCTION

Post-stroke depression (PSD) is a common consequence of stroke that occurs within 3 months of a stroke in 30% of patients (Paolucci et al., 2006; Ayerbe et al., 2013) and is often co-morbid with anxiety disorders and cognitive impairment (Wolfe et al., 2011; Galligan et al., 2016). Patients that suffer PSD have a greater risk of suicide and increased overall mortality within 10 years (Ayerbe et al., 2014; Pompili et al., 2015). PSD is typically treated with serotonin-specific reuptake inhibitors (SSRIs) like fluoxetine (FLX), but SSRIs require chronic treatment (3-4 weeks) to increase serotonin (5-HT) and improve depressive behavior (Mead et al., 2012). In addition, SSRI treatment may enhance cognitive and motor recovery (Jorge et al., 2010; Chollet et al., 2011; Flaster et al., 2013; Robinson and Jorge, 2016). About 50% of depressed patients respond to SSRIs but remission is seen in only 30% and adverse events include hemorrhage and increased mortality (Ayerbe et al., 2014; Mortensen et al., 2014). Hence improved approaches to more effectively treat PSD are needed to enhance stroke recovery.

PSD patients often undergo concurrent rehabilitation therapy involving exercise that may also have an antidepressant effect, but clinical data remain unclear (Rimer et al., 2012). However, combining SSRI and exercise may have enhanced antidepressant activity and may improve cognitive and motor recovery (Jorge et al., 2010; Chollet et al., 2011; Flaster et al., 2013; Robinson and Jorge, 2016). In rodents, wheel running exercise enhances hippocampal BDNF, neurogenesis, synaptic plasticity (Luo et al., 2007; Duman et al., 2008), and could augment SSRI actions through activation of the 5-HT system (Greenwood et al., 2003; Castren and Hen, 2013). However, it remains unclear to what

extent SSRI and exercise may interact in enhancing recovery from PSD (Ayerbe et al., 2015).

In order to address the actions of SSRIs or exercise in recovery from PSD, we sought to develop a highly specific mouse model of chronic PSD. Middle cerebral artery occlusion rodent models can result in post-stroke depression but only after stressors that they induce depression, and cause variable lesions and sensorimotor impairments that may confound evaluation of behavioral phenotypes. To refine the PSD model, we used unilateral microinjection of the vasoconstrictor endothelin-1 (ET1) into the left medial prefrontal cortex (mPFC) to produce a small ischemic lesion that resulted in a robust PSD phenotype without sensorimotor impairment (Vahid-Ansari et al., 2016). The left mPFC was targeted based on evidence that ischemic lesions affecting the left mPFC or its projections are associated with depression-like behavior following stroke (Kronenberg et al., 2014). While medial cerebral artery strokes that affect the mPFC are rare, our model mimics more common disruption to the anxiety-depression circuitry. The PSD mice display robust anxiety and depression phenotypes at 1 wk post stroke, persisting for at least 6 wks post stroke with no improvement, providing a useful model to study recovery.

Here, we address whether chronic treatment with voluntary exercise and/or FLX results in recovery from post-stroke anxiety- and depression-like behaviors, as well as cognitive impairment in this model. We have also examined brain-wide changes FosB expression as a marker of chronic activation (Nestler, 2015) in specific cell types following PSD and treatment. Our results indicate that recovery is associated with increased FosB-positive cells at particular nodes of the anxiety-depression circuitry including the mPFC, nucleus accumbens (NAc), lateral septal nuclei (LSN), amygdala

(amyg) and dorsal raphe (DR). To address potential mechanisms, we examined the lesion site and find that effective treatment induces microglial remodeling of the lesion site, enhancing microglial and astrocytic invasion of the lesion site. These results indicate that chronic SSRI, alone or with exercise, promotes remodeling of the lesion site and activation of the PFC-raphe-limbic circuitry to mediate recovery from PSD.

MATERIALS AND METHODS

Animals

One hundred and four 11 week-old male C57/BL6 (Charles River Laboratories, Montreal, QC, Canada) weighing 25–28 g at the time of surgery were used in this study. Mice were single-housed in standard Plexiglass cages on a 12/12 h light/dark cycle with *ad libitum* access to food and water. Animals were allowed to acclimate to the housing facility for 2 weeks prior to surgery.

Mice were behaviourally tested in different cohorts of 4-32 after surgery and/or chronic treatment post surgery, and some were perfused for post-mortem histology (Table 1). One cohort of PSD mice (n=4) was used 4 days post-stroke for histology. Another group of mice (n=20) was equally divided into sham control and stroke and used 6-wk post-surgery for histology studies.

For the combined treatment study, a group of mice (n=24; 12/group) was randomly assigned to either sham control (n=12) surgery and (subjected to fixed wheels and sucrose 1% (vehicle) or stroke group (n=12) subjected to voluntary exercise and 18 mg/kg/day fluoxetine hydrochloride (FLX) (Cedarlane, Hornby, ON, Canada) (Samuels et al., 2015; Santarelli et al., 2003), starting 1-wk post surgery. Voluntary exercise constituted free access to running wheels in the home cages and the number of wheel

rotations/day was monitored. Next, 2 separate cohorts were used for several behavioural assays and histology after chronic monotherapy (for 3-4 weeks). These cohorts of mice (n=32/cohort; 8/group) were randomly divided into 3 stroke groups and one sham control group. At 1-wk post surgery, the sham and PSD mice received sucrose 1% and fixed wheels (sham or vehicle groups, respectively), sucrose 1% and running wheels (exercise) or fluoxetine (18 mg/kg, po) and fixed wheels (FLX). FLX was prepared in opaque bottles to protect it from light and liquid consumption was carefully monitored. The University of Ottawa Animal Care Committee approved all experimental procedures in accordance with guidelines established by the Canadian Council of Animal Care.

Table 1. Mouse cohorts for behavioral testing.

cohorts	n values	Experiments				
		MRI	behavior	treatment	behavior	histology
			7ds post- surgery	3-4 wks	post-treatment	
			EPM	FLX, Exc, veh	EPM, OF, FST, TS, NSF	
1	4	+	-	-	-	+
2	20	+	EPM	-	EPM	+
3	24	+	EPM	FLX-Exc	EPM, OF, FST, TS, NSF	+
4	32	+	EPM	FLX/Exc/veh	EPM, OF, FST, TS, NSF, MWM	+
5	24	+	EPM	FLX/Exc/veh	EPM, OF, FST, TS, NSF	+

ET-1-induced PSD model

PSD was induced as described previously (Vahid-Ansari et al., 2016), by microinjection of 1 µl of ET-1 (2 µg/µl = 800 pmol/µl) at two sites: first, anterior–posterior (AP), 2.0; medial–lateral (ML), +0.5; dorsoventral (DV), − 2.4; second, (AP) 1.5; (ML) +0.5; (DV) − 2.6. Sham control mice underwent the same procedure except that the ET-1 was replaced with vehicle (sterile water). The mice were placed in a 37°C incubator to maintain body temperature until they regained mobility and were treated with 0.1 ml of 2% transdermal bupivacaine (Chiron, Guelph, ON, Canada) as a topical anesthetic and 2 injections of buprenorphine (0.03 mg/kg, s.c.; Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) for pain management in 3 hours post-surgery. At 4 days post stroke, lesions were visualized in vivo using a 7T GE/Agilent MRI in a subset of mice (4/cohort) at the University of Ottawa Preclinical Imaging Core Facility as described (Vahid-Ansari et al., 2016).

Immunofluorescence

For immunofluorescent studies, mice were euthanized by Euthanyl (149.5 mg/kg i.p., Bimeda-MTC Health Inc., Cambridge, Canada) and perfused by cardiac puncture with chilled PBS and then with 4% PFA for fixation. Whole brains were isolated, cryo-protected overnight in 20% sucrose and frozen at -80°C. Coronal brain slices (20-µm thickness) were prepared using coordinates summarized in Table 2 (Paxinos and Franklin, 2008). Slices were thaw-mounted on Superfrost slides (Thermo-Fisher) and kept at -80°C. The sections were washed 3 × in PBS, blocked 1 h in PBS with 1% BSA, 10% NDS, 0.1% Triton X-100, followed by 24h incubation (unless indicated otherwise) at 22°C with primary antibodies listed in Table 3. The sections were then washed three

times in PBS and incubated for 1h in secondary antibodies at 22°C in blocking solution. Images were acquired with the Axiovision imaging software on a Zeiss Axio Observer D1 microscope under 10X and 20X magnification (n=4/group).

Table 2. Coordinates relative to Bregma of regions assessed by immunofluorescence

Brain region	distance from Bregma
Medial prefrontal cortex; mPFC	1.7
Cingulate cortex; CGctx	1.7
Pre-infra/limbic; PI	1.7
Nucleus accumbens; NAc	1.1
Lateral septal nucleus; LSN	0.5
Lateral habenular nucleus; LHbl	-2.07
Hippocampus; hippo	-1.7
CA1	-1.7
CA2	-1.7
CA3	-1.7
dentate gyrus; DG	-1.7
Amygdala; amyg	-2.06
Dorsal raphe; DR	-4.36 to -4.72
DDR	-4.36 to -4.72
VDR	-4.36 to -4.72
LDR	-4.36 to -4.72
VLDR	-4.36 to -4.72

Table 3. Primary and secondary antibodies for immunofluorescence staining

A

primary antibody	host	concentration	company	cat#
CaMKIIα	R	1:100	Santa Cruz	sc-9035
FosB	M	1:500	Abcam	11959
FosB	R	1:500	Santa Cruz	sc-48
GAD 67	M	1:500	MilliPore	135406
TPH	Sh	1:100	MilliPore	ab1541
VGLuT3	GP	1:100	MilliPore	ab542ZI

B

secondary antibody	concentration	company	cat#
D/GP cy5	1:250	Jackson	706-175-148
D/M cy3	1:250	Jackson	715-163-160
D/R 488	1:1000	life Technology	A-21206
D/Sh cy3	1:200	Jackson	713-165-003
G/GP 594	1:200	life Technology	A-11076
G/M cy5	1:800	abcam	ab6563
G/R 594	1:200	life Technology	A-11037

C

host
D/donkey
R/rabbit
M/mouse
Sh/sheep
GP/guinea pig

Behavioral assays

Behavioral tests were performed following either at 1-wk post-surgery or following 3 weeks of treatment (4 wk post-stroke). Mice were housed in 12h/12h light/dark conditions (lights on at 8:00 AM) and tests were performed beginning at 10:00 AM, after at least 1 h of habituation to the testing room. Testing was performed under white light illumination or red light for the forced swim test (FST). For all cohorts, elevated plus maze (EPM) was done 1 week post-surgery (pre-treatment) to verify PSD phenotype. Notably, mice were kept under treatment until the last test and perfusion. At 3 wk post-treatment the following tests were done in the following order while mice kept under treatment: open field (OF), FST, tail suspension (TS), novelty-suppressed feeding (NSF), second EPM and Morris water maze (MWM) test, according to the timeline. Throughout the testing and behavioral analyses, experimenters were blind to the treatment groups.

Elevated plus maze

Mice were placed in the center of an EPM (20-cm high, ~ 6-cm wide and ~ 75-cm long; Noldus, The Netherlands) with the head toward the closed arm and allowed to explore the maze for 10 min, videotaped and the time spent in closed and open arms analyzed (Ethovision 10, Noldus IT).

Open field test

Mice were placed in a corner of the arena (45-cm long x 45-cm high) for 10 min at light levels of 300 lux, videotaped and the time spent in the center (24 × 15 cm) and corners (squares with 10-cm sides) analyzed (Ethovision 10, Noldus IT).

Forced swim test

Each mouse was placed into clear plastic cylinder (22 cm diameter x 37-cm deep) filled with 4 l of water (24°C), videotaped for 6 min under red lighting and the duration of immobility time quantified using video-tracking software from Med Associates (Ethovision XT).

Tail suspension test

The tail of the mouse was taped to a horizontal bar in a TS box (Med Associates, VT) for 6 min. Immobility duration was measured using an automated detection device (ENV-505TS Load-Cell Amplifier Med 28 Associates, Fairfax, VT, USA) and quantified using Ethovision XT software. The lower threshold (3) was set and the forces recorded below the threshold were considered immobility.

Novelty suppressed feeding (NSF) test

Mice deprived of food for 16 h were individually placed in an arena (45-cm long x 45-cm high; 100 lux) with a food pellet placed in the center. The latency of the mice to begin eating food was recorded manually and immediately after mice chewed the food or after 10 min had expired for the trial, the mice were removed from the arena and placed in their home cage and the latency to feed and amount of food consumed in 5 min was measured.

Morris water maze (MWM) test

A cued version of the MWM was used to evaluate spatial learning and memory (Vorhees and Williams, 2006) in PSD and PSD treated mice. The maze used was a circular pool (132 cm in diameter) filled with non-toxic white colored water (24±1°C). The platform diameter was 10 cm and was located 24 cm from the edge of the pool, hidden 1 cm beneath the surface of the water. Each 1-min trial was started by placing a randomly

chosen mouse in one of four starting locations in the pool with its head outward. If the mouse found the platform within 60 s, it was left to stay on the platform for 5 s; if it did not find the platform, it was gently guided to the platform by the experimenter. Between the trials, all mice were placed back in their home cages while tucked in a towel in order to avoid direct contact with the experimenter. All trials were tracked using an overhead video camera and recorded automatically by an Ethovision digital tracking system (Noldus) to assess path line and latency to escape from the water. Four trials per day were conducted on each mouse with a 30-min inter-trial interval for 10 days (at which time the sham control group consistently reached the platform). On the next day (probe day), the platform was removed and the amount of time the mouse spent in the quadrant where the platform used to be was measured.

Statistical analyses

All analyses were done using the Statistical Package for the Social Sciences (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Data are expressed as mean ± SEM. Data comparing the sham vs. stroke mice or pre- vs post-treatment on one outcome measure were analyzed using an unpaired t-test. Data comparing the immunopositive cell population within one area were also analyzed using an unpaired t-test. Statistical analyses were performed using an analysis of variance (ANOVA) when comparing data across different conditions. Post-hoc comparisons were made with Tukey's multiple comparisons test.

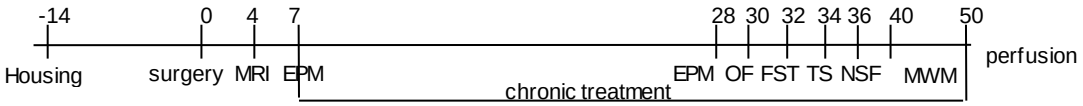
Results

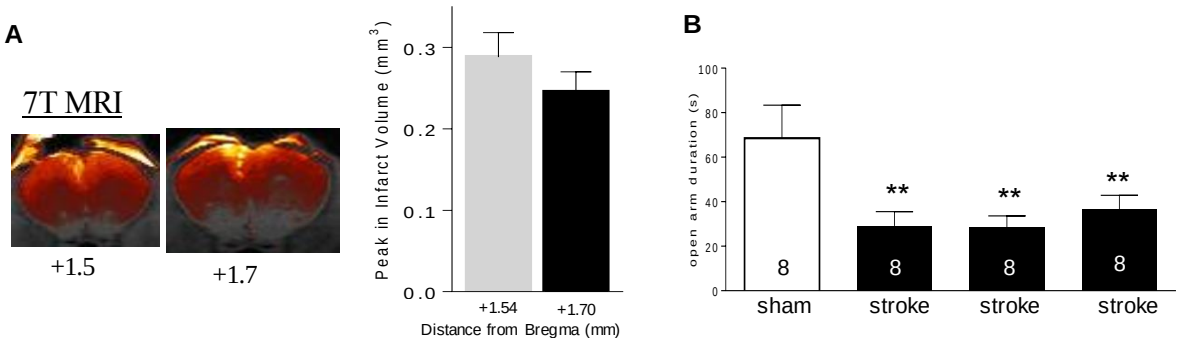
Chronic SSRI but not exercise restores depression/anxiety phenotypes in PSD mouse

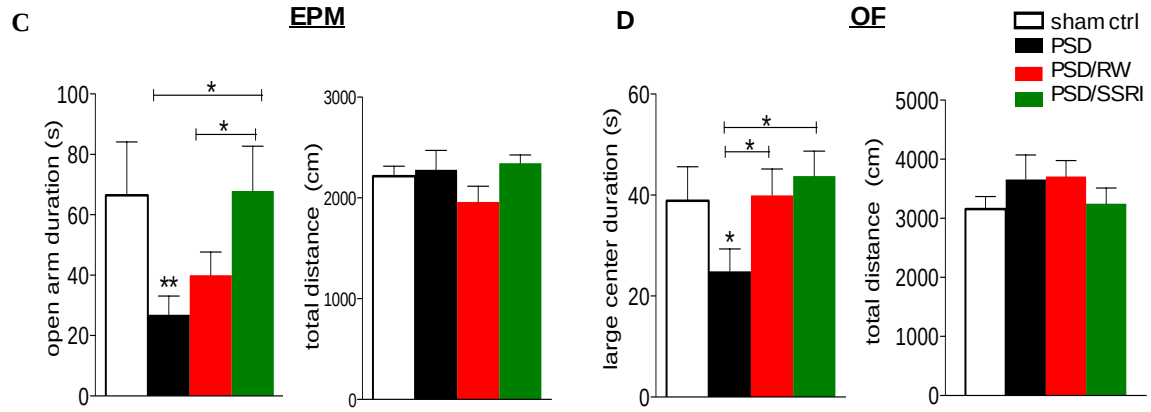
As a model of persistent post-stroke anxiety and depression, adult male C57BL6 mice received an ischemic lesion in the left mPFC to generate the PSD mouse (Vahid-Ansari et al., 2016). At 4 days post-stroke the lesion was verified in a subset of mice using MRI (n=4) (timeline, Fig. 1A) to visualize and confirm successful and accurate lesion of the left mPFC in vivo. The MRI data showed consistent lesions, with similar average volume to that obtained from previous cohorts (Vahid-Ansari et al., 2016). The anxiety phenotype was verified at 1 week post-stroke using the EPM assay in which stroke groups showed significant reductions in open arm duration compared to sham ctrl group (Fig. 1B) and then treatment was begun. The SSRI fluoxetine was administered orally as described to obtain a clinically relevant concentration, and exercise was free running wheel with fixed wheel as control. After 3 weeks of treatment the mice were tested in anxiety and depression tests over 2 weeks while maintaining treatment. Compared to sham, the stroke mice displayed significant anxiety (EPM, OF and NSF tests; Fig. 1C-E) and depression-like (FST, TST; Fig. 1F-G) behavior, consistent with our previous results (Vahid-Ansari et al., 2016). Chronic SSRI reversed the anxiety phenotype to sham control levels in all tests, increasing open arm time in the EPM, time in centre in the OF, and reducing latency to feed in the NSF test.

The mice in PSD-voluntary RW group ran similarly using the running wheels, as measured by distance (72660 ± 3280 m/mouse (mean ± SEM; N = 8) for 3 weeks. However, exercise had no effect in the EPM and NSF tests, but did reverse centre time to

sham levels in the OF test (Fig. 1D). As controls there, was no change in distance travelled in the EPM or OF tests (Fig. 1C, D), or in home cage latency to feed (Fig. 1E) or food consumed (not shown) in the NSF test. Thus, chronic SSRI alone completely reversed the post-stroke anxiety phenotype, while exercise had little effect. Similarly, chronic SSRI but not exercise reversed the depression-like behavior in the FST and TST, reducing immobility duration to sham control levels (Fig. 1F, G).


-14
0
4
7
28
30
32
34
36
40
50
Housing
surgery
MRI
EPM
EPM
OF
FST
TS
NSF
MWM
perfusion
chronic treatment


A
7T MRI
+1.5
+1.7
Peak in Infarct Volume (mm3)
0.3
0.2
0.1
0.0
+1.54
+1.70
Distance from Bregma (mm)
B
open arm duration (s)
100
80
60
40
20
0
8
8
8
8
**
**
**
sham
stroke
stroke
stroke


C
EPM
open arm duration (s)
total distance (cm)
D
OF
large center duration (s)
total distance (cm)
sham ctrl
PSD
PSD/RW
PSD/SSRI

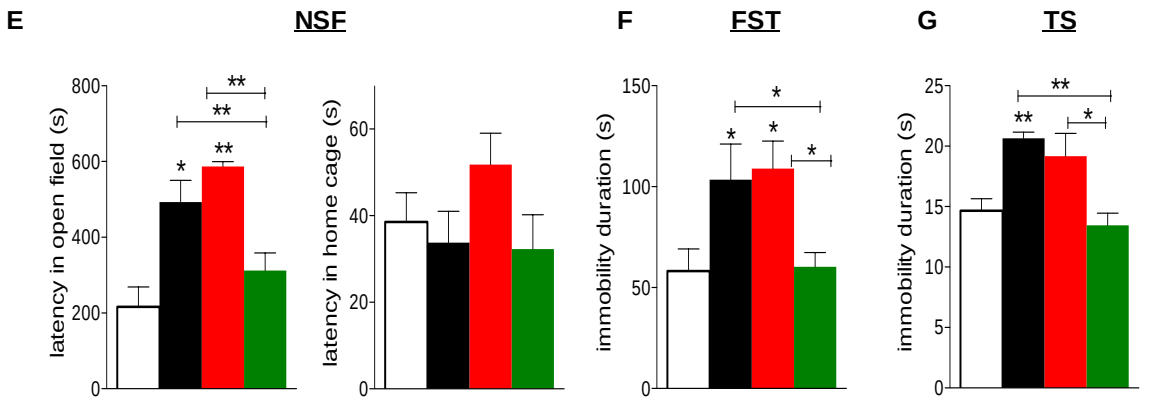

E
NSF
latency in open field (s)
latency in home cage (s)
F
FST
immobility duration (s)
G
TS
immobility duration (s)

Figure 1. Chronic SSRI, but not exercise, reverses behavioral phenotype in PSD mice

Timeline: Mice were individually housed 14 d prior to surgery, microinjected with vehicle (sham) or ET-1 (PSD) in the left mPFC (surg, day 0); at 4d post stroke, lesions were verified by MRI (1A), and at 7d anxiety phenotype verified by EPM in which stroke groups showed significant reductions in open arm duration compared to sham ctrl group (1B). From 7 d onwards treatment was with fluoxetine (18 mg/kg, p.o., SSRI) or free running wheel (RW), with fixed wheel (FW) and vehicle as control. Behavioral assays were done from d 28-40 followed by MWM (see Fig. 3). C. EPM. PSD reduced open arm time compared to sham, indicative of an anxiety phenotype; this was reversed by chronic fluoxetine (PSD/SSRI), but not exercise (PSD/RW). As control for locomotion, total distance travelled did not differ between groups. D. OF. PSD reduced large center duration and SSRI or exercise reversed this anxiety phenotype, with no change in distance travelled. E. NSF. PSD increased latency to feed in open field, and SSRI but not exercise reversed this phenotype. As control for hunger, the latency to feed in home cage did not differ between groups. F. FST. Immobility time in PSD vs. sham group was increased indicating behavioral despair, and this was reversed by SSRI but not exercise. G. TS. Immobility increase in PSD was reversed by SSRI but not exercise. Data represent mean ± SEM, n=8/group, $*p < 0.05$, $**p < 0.01$.

Stroke patients with PSD often receive physical rehabilitation and SSRI as treatment. In the PSD mouse, we addressed whether there was any benefit in combining chronic SSRI and exercise (Fig. 2). Interestingly, in the EPM test, combination treatment significantly increased open arm time and reduced corner time compared to sham control (Fig. 2A), suggesting an augmented anti-anxiety effect in this test compared to SSRI treatment alone. As observed for chronic SSRI alone, combination treatment reversed the anxiety phenotype to sham control level in the OF and NSF tests (Fig. 2B, C), and also reversed depression-like behavior in the FST and TST to sham control levels (Fig. 2D, E). Thus, chronic SSRI treatment, without or with exercise, reversed anxiety- and depression-like phenotypes in the PSD mouse.

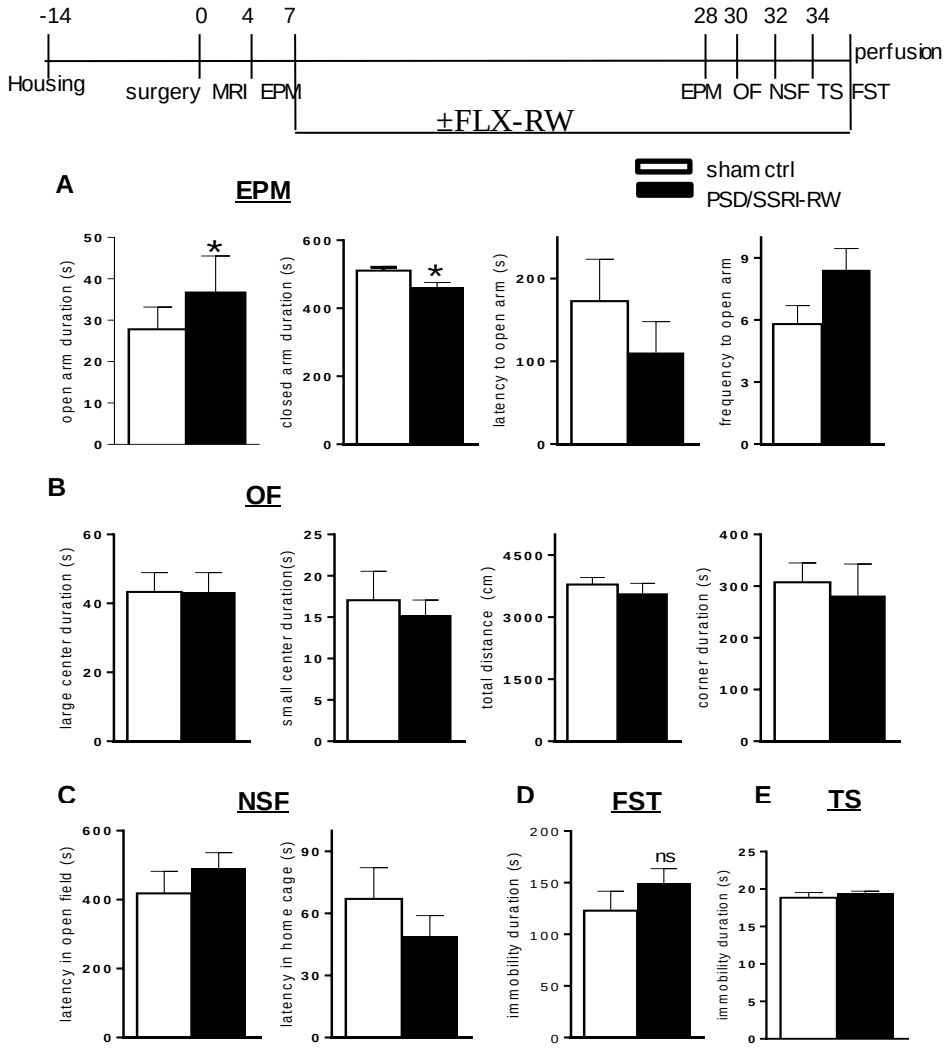


Figure 2. Combination SSRI-exercise treatment normalizes behavior of PSD mice.

Mice injected with ET-1 to induce ischemia in the left mPFC and treated with fluoxetine and running wheel (PSD/SSRI-RW) were compared to sham-injected mice with vehicle and fixed wheel (Sham), with timeline as described in Fig. 1. ctrl/veh-FW. A. EPM. Compared to sham, the PSD/SSRI-RW mice showed increased time spent in open arms, indicating reduced anxiety. There was no difference in latency or frequency to enter the open arm compared to Sham. B. OF. PSD/SSRI-RW mice showed similar times spent in both large and small center and in corner duration compared to Sham, and the same total activity in open arena. C. NSF. No difference was seen in latency to approach food in new arena or to feed in home cage. D. FST. No difference in immobility time was seen between groups. E. TS. No difference in immobility time was seen between groups. Data represent mean ± SEM; n=12/group, *$p < 0.05$

Cognitive impairment in PSD mouse: reversal by chronic SSRI treatment

Cognitive impairment, including decline in learning and memory, is common in PSD patients. Since the mPFC is implicated in cognitive function, we tested whether spatial learning and memory were impaired in the PSD vs. sham control mice using a 10-day MWM test (Fig. 3, timeline). Compared to test day 1, the sham controls show progressive reduction in time to reach the platform from day 5 onwards, while the PSD mice displayed no consistent improvement indicating a complete inability to learn the task (Fig. 3A, B). The SSRI-treated PSD displayed rapid response in one day, with initial improvement greater than sham controls (days 2-4), indicating more rapid learning. Exercised PSD mice showed no improvement compared to PSD mice, and displayed significantly longer latency compared to sham mice from day 4 onwards. On day 11, in the probe test with the platform removed, the PSD and exercised PSD mice showed a reduced time in the target quadrant compared to the Sham and SSRI-treated PSD mice, which spent the majority of time in the correct quadrant, indicating that the SSRI-treated mice retained spatial memory. In two other cohorts (n=20) without the fixed wheel, we observed a similar impairment in spatial learning within 1 wk post-stroke with minimal improvement in the MWM over 10 days. Thus chronic SSRI, but not exercise, reversed the post stroke impairment and enhanced spatial learning and memory capacity.

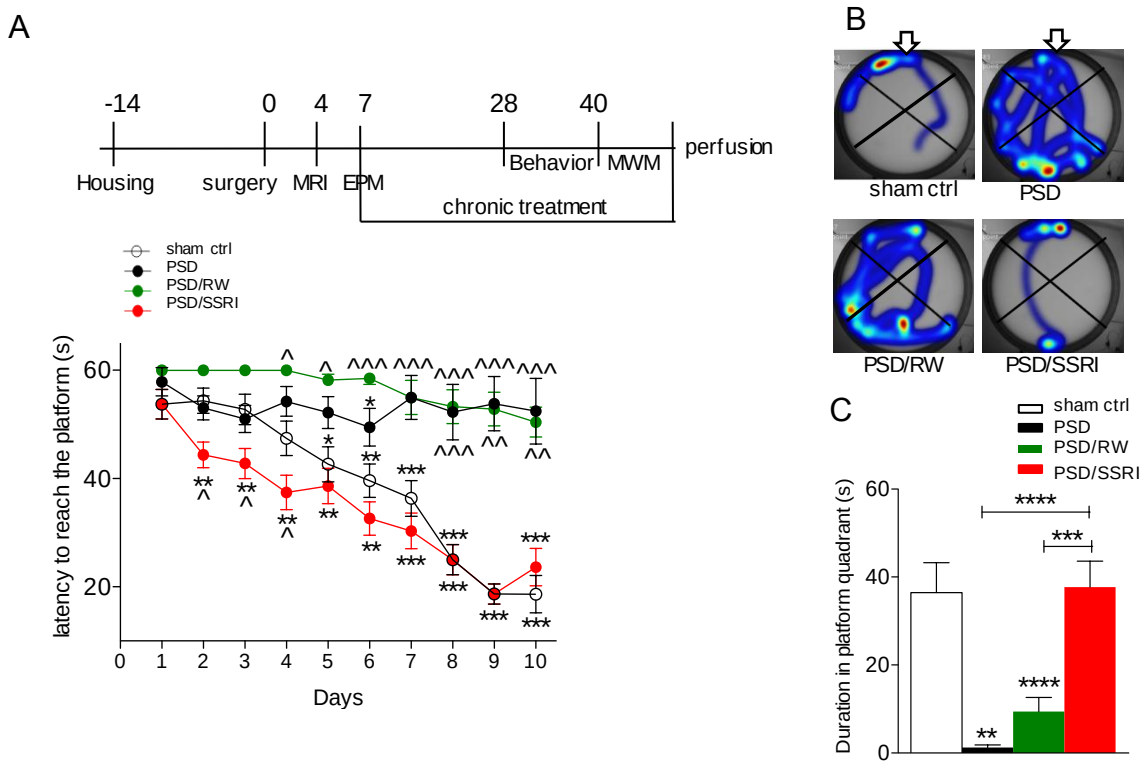


Figure 3. Chronic SSRI, but not exercise, reverses spatial learning and memory in PSD mice.

Spatial learning and memory were tested in the PSD mice treated with fluoxetine (SSRI) or running wheel (RW) vs. sham control with fixed wheel or vehicle as controls, using the MWM test and following the timeline shown. A. Acquisition. The latency to reach the hidden platform was measured each test day. Compared to Sham the PSD and PSD/RW mice showed a nearly complete inability to locate the platform with the 60 s test, while the PSD/SSRI mice showed a significantly reduced latency to reach the platform from day 2-10, that was reduced compared to sham from day 2-4 suggesting enhanced spatial learning. C. Probe test. Compared to sham, PSD mice showed reduced duration in the target quadrant, and this was reversed in the PSD/SSRI group, but not in the PSD/RW mice. Data represent mean ± SEM in n=8/group,*p<0.05, **p<0.01, ***p<0.001 vs. day 1,^p<0.05, ^^p<0.01, ^^^p<0.001 vs. sham ctrl

Contra-lesional corticolimbic neuronal activation following PSD

We previously showed that between 2-6 weeks post-stroke, the ischemic lesion of the left mPFC refills with cells, mainly NeuN-positive neuronal cells (Vahid-Ansari et al., 2016). However, the role of these cells is unclear given the lack of any improvement in anxiety, depression or cognitive phenotypes at 6 weeks post stroke. To address the chronic impact of left mPFC stroke on brain activity, brain sections from PSD vs. sham control mice were obtained at 6 weeks post stroke and immunostained for FosB (Fig. 4A), a marker of acute and chronic cellular activation (Vialou et al., 2015. In PSD compared to sham, in regions with the most FosB-labeled cells the number of FosB-stained cells was significantly increased in several regions including the left and right mPFC (CG and PI, Fig. 4B), nucleus accumbens (NAc), lateral septum (LSN) and lateral habenula (LHb), amygdala (Amy), ventral hippocampus (Hip), dentate gyrus (DG) and the dorsal raphe (DR) (Fig. 4C). In several of these regions, this increase was significantly greater on the right vs. left (ipsi-lesional) side, including CG, PI, LSN, DG, amygdala (Fig. 4C). These results show that left mPFC ischemia induces a persistent activation of the contra-lesional mPFC and downstream limbic targets implicated in anxiety, depression and spatial memory.

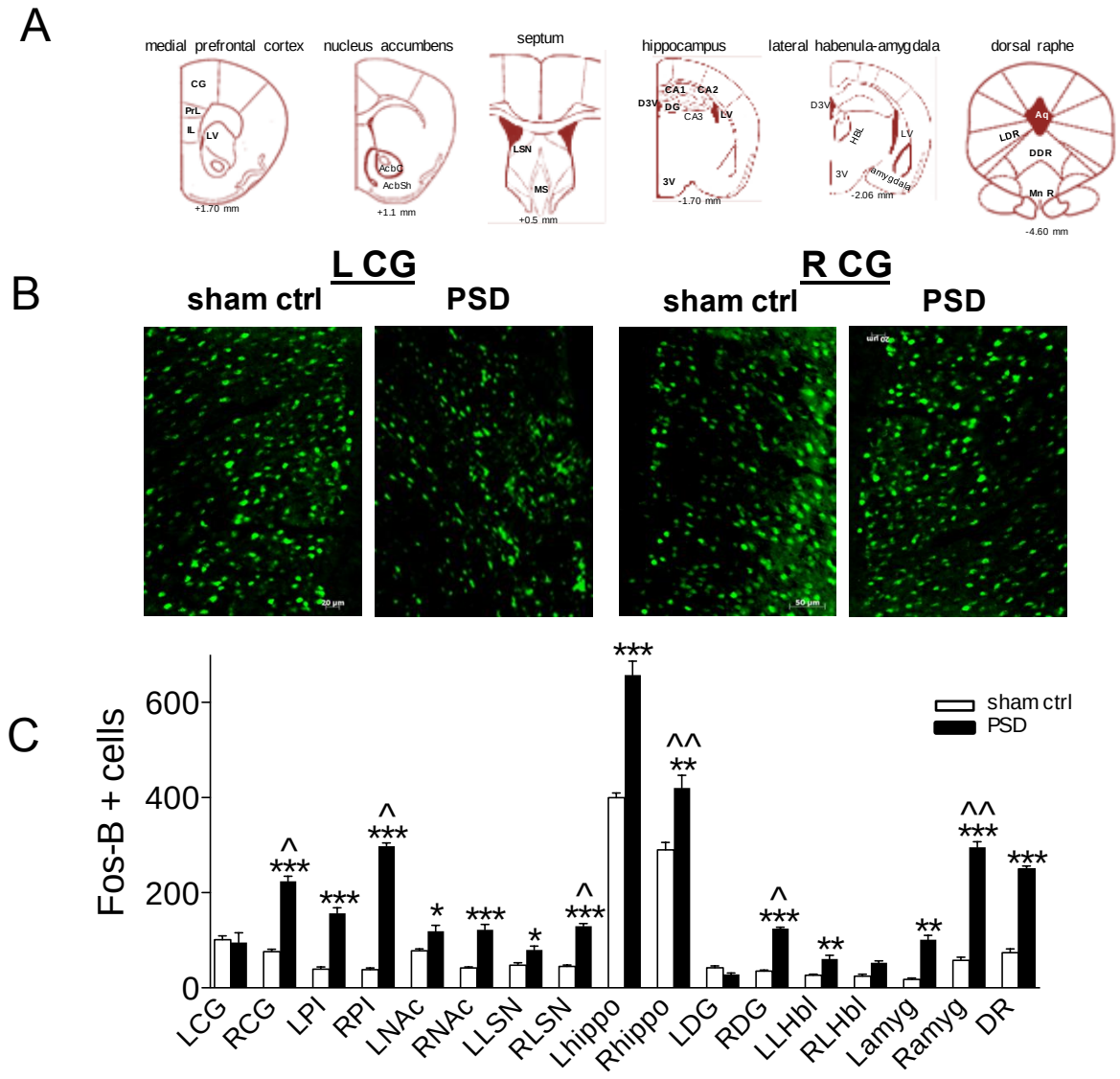


Figure 4. Increased FosB expression in corticolimbic areas of PSD mice.

Following behavioral assays at 6 weeks post stroke (see Fig. 1 timeline) and 1 day following the last assay mice were perfused, brains sectioned and immunostained for FosB. The number of FosB-positive cells quantified in regions where activation was seen. A. Schematic representation of the sections in which FosB activity was observed. B. FosB Immunofluorescence. Representative photomicrographs of FosB immunohistochemistry in CG left (L) vs. right (R) in sham ctrl and PSD. Images were taken under 20X magnification, Scale bar, 20 µm. C. Quantification of FosB-positive cells. FosB-labeled cells were quantified in left (L) and right (R) side, and were increased in PSD vs. sham control in R cingulate gyrus (CG) and pre/infra-limbic (PL/IL) of mPFC, hippocampus, nucleus accumbens (NAc), lateral septum (LSN), amygdala

(amyg), lateral habenula (LHBL) and dorsal raphe (DR) compared to sham control. In several areas, FosB-positive cells were significantly more increased in right vs. left side in the PSD group. Data represent mean ± SEM in n=4/group,*p<0.05, **p<0.01, ***p<0.0001 vs sham ctrl; ^p<0.05, ^^p<0.01 vs contralateral side.

In order to further identify which cell types are being activated after stroke and when, we compared FosB-stained cells in brains from vehicle-treated PSD mice at 4-d or 6-wk post stroke (Fig. 5). To identify the cell types activated, co-immunostaining for FosB with neuronal markers for 5-HT (TPH), glutamate (CAMKIIα or VGLuT3) or GABA (GAD67) subtypes was quantified. At 4 d post stroke, the lesioned left mPFC showed no cells (Vahid-Ansari et al., 2016), and hence no FosB staining and most regions showed very low (<30) FosB+ cells/region bilaterally, except for CA1, DR, and Amy. Compared to 4 d, at 6 wk post stroke FosB-labeled cells were strongly increased in these regions, in left and especially right sides. In the mPFC (CG and PI), NAc, LSN and right CA2/ CA3, DG, the increase in FosB-labeled cells was mainly in CAMKIIα-labeled cells, suggesting a hyper-activation of contra-lesional corticolimbic pyramidal neurons. By contrast, in the LHb mainly GABAergic cells were activated consistent with their inverse activity in depression phenotypes (Warden et al., 2012).

Treatment-induced plasticity of corticolimbic activity post-stroke

Effective SSRI or combination treatments elicited similar changes in FosB-stained cells in several brain regions compared to control (PSD) or exercise alone. In areas in which PSD induced strong FosB activation (CG, PI and NAc), this correlated with increased activation of pyramidal (FosB/CAMK+) neurons, especially on the right side. In the CG and PI, effective treatments but not exercise alone strongly reduced these changes, especially on the right compared to left side to balance pyramidal neuron activation. Oppositely in the left PI, exercise alone reduced FosB and FosB/CAMK cells more than effective treatments to maintain an imbalance of left/right activity. In areas where FosB-stained cells were less prominently increased following PSD, like LSN and Amygdala,

effective treatments induced FosB mainly via increased FosB/GAD67-positive cells, while exercise alone had no effect except for GABA activation in right amygdala. Thus in key areas implicated in cortical control of anxiety (LSN/Amygdala) and depression (mPFC, NAc), the effective treatments reduced the pyramidal neuron hyperactivity or activated GABAergic inhibitory neurons, especially on the right contralesional side.

There were some differences in the effects on neuronal activation of SSRI vs. PSD or the other treatments. SSRI alone uniquely recruited GABAergic activation in left PI, NAc and Amygdala as well as right hippocampal CA2/3 regions. In these regions, exercise alone often had opposite effects to reduce ipsilateral GABAergic or increase contralatereral glutamatergic activation, reversing the left-right balance. The combination treatment uniquely and strongly induced FosB- and FosB/GAD67-positive in the hippocampal DG reducing CA1 pyramidal and interneuron activation, more strongly on the left side. Since the hippocampus is implicated in spatial memory and adult neurogenesis, these results suggest that the combination treatment may produce more robust behavior improvements compared to SSRI alone through synergistic actions within the hippocampus.

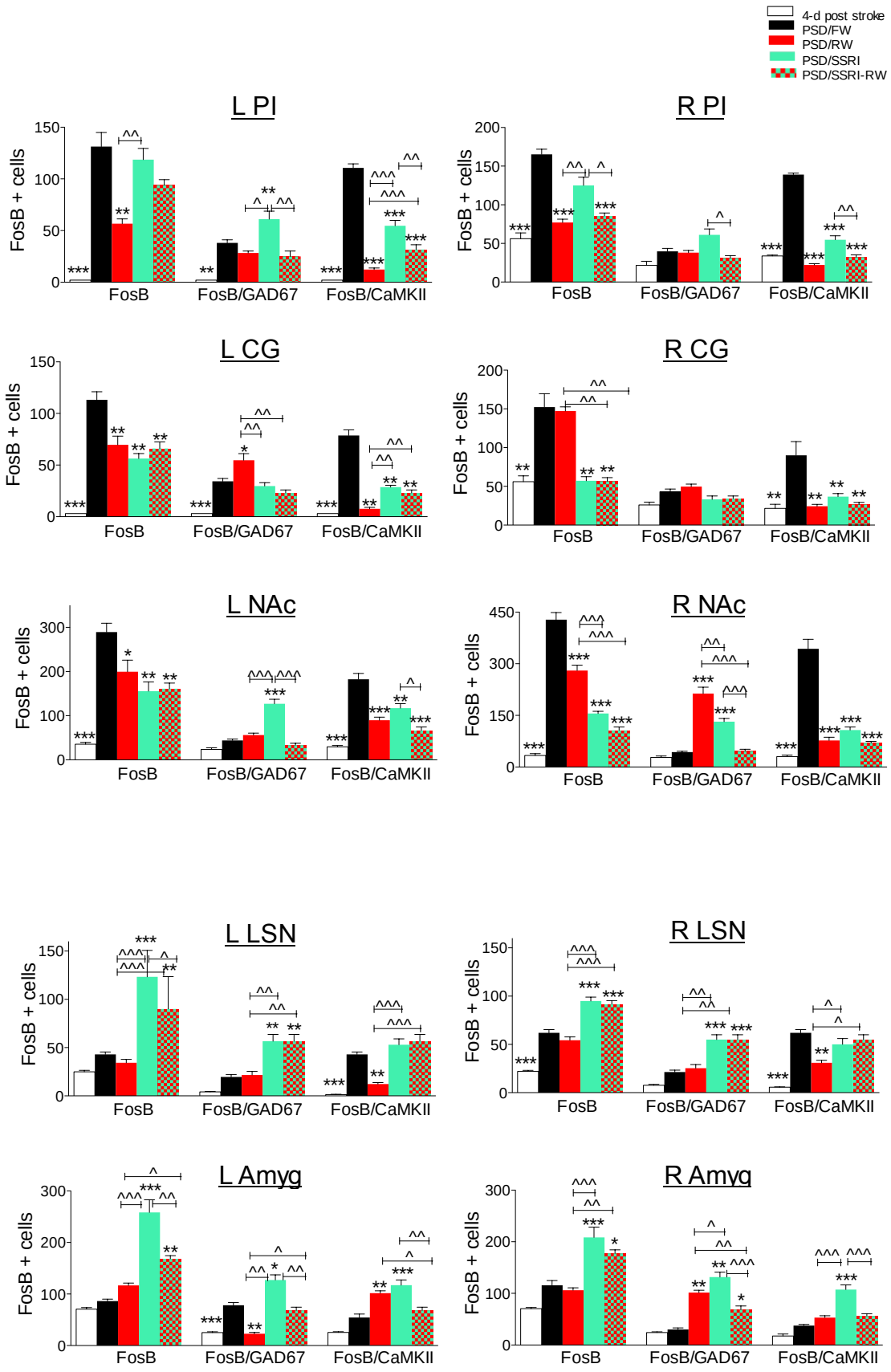

4-d post stroke
PSD/FW
PSD/RW
PSD/SSRI
PSD/SSRI-RW
L PI
R PI
L CG
R CG
L NAc
R NAc
L LSN
R LSN
L Amyg
R Amyg
FosB + cells
FosB
FosB/GAD67
FosB/CaMKII

Figure 5. Treatment-induced changes in the activity of different FosB-positive cells in PSD mice.

Sections were prepared from PSD mice at 4 d post-stroke (4 d) or at 6 weeks post-stroke after control (vehicle/fixed wheel, PSD/FW), fluoxetine/fixed wheel (PSD/SSRI), running wheel (PSD/RW) or both (PSD/SSRI-RW), timeline Figs. 1 and 2. Sections were co-stained for FosB, GAD67 and CAMKIIα and total FosB-positive, FosB-positive GABAergic (FosB/GAD67) or pyramidal (FosB/CAMKIIα) neurons were quantified on ipsilateral left (L) and contralateral right (R) side. Shown is data from mPFC (PI, CG), NAc, LSN, LAmyg. Compared to PSD/FW (at 6 weeks), at 4d post-stroke, no FosB was detected in left PI, CG consistent with absence of cells acutely following the lesion, while FosB was very low in right PI, CG and in NAc. Comparing treated to vehicle-treated PSD mice, PI-CG-NAc showed similar patterns of reductions in FosB-stained cells while and LSN-Amyg showed similar increases, especially for the effective treatments (SSRI or SSRI-RW). Data represent mean ± SEM in n=4/group, *$p<0.05$, **$p<0.01$, ***$p<0.001$ two way ANOVA Tukey's post-hoc vs PSD/FW. ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs other group as indicated by bars.

In the dorsal raphe (Fig. 6A, C) and raphe (Fig. 6B, D) FosB-positive cells increased from 4 d to 6 weeks post stroke, including 5-HT, GABA and glutamate cells, while total TPH-positive cells were unchanged (Fig. 6C). Chronic fluoxetine had little effect on the number of Fos/TPH-positive cells, but induced FosB activity most prominently in TPH-negative cells (Fig. 6A, yellow arrow), particularly in GAD67/FosB positive cells of the ventral raphe (Fig. 6B, yellow arrowhead) while exercise greatly reduced both (Fig. 6D). Conversely, with chronic exercise the number of FosB/VGluT3 cells remained high, but FosB/TPH and FosB/GAD67 cells were greatly reduced. Thus, effective chronic treatments were able to recruit GABAergic raphe cells while maintaining activation of 5-HT neurons.

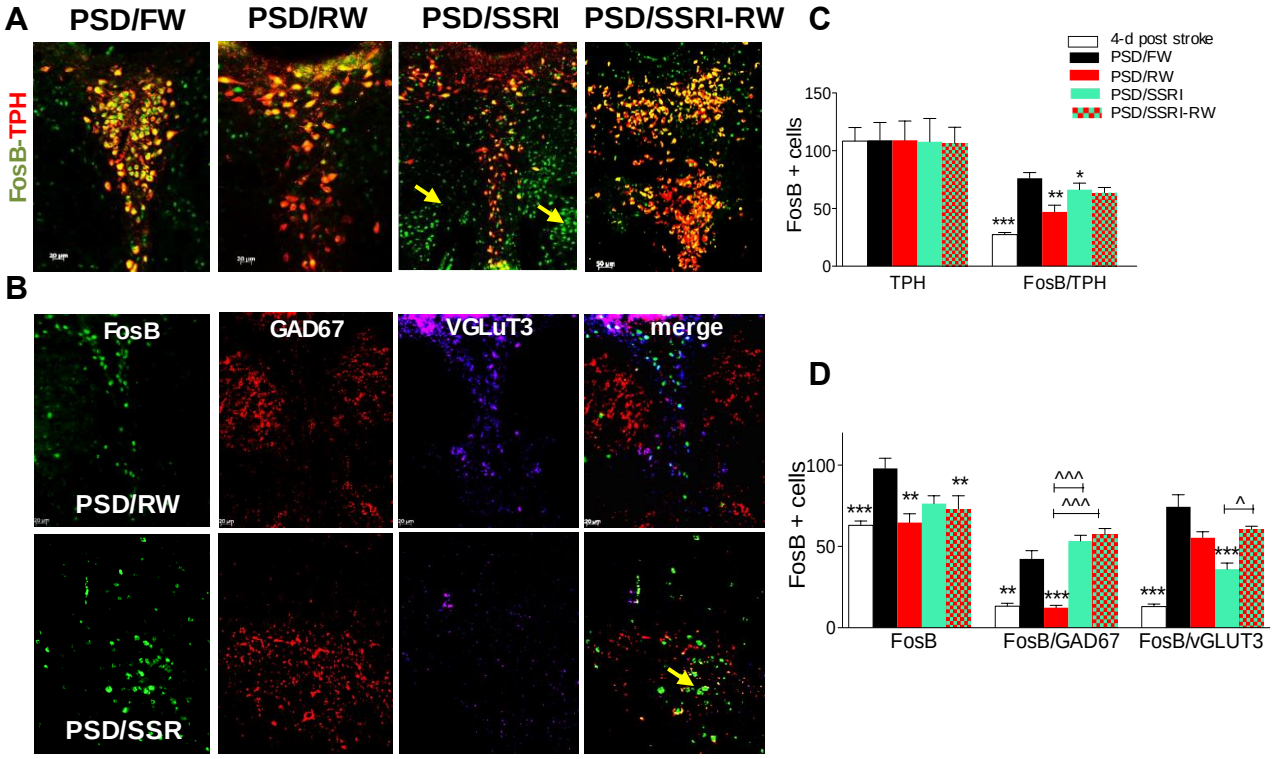


Figure 6. Treatment-induced changes in FosB-positive dorsal raphe cell activity.

Sections of dorsal raphe were prepared from control PSD mice (PSD/FW) or PSD mice treated with running wheel (PSD/RW), fluoxetine (PSD/SSRI), or both (PSD/SSRI-RW), and stained for FosB, TPH, GAD67, and VGLuT3 to identify FosB-positive 5-HT, GABAergic, and glutamatergic neurons. A. FosB/TPH co-staining. Merged images show FosB (green), TPH (red) and co-stained (yellow) cells in dorsal raphe. Arrows show TPH- FosB+ cells (possibly GABAergic, see panel B) in ventrolateral dorsal raphe. Images were taken under 40X magnification, Scale bar, 50 µm. B. FosB-GAD67-vGlut3 co-staining in PSD/RW vs. PSD/SSRI dorsal raphe. Images of FosB (green), GAD67 (red), VGluT3 (magenta) and merged images, 20X magnification, scale bar, 20 µm. Arrow shows FosB only + cells in PSD/SSRI-treated samples. PSD/SSRI induced higher activation of GAD67 positive cells in DR (FosB/GAD67) compared to vGLuT3 positive cells, while PSD/RW induced-higher activation of VGLuT3 positive cells in DR. C. Quantification of total TPH+ and FosB/TPH cells in dorsal raphe. Exercise reduced the number of FosB/TPH cells (PSD/RW vs. PSD/FW), while SSRI or SSRI-RW had minimal effect with no change in the number of TPH-positive cells. D. Quantification of FosB+, FosB/GAD67+ and FosB/VGLuT3+ cells in dorsal raphe. Compared to control (PSD/FW), in PSD/RW mice but not PSD/SSRI or SSRI-RW mice, a reduction in FosB/GAD67 cells was seen. Data represent mean ± SEM in n=4/group, *p<0.05, **p<0.01, ***p<0.001 two way ANOVA Tukey's post-hoc vs PSD/FW. ^p<0.05, ^^p<0.01, ^^^p<0.001 vs other group as indicated by bars.

Discussion

Chronic fluoxetine promotes recovery from PSD phenotypes

Current treatments for PSD, including chronic antidepressant treatment, are not effective in a majority of patients. In order to provide insight into effective treatments for PSD and associated anxiety and cognitive impairments, we have taken advantage of our PSD mouse model. Evidence from human imaging studies suggests alterations in the activity of the PFC-limbic circuitry are associated with anxiety disorders and major depression (Ressler and Mayberg, 2007; Krishnan and Nestler, 2008; Northoff et al., 2011) in major depression. Therefore, we targeted mPFC as a central node of the mPFC-subcortical limbic circuitry (Riga et al., 2014) to model PSD phenotypes (Singh et al., 2000; Yang et al., 2015). While lesion of the mPFC from anterior cerebral artery stroke is rare, much more common large ischemic or white matter strokes appear to disrupt multiple nodes and connections within the anxiety-depression circuitry, resulting in PSD (Lassalle-Lagadec et al., 2012; Liao et al., 2013). Previous rodent models of PSD have used large MCAO strokes, but in combination with chronic mild stress to elicit anxiety, depression and cognitive phenotypes (Kim et al., 2015). Hence, these are mixed models of stress- and ischemia-induced phenotypes, while our mPFC lesion selectively models ischemia-induced behavioral and cognitive outcomes.

Having previously shown a robust anxiety and depression phenotype that persists for at least 6 weeks (Vahid-Ansari et al., 2016), here we also found severe deficits in spatial learning and memory. In contrast to unilateral ischemia, others have shown that bilateral ET-1-induced stroke in rodent mPFC induces impairments in spatial learning, but with a subtle effect on anxiety behavior (Deziel et al., 2015; Livingston-Thomas et

al., 2015; Zhou et al., 2016). Compared to bilateral strokes, unilateral mPFC lesion may dis-inhibit the contralateral mPFC to drive the behavioral phenotype, as suggested by our FosB activation data. Consistent with this, left but not right middle cerebral artery occlusion (MCAO) in rats induced depression and anxiety-like behavior (Kronenberg et al., 2012). Clinical studies suggest that unilateral ischemia affecting the left vs. right mPFC is preferentially associated with PSD (Terroni et al., 2011; Murakami et al., 2013; Robinson and Jorge, 2016), although the opposite is seen (Wei et al., 2015). Ischemia of the left PFC is also associated with cognitive impairment (Bolla-Wilson et al., 1989; Potter et al., 2007), indicating the usefulness of unilateral lesions in mouse mPFC to model post-stroke cognitive impairment.

Our findings indicate that, in addition to behavioral recovery, learning and memory deficits were reversed by chronic treatment with SSRI. Similarly, in the rat left MCAO model, SSRI treatment was effective to reverse anxiety and depression-like behaviors (Kronenberg et al., 2012). SSRI treatment of PSD patients improves both behavioral and cognitive recovery as shown in clinical studies (Zhang et al., 2013; Robinson and Jorge, 2016). Chronic SSRI treatment may have additional cognitive benefits in PSD patients with strokes that affect PFC function (Flaster et al., 2013). In particular, chronic fluoxetine may improve spatial memory in part by enhancing CA1 LTP (Mlinar et al., 2015). Our results indicate that mice with left mPFC lesion respond to chronic SSRI with improvements in anxiety, depression and cognitive function.

Strikingly, we find that while SSRI treatment reversed anxiety and depression-like behavior and the cognitive deficit, exercise had minimal therapeutic effect on its own in the PSD mouse. On the other hand, the combination of SSRI and exercise was

effective, and even reduced anxiety to below sham control levels. Like SSRIs, free running wheel exercise may have beneficial effects in ischemic animals, such as enhancing brain growth factors and hippocampal neurogenesis (Kleim et al., 2002; Swain et al., 2003; Will et al., 2004). In mice, running mediates the neurogenic and neurotrophic effects of enriched environment (Kobilo et al., 2011). Both chronic SSRI and exercise increase hippocampal neurogenesis in mice post-MCAO associated with improvement in the MWM test (Luo et al., 2007; Li et al., 2009). Targeted exercise can also enhance post-stroke cortical plasticity and sensorimotor recovery (Overman and Carmichael, 2014). However, exercise alone was insufficient to reverse the phenotypes in our PSD model. Similarly, in stroke patients intense exercise may have a mild effect in PSD recovery (Eng and Reime, 2014), but early (started at 18h post stroke) rehabilitation can actually be detrimental to functional recovery (Bernhardt et al., 2015).

Chronic SSRI reverses PSD-induced imbalance in neuronal activation

To provide insight into the chronic changes in brain activity following unilateral ischemic lesion and treatment, we mapped FosB-positive cells. However, we cannot rule out activation of other regions that do not express FosB, and could not detect reductions in activity in these regions. We found that brain-wide FosB activity was relatively low at 4 days post stroke, while at 6 weeks post stroke there was a parallel change in the activity of the mPFC and several of its limbic and raphe targets compared to sham control, consistent with the emergence of the behavioral phenotype. Similarly, following ET-1-induced ischemia in rat sensorimotor cortex, FosB-labeled cells increased between 9-17 days post ischemia in contralesional cortex and perilesional striatal regions (Clarke et al., 2014). This suggests that ischemia-induced acute inactivation gradually evolves to a

hyper-activation of cortical targets that appears to drive the behavioral phenotype. Importantly, ischemia of the left mPFC resulted in a preferential activation of the contralateral right brain, including the right mPFC, NAc, amygdala, and LS. This suggests a contralateral inhibition model similar to that proposed for motor cortex lesions (Dijkhuizen et al., 2003; Silasi and Murphy, 2014), in which the loss of projections from the left side dis-inhibits the right mPFC, which is propagated to its targets.

Consistent with this idea, PSD mice displayed a greater number of FosB/CaMKII+ pyramidal cells in the right mPFC, NAc, lateral septum, and hippocampal regions (CA3, DG). There is a strong glutamatergic projection from mPFC to NAc that could mediate coupling between these regions in depression-like behavior (Russo and Nestler, 2013). FosB induction in mPFC is implicated in stress susceptibility leading to anxiety- and depression-like behavior (Vialou et al., 2014). Conversely, chronic antidepressant treatment reduced the number of FosB positive pyramidal cells in these areas in the PSD mice, an effect of fluoxetine that is observed in human PFC (Komlosi et al., 2012). Although chronic antidepressant treatment increases FosB levels in stressed mice, the induced FosB is rendered inactive, consistent with a beneficial effect of reduced FosB activity in the nucleus accumbens (Robison et al., 2014; Vialou et al., 2015). Several other regions showed differential activation for effective (SSRI±exercise) vs. non-effective treatment (exercise only) in the PSD mice. The lateral septum receives dense 5-HT innervation and is particularly sensitive to SSRIs (Sheehan et al., 2004; Hai et al., 2016) and in PSD mice chronic SSRI treatment induced GABAergic FosB, consistent with a chronic anxiolytic role for septal interneurons (Trent and Menard, 2010; Anthony et al., 2014; Parfitt et al., 2017). A similar response to SSRI was seen in the

amygdala, driven by increased GABAergic activation which may mediate an anti-anxiety effect (Jennings et al., 2013; Nieh et al., 2013), but weaker for combination treatment. In contrast, stimulation of mPFC afferents to the lateral habenula results in acute depression-like responses thought to involve in part glutamatergic projections to the raphe (Warden et al., 2012; Pollak Dorocic et al., 2014). In PSD mice, CAMKII+ FosB was absent, consistent with silencing of lateral habenula efferent activity that may be a compensatory anti-depressant or anti-anxiety response (Dolzani et al., 2016). Although all treatments reversed this inhibition, exercise had the largest effect while this was suppressed in effective treatments to promote recovery in the PSD model. Oppositely, stimulation of mPFC projections to the DR results in acute antidepressant response (Warden et al., 2012). We found that chronic effective treatments increased raphe GABAergic activation compared to exercise. GABAergic interneurons respond to aversive stimuli and act locally to inhibit 5-HT neurons (Li et al., 2016), but some project to mPFC and NAc and may reduce forebrain activation (Bang and Commons, 2012). The lack of increase in 5-HT neurons may also reflect autoinhibition by 5-HT1A receptors in the presence of SSRI treatment. In the hippocampus, similar treatment response patterns were seen in CA1-3, with exercise activating GABAergic neurons at ipsilateral, while SSRI activated GABAergic neurons at contralateral side; in both cases combination treatment showed weak or reduced activation. Raphe 5-HT/glutamate neurons have been shown to activate hippocampal interneurons (Varga et al., 2009) and could contribute to this effect of SSRI treatment. The net effect of active treatment was to balance left vs. right hippocampal activity. However, unlike other treatments, combination treatment

strongly induced FosB in DG (especially left DG), a site of neurogenesis that may contribute to recovery of anxiety or cognition (Kheirbek et al., 2013).

In summary, while PSD leads to a lateralized activation of the right mPFC and several limbic targets, effective treatments were able to selectively reverse these changes to balance left vs. right side activation. Furthermore, effective treatments targeted GABAergic cells within the mPFC, NAc, lateral septum, amygdala and raphe to reduce activation and normalize behavior. While lateralization is not well appreciated in rodents, there are examples for its role in chronic behavior, including left CA3 hippocampal-dependent long-term memory (Shipton et al., 2014).

Conclusion

Using our PSD mouse model, we find that chronic SSRI or SSRI and exercise mediate recovery of anxiety and depression phenotypes, while exercise alone had minimal effect. Chronic SSRI also reversed the memory impairment induced by LmPFC lesion. These changes were associated with alterations in FosB positive cell in the mPFC-limbic network SSRI±exercise functions may promote recovery at the lesion site, and throughout the neural circuitry that becomes dysregulated following the stroke. In addition, the effects of SSRI treatment at the lesion site may underlie a broad benefit in enhancing stroke recovery observed in stroke patients (Chollet et al., 2011; Mead et al., 2013).

Acknowledgements

We thank Dr. Ania Serefko, Danika Cziranka-Crooks, Rosalie Gauthier and Mirela Barclay for technical assistance with parts of the research. This research was supported by grants to P.R.A. from the Canadian Partnership for Stroke Recovery (CPSR) and the Canadian Institutes of Health Research. F.V.A. was supported by a CPSR studentship award.

Chapter 5

General Discussion

Major depressive disorder is one of the leading causes of disability worldwide and it is believed that it increases the risk and accelerates the progression of cardiovascular or cerebrovascular disease, and also reduces recovery in those disorders (Hakim, 2011). However, by using new strategies to specifically model depression it may be possible to elucidate its common and different underlying mechanisms. Towards this aim, I have developed two models of depression: the cF1ko to model genetic predisposition to major depression and anxiety; and the PSD mouse model of injury associated depression, anxiety and cognitive impairment.

The role of Freud-1, 5-HT1A receptor repressor, in depression and anxiety

Depression is a complex disease that involves both genetic and environmental contributors. Association studies suggest that functional genetic polymorphisms that alter the serotonin system in combination with life stressors can synergistically increase the strength of these associations (Caspi et al., 2003). Increased 5-HT1A autoreceptor expression in depressed patients (Parsey et al., 2010) and post-mortem depressed suicide tissues (Stockmeier et al., 1998; Boldrini et al., 2008) suggest a role for chronic changes, such as altered transcriptional regulation, in the dys-regulation of the 5-HT1A gene. Indeed, the G/C single-nucleotide polymorphism in the serotonin 1a receptor promoter, rs6295, has previously been linked with depression, suicide and antidepressant responsiveness (Le Francois et al. 2008; Donaldson et al, 2016). To test this model, I targeted Freud-1 which had been shown to be a powerful repressor of the 5-HT1A gene in vitro (Ou et al. 2003; Rogaeva et al., 2007). Therefore, I generated the cF1ko mouse model in which Freud-1 is conditionally knocked out only in 5-HT cells in adulthood to assess the depression/anxiety phenotype. Briefly, I showed that lack of Freud-1 in 5-HT

cells results in significantly increased functional 5-HT1A autoreceptor levels with: increased 5-HT1A binding in raphe; augmented hypothermia response to DPAT; and resulting in reduced activity of 5-HT neurons (as measured by FosB), and reduced 5-HT levels in raphe. Mice lacking Freud-1 in adult 5-HT neurons developed a robust depression and anxiety phenotype and were completely resistant to chronic fluoxetine treatment. Taken together, these results point to the major role of Freud-1-induced changes in 5-HT1A autoreceptor expression and 5-HT neuronal activity associated with SSRI-resistant depression-like/anxiety-like phenotype. This model fits the clinical data that associate the rs6295 polymorphism with increased 5-HT1A autoreceptor expression, major depression and anxiety disorders, and importantly resistance to SSRI treatment. Interestingly there is evidence that the rs6295 polymorphism also confers resistance to atypical antipsychotic treatment for negative symptoms of schizophrenia, suggesting a role for the 5-HT1A receptor or 5-HT system.

I also showed that the behavioral phenotype in cF1ko mice is dependent on 5-HT1A autoreceptors by knockout of both Freud-1 and 5-HT1A autoreceptors in 5-Ht neurons, which prevented Freud-1 induced anxiety and depression phenotype. However, in this study I did not address other genetic modifications that may be responsible for the behavioral outcomes apart from the persistent higher expression of 5-HT1A autoreceptor in cF1ko. Freud-1/CC2D1A has been implicated in transcriptional regulation of both serotonin and dopamine receptors in the rodents and human brain (Ou et al. 2003; Rogaeva et al. 2007; Szewczyk et al. 2010). In cF1ko, Freud-1 is only removed from 5-HT cells while it remains intact in dopamine cells to negatively regulate the expression of D2 inhibitory receptors (Rogaeva and Albert, 2007). Since D2 receptors appear to exert a

paradoxical excitatory effect in 5-HT neurons (Aman et al. 2007), it can be postulated that the Freud-1 inhibitory role on D2 receptor expression is less pronounced and results in increased dopaminergic signaling in 5-HT neurons in cF1ko. This would tend to counteract the increase inhibition mediated by 5-HT1A receptors, but may explain the paradoxical anti-depressed state seen when both Freud-1 and 5-HT1A autoreceptors are knocked out.

Freud-1 was identified in a genetic screen for NF-κB activators and activates NF-κB pathway (Zhao et al., 2010). Gene deletion of Freud-1 leads to dysregulation of NF-κB signaling to synaptic plasticity and transmission in cortical development (Manzini et al., 2014). Lack of the Freud-1 in 5-HT neurons might contribute to changes in morphology and also synaptic density of the 5-HT neurons, which in turn results in dysregulation of 5-HT system activity. Similarly, Oaks et al., 2016 showed that conditional removal of Freud-1 in post-natal cortical pyramidal neurons led to abnormal cortical dendrite organization and a reduction in dendritic spine density associated with deficits in neuronal plasticity and in spatial learning and memory, reduced sociability, hyperactivity and anxiety. However, these effects of Freud-1 deletion appear to be developmental, and it is unclear whether deletion of Freud-1 in adulthood would affect neuronal structure or function. Furthermore, the 5-HT1A receptor transcription is induced by NF-κB (Wissink et al., 2000), and loss of Freud-1 would reduce this pathway: but since we saw 5-HT1A up-regulation it suggests that the Freud-1- NF-κB pathway is not active in adult 5-HT neurons. While I did not observe changes in the number of TPH-positive cells (suggesting no loss of 5-HT neurons), I did not address whether the dendrites or spines of 5-HT neurons are altered.Therefore, in cF1ko, 5-HT transmission is

mainly altered due to overexpression of 5-HT1A autoreceptors, but further studies are needed to determine whether synaptic reorganization may also occur.

It is notable that the 5-HT1A receptor gene is regulated by a number of identified transcription factors. For example, PET-1 is required for 5-HT1A receptor expression specifically in serotonin neurons due to its exclusive expression in these neurons (Liu et al., 2010; Jacobson et al., 2011) and directly activates the 5-HT1A promoter (Jacobsen et al., 2011). Other transcription factors that may dynamically regulate 5-HT1A receptors in the brain include Deaf1 and Hes1/5 (Lemonde et al., 2003; Jacobsen et al., 2008). Gene knockout of Hes1 in mice results in premature embryonic over-expression of 5-HT1A receptors in the midbrain (Jacobsen et al., 2008). Deaf1 represses 5-HT1A receptor expression in raphe cells (Lemonde et al., 2003), yet enhances its transcription in some non-serotonergic cells (Czesak et al., 2006) like PFC and hippocampal CA1 pyramidal neurons (Lemonde et al., 2003; Szewczyk et al., 2009). In a clinical study, Goswami et al. (2010) reported that Deaf1 and REST, were significantly increased in DR neurons of female MDD subjects compared to female control subjects. In cF1ko, the constitutive expression of other 5-HT1A receptor regulators was not targeted. Whether lack of Freud-1 is paralleled by changes in other 5-HT1A regulators was not addressed in this report. However, whether loss of Freud-1 induces changes in other regulators' functions to presumably re-balance the 5-HT system function could be further investigated.

Freud-1 itself is regulated by multiple factors. Freud-1 expression appears to be stress-sensitive, as chronic stress in rats reduced Freud-1 levels in PFC while increasing 5-HT1A receptor levels, perhaps representing a compensatory adaptation to the stress (Iyo et al., 2009). In humans, Freud-1 and 5-HT1A protein levels were reduced in PFC of

depressed subjects, particularly younger subjects, suggesting role for Freud-1 in early stages of depression (Szewczyk et al., 2010). While Freud-1 protein was reduced, Freud-1 is inhibited by calcium-CaMK and could downregulate 5-HT1A expression in response to reduced calcium levels (Ou et al., 2003). Since CaMKII is primarily expressed in pyramidal neurons, calcium-mediated inactivation of Freud-1 may be more specific to these neurons compared to interneurons, leading to calcium-mediated up-regulation of 5-HT1A heteroreceptors specifically in pyramidal neurons (Albert et al., 2014). Calcium-CaMK mediated inhibition of Freud-1 could mediate antidepressant-induced down-regulation of the 5-HT1A receptor. For example, treatment with fluoxetine is thought to increase extracellular 5-HT levels in the raphe, which would activate 5-HT1A autoreceptor signalling to inhibit neuronal calcium entry. This would lead to an activation of Freud-1 and repression of 5-HT1A autoreceptors, to down-regulate transcription of the 5-HT1A gene. However, in the absence of Freud-1, the 5-HT1A autoreceptors would not down-regulate and this could explain the fluoxetine-resistant phenotype in the cF1ko mice.

To address whether the behavioral phenotype induced by loss of Freud-1 is dependent on the increased level of 5-HT1A autoreceptors, I generated the cF1/1A double knockout in which both Freud-1 and 5-HT1A autoreceptors were conditionally removed only in 5-HT cells. In agreement with previous results (Richardson-Jones et al., 2010), knockout of the 5-HT1A autoreceptor in adults did not alter anxiety or depression behavior. However, lack of Freud-1 in 5-HT1A WT background induces depression/anxiety phenotype. Knocking out both Freud-1 and 5-HT1A (dko), only in raphe 5-HT cells, induces a stress resilient phenotype with no changes in anxiety

phenotype. This result indicates the role of 5-HT1A autoreceptor in Freud-1-induced pro-depressant phenotype. Furthermore, lack of 5-HT1A autoreceptors blunted the cF1ko-induced anxiety in dko suggesting that the cF1ko phenotype is dependent on 5-HT1A autoreceptor.

Previous animal studies showed that 5-HT1A receptors are generally involved in modulation of both depression and anxiety behavior (Klemenhagen et al, 2006, Albert et al., 2014). Global 5-HT1A knock-out mice show a strong anxiety-like phenotype. Tissue-specific conditional expression of 5-HT1A receptors in the hippocampus and cortex during the critical period from postnatal day P5 to P21 can rescue this phenotype (Gross et al., 2002). Conversely, transgenic mice that overexpress 5-HT1A receptors during embryonic and early postnatal development P1.5 display reduced anxiety-like behaviors (Kusserow et al., 2004). Taken together, these results indicate that normal behavior is mediated through the harmonized activity of both 5-HT1A auto- and heteroreceptors, and that an imbalance of either can alter anxiety and depression behaviors. Transgenic mouse models help to dissect the role of 5-HT1A auto- vs. heteroreceptors at the different stages of development; however the role of compensatory mechanisms to promote resilience or recovery should not be neglected. In this regard, it would be interesting to further assess the possible changes in 5-HT1A heteroreceptor function in the cF1ko and dko phenotypes. For example it would be interesting to assess the activity of GABAergic cells in the 5-HT1A ko that may compensate for the loss of autoreceptor inhibition. In addition, glutamatergic input from PFC may be reduced. These assessments would suggest a possible compensatory alteration in the 5-HT neuronal network implicated in depression/anxiety in cF1ko model.

Freud-1 conditional knockout mouse: a novel fluoxetine treatment resistant model

The three-week latency for the therapeutic effects of SSRI treatment is thought to involve desensitization of 5-HT1A autoreceptors to release 5-HT neurons from recurrent inhibition (Pineyro and Blier, 1999; Albert et al., 2011; Descarries and Riad, 2012). The loss of Freud-1 may render 5-HT neurons resistant to desensitization by chronic SSRI treatment. 5-HT1A receptors couple to inhibition of intracellular calcium levels (Albert et al., 1990; Penington, et al., 1990), which could activate Freud-1 DNA binding and repression (Ou et al., 2000), leading to 5-HT1A autoreceptor desensitization. Quantitative measurements of the level of 5-HT1A auto and heteroreceptors in cF1ko chronically treated with fluoxetine showed no detectable changes in the level of 5-HT1A binding in DR/PFC/hippocampus compared to cF1ko vehicle-treated group. However, a significant reduction in 5-HT1A level was observed in MR (data not shown), indicating different 5-HT1A autoreceptor sensitivity in MR and DR to Freud-1-dependent down-regulation, or insufficient time for 5-HT1A down-regulation in response to fluoxetine (3 weeks, po, 18 mg/kg). To further address these possibilities, changes in fluoxetine treatment paradigm (e.g. treatment duration, doses) could be applied. Other repressors, such as Deaf1, could mediate the Freud-1-independent down-regulation of 5-HT1A autoreceptors in MR. In this regard, the G(-1019) allele that associates with up-regulation of 5-HT1A autoreceptor in human depressed subjects (Parsey et al., 2010), is also associated with resistance to SSRI treatment (LeFrancois et al. 2008, Newman-Tancredi and Albert, 2012). Since Deaf1 cannot bind to this allele, it could suggest a role for Deaf1 in fluoxetine-mediated down-regulation of the 5-HT1A autoreceptor.

As a model of fluoxetine-resistant anxiety and depression, the cF1ko mouse provides a model to test for other antidepressant treatments that may augment SSRI treatment, or bypass the 5-HT system. For example, it would be interesting to test whether antidepressants that target 5-HT and other systems (e.g., imipramine, SNRIs) are effective in the cF1ko mice. Other treatments, like the subanesthetic doses of the non-competitive NMDA receptor antagonist ketamine are able to rapidly but transiently induce the antidepressant effects in patients with TRD (Kapur and Seeman, 2002), by perhaps bypassing the 5-HT system (Duman et al., 2016). In pre-clinical studies in male rhesus monkeys, ketamine selectively enhances serotonergic transmission by inhibition of SERT activity resulting in transiently increases in serotonin levels in the extracellular fluid of the prefrontal cortex (Yamamato et al., 2013). How the transient combination of SERT and ketamine enhance each other's antidepressant actions is under investigation. To my knowledge, because of the lack of reliable TRD model, the effects of ketamine have not been used in an appropriate model. Therefore, using ketamine for the improvement of the TRD behavior in cF1ko provides an opportunity to better investigate different features in TRD patients for recovery. The cF1ko provides a useful model of treatment-resistant depression associated with genetic dys-regulation of the 5-HT1A autoreceptor. This model could be useful to identify novel and effective treatments for depressed subjects with genetic predisposition, particularly those with polymorphisms affecting the 5-HT system.

Post-Stroke Depression and 5-HT system

PSD is associated with anxiety and cognitive decline and is common after stroke (Carota et al., 2005; Pollock et al., 2012). Current antidepressant treatments (SSRIs) result in

remission in only 30% of depressed patients hence a better understanding of stroke induced impairment leading to depression and how these can be ameliorated is needed. To better study the functional changes in neural circuitry responsible for PSD phenotypes and upon successful treatment, I developed a preclinical ischemic model which mimics PSD in stroke survivors (Vahid-Ansari et al., 2016). Unilateral microinjection of the vasoconstrictor, ET-1, into the left mPFC was used to induce focal ischemia, which resulted in persistent depression, anxiety, and cognitive impairment phenotypes in the absence of motor deficits. The mPFC was targeted to disrupt the mPFC-subcortical circuitry to model PSD phenotypes (Singh et al., 2000; Yang et al., 2015). In agreement, evidence from human imaging studies suggests that different sub-populations of PFC neurons with different targets mediate anxiety, depression and cognitive function (Ressler et al., 2007; Krishnan and Nestler, 2008; Savitz and Devertz, 2009). Several limbic areas implicated in behavior, including the nucleus accumbens, amygdala, hippocampus, hypothalamus, are highly interconnected with the mPFC. Clinical fMRI studies have shown changes in both structure and function of these areas in major depression (Ressler and Mayberg, 2007; Northoff et al., 2011). The mPFC also projects to midbrain dorsal raphe and can regulate its activity (Pollak Dorocic et al., 2014; Weissbourd et al., 2014; Celada et al., 2001; Geddes et al., 2016). Based on both serotonin depletion studies and the observed efficacy of SSRIs, the serotonin system has been long linked to depression (aan het Rot et al., 2009). Therefore, I hypothesized that constitutive changes in mPFC-DR circuitry underlie PSD and successful interventions restore its normal activity.

Although the anatomical connections between PFC-DR have been known for many years (Maclean et al., 1952), the specific neuronal populations responsible for

functional connectivity within this circuit, or how altered activity in one area affects the others is not well understood. For example, in healthy rats, selectively activating inputs from mPFC that target unidentified neurons in DR results in an antidepressant response in the FST (Warden et al. 2012). However it is unknown how this circuitry is modified in depression, or which DR neurons (5-HT, GABA, glutamate) are targeted. It can be postulated that pharmacological treatments that activate specific subpopulations of mPFC can efficiently affect depression-related network to recover depression and perhaps the associated behaviors like anxiety and impairments in cognition. Here, I showed that in a PSD model, the activity of limbic areas has been perturbed, and that SSRI alone or combined with exercise treatment, which engages the serotonergic system to alter corticolimbic activity, resulted in recovery from PSD.

The mechanisms by which antidepressants can alleviate the depressive symptoms after stroke remain unclear. Fluoxetine improves the ischemic brain injury by reducing inflammatory reaction, promoting the expression of regenerative and neuroprotective proteins and controlling excessive post-stroke excitation (Lim et al., 2009; Shin et al., 2009). SSRIs, which increase 5-HT levels throughout the brain, are also believed to facilitate neuronal regeneration resulting in the plasticity of neurons (Russo-Neustadt et al., 2000; Coppell et al., 2003). As a complementary treatment, exercise has been considered protective for PSD and task/goal-oriented rehabilitation reduces the risk of developing PSD (Hou et al., 2013). Furthermore, exercise programs appear to enhance recovery from PSD (Eng et al., 2014). However, in the latter study, it was not clear what proportion of PSD patients were also receiving antidepressant treatment. Thus, although the combination of SSRI and exercise may be beneficial for PSD, it is not clear whether

these treatments have synergistic effects to reverse PSD phenotypes. My results indicate that in a mouse PSD model, the major benefit is due to SSRI treatment, which on its own produced complete recovery. However, in some tests the combination of exercise and SSRI treatment appeared to produce improvements greater than the sham control. It would be important to address in clinical studies whether there is any benefit to exercise ± SSRI therapy for PSD.

Recovery from PSD is associated with region-dependent activity changes

In order to address chronic changes in neuronal activity in the PSD mouse and associated with treatments I examined the expression of FosB. Expression of the immediate early gene product FosB (particularly delta- or delta-delta-FosB) can be an indirect correlate of chronic changes in neuronal activity (Vialou et al., 2015; Vahid-Ansari et al., 2017). FosB-positive cells are widely distributed in PSD brain at 6 weeks post-stroke and were more prominently increased in the right brain, including the cingulate cortex and pre/infra-limbic prefrontal cortex, amygdala, septum, lateral habenula, and in the dorsal raphe compared to the sham control. Similarly, in unilateral sensorimotor MCAO in rats, a preferential activation of the contralateral cortex is seen; however, recovery appears to involve the reactivation of the ipsilateral side (Dijkhuizen et al., 2003). The function of the cortical and other brain areas relies on complex local circuits of interconnected excitatory glutamate and inhibitory GABA neurons, with GABA playing a strong network regulatory role (Freund, 2003; Fino et al., 2013). In PSD brain, there was a significant increase in the activity of FosB/CaMKIIα+ cells at the contralateral side in regions like mPFC; septum; amygdala; hippocampal regions and DR. These results point to a loss of contralateral inhibition of the right mPFC that results in its hyperactivation,

and that is transmitted to downstream through the corticolimbic network. Figure 1A (appendix) summarizes the asymmetric activation of right vs. left side induced in PSD. In PSD brain, there were also significant changes in raphe 5-HT cell activity, as well as in non-5-HT cells, as a key target of mPFC projections. In addition, in PSD brain, the lesion site becomes refilled with neuronal cells (Vahid-Ansari et al., 2016). Although these neurons are functional as indicated by FosB activity (Vahid-Ansari and Albert, 2017), they are not effectively integrated into the network, as evidenced by the persistent PSD phenotypes. Thus PSD results in neuronal activity changes, particularly in the right mPFC and its downstream targets.

Fluoxetine promotes recovery from PSD phenotypes

Evidence from clinical studies suggests that SSRIs, which were employed in initial studies of patients diagnosed with PSD, also improve cognitive and functional recovery (Flaster et al., 2013). However, how SSRIs improve cognitive function is unclear. In the PSD mouse, we found that chronic FLX treatment improved not only anxiety and depression-like phenotypes, but also reversed the cognitive impairment seen in these mice. We also saw widespread changes in FosB-labeled cells in the FLX-treated mice which indicate that FLX was able to normalize the PSD-induced activation of the corticolimbic network implicated in the behavioral and cognitive changes. Along with the corticolimbic network activity changes in PSD/SSRI brain reported here, previous studies found other beneficial roles for FLX. FLX has been shown to influence neurogenesis in the cerebral cortex and hippocampal area (Banasr et al., 2004; Encinas et al., 2006) and enhances the synaptogenesis and axonal sprouting during acute and subacute phases of stroke recovery (Cramer et al., 2008). Although we did not assess

these parameters in our model, the presence at the lesion site of NeuN+ along with the FosB/CamKIIα+ or FosB/GAD67+ cells could reflect in part the migration of newborn neurons to the lesion site or spread from adjacent perilesional cortex following shrinkage or remodeling of the lesion site. After effective chronic SSRI treatment, these neurons may integrate into the corticolimbic circuitry perhaps through induced synaptic reorganization, as has been reported in the adult visual cortex following SSRI treatment (Maya Vetencourt et al., 2008). Integrated neuronal cells are continuously in cross-talk with glial cells to maintain the activity of brain regions. Several studies have found the resultant induction of signaling pathways in glia and neurons after 2 weeks treatment with fluoxetine. Interestingly, in a different model of depression, some suggest that the major chronic impact of SSRIs in the fluoxetine-treated mice is on astrocytes in addition to improving behavioural symptoms (Gosselin et al., 2009). In a co-culture system, Dhami et al., 2013 demonstrated that pre-treatment of microglia with the antidepressants fluoxetine improves the viability of weakened neurons through the reduction release of glutamate from activated microglia in a model of ischemic insult (Dhami et al., 2013; Hale and Lowry, 2011). Taken together, in PSD brain, fluoxetine might protect neurons from microglia-mediated glutamate release at the lesion site and perhaps provide a rich niche for the new cells to better function.

In PSD mice, the activity of the DR was also affected. The DR is a heterogeneous structure (Hale and Lowry, 2011; Jasinska et al., 2012), therefore neuronal activation of different cell types within DR was also assessed. The FosB studies showed changes in the activity of serotonin and some non-serotonin cells in DR of PSD mice. Although no changes in the number of TPH-positive cells was found under different conditions, a

significant increase in FosB/TPH-stained cells was observed in PSD brains. In DR, increased activation of serotonin system was seen, along with the increases in the activity of VGLuT3+ and GAD67+ cells in PSD mice. Chronic treatment with SSRI alone or in combination with RW significantly enhanced only the activity of GAD67+ cells while decreasing the activity of VGLuT3+ cells. The VGLuT3 DR neurons project to mPFC, amygdala and striatum/NAc (Gagnon et al., 2014), and thus could activate these regions. Therefore, it can be postulated that higher activation of serotonin system reversed the lower activity of interneurons resulting in inhibition of over-activated glutamatergic cells in PSD DR. In fact, fluoxetine's GABA-enhancing effects have been already shown in different depression models (Zhong and Yan, 2004; Walia and Gilhotra, 2017) and SSRIs act in part to restore the disrupted GABAergic activity in different parts of the brain (Komlosi et al., 2012).

In addition to DR, PSD mice display a greater number of FosB/CAMKIIα-positive glutamatergic cells in areas mapped in the limbic circuitry. In some areas like hippocampal regions (CA3, DG), mPFC, NAc, and septum these cells were more pronounced on the contralateral side compared to the lesion side. Both SSRI and SSRI-RW enhanced the GAD67-positive interneuron activity while reducing the over-activity of total or CaMKII-associated FosB in the same areas. The reversal of the PSD-induced imbalance of activity was also associated with recovery from PSD. In fact, because FLX increases synaptic 5-HT levels brain-wide, the recovery from PSD suggests that the beneficial effects of either FLX alone or with exercise are dependent on the 5-HT system, which underlines the primary role of the 5-HT-PFC circuitry in an effective treatment. 5-HT neurons send axons to almost all brain regions and modulate the activity of various

populations of neurons by synaptic contacts that are either excitatory or inhibitory. The function of 5-HT system is mediated through different receptors and among those the 5-HT1A receptor has a key role in SSRI treatment. For example, there are two major sub-populations of 5-HT1A heteroreceptors in the PFC which inhibits the activation of pyramidal glutamatergic neurons and interneurons, respectively (Santana et al., 2004; Amargos-Bosch et al., 2004). In cortical neurons, 5-HT1A receptor activation inhibits NMDA currents (Yuen et al., 2008), and reduces CAMKII-induced AMPA phosphorylation (Cai et al., 2002), resulting in an inhibition of neuronal activity. A similar circuitry to that proposed for PFC may exist in the hippocampal CA1 region (Varga et al., 2009). Optogenetic stimulation of raphe projections inhibits CA1 pyramidal neurons via 5-HT1A receptors on these neurons; in addition, raphe projections innervate interneurons inhibiting them via 5-HT1A receptors.

In addition to the mPFC, SSRI treatment altered the activity of the amygdala and lateral septum. The medial amygdala and lateral septum (Herdade et al., 2006; Viana Mde et al., 2008), which receive massive 5-HT projections from DR, also play an important role in modulation of defensive behavior, stress/anxiety and behavioral inhibition. Deep brain stimulation (DBS) of the dorsal DR induced anxiolytic effects, which was associated with altered neuronal activity in the medial amygdala, lateral septum and cingulate cortex (Wscieklica et al., 2017). Relatively, 5-HT1A agonist or GABA/benzodiazepine compounds directly injected to LS showed anxiogenic vs. anxiolytic effects on inhibitory avoidance (Viana Mde et al., 2008), suggesting opposite roles for 5-HT and GABA on anxiety in LS. Thus 5-HT1A-induced modulation of

GABAergic interneurons may play key roles in the action of SSRI treatment on distinct behaviors by targeting different brain regions.

Exercise failed to produce changes in PSD neuronal activity

Rehabilitation treatment in form of exercise is highly recommended for stroke survivors to promote their recovery (Jeong et al., 2014). A large body of work suggests that exercise can improve health, behavior and facilitate neurogenesis in areas of the brain that are notably impacted by stroke. Exercise ranges from light goal directed activity to moderately intense walking or immobile bike riding. Indeed, exercise is beneficial for decreasing risk factors for a wide range of disturbances in emotion and cognitive impairments (Phillips, 2017). Although how exercise exerts these benefits is debatable, some studies implicate activation of the 5-HT system. For example pedaling exercise improves negative emotion associated with activation of the serotonergic system and changes in anterior cingulate cortex (Ohmatsu et al., 2014).

In animal models, the beneficial effects of exercise on neurological maintenance and cognition have been widely shown. The impact of exercise on stroke involves increases in growth factors, neurogenesis, angiogenesis and many other factors (Will et al., 2004; Swain et al., 2003; Kleim et al., 2002). Treadmill running in animals reduces microgliosis and inhibits the release of pro-inflammatory cytokines resulting in reductions in lesion size and cell death in the hippocampus and improves short-term memory following cerebral ischemia (Austin et al., 2014; Lovatel et al., 2014; Sim et al., 2005; Sim et al., 2004). On the negative side, forced running exercise can increase the serum levels of the stress hormone corticosterone and induce a chronic stress response, which may result in detrimental effects in the event of a brain injury (Leasure et al., 2008;

Brown et al., 2007). In this report, voluntary exercise was applied to reverse the anxiety and depression phenotypes because its effects include increases in growth factors and neurogenesis, beneficial factors for PSD. Voluntary wheel running induces neurogenesis in the dentate gyrus resulting from enhanced proliferation and differentiation of neurons, in rodents. It also causes newborn neurons to integrate into the hippocampal structure leading to a reversal of depression-related behaviour and cognitive deficits (Kobilo et al., 2011; van Praag et al., 2005; van Praag et al., 1999; So et al., 2017; Uda et al., 2006). The newly-integrated neurons in hippocampus display a lower excitability threshold resulting in plasticity enhancement and improvement in learning and memory (Creer et al., 2010). In this mouse PSD model, exercise induced changes in PSD neuronal activity as detected by FosB, however it was not effective to reverse the PSD phenotypes. Exercise, in a brain-region dependent manner, induced significant decreases in the overall activity of areas like bilateral mPFC, NAc, septum, CA2/CA3 while significantly enhancing excitation in bilateral lateral habenula, left amygdala and right CA1 and CA2 regions, with no notable changes in DR excitation activity (except strong reduction in FosB/GABA cells). In contrast, exercise in combination with SSRI synergistically changed the PSD neuronal activity in areas like bilateral DG, well known for neurogenesis. It would be interesting to test in future studies whether neurogenesis was increased by SSRI/RW. Otsuka et al. (2015) showed that chronic voluntary exercise in combination with food supplementation reverses the depression and anxiety induced by either chronic uncontrollable stress or chronic social defeat in adult C57BL/6. By contrast, in normal C57BL/6J mice chronic voluntary running wheel alone reduces depressive-like symptoms, increases VGF and BDNF proteins in hippocampus and alters

synaptic plasticity gene profile activity (Hunsberger et al., 2007). Additionally, the results of Kim and Han (2016) studies suggest that both chronic stress (repeated restraint) and exercise commonly and similarly activate lateral habenula and hippocampus. However, the effects produce opposite outcomes on mood behaviors. It likely shows the role of lateral habenula in stress and the role of hippocampus in exercise-induced antidepressant effects. Taken together, from previous studies, it can be postulated that exercise induces antidepressant effects only in some distinct depression models. To promote recovery from emotional disturbances and cognitive impairments like in PSD, exercise should be applied in combination with compounds like SSRIs.

Relevant to successful clinical approaches, here I have shown that the combination of SSRI±exercise mediates a full recovery of a reliable PSD mouse model. I have identified the key brain regions that are affected by the stroke. I have also determined how their activity changes with successful treatment. In future studies it would be interesting to directly target serotonin projections in forebrain regions implicated in PSD using optogenetic mice expressing the light-sensitive ion channel, Channelrhodopsin (ChR2) and test whether this mediates rapid behavioural improvement following PSD. It would also be interesting to test whether this optogenetic approach can bypass the 5-HT1A auto-inhibitory regulation of 5-HT system induced in the cF1ko model by direct stimulation of 5-HT cells in dorsal raphe or at specific 5-HT targets, like the mPFC. These studies would provide valuable knowledge to design brain stimulation approaches for stroke patients to improve their recovery from PSD and improve their stroke outcome as rapid and efficient as possible.

Chapter 6

Conclusion

In clinical trials and preclinical mouse models, changes in both neuronal structure and function have been observed in limbic regions in depressive behaviour. Alterations in serotonergic neurotransmission in these areas have been also widely proposed in the etiology of depression and anxiety. To better understand common and unique alterations in both genetic and injury related depression, I have generated and investigated two relevant mouse models. The two mouse models exemplify a serotonin-related genetic alteration (Flx-Freud-1 mice) that leads to increased 5-HT1A autoreceptor expression as seen in major depression, resulting in both anxiety and depression phenotypes; and a unilateral ischemic lesion model that leads to depression, anxiety phenotypes along with cognitive impairments in form of deficits in spatial learning and memory. The results of both genetic and stroke models reported here show that changes in the 5-HT system contribute to widespread dysregulation of the corticolimbic circuitry implicated in depression and anxiety.

First strategy: genetic model

In the raphe-specific Freud-1 conditional knockout mouse model, loss of the transcriptional repressor Freud-1 in serotonin neurons in early adulthood induced overexpression of 5-HT1A autoreceptors, reduced raphe serotonin levels and led to anxiety- and depression-like behaviors that were resistant to chronic SSRI treatment. The new approach in this model reveals that changes in the 5-HT1A transcriptional regulator Freud-1 in adulthood can be associated with SSRI-resistant phenotypes. These behaviors were dependent on the induction of 5-HT1A autoreceptors since on the raphe-5-HT1A knockout background, raphe knockout of Freud-1 blocked or reversed the behavioral phenotype. This mouse model is relevant to the changes seen in major depression

associated with the rs6295 C(-1019)G 5-HT1A promoter polymorphism, which disrupts repression by Deaf1 (Lemonde et al., 2003), leading to a marked 2-fold increase in 5-HT1A autoreceptors in depressed subjects (Parsey et al., 2010). This genetic alteration in 5-HT1A gene expression has been associated with major depression, but also with anxiety disorders and with a reduced response to chronic SSRI treatment (Le Francois et al., 2008). However, although meta-analyses have support these associations, they are not always consistent. The phenotype of the cF1ko mouse provides important validation for the concept that transcriptional dys-regulation of a key target gene in the serotonin system can induce behavioral alterations and changes in SSRI response. These studies suggest that it may be possible to ultimately harness functional polymorphisms that affect transcription as a component of diagnosis of anxiety or depression subtypes and to predict treatment response. This could lead to a more tailored approach to the categorization and treatment of major depression and related illnesses.

Second strategy: LmPFC stroke lesion

In ischemic mouse model, there was a strong anxiety and depression phenotype as well as impairment in spatial learning and memory, and SSRI treatment alone was effective to reverse these phenotypes. These data suggest that while genetic alteration of the 5-HT system can confer SSRI resistance, targeting the mPFC appears to retain sensitivity to SSRI. Although the stroke in the PSD mouse specifically targeted the left mPFC, over time there was increased activity that permeated throughout the corticolimbic circuitry implicated in anxiety and depression behaviors. In particular, there was greater activation on the right (contralateral) side, and treatment with SSRI reduced and balanced the right and left activation, primarily through recruiting activation of GABAergic neurons and

reducing activity of glutamatergic projection neurons. This result suggests two possibilities that could underlie PSD: either an excessive excitation at the contralateral side or excessive inhibition at the ipsilateral side to the lesion. Interestingly, both could be optogenetically targeted for efficient treatment. The effect of SSRI suggests that serotonin is able to enhance interneuronal activity to reduce pyramidal excitation in this model of injury. This hypothesis could be directly tested by targeting interneurons for optogenetic stimulation, or targeting pyramidal neurons for inhibition. For example by crossing CaMKII-Cre and flx-STOP-Arch-GFP mice, CamKII-Arch mice which express Arch in forebrain glutamatergic neurons will be generated. In these mice, green light-induced hyperpolarization will inhibit the activity of pyramidal glutamate neurons upon. The light fiber implantation could be performed in the right mPFC to directly normalize the top-down glutamate neurons activity.

Similarly, a preliminary FosB study (data not shown) showed significant changes in the septum, NAc and amygdala neuronal activity in cF1ko compared to F1WT. This suggests that in cF1ko model, dysregulation in 5-HT system affects the activity of some brain regions implicated in depression and anxiety. Ultimately these studies of regional activation may provide a mechanistic basis for region-specific targeting as a useful strategy for brain stimulation approaches like deep brain stimulation in treatment resistant depressed patients.

References

Aan het Rot M., Mathew S.J., Charney D.S. (2009) Neurobiological mechanisms in major depressive disorder. CMAJ 180, 305–313. doi:10.1503/cmaj.080697.

Abbas S.Y., Nogueira M.I., Azmitia E.C. (2007) Antagonist-induced increase in 5-HT1A-receptor expression in adult rat hippocampus and cortex. Synapse. Jul;61(7):531-9

Abeysinghe H.C., Bokhari L., Dusting G.J., Roulston C.L. (2014) Brain remodelling following endothelin-1 induced stroke in conscious rats. PLoS One 9(5): e97007.

Abrams J.K, Johnson P.L., Hollis J.H., Lowry C.A. (2004) Anatomic and functional topography of the dorsal raphe nucleus. Ann N Y Acad Sci 1018: 46–57

Albert P.R. (2012) Transcriptional regulation of the 5-HT1A receptor: implications for mental illness. Philos Trans R Soc Lond B Biol Sci. 367(1601): 2402–2415. doi: 0.1098/rstb.2011.0376.

Albert P.R., Francois B.L. (2010) Modifying 5-HT1A receptor gene expression as a new target for antidepressant therapy. Front Neurosci 4:35.

Albert P.R., Fiori L.M. (2014) Transcriptional dys-regulation in anxiety and major depression: 5-HT1A gene promoter architecture as a therapeutic opportunity. Curr Pharm Des 20:3738-3750.

Albert P.R., Le François B., Millar A.M. (2011) Transcriptional dysregulation of 5-HT1A autoreceptors in mental illness. Mol Brain. 2011 May 27;4:21. doi: 10.1186/1756-6606-4-21. Review.

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

Albert P.R., Tiberi M. (2001) Receptor signaling and structure: insights from serotonin-1 receptors. Trends Endocrinol. Metab. 12, 453–460. doi: 10.1016/S1043-2760(01)00498-2

Albert P.R., Vahid-Ansari F., Luckhart C. (2014) Serotonin-prefrontal cortical circuitry in anxiety and depression phenotypes: pivotal role of pre- and post-synaptic 5-HT1A receptor expression. Front Behav Neurosci. Jun 6;8:199. doi: 10.3389/fnbeh.2014.00199. eCollection 2014. Review.

Albert P.R., Zhou Q.Y. VanTol H.H., Bunzow J.R., Civelli O. (1990) Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine 1A receptor gene. J. Biol.Chem. 265, 5825–5832.

Almeida O.P., Xiao J. (2007) Mortality associated with incident mental health disorders after stroke. Aust N Z J Psychiatry 41(3): 274-281.

Aman T.K., Shen R.Y., Haj-Dahmane S. (2007) D2-like dopamine receptors depolarize dorsal raphe serotonin neurons through the activation of nonselective cationic conductance. J Pharmacol Exp Ther 320:376-385.

Amargos-Bosch M., Bortolozzi A., Puig M. V., Serrats J., Adell A., Celada P., et al. (2004) Co-expression and in vivo interaction of serotonin1A and serotonin2A receptors

in pyramidal neurons of prefrontal cortex. Cereb. Cortex 14, 281–299 10.1093/cercor/bhg128.

Andrade R., Huereca D., Lyons J.G., Andrade E.M., McGregor K.M. (2015) 5-HT1A Receptor-Mediated Autoinhibition and the Control of Serotonergic Cell Firing. ACS Chem Neurosci 6:1110-1115.

Anthony T.E., Dee N., Bernard A., Lerchner W., Heintz N., Anderson D.J. (2014) Control of stress-induced persistent anxiety by an extra-amygdala septohypothalamic circuit. Cell 156:522-536

Ansorge M.S., Zhou M., Lira A., Hen R., Gingrich J.A. (2004) Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science 306 879-81.

Audero E., Mlinar B., Baccini G., Skachokova Z.K., Corradetti R., Gross C. (2013) Suppression of serotonin neuron firing increases aggression in mice. J. Neurosci. 33 8678-88.

Astrom M., Adolfsson R., Asplund K. (1993) Major depression in stroke patients. A 3-year longitudinal study. Stroke 24(7): 976-982.

Atteih S., Mellon L., Hall P., Brewer L., Horgan F., Williams D., et al. (2015) Implications of stroke for caregiver outcomes: findings from the ASPIRE-S study. International journal of stroke : official journal of the International Stroke Society 10(6): 918-923.

Austin M.W., Ploughman M., Glynn L., Corbett D. (2014). Aerobic exercise effects on neuroprotection and brain repair following stroke: a systematic review and perspective. Neurosci. Res. 87, 8e15.

Ayerbe L., Ayis S., Crichton S., Wolfe C.D., Rudd A.G. (2013) The natural history of depression up to 15 years after stroke: the South London Stroke Register. Stroke 44:1105-1110.

Ayerbe L., Ayis S., Crichton S.L., Rudd A.G., Wolfe C.D. (2014) Explanatory factors for the increased mortality of stroke patients with depression. Neurology 83:2007-2012.

Ayerbe L., Ayis S.A., Crichton S., Rudd A.G., Wolfe C.D. (2015) Explanatory factors for the association between depression and long-term physical disability after stroke. Age Ageing 44:1054-1058.

Bang S.J., Commons K.G. (2012) Forebrain GABAergic projections from the dorsal raphe nucleus identified by using GAD67-GFP knock-in mice. J Comp Neurol 520:4157-4167.

Bang S.J., Jensen P., Dymecki S.M., Commons K.G. (2012) Projections and inter connections of genetically defined serotonin neurons in mice. Eur. J. Neurosci. 35,85–96. doi:10.1111/j.1460-9568.2011.07936.x.

Banasr M., Hery M., Printemps R., Daszuta A. (2004) Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-

HT receptor subtypes in the dentate gyrus and the subventricular zone. Neuropsychopharmacology 29(3):450–460.

Barker-Collo S.L. (2007) Depression and anxiety 3 months post stroke: Prevalence and correlates. Arch Clin Neuropsychol 22(4): 519-531.

Barnes N.M., Sharp T. (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38, 1083–1152. doi:10.1016/S0028-3908(99)00010-6.

Basel-Vanagaite L., Attia R., Yahav M., Ferland R.J., Anteki .L, Walsh C.A., Olender T., Straussberg R., Magal N., Taub E., Drasinover V., Alkelai A., Bercovich D., Rechavi G., Simon A.J., Shohat M. (2006) The CC2D1A, a member of a new gene family with C2 domains, is involved in autosomal recessive non-syndromic mental retardation. J Med Genet 43:203-210.

Béïque J.C., Campbell B., Perring P., Hamblin M.W., Walker P., Mladenovic L., Andrade R. (2004) Serotonergic regulation of membrane potential in developing rat prefrontal cortex: coordinated expression of 5-hydroxytryptamine (5-HT)1A, 5-HT2A, and 5-HT7 receptors. J Neurosci. 2004 May 19;24(20):4807-17.

Belmer A., Klenowski P.M., Patkar O.L., Bartlet S.E. (2017) Mapping the connectivity of serotonin transporter immunoreactive axons to excitatory and inhibitory neurochemical synapses in the mouse limbic brain. Brain Struct Funct 222:1297–1314. doi:10.1007/s00429-016-1278-x.

Belmer A., Lanoue V., Patkar O.L., Bartlet S.E. (2016) Excitatory/inhibitory balance of serotonergic axon connectivity in the brain. J of neurology and neuromedicine 1(9):18-2 **Belzung C**., Griebel G. (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 125(1-2): 141-149.

Belzung C., Turiault M., Griebel G. 92014) Optogenetics to study the circuits of fear- and depression-like behaviors: a critical analysis. Pharmacol Biochem Behav 122: 144-157.2.

Bernhardt J., Langhorne P., Lindley R.I., Thrift A.G., Ellery F., Collier J., Churilov L., Moodie M., Dewey H., Donnan G. (2015) Efficacy and safety of very early mobilisation within 24 h of stroke onset (AVERT): a randomised controlled trial. Lancet 386:46-55.

Blanco C., Raza M.S., Schneier F.R., Liebowitz M.R., (2003) The evidence-based pharmacological treatment of social anxiety disorder. Int J Neuropsychopharmacology 6:427-442.

Blier P., El Mansari M. (2013) Serotonin and beyond: therapeutics for major depression. Philos Trans R Soc Lond B Biol Sci 368:20120536.

Blier P., Pineyro G., el Mansari M., Bergeron R., de Montigny C. (1998) Role of somatodendritic 5-HT autoreceptors in modulating 5-HT neurotransmission. Ann N Y Acad Sci 861:204-216.

Blier P., Ward N.M. (2003) Is there a role for 5-HT(1A) agonists in the treatment of depression? Biol Psychiatry 53(3):193-203.

Boldrini M., Underwood M.D., Mann J.J., Arango V. (2008). Serotonin-1A autoreceptor binding in the dorsal raphe nucleus of depressed suicides. J Psychiatr Res 42: 433-442.

Bolla-Wilson K., Robinson R.G., Starkstein S.E., Boston J., Price T.R. (1989) Lateralization of dementia of depression in stroke patients. Am J Psychiatry 146:627-634.

Booij L., Tremblay R.E., Leyton M., Seguin J.R.,Vitaro F., Gravel P., et al. (2010) Brain serotonin synthesis in adult males characterized by physical aggression during childhood: a21-year longitudinal study. PLOS ONE 5:e11255. doi: 10.1371/journal.pone.0011255.

Booij L., Tremblay R.E., Szyf M., Benkelfat C. (2015) Genetic and early environmental influences on the serotonin system: consequences for brain development and risk for psychopathology. J Psychiatry Neurosci 40:5-18.

Bortolozzi A., Castane A., Semakova J., Santana N., Alvarado G., Cortes R Ferres-Coy A., Fernandez G., Carmona M.C., Toth M., Perales J.C., Montefeltro A., Artigas F. (2012) Selective siRNA-mediated suppression of 5-HT1A autoreceptors evokes strong anti-depressant-like effects. Mol Psychiatry 17 612-23.

Bouaziz E., Emerit M.B., Vodjdani G., Gautheron V., Hamon M., Darmon M., Masson J. (2014) Neuronal Phenotype Dependency of Agonist-Induced Internalization of the 5-HT1A Serotonin Receptor. J Neurosci 34:282-294.

Bouët V., Freret T., Toutain J., Divoux D., Boulouard M., Schumann-Bard P. (2007) Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. Exp Neurol. 203(2):555-67.

Branchi I., Santarelli S., Capoccia S., Poggini S., D'Andrea I., Cirulli F., Alleva, E. (2013) Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PlOS One 8(4), 1-9.

Brookes R.L., Herbert V., Lawrence A.J., Morris R.G., Markus H.S. (2014) Depression in small-vessel disease relates to white matter ultrastructural damage, not disability. Neurology 83(16): 1417-1423.

Brown D.A., Johnson M.S., Armstrong C.J., Lynch J.M., Caruso N.M., Ehlers L.B., Fleshner M., Spencer R.L., Moore R.L. (2007) Short-term treadmill running in the rat: what kind of stressor is it?, 1985 J. Appl. Physiol. 103 (6), 1979e1985.

Brown, G.L., Ebert, M.H., Goyer, P.F., Jimerson, D.C., Klein, W.J., Bunney, W.E., Goodwin F.K. (1982) Aggression, suicide, and serotonin: relationships to CSF amine metabolites. Am J Psychiatry 139(6):741-6.

Burkhard P., Stetefeld J., Strelkov S.V. (2001) Coiled coils: a highly versatile protein folding motif. Trends Cell Biol 11:82–88.

Cai X., Gu Z., Zhong P., Ren Y., Yan Z. (2002) Serotonin 5-HT1A receptors regulate AMPA receptor channels through inhibiting Ca2+/calmodulin-dependent kinase II in prefrontal cortical pyramidal neurons. J. Biol. Chem. 277, 36553–36562 10.1074/jbc.M203752200.

Calizo L.H., Akanwa A., Ma X., Pan Y.Z., Lemos J.C., Craige C., Heemstra L.A., Beck S.G. (2011) Raphe serotonin neurons are not homogenous: electrophysiological, morphological and neurochemical evidence. Neuropharmacology 61:524-543.

Campbell Burton C.A., Holmes J., Murray J., Gillespie D., Lightbody C.E., Watkins C.L., et al. (2011) Interventions for treating anxiety after stroke. The Cochrane database of systematic reviews (12): Cd008860.

Capizzano A.A., Jorge R.E., Robinson R.G. (2010) Limbic metabolic abnormalities in remote traumatic brain injury and correlation with psychiatric morbidity and social functioning. J Neuropsychiatry Clin Neurosci. 2010 Fall; 22(4):370-7. doi: 10.1176/appi.neuropsych.22.4.370.

Carmichael S.T. (2005) Rodent models of focal stroke: size, mechanism, and purpose. NeuroRx 2(3): 396-409.

Carobrez A.P., Bertoglio L.J. (2005) Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neurosci Biobehav Rev 29(8): 1193-1205.

Carota A., Berney A., Aybek S., Iaria G., Staub F., Ghika-Schmid F., Annable L., Guex P., Bogousslavsky J. (2005) A prospective study of predictors of poststroke depression. Neurology. Feb 8;64(3):428-33.

Caspi A., Moffitt T.E. (2006) Gene-environment interactions in psychiatry: joining forces with neuroscience. Nature Reviews. Neuroscience 7(7), 583–90.

Caspi A., Hariri A. R., Holmes A., Uher R., Moffitt T.E. (2010) Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. The American Journal of Psychiatry 167(5), 509–27.

Caspi A., Sugden K., Moffitt T.E., Taylor A., Craig I.W., Harrington H. et al. (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 386–9.

Castagne V., Moser P., Roux S., Porsolt R.D. (2011) Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. Curr Protoc Neurosci Chapter 8: Unit 8.10A.

Castren E., Hen R. (2013) Neuronal plasticity and antidepressant actions. Trends Neurosci 36:259-267.

Celada P., Puig M.V., Casanovas J.M., Guillazo G., Artigas F. (2001) Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin-1A, GABA(A), and glutamate receptors. J Neurosci 21:9917-9929.

Celada P., Bortolozzi A., Artigas, F. (2013) Serotonin 5-HT1A receptors as targets for agents to treat psychiatric disorders: rationale and current status of research. CNS Drugs 27(9), 703–16.

Celada P., Puig M., Amargos-Bosch M., Adell A., Artigas F. (2004) The therapeutic role of 5-HT (1A) and 5-HT (2A) receptors in depression. J. Psychiatry Neurosci. 29,252–265.

Chen C.L., Tang F.T., Chen H.C., Chung C.Y., Wong M.K. (2000) Brain lesion size and location: effects on motor recovery and functional outcome in stroke patients. Archives of physical medicine and rehabilitation 81(4): 447-452.

Chau D.T., Fogelman Ph., Nordanskog P., Drevets W.C., Hamilton J.P. (2017) Distinct neural-functional effects of treatments with selective serotonin reuptake inhibitors, electroconvulsive therapy, and transcranial magnetic stimulation and their relations to regional brain function in major depression: A Meta-analysis. Biological Psychiatry: Cognitive Neuroscience and Neuroimaging, 2:318–326. doi.org/10.1016/j.bpsc.2017.01.003.

Chollet F., Tardy J., Albucher J.F., Thalamas C., Berard E., Lamy C., et al. (2011) Fluoxetine for motor recovery after acute ischaemic stroke (FLAME): a randomised placebo-controlled trial. Lancet Neurol 10:123-30.

Clarke J., Langdon K.D., Corbett D. (2014) Early poststroke experience differentially alters periinfarct layer II and III cortex. J Cereb Blood Flow Metab 34:630-637.

Commons K.G., Connolley K.R., Valentino R.J. (2003) A neurochemically distinct dorsal raphe-limbic circuit with a potential role in affective disorders. Neuropsychopharmacology. Feb;28(2):206-15.

Cordova C.A., Jackson D., Langdon K.D., Hewlett K.A., Corbett D. (2014) Impaired executive function following ischemic stroke in the rat medial prefrontal cortex. Behav Brain Res 258: 106-111.

Coppell A.L., Pei Q., Zetterström T.S. (2003) Bi-phasic change in BDNF gene expression following antidepressant drug treatment. Neuropharmacology. Jun;44(7):903-10.

Coppen A. (1967) The biochemistry of affective disorders. British Journal of Psychiatry, vol. 113, no. 504, pp. 1237–1264, 1967.

Covington H.E., 3rd, Lobo M.K., Maze I., Vialou V., Hyman J.M., Zaman S., et al. (2010) Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. J Neurosci 30(48): 16082-16090.

Cramer S.C. (2008) Repairing the human brain after stroke, II: restorative therapies. Ann Neurol. 63(5):549–560.

Crawley J.N. (2008) Behavioral phenotyping strategies for mutant mice. Neuron 57:809–818.

Crawley J.N. (2007) What is wrong with my mouse?: behavioral phenotyping of transgenic and knockout mice. Wiley-Liss: Hoboken, NJ.

Creer D. J., Romberg C., Saksida L. M., Praag H. van, Bussey T. J. (2010) Running enhances spatial pattern separation in mice," Proceedings of the National Academy of Sciences of the United States of America, vol. 107, no. 5, pp. 2367– 2372.

Cryan J.F., Holmes A. (2005) The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov 4(9): 775-790.

Czesak M., Le Francois B., Millar A.M., Deria M., Daigle M., Visvader J.E., Anisman H., Albert P.R. (2012) Increased serotonin-1A (5-HT1A) autoreceptor expression and reduced raphe serotonin levels in deformed epidermal autoregulatory factor-1 (Deaf-1) gene knock-out mice. J Biol Chem 287:6615-6627.

Czesak M., Lemonde S., Peterson E.A., Rogaeva A., Albert P.R. (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci. Feb 8;26(6):1864-71.

de Groot J.C., de Leeuw F.E., Oudkerk M., Hofman A., Jolles J., Breteler M.M. (2000) Cerebral white matter lesions and depressive symptoms in elderly adults. Arch Gen Psychiatry 57(11): 1071-1076.

de Leeuw F.E., de Groot J.C., van Gijn J. (2001) Cerebral white matter lesions in the elderly: vascular risk factors and cognitive consequence. Ned Tijdschr Geneeskd. Oct 27;145(43):2067-71. Review.

Deverts W.C., (2000) Functional anatomical abnormalities in limbic and prefrontal cortical structures in major depression. Progress in brain research. 126:413-43. doi.org/10.1016/S0079-6123(00)26027-5.

Descarries L., Riad M. (2012) Effects of the antidepressant fluoxetine on the subcellular localization of 5-HT1A receptors and SERT. Philos Trans R Soc Lond B Biol Sci. 5;367(1601):2416-25. doi: 10.1098/rstb.2011.0361. Review.

Deziel R.A., Ryan C.L., Tasker R.A. (2015) Ischemic lesions localized to the medial prefrontal cortex produce selective deficits in measures of executive function in rats. Behav Brain Res 293:54-61.

Dhami K.S., Churchward M.A., Baker G.B., Todd K.G. (2013) Fluoxetine and citalopram decrease the microglial release of glutamate and D-serine to promote cortical neuronal viability following ischemic insult. , Molecular and Cellular Neuroscience 56: 365–374.

Dijkhuizen R.M., Singhal A.B., Mandeville J.B., Wu O., Halpern E.F., Finklestein S.P., Rosen B.R., Lo E.H. (2003) Correlation between brain reorganization, ischemic damage, and neurologic status after transient focal cerebral ischemia in rats: a functional magnetic resonance imaging study. J Neurosci. Jan 15;23(2):510-7.

Dolzani S.D., Baratta M.V., Amat J., Agster K.L., Saddoris M.P., Watkins L.R., Maier S.F. (2016) Activation of a Habenulo-Raphe Circuit Is Critical for the Behavioral and Neurochemical Consequences of Uncontrollable Stress in the Male Rat. eNeuro 3.

Donaldson Z.R., Nautiyal K.M., Ahmari S.E., Hen R. (2013) Genetic approaches for understanding the role of serotonin receptors in mood and behavior. Curr. Opin. Neurobiol 23,399–406. doi:10.1016/j. conb.2013.01.011.

Donaldson Z.R., le Francois B., Santos T.L., Almli L.M., Boldrini M., Champagne F.A., Arango V., Mann J.J., Stockmeier C.A., Galfalvy H., Albert P.R., Ressler K.J., Hen R. (2016) The functional serotonin 1a receptor promoter polymorphism, rs6295, is associated with psychiatric illness and differences in transcription. Transl Psychiatry 6:e746.

Donaldson Z.R., Piel D.A., Santos T.L., Richardson-Jones J., Leonardo E.D., Beck S.G., Champagne F.A., Hen R. (2014) Developmental effects of serotonin 1A autoreceptors on anxiety and social behavior. Neuropsychopharmacology 39:291-302.

Dorocic I.P., Furth D., Xuan Y., Johansson Y., Pozzi L., Silberberg G., Carlé nM., Meletis K. (2014) A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei. Neuron 83:663–678.

du Jardin K.G., Muller H.K., Elfving B., Dale E., Wegener G., Sanchez C. (2016) Potential involvement of serotonergic signaling in ketamine's antidepressant actions: A critical review. Prog Neuropsychopharmacol Biol Psychiatry 71:27-38.

Duman C.H., Schlesinger L., Russell D.S., Duman R.S. (2008) Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. Brain Res. Mar 14;1199:148-58. doi: 10.1016/j.brainres.2007.12.047.

Duman R.S., Aghajanian G.K. (2012) Synaptic dysfunction in depression: potential therapeutic targets. Science 5;338(6103):68-72. doi:10.1126/science.1222939. Review.

Duman R.S., Aghajanian G.K., Sanacora G., Krystal J.H. (2016) Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants. Nat Med 22:238-249.

Dunlop B.W., Rajendra J.K., Craighead W.E., Kelley M.E., McGrath C.L., Choi K.S., Kinkead B., Nemeroff C.B., Mayberg H.S. (2017) Functional connectivity of the subcallosal cingulate cortex and differential outcomes to treatment with cognitive-behavioral therapy or antidepressant medication for major depressive disorder. Am J Psychiatry: appiajp201616050518.

Encinas J.M., Vaahtdkari A., Enikolopov G. (2006) Fluoxetine targets early progenitor cells in the adult brain. Proc Natl Acad Sci USA. 103(21):8233–8238.

Eng J.J., Reime B. (2014) Exercise for depressive symptoms in stroke patients: a systematic review and meta-analysis. Clin Rehabil 28:731-739.

Erdmann G., Schutz G., Berger S. (2007) Inducible gene inactivation in neurons of the adult mouse. BMC Neurosci. 2007 Aug 2;8:63.

Esparrago Llorca G., Castilla-Guerra L., Fernandez Moreno M.C., Ruiz Doblado S., Jimenez Hernandez M.D. (2015) Post-stroke depression: an update. Neurologia 30(1): 23-31.

Evans D.L., Charney D.S., Lewis L., Golden R.N., …, Sunderland T., Tibbits P.Jr., Valvo W.J. (2005) Mood Disorders in the Medically Ill: Scientific Review and Recommendations. Biol Psychiatry 58(3):175-89. doi: 10.1016/j.biopsych.2005.05.001.

Ferres-Coy A., Santana N., Castane A., Cortes R., Carmona M.C., Toth M., Montefeltro A., Artigas F., Bortolozzi A. (2013) Acute 5-HT(1A) autoreceptor knockdown increases antidepressant responses and serotonin release in stressful conditions. Psychopharmacology (Berl) 225 61-74.

Fino E., Packer A.M., Yuste R. (2013) The logic of inhibitory connectivity in the neocortex. Neuroscientist. `9(3):228-37. doi: 10.1177/1073858412456743. Review.

Fisher M., Feuerstein G., Howells D.W., Hurn P.D., Kent T.A., Savitz S.I., et al. (2009) Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 40(6): 2244-2250.

Flaster M., Sharma A.,Rao M. (2013) Poststroke Depression: A review emphasizing the role of prophylactic treatment and synergy with treatment for motor recover topics in stroke rehabilitation. Mar-Apr;20(2):139-50. doi: 10.1310/tsr2002-139.

Flores J.A., El Banoua F., Gala n-Rodrí guez B., Fernandez-Espejo E. (2004) Opiate anti-nociception is attenuated following lesion of largedopamine neurons of the periaqueductal gray: critical role for D1 (not D2) dopamine receptors. Pain 110:205–214.

Franklin K.B.J., Paxinos G. (2008) The mouse Brain in Stereotaxic Coordinates, 3 rd Edn.New York, NY:Academic Press.

Freund T.F. (2003) Interneuron Diversity series: Rhythm and mood in perisomatic inhibition. Trends Neurosci. 26(9):489-95.

Fuxe K., Cintra A., Andbjer B., Anggard E., Goldstein M., Agnati L.F. (1989) Centrally administered endothelin-1 produces lesions in the brain of the male rat. Acta Physiol Scand 137(1): 155-156.

Gagnon D., Parent M. (2014) Distribution of VGLUT3 in highly collateralized axons from the rat dorsal raphe nucleus as revealed by single-neuron reconstructions. PLoS One 9:e87709.

Galligan N.G., Hevey D., Coen R.F., Harbison J.A. (2016) Clarifying the associations between anxiety, depression and fatigue following stroke. J Health Psychol 21(12) 2863

–2871. DOI: 10.1177/1359105315587140.

Garcia-Garcia A.L., Newman-Tancredi A., Leonardo E.D. (2014) 5-HT(1A) receptors in mood and anxiety: recent insights into autoreceptor versus heteroreceptor function. Psychopharmacology (Berl) 231:623-636.

Geddes S.D., Assadzada S., Lemelin D., Sokolovski A., Bergeron R., Haj-Dahmane S., Beique J.C. (2016) Target-specific modulation of the descending prefrontal cortex inputs to the dorsal raphe nucleus by cannabinoids. Proc Natl Acad Sci U S A 113:5429-5434.

Geddes S.D., Assadzada S., Sokolovski A., Bergeron R., Haj-Dahmane S., Beique J.C. (2015) Time-dependent modulation of glutamate synapses onto 5-HT neurons by antidepressant treatment. Neuropharmacology 95:130-143.

Ghose S.S., Williams L.S., Swindle R.W. (2005) Depression and other mental health diagnoses after stroke increase inpatient and outpatient medical utilization three years poststroke. Med Care 43(12): 1259-1264.

Glenn M.J., Lehmann H., Mumby D.G., Woodside B. (2005) Differential fos expression following aspiration, electrolytic, or excitotoxic lesions of the perirhinal cortex in rats. Behav Neurosci. Jun;119(3):806-13.

Gong Q., He Y. (2015) Depression, neuroimaging and connectomics: a selective overview. Biol Psychiatry 77(3): 223-235.

Goodwin F.K. (1982) Aggression, suicide, and serotonin: Relationships to CSF amine metabolites. Am J Psychiatry 139, 741–746.

Goodwin R.D., Devanand D.P. (2008) Stroke, depression, and functional health outcomes among adults in the community. Journal of geriatric psychiatry and neurology 21(1): 41-46.

Gosselin R.D., Gibney S., O'Malley D., Dinan T.G., Cryan J.F. (2009). Region specific decrease in glial fibrillary acidic protein immunoreactivity in the Brain of a rat model of depression. Neuroscience 159, 915–925.doi:10.1016/j. neuroscience. 2008.10.018

Goswami D.B., May W.L., Stockmeier C.A., Austin M.C. (2010) Transcriptional expression of serotonergic regulators in laser-captured microdissected dorsal raphe neurons of subjects with major depressive disorder: sex-specific differences. J Neurochem 112:397-409.

Gras C., Herzog E., Bellenchi G.C., Bernard V., Ravassard P., Pohl M., Gasnier B., Giros B., El Mestikawy S. (2002) A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J Neurosci 22:5442-5451.

Greenwood B.N., Foley T.E., Day H.E., Campisi J., Hammack S.H., Campeau S., Maier S.F., Fleshner M. (2003) Freewheel running prevents learned helplessness/behavioral depression: role of dorsal raphe serotonergic neurons. J Neurosci 23:2889-2898.

Groenewegen H.J., Wright C.I., Uylings H.B. (1997) The anatomical relationships of the prefrontal cortex with limbic structures and the basal ganglia. Journal of psychopharmacology (Oxford, England) 11(2): 99-106.

Gross C., Hen R. (2004) Genetic and environmental factors interact to influence anxiety. Neurotox Res. 2004;6(6):493-501. Review.

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

Hackett M.L., Yang M., Anderson C.S., Horrocks J.A., House A. (2008) Interventions for treating depression after stroke. Cochrane Database Syst Rev. Oct 8;(4):CD003437. doi: 10.1002/14651858.CD003437.pub3. Review.

Hadjighassem M.R., Austin M.C., Szewczyk B., Daigle M., Stockmeier C.A., Albert P.R. (2009) Human Freud-2/CC2D1B: a novel repressor of postsynaptic serotonin-1A receptor expression. Biol Psychiatry 66:214-222.

Hai A., Cai L.X., Lee T., Lelyveld V.S., Jasanoff A. (2016) Molecular fMRI of Serotonin Transport. Neuron 92:754-765.

Hakim A.M. (2011) Depression, strokes and dementia: new biological insights into an unfortunate pathway. Cardiovascular psychiatry and neurology 2011: 649629.

Hale M.W., Hay-Schmidt A., Mikkelsen J.D., Poulsen B., Bouwknecht J.A., Evans A.K., Stamper C.E., Shekhar A., Lowry C.A. (2008) Exposure to an open-field arena increases c-Fos expression in a subpopulation of neurons in the dorsal raphe nucleus, including neurons projecting to the basolateral amygdaloid complex. Neuroscience. Dec 10;157(4):733-48. doi: 10.1016/j.neuroscience.2008.09.050.

Hale M.W., Lowry C.A. (2011) Functional topography of mid- brain and pontine serotonergic systems: implications for synaptic regulation of serotonergic circuits. Psychopharmacology 213, 243–264. doi:10.1007/s00213-010- 2089-z.

Hale M.W., Shekhar A., Lowry, C.A. (2012) Stress-related serotonergic systems: implications for symptomatology of anxiety and affective disorders. Cell. Mol. Neurobiol. 32, 695–708. doi:10.1007/s10571-012- 9827-1.

Hamani C., Diwan M., Macedo C.E., Brandao M.L., Shumake J., Gonzalez-Lima F., Raymond R., Lozano A.M., Fletcher P.J., Nobrega J.N. (2010) Antidepressant-like effects of medial prefrontal cortex deep brain stimulation in rats. Biol Psychiatry 67:117-124.

Hascoet M., Bourin M. (1998) A new approach to the light/dark test procedure in mice. Pharmacol Biochem Behav 60(3): 645-653.

Heisler L.K., Chu H.M., Brennan T.J., Danao J.A., Bajwa P., Parsons L.H. et al. (1998) Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc Natl Acad Sci 95: 15049–15054.

Hendricks T.J., Fyodorov D.V., Wegman L.J., Lelutiu N.B., Pehek E.A., Yamamoto B. et al. (2003). Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 37, 233–247. doi:10.1016/S0896-6273(02)01167-4.

Herdade K.C., Strauss C.V., Zangrossi Jr. H., Viana M.B. (2006) Effects of medial amygdala inactivation on a panic-related behavior Behav. Brain Res., 172, pp. 316–323.

Hesselgrave, N., Parsey, R.V. (2013) Imaging the serotonin 1A receptor using [11 C] WAY100635 in healthy controls and major depression. Phil Trans R Soc B, 368.

Hioki H., Nakamura H., Ma Y.F., Konno M., Hayakawa T., Nakamura K.C., Fujiyama F., Kaneko T. (2010) Vesicular glutamate transporter 3-expressing nonserotonergic projection neurons constitute a sub region in the rat midbrain raphe nuclei. J Comp Neurol 518:668–686.

Holmes A., Murphy D.L., Crawley J.N. (2002) Reduced aggression in mice lacking the serotonin transporter. Psychopharmacology (Berl) 161 160-7.

Holmes A., Murphy D.L., Crawley J.N. (2003) Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. Biol. Psychiatry 54 953-9.

Hoogenboom W.S., Perlis R.H., Smoller J.W., Zeng-Treitler Q., Gainer V.S., Murphy S.N., Churchill S.E., Kohane I.S., Shenton M.E., Iosifescu D.V. (2014) Limbic system white matter microstructure and long-term treatment outcome in major depressive disorder: a diffusion tensor imaging study using legacy data. World J Biol Psychiatry. 2014 Feb;15(2):122-34. doi: 10.3109/15622975.2012.669499.

Horie N., Maag A.L., Hamilton S.A., Shichinohe H., Bliss T.M., Steinberg G.K.. (2008) Mouse model of focal cerebral ischemia using endothelin-1. J Neurosci Methods 173(2): 286-290.

Hou W.H., Liang H.W., Hsieh C.L., Hou C.Y., Wen P.C., Li C.Y. (2013) Effects of stroke rehabilitation on incidence of poststroke depression: A population-based cohort study. J Clin Psychiatry. 74: e859–866,

Hoyer D., Hannon J.P., Martin G.R. (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav. 71(4):533-54.

Hunsberger J.G., Newton S.S., Bennett A.H., Russell D.S., Salton S.R., Duman R.S. (2007) Antidepressant actions of the exercise-regulated gene VGF. Nature Medicine vol. 13, no. 12, pp. 1476–1482.

Hurley K.M., Herbert H., Moga M.M., Saper C.B. (1991) Efferent projections of the infralimbic cortex of the rat. J. Comp. Neurol. 308, 249–276. doi: 10.1002/cne.903080210.

Husaini B., Levine R., Sharp L., Cain V., Novotny M., Hull P., et al. (2013) Depression increases stroke hospitalization cost: an analysis of 17,010 stroke patients in 2008 by race and gender. Stroke research and treatment 2013: 846732.

Hyman S.E. (2000) Mental Illness: Genetically Complex Disorders of Neural Circuitry and Neural Communication. Neuron 28:2, November, 321-323

Iyo A.H., Kieran N., Chandran A., Albert P.R., Wicks I., Bissette G., Austin M.C. (2009) Differential regulation of the serotonin 1 A transcriptional modulators five prime repressor element under dual repression-1 and nuclear-deformed epidermal autoregulatory factor by chronic stress. Neuroscience. 10;163(4):1119-27. doi: 10.1016/j.

Jacobsen J.P., Medvedev I.O., Caron, M.G. (2012a) The 5-HT deficiency theory of depression: perspectives from a naturalistic 5-HT deficiency model, the tryptophan

hydroxylase 2 Arg 439 His knock in mouse. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367,2444–2459. doi:10.1098/rstb.2012.0109.

Jacobsen J.P., Siesser W.B., Sachs B.D., Peterson S., Cools M.J., Setola V., Folgering J.H., Flik G., Caron M.G. (2012) Deficient serotonin neurotransmission and depression-like serotonin biomarker alterations in tryptophan hydroxylase 2 (Tph2) loss-of-function mice. Mol Psychiatry 17 694-704.

Jacobsen K.X., Czesak M., Deria M., Le Francois B., Albert P.R. (2011) Region-specific regulation of 5-HT1A receptor expression by Pet-1-dependent mechanisms in vivo. J Neurochem 116:1066-1076.

Jacobsen K.X., Vanderluit J., Slack R.S., Albert P.R. (2008) HES1 regulates 5-HT1A receptor gene transcription at a functional polymorphism: essential role in developmental expression. Mol. Cell. Neurosci. 38, 349–35810.1016/j.mcn.2008.03.007. doi:10.1016/j.mcn.2008.03.007.

Jans L.A., Riedel W.J., Markus C.R., Blokland A. (2007) Serotonergic vulnerability and depression: assumptions, experimental evidence and implications. Mol. Psychiatry 12, 522–543. doi:10.1038/sj.mp.4001920.

Jasinska A.J., Lowry C.A., Burmeister M. (2012) Serotonin transporter gene, stress and raphe-raphe interactions: a molecular mechanism of depression. Trends Neurosci. 35(7):395-402. doi: 10.1016/j.tins.2012.01.001.

Jennings J.H., Sparta D.R., Stamatakis A.M., Ung R.L., Pleil K.E., Kash T.L., Stuber G.D. (2013) Distinct extended amygdala circuits for divergent motivational states. Nature 496:224-228.

Jeong Y.-J., Kim W.-Ch., Kim Y.-Sh, Choi K.-W., Son S.-Y., Jeong Y.-G. (2014) the relationship between rehabilitation and changes in depression in stroke patients. J. Phys. Ther. Sci. 26: 1263–1266.

Jorge R.E., Acion L., Moser D., Adams H.P. Jr., Robinson R.G. (2010) Escitalopram and enhancement of cognitive recovery following stroke. Arch Gen Psychiatry 67:187-196

Jorge R.E., Robinson R.G., Arndt S., Starkstein S. (2003) Mortality and poststroke depression: a placebo-controlled trial of antidepressants. Am J Psychiatry 160:1823-9

Juang H.-T., Chen P.-C., Chien K.-L. (2015) Using antidepressants and the risk of stroke recurrence: report from a national representative cohort study. BMC Neurol. 2015; 15: 86. doi:10.1186/s12883-015-0345-x

Kalueff A.V., Gallagher P.S., Murphy D.L. (2006) Are serotonin transporter knockout mice 'depressed'?: hypoactivity but no anhedonia. Neuroreport 17 1347-51.

Kapur S., Seeman P. (2002) NMDA receptor antagonists ketamine and PCP have direct effects on the dopamine D(2) and serotonin 5-HT(2)receptors-implications for models of schizophrenia. Mol Psychiatry. 2002;7(8):837-44

Karg K., Burmeister M., Shedden K., Sen S. (2011) The serotonin trans- porter promoter variant (5-HTTLPR), stress, and depression meta-analysis revisited: evidence of genetic moderation. Arch. Gen. Psychiatry 68, 444–454. doi: 10.1001/archgenpsychiatry.2010.189.

Kato M., Serretti A., Nonen S., Takekita Y., Wakeno M., Azuma J., Kinoshita T. (2015) Genetic variants in combination with early partial improvement as a clinical utility predictor of treatment outcome in major depressive disorder: the result of two pooled RCTs. Transl Psychiatry 5:e513.

Kazakov V.N., Kravtsov P.Y., Krakhotkina E.D., Maisky V.A. (1993) Sources of cortical, hypothalamic and spinal serotonergic projections: topical organization within the nucleus raphe dorsalis. Neuroscience 56, 157–164. doi: 10.1016/0306-4522(93)90570-6.

Kessler R.C. (2003) Epidemiology of women and depression. J Affect Disord 74:5–13.

Kessler R.C., Bromet E.J. (2013) The epidemiology of depression across cultures. Annu Rev Public Health 34:119-138.

Kheirbek M.A., Drew L.J., Burghardt N.S., Costantini D.O., Tannenholz L., Ahmari S.E., Zeng H., Fenton A.A., Hen R. (2013) Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. Neuron 77:955-968.

Kim J.C., Cook M.N., Carey M.R., Shen C., Regehr W.G., Dymecki S.M. (2009) Linking genetically defined neurons to behavior through a broadly applicable silencing allele. Neuron 63 305-15.

KimT.K., Han P.L. (2016) Chronic stress and moderate physical exercise prompt widespread common activation and limited differential activation in specific brain regions. Neurochemistry International 99 (2016) 252e261.

Kim Y.R., Kim H.N., Pak M.E., Ahn S.M., Hong K.H., Shin H.K., Choi B.T. (2015) Studies on the animal model of post-stroke depression and application of antipsychotic aripiprazole. Behav Brain Res 287:294-303.

Kimura M., Robinson R.G. Kosier J.T (2000) Treatment of cognitive impairment after poststroke depression, A Double-Blind Treatment Trial. Stroke 31:1482-1486. doi.org/10.1161/01.STR.31.7.1482.

Kishi T., Yoshimura R., Fukuo Y., Okochi T., Matsunaga S., Umene-Nakano W., et al. (2013) The serotonin 1A receptor gene confer susceptibility to mood disorders: results from an extended meta-analysis of patients with major depression and bipolar disorder. Eur. Arch. Psychiatry Clin. Neurosci. 263,105–118. doi: 10.1007/s00406-012-0337-4.

Kleim J.A., Cooper N.R., andenberg P.M. (2002) Exercise induces angiogenesis but does not alter movement representations within rat motor cortex. Brain Res; 934: 1–6.

Klein J., Winter C., Coquery N., Heinz A., Morgenstern R., Kupsch A., et al. (2010) Lesion of the medial prefrontal cortex and the subthalamic nucleus selectively affect depression-like behavior in rats. Behav Brain Res 213(1): 73-81.

Klemenhagen K.C., Gordon J.A., David D.J., Hen R., Gross C.T. (2006) Increased fear response to contextual cues in mice lacking the 5-HT1A receptor. Neuropsychopharmacology 31(1):101-11.

Kobilo T., Liu Q.R., Gandhi K., Mughal M., Shaham Y., van Praag H. (2011) Running is the neurogenic and neurotrophic stimulus in environmental enrichment. Learn Mem 18:605-609.

Kocsis B., Varga V., Dahan L., Sik, A. (2006) Serotonergic neuron diver- sity: identification of raphe neurons with discharges time-locked to the hippocampal theta rhythm. Proc. Natl. Acad. Sci. U.S.A. 103, 1059–1064. doi: 10.1073/pnas.0508360103.

Komlósi G., Molnár G., Rózsa M., Oláh S., Barzó P., Tamás G. (2012) Fluoxetine (Prozac) and Serotonin Act on Excitatory Synaptic Transmission to Suppress Single Layer 2/3 Pyramidal Neuron-Triggered Cell Assemblies in the Human Prefrontal Cortex. J Neurosci. 14; 32(46): 16369–16378.doi: 10.1523/JNEUROSCI.2618-12.2012.

Kotila M., Numminen H., Waltimo O., Kaste M. (1998) Depression after stroke: results of the FINNSTROKE Study. Stroke 29(2): 368-372.

Kraemer M., Schormann T., Hagemann G., Qi B., Witte O.W., Seitz R.J. (2004) Delayed shrinkage of the brain after ischemic stroke: preliminary observations with voxel-guided morphometry. Journal of neuroimaging: official journal of the American Society of Neuroimaging 14(3): 265-272.

Krishnan V., Nestler E.J. (2008) The molecular neurobiology of depression. Nature, 16;455(7215):894-902.

Kronenberg G., Balkaya M., Prinz V., Gertz K., Ji .S, Kirste I., et al. (2012) Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression. Biol Psychiatry 72:273-81

Kronenberg G., Gertz K., Heinz A., Endres M. (2014) Of mice and men: modelling post-stroke depression experimentally. British journal of pharmacology 171(20): 4673-4689.

Kushwaha N., Albert P.R. (2005) Coupling of 5-HT1A autoreceptors to inhibition of mitogen-activated protein kinase activation via G beta gamma subunit signaling. Eur. J. Neurosci. 21,721–732. doi:10.1111/j.1460-9568.2005. 03904.x.

Kusserow H., Davies B., Hortnagl H., Voigt I., Stroh T., Bert B., Deng D.R., Fink H., Veh R.W., Theuring F. (2004) Reduced anxiety-related behaviour in transgenic mice overexpressing serotonin 1A receptors. Brain Res. Mol. Brain Res. 129 104-16.

Lacroix L., Broersen L.M., Weiner I., Feldon J. (1998) The effects of excitotoxic lesion of the medial prefrontal cortex on latent inhibition, prepulse inhibition, food hoarding, elevated plus maze, active avoidance and locomotor activity in the rat. Neuroscience 84(2): 431-442.

Lam R.W. (2012) Depression (2nd Edition.). Oxford University Press.

Lanfumey L., Hamon M. (2004) 5-HT1A receptors. Curr Drug Targets CNS Neurol Disord. 3(1):1-10. Review.

Lanfumey L., Mongeau R., Cohen-Salmon C., Hamon M. (2008) Corticosteroid-serotonin interactions in the neurobiological mechanisms of stress-related disorders. Neurosci. Bio behav. Rev. 32,1174–1184. doi: 10.1016/j.neubiorev.2008.04.006.

Lassalle-Lagadec S., Sibon I., Dilharreguy B., Renou P., Fleury O., Allard M. (2012) Subacute default mode network dysfunction in the prediction of post-stroke depression severity. Radiology 264:218-224.

Leary M.C., Saver J.L. (2003) Annual incidence of first silent stroke in the United States: a preliminary estimate. Cerebrovasc Dis 16(3): 280-285.

Leasure J.L., Jones M. (2008) Forced and voluntary exercise differentially affect brain

and behavior. Neuroscience 156 (3), 456e465.

Lee H.S., Eum Y.J., Jo S.M., and Waterhouse B.D. (2007) Projection patterns from the amygdaloid nuclear complex to subdivisions of the dorsal raphe nucleus in the rat. Brain Res. 1143, 116–125. doi: 10.1016/j.brainres.2007.01.081.

Lee H.S., Kim M.-A., Valentino R.J., Waterhouse B.D. (2003) Glutamatergic afferent projections to the dorsal raphe nucleus of the rat. Brain Res. 963, 57–71. doi: 10.1016/S0006-8993(02)03841-6.

Le François B., Czesak M., Steubl D., Albert P.R. (2008) Transcriptional regulation at a HTR1A polymorphism associated with mental ill-ness. *Neuropharmacology* 55, 977–985.doi:10.1016/j. neuropharm. 06.046.

Lehmann H.E., Cahn C.H., De Verteuil R.L. (1958) The treatment of depressive conditions with imipramine (G 22355). Can Psychiatr Assoc J 3:155-164.

Lemonde S., Rogaeva A., Albert P.R. (2004a) Cell type-dependent recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene. J Neurochem 88:857-868.

Lemonde S., Du L., Bakish D., Hrdina P.D., Albert P.R. (2004b) Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int J Neuropsychopharmacol 7:501-506.

Lemonde S., Turecki G., Bakish D., Du L., Hrdina P.D., Bown C.D., Sequeira A., Kushwaha N., Morris S.J., Basak A., Ou X.M., Albert P.R. (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci. 2003 Sep 24;23(25):8788-99.

Lenicov F.R., Lemonde S., Czesak M., Mosher T.M., Albert P.R. (2007) Cell-type specific induction of tryptophanhydroxylase-2 transcription by calcium mobilization. J. Neurochem. 103,2047–2057. doi:10.1111/j.1471- 4159.2007.04903.x.

Lesch K.P., Araragi N., Waider J., van den Hove D., Gutknecht L. (2012) Targeting brain serotonin synthesis: insights into neurodevelopmental disorders with long-term

outcomes related to negative emotionality, aggression and anti-social behaviour. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367,2426–2443. doi: 10.1098/rstb.2012.0039.

Li L., Harms K.M., Ventura P.B., Lagace D.C., Eisch A.J., Cunningham L.A. (2010) Focal cerebral ischemia induces a multilineage cytogenic response from adult subventricular zone that is predominantly gliogenic. Glia 58(13): 1610-1619.

Li W.L., Cai H.H., Wang B., Chen L., Zhou Q.G., Luo C.X., Liu N., Ding X.S., Zhu D.Y. (2009) Chronic fluoxetine treatment improves ischemia-induced spatial cognitive deficits through increasing hippocampal neurogenesis after stroke. J Neurosci Res. Jan;87(1):112-22. doi: 10.1002/jnr.21829.

Li Y., Zhong W., Wang D., Feng Q., Liu Z., Zhou J., Jia C., Hu F., Zeng J., Guo Q., Fu L., Luo M. (2016) Serotonin neurons in the dorsal raphe nucleus encode reward signals. Nat Commun 7:10503.

Liao Y., Huang X., Wu Q., Yang C., Kuang W., Du M., et al. (2013) Is depression a disconnection syndrome? Meta-analysis of diffusion tensor imaging studies in patients with MDD. Journal of psychiatry & neuroscience : JPN 38(1): 49-56.

Lim C.M., Kim S.W., Park J.Y., Kim C., Yoon S.H., Lee J K. (2009) Fluoxetine affords robust neuroprotection in the post-ischemic brain via its anti-inflammatory effect. J Neurosci Res 87: 1037–1045.

Liu C., Maejima T., Wyler S.C., Casadesus G., Herlitze S., Deneris E.S. (2010) Pet-1 is required across different stages of life to regulate serotonergic function. Nat Neurosci 13 1190-8.

Livingston-Thomas J.M., Jeffers M.S., Nguemeni C., Shoichet M.S., Morshead C.M.,Corbett D. (2015) Assessing cognitive function following medial prefrontal stroke in the rat. Behav Brain Res. 1;294:102-10. doi: 10.1016/j.bbr.2015.07.053.

Llado-Pelfort L., Santana N., Ghisi, V. Artigas, F. and Celada, P. (2012). 5-HT1A receptor agonists enhance pyramidal cell firing in prefrontal cortex through a preferential action on GABA interneurons. Cereb. Cortex 22, 1487–1497. doi: 10.1093/cercor/bhr220

Lo Iacono L. and Gross C. (1998) Alpha-Ca2þ/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. J Neurosci 28: 6250–6257.

Lovatel G.A., Bertoldi K., Elsnerb V.R., Piazza F.V., …, Sequeria I.R. (2014) Long-term effects of pre and post-ischemic exercise following global cerebral ischemia on astrocyte and microglia functions in hippocampus from Wistar rats. Brain Res. 1587, 119e126.

Lowry C.A. (2002) Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. J. Neuroendocrinol. 14, 911–923. doi:10.1046/j.1365- 2826.2002.00861.x.

Lowry C.A., Hale M.W., Evans A.K., Heerkens J., Staub D.R., Gasser P.J., et al. (2008) Serotonergic systems, anxiety, and affective disorder. Ann.N.Y.Acad.Sci. 1148, 86–94. doi:10.1196/annals. 1410.004.

Lowry C.A., Johnson P.L., Hay-Schmidt A., Mikkelsen, J., Shekhar, A. (2005) Modulation of anxiety circuits by serotonergic systems. Stress 8, 233–246. doi: 10.1080/10253890500492787.

Lozano A.M., Giacobbe P., Hamani C., Rizvi S.J., Kennedy S.H., Kolivakis T.T., Debonnel G., Sadikot A.F., Lam R.W., Howard A.K., Ilcewicz-Klimek M., Honey C.R., Mayberg H.S (2012) A multicenter pilot study of subcallosal cingulate area deep brain stimulation for treatment-resistant depression. J Neurosurg 116:315-322.

Luckhart C., Philippe T.J., Le Francois B., Vahid-Ansari F., Geddes S.D., Beique J.C., Lagace D.C., Daigle M., Albert P.R. (2016) Sex-dependent adaptive changes in serotonin-1A autoreceptor function and anxiety in Deaf1-deficient mice. Mol Brain 9:77.

Luo C.X., Jiang J., Zhou Q.G., Zhu X.J., Wang W., Zhang Z.J., Han X., Zhu D.Y. (2007) Voluntary exercise-induced neurogenesis in the postischemic dentate gyrus is associated with spatial memory recovery from stroke. J Neurosci Res. Jun;85(8):1637-46.

Luo M., Zhou J., Liu Z. (2015) Reward processing by the dorsal raphe nucleus: 5-Ht and beyond. Learning & Memory 22:452–460. doi/10.1101/lm.037317.114.

MacLean P.D. (1952) Some psychiatric implications of physiological studies on frontotemporal portion of limbic system (Visceral brain) Clin. Neurophysiol. 4, 407–418.

Mann J.J. (1999) Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. Neuropsychopharmacology 21:99S-105S.

Manzini M.C., Xiong L., Shaheen R., Tambunan D.E., Di Costanzo S., Mitisalis V., Tischfield D.J., Cinquino A., Ghaziuddin M., Christian M., Jiang Q., Laurent S., Nanjiani Z.A., Rasheed S., Hill R.S., Lizarraga SB, Gleason D, Sabbagh D, Salih MA, Alkuraya F.S., Walsh C.A. (2014) CC2D1A regulates human intellectual and social function as well as NF-kappaB signaling homeostasis. Cell Rep 8:647-655.

Mark V.W., Taub E., Perkins C., Gauthier L., Uswatte G. (2008) MRI infarction load and CI therapy outcomes for chronic post-stroke hemiparesis. Restorative neurology and neuroscience 26(1): 13-33.

Maya Vetencourt J.F., Sale A., Viegi A., Baroncelli L., De Pasquale R., O'Leary O.F., Castrén E., Maffei L. (2008) The antidepressant fluoxetine restores plasticity in the adult visual cortex. Science. Apr 18;320(5874):385-8. doi: 10.1126/science.1150516.

Mayo N.E., Korner-Bitensky N.A., Becker R. (1991) Recovery time of independent function post-stroke. American journal of physical medicine & rehabilitation/Association of Academic Physiatrists 70(1): 5-12.

McCann S.K., Irvine C., Mead G.E., Sena E.S., Currie G.L., Egan K.E., et al. (2014) Efficacy of antidepressants in animal models of ischemic stroke: a systematic review and meta-analysis. Stroke 45(10): 3055-3063.

McGrath J.J., Saha S., Al-Hamzawi A., Andrade L., Benjet C., Bromet E.J., Browne M.O., Caldas de Almeida J.M., Chiu W.T., Demyttenaere K., Fayyad J., Florescu S., de Girolamo G., Gureje O., Haro J.M., Ten Have M., Hu C., Kovess-Masfety V., Lim C.C., Navarro-Mateu F., Sampson N., Posada-Villa J., Kendler K.S., Kessler R.C. (2016) The

bidirectional associations between psychotic experiences and DSM-IV mental disorders. Am J Psychiatry 173:997–1006.

Mead G.E., Hsieh C.F., Lee R., Kutlubaev M.A., Claxton A., Hankey G.J., et al. (2012) Selective serotonin reuptake inhibitors (SSRIs) for stroke recovery. The Cochrane database of systematic reviews 11: Cd009286.

Mead G.E., Hsieh C.F., Lee R., Kutlubaev M., Claxton A., Hankey G.J., Hackett M. (2013) Selective serotonin reuptake inhibitors for stroke recovery: a systematic review and meta-analysis. Stroke 44:844-850.

Meltzer H.Y., Massey B.W., Horiguchi M. (2012) The role of 5-HT1A receptors in phencyclidine (PCP)-induced novel object recognition (NOR) deficit in rats. Psychopharmacology (Berl). 2012 May;221(2):205-15. doi: 10.1007/s00213-011-2561-4.

Metz G.A., Whishaw I.Q. (2009) The ladder rung walking task: a scoring system and its practical application. J Vis Exp (28).

Metzger D., Clifford J., Chiba H., Chambon P. (1995) Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci U S A. Jul 18;92(15):6991-5.

Millan M.J. (2004). The role of monoamines in the actions of established and "novel" anti depressant agents: acritical review. Eur. J. Pharmacol. 500,371–384. doi: 10.1016/j.ejphar.2004.07.038.

Mikami K., Jorge R.E., Moser D.J., Arndt S., Jang M., Solodkin A., Small S.L., Fonzetti P, Hegel M.T., Robinson R.G. (2014) Prevention of post-stroke generalized anxiety disorder, using escitalopram or problem-solving therapy. J Neuropsychiatry Clin Neurosci. Fall;26(4):323-8. doi: 10.1176/appi.neuropsych.11020047.

Mlinar B., Stocca G., Corradetti R. (2015) Endogenous serotonin facilitates hippocampal long-term potentiation at CA3/CA1 synapses. J Neural Transm (Vienna) 122:177-185.

Mogha A., Guariglia S.R., Debata P.R., Wen G.Y., Banerjee P. (2012). Serotonin1A receptor-mediated signaling through ERK and PKC alpha is essential for normal synaptogenesis in neonatal mouse hippocampus. Transl. Psychiatry 2 e66. doi:10.1038/tp.2011.58.

Mortensen J.K., Larsson H., Johnsen S.P., Andersen G. (2014) Impact of prestroke selective serotonin reuptake inhibitor treatment on stroke severity and mortality. Stroke. Jul;45(7):2121-3. doi: 10.1161/STROKEAHA.114.005302.

Mosienko V., Bert B., Beis D., Matthes S., Fink H., Bader M., Alenina N. (2012) Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. Transl Psychiatry 2 e122.

Murakami T., Hama S., Yamashita H., Onoda K., Kobayashi M., Kanazawa J., Yamawaki S., Kurisu K. (2013) Neuroanatomic pathways associated with poststroke affective and apathetic depression. Am J Geriatr Psychiatry. Sep;21(9):840-7. doi: 10.1016/j.jagp.2013.01.057.

Narushima K., Kosier J.T., Robinson R.G. (2003) A reappraisal of poststroke depression, intra- and inter-hemispheric lesion location using meta-analysis. J Neuropsychiatry Clin Neurosci 15(4): 422-430.

Naumenko V.S., Osipova D.V., Tsybko A.S. (2010) On the role of selective silencer Freud-1 in the regulation of the brain 5-HT(1A) receptor gene expression. Mol Biol (Mosk) 44:898-903.

Nestler E.J. (2015) FosB: A transcriptional regulator of stress and antidepressant responses. Eur J Pharmacol 753:66-72.

Newman-Tancredi A., Albert P.R. (2012) Gene polymorphism at serotonin 5-HT1A receptors:moving towards personalized medicine for psychosis and mood deficits, in Schizophrenia Research:Recent Advances (Sumiyoshi T, ed), (NewYork, NY:Nova Publishers), 337–358.

Nieh E.H., Kim S.Y., Namburi P., Tye K.M. (2013) Optogenetic dissection of neural circuits underlying emotional valence and motivated behaviors. Brain Res 1511:73-92.

Northoff G., Wiebking Ch., Feinbergc T., Panksepp J. (2011) The 'resting-state hypothesis' of major depressive disorder—A translational subcortical–cortical framework for a system disorder. Neuroscience and Biobehavioral Reviews 35: 1929–1945 doi:10.1016/j.neubiorev.2010.12.007

Nys G.M., van Zandvoort M.J., van der Worp H.B, de Haan E.H., de Kort P..L, Jansen B.P., Kappelle L.J. (2006) Early cognitive impairment predicts long-term depressive symptoms and quality of life after stroke. J Neurol Sci 247(2):149-156.

Oaks A.W., Zamarbide M., Tambunan D.E., Santini E., Di Costanzo S., Pond H.L., et al (2016) Cc2d1a Loss of Function Disrupts Functional and Morphological Development in Forebrain Neurons Leading to Cognitive and Social Deficits. Cereb Cortex.

Ogawa S.K., Cohen J.Y., Hwang D., Uchida N., Watabe-Uchida M. (2014) Organization of monosynaptic inputs to the serotonin and dopamine neuromodulatory systems. Cell Rep 8:1105-1118.

O'Hearn E., Molliver M.E. (1984). Organization of raphe-cortical projections in rat: a quantitative retrograde study. Brain Res. Bull. 13, 709–726. doi:10.1016/0361-9230(84)90232-6.

Ohmatsua S., Nakanoa H., Tominagab T., Terakawad Y., Muratab T., Moriokaa Sh. (2014) Activation of the serotonergic system by pedaling exercise changes anterior cingulate cortex activity and improves negative emotion. Behavioural Brain Research 270:112–117.

Overman J.J., Carmichael S.T. (2014) Plasticity in the injured brain: more than molecules matter. Neuroscientist 20:15-28.

Otsuka A., Shiuchi T., Chikahisa S., Shimizu N., Sei H. (2015) Voluntary exercise and increased food intake after mild chronic stress improve social avoidance behavior in mice. Physiology & Behavior, vol. 151, pp. 264–271.

Otsuka T., Nishii A., Amemiya S., Kubota N., Nishijima T., Kita I. (2016) Effects of acute treadmill running at different intensities on activities of serotonin and corticotropin-releasing factor neurons, and anxiety-and depressive-like behaviors in rats. Behavioural Brain Research 298: 44–51.

Ou X.M., Jafar-Nejad H., Storring J.M., Meng J.H., Lemonde S., Albert P.R. (2000) Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene. J Biol Chem. 275:8161–8168. doi: 10.1074/jbc.275.11.816.

Ou X.M., Lemonde S., Jafar-Nejad H., Bown C.D., Goto A., Rogaeva A., Albert P. R. (2003) Freud-1: A neuronal calcium-regulated repressor of the 5-HT1A receptor gene. J Neurosci 23(19): 7415-7425.

Page S.J., Gauthier L.V., White S. (2013) Size doesn't matter: cortical stroke lesion volume is not associated with upper extremity motor impairment and function in mild, chronic hemiparesis. Archives of physical medicine and rehabilitation 94(5): 817-821.

Paolucci S., Gandolfo C., Provinciali L., Torta R., Toso V. (2006) The Italian multicenter observational study on post-stroke depression (DESTRO). J Neurol 253(5): 556-562.

Paradiso S., Anderson B.M., Boles Ponto L.L., Tranel D., Robinson R.G. (2011) Altered neural activity and emotions following right middle cerebral artery stroke. J Stroke Cerebrovasc Dis. Mar-Apr; 20(2): 94-104. doi: 10.1016/j.jstrokecerebrovasdis.2009.11.005.

Parfitt G.M., Nguyen R., Bang J.Y., Aqrabawi A.J., Tran M.M., Seo D.K., Richards B.A., Kim J.C. (2017) Bidirectional Control of Anxiety-Related Behaviors in Mice: Role of Inputs Arising from the Ventral Hippocampus to the Lateral Septum and Medial Prefrontal Cortex. Neuropsychopharmacology 42:1715-1728.

Parks C.L., Robinson P.S., Sibille E., Shenk T., Toth M. (1998) Increased anxiety of mice lacking the serotonin1A receptor. Proc Natl Acad Sci U S A 95 10734-9.

Parsey R.V., Ogden R.T., Miller J.M., Tin A., Hesselgrave N., Goldstein E., Mikhno A., Milak M., Zanderigo F., Sullivan G.M., Oquendo M.A., Mann J.J. (2010) Higher serotonin 1A binding in a second major depression cohort: modeling and reference region considerations. Biol Psychiatry. 15;68(2):170-8. doi: 10.1016/j.

Pathak Y., Salami O., Baillet S., Li Z., Butson C.R. (2016) Longitudinal Changes in Depressive Circuitry in Response to Neuromodulation Therapy. Front Neural Circuits. Jul 29;10:50. doi: 10.3389/fncir.2016.00050.

Paxinos G., Franklin K.B.J. (2008) The Mouse Brain in Stereotaxic Coordinates, 2nd Edition. San Diego, CA: Academic Press.

Penington N.J., Kelly J.S. (1990) Serotonin receptor activation reduces calcium current in an acutely dissociated adult central neuron. Neuron 4, 751–75810.1016/0896-6273(90)90201-P doi:10.1016/0896-6273(90)90201-P.

Penington N.J., Kelly J.S., Fox A.P. (1991) A study of the mechanism of Ca2+ current inhibition produced by serotonin in rat dorsal raphe neurons. J Neurosci 11:3594-3609.

Peyron C., Petit J.M., Rampon C., Jouvet M., Luppi P.H. (1998) Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. Neuroscience. Jan;82(2):443-68.

Phillips C. (2017) Physical activity modulates common neuroplasticity substrates in major depressive and bipolar disorder. Neural Plasticity Volume 2017, Article ID 7014146, 37 pages doi.org/10.1155/2017/7014146.

Piñeyro G. (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev. 51(3):533-9.

Piñeyro G., Blier P. (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev 51:533-591.

Piszczek L., Schlax K., Wyrzykowska A., Piszczek A., Audero E., Thilo Gross C. (2013) Serotonin 1A auto-receptors are not sufficient to modulate anxiety in mice. Eur. J. Neurosci. 38(4):2621-7. doi: 10.1111/ejn.12260.

Pletscher A., (1991) The discovery of antidepressants: a winding path. Experientia. vol. 47, no. 1, pp. 4–8, 1991.

Pollock A., St George B., Fenton M., Firkins L. (2012) Top ten research priorities relating to life after stroke. Lancet Neurol.11:209.

Pollak Dorocic I., Furth D., Xuan Y., Johansson Y., Pozzi L., Silberberg G., Carlen M., Meletis K. (2014) A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei. Neuron 83:663-678.

Pompili M., Venturini P., Lamis D.A., Giordano G., Serafini G., Belvederi Murri M., Amore M., Girardi P. (2015) Suicide in stroke survivors: epidemiology and prevention. Drugs Aging 32:21-29.

Popova N.K., Naumenko V.S. (2013) 5-HT1A receptor as a key player in the brain 5-HT system. Rev Neurosci. 24(2):191-204. doi: 10.1515/revneuro-2012-0082. Review.

Potter G.G., Blackwell A.D., McQuoid D.R., Payne M.E., Steffens D.C., Sahakian B.J., Welsh-Bohmer K.A., Krishnan K.R. (2007) Prefrontal white matter lesions and prefrontal task impersistence in depressed and nondepressed elders. Neuropsychopharmacology 32:2135-2142.

Praag H. van, Kempermann G., Gage F. H. (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature Neuroscience, vol. 2, no. 3, pp. 266–270, 1999

Praag H. van, Shubert T., Zhao C., Gage F.H. (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. The Journal of Neuroscience, vol. 25, no. 38, pp. 8680–8685.

Price J.L., Drevets W.C. (2012) Neural circuits underlying the pathophysiology of mood disorders. Trends in cognitive sciences 16(1): 61-71.

Puig M.V., Gulledge A.T. (2011) Serotonin and prefrontal cortex function: neurons, networks, and circuits. Mol. Neurobiol. 44,449–464. doi: 10.1007/s12035-011-8214-0.

Rajashekaran P., Pai K., Thunga R., Unnikrishnan B. (2013) Post-stroke depression and lesion location: A hospital based cross-sectional study. Indian J Psychiatry. Oct;55(4):343-8. doi: 10.4103/0019-5545.120546.

Ramboz S., Oosting R., Amara D.A., Kung H.F., Blier P., Mendelsohn M., Mann J.J., Brunner D., Hen R. (1998) Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci U S A 95 14476-81.

Ressler K.J., Mayberg H.S. (2007) Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. Nat Neurosci. 10:1116-1124.

Reynolds G.P., Arranz B., Templeman L.A., Fertuzinhos S., San L. (2006) Effect of 5-HT1A receptor gene polymorphism on negative and depressive symptom response to antipsychotic treatment of drug-naive psychotic patients. Am J Psychiatry 163:1826-1829.

Richardson-Jones J.W., Craige C.P., Guiard B.P., Stephen A., Metzger K.L., Kung H.F., Gardier A.M., Dranovsky A., David D.J., Beck S.G., Hen R., Leonardo E.D. (2010) 5-HT(1A) Autoreceptor Levels Determine Vulnerability to Stress and Response to Antidepressants. Neuron 65 40-52.

Richardson-Jones J.W., Craige C.P., Nguyen T.H., Kung H.F., Gardier A.M., Dranovsky A., David D.J., Guiard B.P., Beck S.G., Hen R., Leonardo E.D. (2011) Serotonin-1A autoreceptors are necessary and sufficient for the normal formation of circuits underlying innate anxiety. J. Neurosci. 31 (600)8-18.

Riga D., Matos M.R., Glas A., Smit A.B., Spijker S., Van den Oever M.C. (2014) Optogenetic dissection of medial prefrontal cortex circuitry. Frontiers in systems neuroscience 8: 230.

Riley J.D., Le V., Der-Yeghiaian L., See J., Newton J.M., Ward N.S., et al. (2011) Anatomy of stroke injury predicts gains from therapy. Stroke 42(2): 421-426.

Rimer J., Dwan K., Lawlor D.A, Greig C.A., McMurdo M., Morley W., Mead G.E. (2012) Exercise for depression. Cochra.ne Database Syst Rev 7:Cd004366.

Robison A.J., Vialou V., Sun H.S., Labonte B., Golden S.A., Dias C., Turecki G., Tamminga C., Russo S., Mazei-Robison M., Nestler E.. (2014) Fluoxetine epigenetically alters the CaMKIIalpha promoter in nucleus accumbens to regulate DeltaFosB binding and antidepressant effects. Neuropsychopharmacology 39:1178-1186.

Robinson R.G., Jorge R.E. (2016) Post-Stroke Depression: A Review. Am J Psychiatry 173(3): 221-231.

Rogaeva A., Albert P.R. (2007) The mental retardation gene CC2D1A/Freud-1 encodes a long isoform that binds conserved DNA elements to repress gene transcription Eur J Neurosci. 26(4):965-74.

Rogaeva A., Ou X.M., Jafar-Nejad H., Lemonde S., Albert P.R. (2007) Differential repression by Freud-1/CC2D1A at a polymorphic site in the dopamine-D2 receptor gene. J Biol Chem 282:20897-20905.

Roome R.B., Bartlett R.F., Jeffers M., Xiong J., Corbett D., Vanderluit .JL. (2014) A reproducible Endothelin-1 model of forelimb motor cortex stroke in the mouse. J Neurosci Methods 2014; 233: 34-44.

Rush A.J., Warden D., Wisniewski S.R., Fava M., Trivedi M.H., Gaynes B.N., et al. (2009) STAR∗D: revising conventional wisdom. CNS Drugs 23, 627–647. doi: 10.2165/00023210-200923080-00001.

Russo-Neustadt A.A., Beard R.C., Huang Y.M., Cotman C.W. (2000) Physical activity and antidepressant treatment potentiate the expression of specific brain-derived neurotrophic factor transcripts in the rat hippocampus. Neuroscience. 101: 305–312.

Russo S.J/, Nestler EJ (2013) The brain reward circuitry in mood disorders. Nat Rev Neurosci 14:609-625.

Sachs B.D., Jacobsen J.P., Thomas T.L., Siesser W.B., Roberts W.L., Caron M.G. (2013) The effects of congenital brain serotonin deficiency on responses to chronic fluoxetine. Transl Psychiatry 3 e291.

Samuels B.A., Anacker C., Hu A., Levinstein M.R., Pickenhagen A., Tsetsenis T., Madronal N., Donaldson Z.R., Drew L.J., Dranovsky A., Gross C..T, Tanaka K.F., Hen R. (2015) 5-HT1A receptors on mature dentate gyrus granule cells are critical for the antidepressant response. Nat Neurosci 18:1606-1616.

Santana N., Bortolozzi A., Serrats J., Mengod G., Artigas F. (2004) Expression of serotonin1A and serotonin2A receptors in pyramidal and GABAergic neurons of the rat prefrontal cortex. Cereb. Cortex 14, 1100–1109 10.1093/cercor/bhh070.

Santarelli L., Saxe M., Gross C., Surget A., Battaglia F., Dulawa S., et al. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science (New York, NY) 301(5634): 805-809.

Sato S., Yamakawa Y., Terashima Y., Ohta H., Asada T. (2006) Efficacy of milnacipran on cognitive dysfunction with post-stroke depression: preliminary open-label study. Psychiatry Clin Neurosci. Oct;60(5):584-9.

Savitz J.B., Drevets W.C. (2009) Imaging phenotypes of major depressive disorder: genetic correlates. Neuroscience 164(1), 300–30.

Schallert T., Fleming S.M., Leasure J.L., Tillerson J.L., Bland S.T. (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39(5): 777-787.

Sheehan T.P., Chambers R.A., Russell D.S. (2004) Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res Brain Res Rev 46:71-117.

Schildkraut J.J. (1965) The catecholamine hypothesis of affective disorders: a review of supporting evidence. The American journal of psychiatry vol. 122, no. 5, pp. 509–522, 1965.

Schiller D., Weiner I. (2004) Lesions to the basolateral amygdala and the orbitofrontal cortex but not to the medial prefrontal cortex produce an abnormally persistent latent inhibition in rats. Neuroscience 128(1): 15-25.

Selim M.H., Molina C.A. (2012) Poststroke treatment with selective serotonin reuptake inhibitors: a journey from sadness to motor recovery. Stroke. Nov;43(11):3154-5. doi: 10.1161/STROKEAHA.112.657635.

Shao B., Zhou Y.L., Wang H., Lin Y.S. (2015) The role of calcitonin gene-related peptide in post-stroke depression in chronic mild stress-treated ischemic rats. Physiol Behav. 139:224-30. doi: 10.1016.

Shen H., Semba K. (1994) A direct retinal projection to the dorsal raphe nucleus in the rat. Brain Res. 635, 159–168. doi: 10.1016/0006-8993(94)91435-4.

Shin T.K., Kang M.S., Lee H.Y., et al. (2009) Fluoxetine and sertraline attenuate post ischemic brain injury in mice. Korean J Physiol Pharmacol.13: 257–263.

Shipton O.A., El-Gaby M., Apergis-Schoute J., Deisseroth K., Bannerman D.M., Paulsen O., Kohl M.M. (2014) Left-right dissociation of hippocampal memory processes in mice. Proc Natl Acad Sci U S A 111:15238-15243.

Silasi G., Murphy T.H. (2014) Stroke and the connectome: how connectivity guides therapeutic intervention. Neuron 83(6): 1354-1368.

Sim Y.J., Kim S.S., Kim J.Y., Shin M.S., Kim C.J. (2004) Treadmill exercise improves short-term memory by suppressing ischemia-induced apoptosis of neuronal cells in gerbils. Neurosci. Lett. 372 (3), 256e261.

Sim Y.J., Kim H., Yoon S.-J, Kim S.-S., … Kim Ch.-J. (2005) Long-term treadmill exercise overcomes ischemia-induced apoptotic neuronal cell death in gerbils. Physiol. Behav. 84 (5), 733e738.

Singh A., Black S.E., Herrmann N., Leibovitch F.S., Ebert P.L., Lawrence J. et al. (2000) Functional and neuroanatomic correlations in poststroke depression: the Sunnybrook Stroke Study. Stroke 31: 637–644.

Souslova T., Mirédin K., Millar A.M., Albert PR (2016) Recruitment by the Repressor Freud-1 of Histone Deacetylase-Brg1 Chromatin Remodeling Complexes to Strengthen HTR1A Gene Repression. Mol Neurobiol. Dec 2. https://www.ncbi.nlm.nih.gov/pubmed/27914010.

Southwick S.M., Vythilingam M., Charney D.S. (2005) The psychobiology of depression and resilience to stress: implications for prevention and treatment. Annu Rev Clin Psychol.1:255-91.

Steinbusch H.W.M. (1981) Distribution of serotonin immunoreactivity in the central nervous system of the rat cell bodies and terminals. Neuroscience 6, 557–618. doi: 10.1016/0306-4522(81)90146-9.

Steinbusch H.W.M., van der Kooy D., Verhofstad A.A., Pellegrino A. (1980) Serotonergic and non- serotonergic projections from the nucleus raphe dorsalis to the caudate-putamen complex in the rat, studied by a combined immunofluorescence and fluorescent retrograde axonal labeling technique. Neurosci. Lett. 19, 137–142. doi:10.1016/0304-3940 (80)90184-6.

Stockmeier C., Shapiro L. , Dilley G.E., Kolli T. N., Friedman L., Rajkowska G. (1998) Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. The Journal of Neuroscience, 18(18), 7394–401.

Storring J.M., Charest A., Cheng P., Albert P.R. (1999) TATA-driven transcriptional initiation and regulation of the rat serotonin 5-HT1A receptor gene. J Neurochem. Jun;72(6):2238-47.

Sullivan G.M., Oquendo M.A., Milak M., Miller J.M., Burke A., Ogden R.T., Parsey R.V., Mann J.J. (2015) Positron Emission Tomography Quantification of Serotonin1A Receptor Binding in Suicide Attempters With Major Depressive Disorder. JAMA Psychiatry 72:169-178.

Svensson M., Lexell J., Deierborg T., (2014) Effects of physical exercise on neuroinflammation, neuroplasticity, neurodegeneration, and behavior: what we can learn from animal models in clinical settings. Neurorehabil Neural Repair 29 (6), 577e589.

Swain R.A., Harris A.B., Wiener E.C., Dutka M.V., Morris H.D., Theien B.E., Konda S., Engberg K., Lauterbur P.C., Greenough W.T. (2003) Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. Neuroscience 117: 1037–1046.

Snyder J.S., Glover L.R., Sanzone K.M., Kamhi J.F., Cameron H.A. (2009) The effects of exercise and stress on the survival and maturation of adult-generated granule cells. Hippocampus. Oct;19(10):898-906. doi: 10.1002/hipo.20552.

Szewczyk B., Albert P.R., Burns A.M., Czesak M., Overholser J.C., Jurjus G.J., Meltzer H.Y., Konick L. C., Dieter L., Herbst N., May W., Rajkowska G., Stockmeier C.A., Austin M.C. (2009) Gender-specific decrease in NUDR and 5-HT1A receptor proteins in the prefrontal cortex of subjects with major depressive disorder. Int. J. Neuropsychopharmacol. 12, 155–168.

Szewczyk B., Albert P.R., Rogaeva A., Fitzgibbon H., May W.L., Rajkowska G., et al (2010). Decreased expression of Freud-1/CC2D1A, a transcriptional repressor of the 5-HT1A receptor, in the prefrontal cortex of subjects with major depression. Int J Neuropsychopharmacol 13: 1089-1101.

Szewczyk B., Kotarska K., Daigle M., Misztak P., Sowa-Kucma M., Rafalo A., Curzytek K., Kubera M., Basta-Kaim A., Nowak G., Albert PR (2014) Stress-induced alterations in

5-HT1A receptor transcriptional modulators NUDR and Freud-1. Int J Neuropsychopharmacol 17:1763-1775.

Takekita Y., Fabbri C., Kato M., Nonen S., Sakai S., Sunada N., Koshikawa Y., Wakeno M., Okugawa G., Kinoshita T., Serretti A. (2015) HTR1A Gene Polymorphisms and 5-HT1A Receptor Partial Agonist Antipsychotics Efficacy in Schizophrenia. J Clin Psychopharmacol 35:220-227.

Tennant K.A., Jones T.A. (2009) Sensorimotor behavioral effects of endothelin-1 induced small cortical infarcts in C57BL/6 mice. J Neurosci Methods 181(1): 18-26.

Terroni L., Amaro E., Iosifescu D.V., Tinone G., Sato J.R., Leite C.C., et al. (2011) Stroke lesion in cortical neural circuits and post-stroke incidence of major depressive episode: a 4-month prospective study. The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry 12(7): 539-548.

Thomas S.A., Lincoln N.B. (2006) Factors relating to depression after stroke. Br J Clin Psychol 45(Pt 1): 49-61.

Trent N.L., Menard J.L. (2010) The ventral hippocampus and the lateral septum work in tandem to regulate rats' open-arm exploration in the elevated plus-maze. Physiol Behav 101:141-152.

Trivedi M.H., Fava M., Wisniewski S.R., Thase M.E., Quitkin F., Warden D., Ritz L., Nierenberg A.A., Lebowitz B.D., Biggs M.M., Luther J.F., Shores-Wilson K., Rush A.J. (2006) Medication augmentation after the failure of SSRIs for depression. N Engl J Med 354:1243-1252.

Trivedi M.H., Rush A.J., Wisniewski S.R., Nierenberg A.A., Warden D., Ritz L., et al. (2006) Evaluation of outcomes with citalopram for depression sing measurement-based care in STAR*D: implications for clinical practice. Am.J. Psychiatry 163,28–40. doi:10.1176/appi.ajp.163.1.28.

Tureyen K., Vemuganti R., Sailor K.A., Dempsey R.J. (2004) Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. J Neurosci Methods 139(2): 203-207.

Uda M., Ishido M., Kami K., M. Masuhara (2006) Effects of chronic treadmill running on neurogenesis in the dentate gyrus of the hippocampus of adult rat," Brain Research, vol. 1104, no. 1, pp. 64–72.

Vahid-Ansari F., Lagace D.C., Albert P.R. (2016) Persistent post-stroke depression in mice following unilateral medial prefrontal cortical stroke. Transl Psychiatry. 2;6(8):e863. doi: 10.1038/tp.2016.124.

Van Bockstaele E.J., Biswas A., Pickel V.M. (1993) Topography of serotonin neurons in the dorsal raphe nucleus that send axon collaterals to the rat prefrontal cortex and nucleus accumbens. Brain Res. Oct 8;624(1-2):188-98.

Varga V., Losonczy A., Zemelman B. V., Borhegyi Z., Nyiri G., Domonkos A., et al. (2009) Fast synaptic subcortical control of hippocampal circuits. Science 326, 449–453 10.1126/science.1178307.

Vaynman S., Ying Z., Gomez-Pinilla F. (2004) Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. Eur J Neurosci. Nov;20(10):2580-90.

Vermeer S.E., Longstreth W.T., Jr., Koudstaal P.J. (2007) Silent brain infarcts: a systematic review. Lancet 6(7): 611-619.

Veenstra-Vanderweele J., Muller C.L., Iwamoto H., Sauer J.E., Owens W.A., Shah C.R., Cohen J., Mannangatti P., Jessen T., Thompson B.J., Ye R., Kerr T.M., Carneiro A.M., Crawley J.N., Sanders-Bush E., McMahon D.G., Ramamoorthy S., Daws L.C., Sutcliffe J.S., Blakely R.D. (2012) Autism gene variant causes hyperserotonemia, serotonin receptor hypersensitivity, social impairment and repetitive behavior. Proc Natl Acad Sci U S A 109 5469-74.

Viana Mde B., Zangrossi Jr. H., Onusic G.M. (2008) 5-HT1A receptors of the lateral septum regulate inhibitory avoidance but not escape behavior in rats Pharmacol. Biochem. Behav., 89 pp. 360–366.

Vialou V., Bagot R.C., Cahill M.E., Ferguson D., Robison A.J., Dietz D.M., et al. (2014) Prefrontal cortical circuit for depression- and anxiety-related behaviors mediated by cholecystokinin: role of DeltaFosB. J Neurosci 34(11): 3878-3887.

Vialou V., Thibault M., Kaska S., Cooper S., Gajewski P., Eagle A., Mazei-Robison M., Nestler E.J., Robison A.J. (2015) Differential induction of FosB isoforms throughout the brain by fluoxetine and chronic stress. Neuropharmacology. 99:28-37. doi: 10.1016/j.neuropharm.

Vinkers C.H., Oosting R.S., van Bogaert M.J., Olivier B., Groenink L. (2010) Early-life blockade of 5- HT(1A) receptors alters adult anxiety behavior and benzodiazepine sensitivity. Biol Psychiatry 67: 309–316.

Vorhees C.V., Williams M.T. (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature Protocols; 1(2): 848-858.

Walia V., Gilhotra N. (2017) GABAergic Influence in the Antidepressant Effect of Fluoxetine in Unstressed and Stressed Mice. Journal of Applied Pharmaceutical Science Vol. 7 (03), pp. 141-147.

Walsh K.H., Mc Dougle C.J. (2004) Pharmacological augmentation strategies for treatment-resistant obsessive-compulsive disorder. Expert Opin Pharmaco ther. 5:2059-2067.

Walther D.J., Peter J.U., Bashammak H.S., Hortnag L.H., Voits M., Fink H, et al. (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science 299, 76. doi:10.1126/science.1078197.

Wang Y., Jin K., Greenberg D.A. (2007) Neurogenesis associated with endothelin-induced cortical infarction in the mouse. Brain Res 1167: 118-122.

Warden M.R., Selimbeyoglu A., Mirzabekov J.J., Lo M., Thompson K.R., Kim S.Y., Adhikari A., Tye K.M., Frank L.M., Deisseroth K (2012) A prefrontal cortex-brainstem neuronal projection that controls response to behavioural challenge. Nature 492:428-432.

Warner-Schmidt J. (2013) Treating the brain deep down: Short-circuiting depression. Nature medicine 19(6): 680-681.

Waterhouse B.D., Border B., Wahl L., Mihailoff G.A. (1993) Topographic organization of rat locus coeruleus and dorsal raphe nuclei: distribution of cells projecting to visual system structures. J. Comp. Neurol. 336, 345–361. doi:10.1002/cne. 903360304.

Waterhouse B.D., Mihailoff G.A., Baack J.C., Woodward D.J. (1986) Topographical distribution of dorsal and median raphe neurons projecting to motor, sensorimotor, and visual cortical areas in the rat. J. Comp. Neurol. 249,460–476,478-481. doi:10.1002/cne.902490403.

Wei N., Yong W., Li X., Zhou Y., Deng M., Zhu H., et al. (2015) Post-stroke depression and lesion location: a systematic review. J Neurol 262(1): 81-90.

Weissbourd B., Ren J., DeLoach K.E., Guenthner C.J., Miyamichi K., Luo L. (2014) Presynaptic partners of dorsal raphe serotonergic and GABAergic neurons. Neuron 83:645–66.

Whiteford H.A., Degenhardt L., Rehm J., Baxter A.J., Ferrari A.J., Erskine H.E., Charlson F.J., Norman R.E., Flaxman A.D., Johns N., Burstein R., Murray C.J., Vos T. (2013) Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet 382:1575-1586.

Whyte E.M., Mulsant B.H., Rovner B.W., Reynolds C.F. (2006) Preventing depression after stroke. International review of psychiatry (Abingdon, England) 18(5): 471-481.

Will B., Galani R., Kelche C., Rosenzweig M.R. (2004) Recovery from brain injury in animals: relative efficacy of environmental enrichment, physical exercise or formal training (1990-2002). Prog Neurobiol. Feb;72(3):167-82.

Williams L.S. (2005) Depression and stroke: cause or consequence? Semin Neurol 2005; 25(4): 396-409.

Williamson M.P. (1994) The structure and function of proline-rich regions in proteins, Biochem J 297:249–260.

Windle V., Corbett D. (2005) Fluoxetine and recovery of motor function after focal ischemia in rats. Brain Res 1044(1): 25-32.

Windle V., Szymanska A., Granter-Button S., White C., Buist R., Peeling J., et al. (2006) An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. Exp Neurol 201(2): 324-334.

Wissink S., Meijer O., Pearce D., van Der Burg B., van Der Saag P.T. (2000) Regulation of the rat serotonin-1A receptor gene by corticosteroids. J Biol Chem 275:1321-1326.

Wolfe C.D., Crichton S.L., Heuschmann P.U., McKevitt C.J., Toschke A.M., Grieve A.P., et al. (2011) Estimates of outcomes up to ten years after stroke: analysis from the prospective South London Stroke Register. PLoS medicine 8(5): e1001033.

Wong D.T., Perry K.W., Bymaster F.P. (2005) Case history: the discovery of Fluoxetine hydrochloride (Prozac). Nat. Rev. Drug Discov. 4, 764–774.

Wscieklicaa T., Silva M.S.C.F., Lemesa J.A., Melo-Thomasb L., Céspedesa I.C., Vianaa M.B. (2017) Deep brain stimulation of the dorsal raphe inhibits avoidance and escape reactions and activates forebrain regions related to the modulation of anxiety/panic. Behavioural Brain Research 321 (2017) 193–200. doi.org/10.1016/j.bbr.2016.11.054.

Wu R.H., Feng C., Xu Y., Hua T., Liu X.Y., Fang M. (2014) Late-onset depression in the absence of stroke: associated with silent brain infarctions, microbleeds and lesion locations. International journal of medical sciences 11(6): 587-592.

Wulsin L., Alwell K., Moomaw C..J, Lindsell C.J., Kleindorfer D.O., Woo D., et al. (2012) Comparison of two depression measures for predicting stroke outcomes. Journal of psychosomatic research 72(3): 175-179.

Xiao-Wei J.I., Chun-Ling W.U., Xing-Chen Wang, J.L., Jian-Zhong B, Dian-Yun W. (2014) Monoamine neurotransmitters and fibroblast growth factor-2 in the brains of rats with post-stroke depression. Exp Ther Med. 8(1): 159–164. doi: 10.3892/etm.2014.1674.

Yamamoto Sh., Ohba H., Nishiyama Sh., Harada N., Kakiuchi T, Tsukada H., Domino E.F. (2013) Subanesthetic Doses of Ketamine Transiently Decrease Serotonin Transporter Activity: A PET Study in Conscious Monkeys. Neuropsychopharmacology 38, 2666–2674; doi:10.1038/npp.2013.176.

Yang S., Hua P., Shang X., Cui Z., Zhong S., Gong G. et al. (2015) A significant risk factor for post stroke depression: the depression-related subnetwork. J Psychiatry Neurosci. 40: 259–268.

Young S.N., Leyton, M. (2002) The role of serotonin in human mood and social interaction. Insight from altered tryptophan levels. Pharmacol.Biochem. Behav. 71,857–865. doi:10.1016/S0091-3057(01)00670-0.

Yuen E. Y., Jiang Q., Chen P., Feng J., Yan Z. (2008) Activation of 5-HT2A/C receptors counteracts 5-HT1A regulation of n-methyl-D-aspartate receptor channels in pyramidal neurons of prefrontal cortex. J. Biol. Chem. 283, 17194–17204 10.1074/jbc.M801713200.

Zhang G., Chen l., Yang L., Hua X., Zhou B.,….., Xu X. (2014) Combined use of spatial restraint stress and middle cerebral artery occlusion is a novel model of post-stroke depression in mice. Behav Brain Res. 1; 260: 162–170. doi: 10.1016/j.bbr.2013.10.047.

Zhang L.S., Hu X.Y., Yao L.Y., Geng Y., Wei L.L., Zhang J.H., Chen W. (2013) Prophylactic effects of duloxetine on post-stroke depression symptoms: an open single-blind trial. Eur Neurol 69:336-343

Zhao M., Li X.D., Chen Z. (2010) CC2D1A, a DM14 and C2 domain protein, activates NF-kappaB through the canonical pathway. J Biol Chem 285:24372-24380.

Zhao M., Raingo J., Chen Z.J., Kavalali E.T. (2011) Cc2d1a, a C2 Domain Containing Protein Linked to Non-Syndromic Mental Retardation, Controls Functional Maturation of Central Synapses. J Neurophysiol 105:1506-1515.

Zhong P., Yan Z. (2004) Chronic antidepressant treatment alters serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. Neuroscience 129:65-73.

Zhou L.Y., Wright T.E., Clarkson A.N. (2016) Prefrontal cortex stroke induces delayed impairment in spatial memory. Behav Brain Res 296: 373–378. doi: 10.1016/j.bbr.2015.08.022.

APPENDIX

Serotonin-prefrontal cortical circuitry in anxiety and depression phenotypes: pivotal role of pre- and post-synaptic 5-HT1A receptor expression

Paul R. Albert,1,* Faranak Vahid-Ansari,1,2 and Christine Luckhart1,2

1 OHRI (Neuroscience) and 2 Department of Cellular and Molecular Medicine
* University of Ottawa Brain and Mind Research Institute
451 Smyth Road
Ottawa, ON Canada K1H-8M5
Front Behav Neurosci. 2014; 8: 199. doi: 10.3389/fnbeh.2014.00199

Abstract

Decreased serotonergic activity has been implicated in anxiety and major depression, and antidepressants directly or indirectly increase the long-term activity of the serotonin system. A key component of serotonin circuitry is the 5-HT1A autoreceptor, which functions as the major somatodendritic autoreceptor to negatively regulate the "gain" of the serotonin system. In addition, 5-HT1A heteroreceptors are abundantly expressed post-synaptically in the prefrontal cortex (PFC), amygdala, and hippocampus to mediate serotonin actions on fear, anxiety, stress, and cognition. Importantly, in the PFC 5-HT1A heteroreceptors are expressed on at least two antagonist neuronal populations: excitatory pyramidal neurons and inhibitory interneurons. Rodent models implicate the 5-HT1A receptor in anxiety- and depression-like phenotypes with distinct roles for pre- and post-synaptic 5-HT1A receptors. In this review, we present a model of serotonin-PFC circuitry that integrates evidence from mouse genetic models of anxiety and depression involving knockout, suppression, over-expression, or mutation of genes of the serotonin system including 5-HT1A receptors. The model postulates that behavioral phenotype shifts as serotonin activity increases from none (depressed/aggressive not anxious) to low (anxious/depressed) to high (anxious, not depressed). We identify a set of conserved transcription factors including Deaf1, Freud-1/CC2D1A, Freud-2/CC2D1B and glucocorticoid receptors that may confer deleterious regional changes in 5-HT1A receptors in depression, and how future treatments could target these mechanisms. Further studies to specifically test the roles and regulation of pyramidal vs. interneuronal populations of 5-HT receptors are needed better understand the role of serotonin in anxiety and depression and to devise more effective targeted therapeutic approaches.

Sex-dependent adaptive changes in serotonin-1A autoreceptor function and anxiety in Deaf1-deficient mice

Christine Luckhart1,2, Tristan J. Philippe1,2, Brice Le François1,2, Faranak Vahid-Ansari1,2, Sean D. Geddes2, Jean-Claude Beique2, Diane C. Lagace2, Mireille Daigle1, Paul R. Albert1,2*

1 OHRI (Neuroscience) and 2 Department of Cellular and Molecular Medicine
*University of Ottawa Brain and Mind Research Institute
451 Smyth Road
Ottawa, ON Canada K1H-8M5
Molecular Brain 2016 9:77,DOI: 10.1186/s13041-016-0254-y

Abstract

The C (-1019) G rs6295 promoter polymorphism of the serotonin-1A (5-HT1A) receptor gene is associated with major depression in several but not all studies, suggesting that compensatory mechanisms mediate resilience. The rs6295 risk allele prevents binding of the repressor Deaf1 increasing 5-HT1A receptor gene transcription, and the Deaf1-/- mouse model shows an increase in 5-HT1A autoreceptor expression. In this study, Deaf1-/- mice bred on a mixed C57BL6-BALB/c background were compared to wild-type littermates for 5-HT1A autoreceptor function and behavior in males and females. Despite a sustained increase in 5-HT1A autoreceptor binding levels, the amplitude of the 5-HT1A autoreceptor-mediated current in 5-HT neurons was unaltered in Deaf1-/- mice, suggesting compensatory changes in receptor function. Consistent with increased 5-HT1A autoreceptor function in vivo, hypothermia induced by the 5-HT1A agonist DPAT was augmented in early generation male but not female Deaf1-/- mice, but was reduced with succeeding generations. Loss of Deaf1 resulted in a mild anxiety phenotype that was sex-and test-dependent, with no change in depression-like behavior. Male Deaf1 knockout mice displayed anxiety-like behavior in the open field and light-dark tests, while female Deaf1-/- mice showed increased anxiety only in the elevated plus maze. These data show that altered 5-HT1A autoreceptor regulation in male Deaf1-/- mice can be compensated for by generational adaptation of receptor response that may help to normalize behavior. The sex dependence of Deaf1 function in mice is consistent with a greater role for 5-HT1A autoreceptors in sensitivity to depression in men.

Deaf1-dependent 5-HT1A receptor gene regulation by MeCP2 controls raphe 5-HT1A autoreceptor activity: MeCP2 regulates 5-HT1A receptor gene expression via a novel Deaf1-dependent methylation-insensitive mechanism

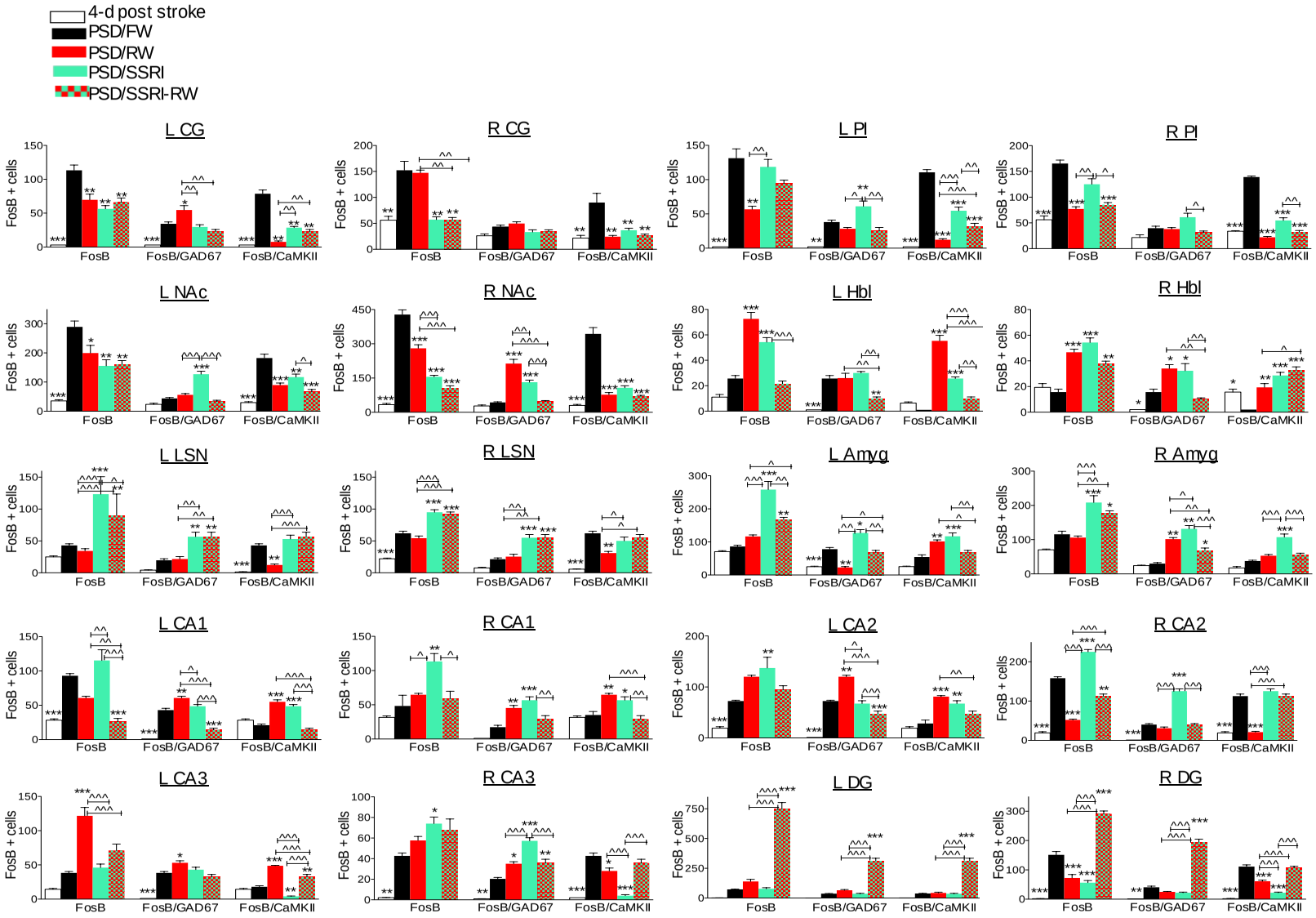
Tristan J. Philippe1,2, Brice Le François1,2, Zoe Donaldson3,4, Faranak Vahid-Ansari1,2 , Valérie Cardin1,2, Mireille Daigle1, Rene Hen3,4, Paul R. Albert1,2*

1OHRI (Neuroscience) and 2Department of Cellular and Molecular Medicine
University of Ottawa Brain and Mind Research Institute Ottawa, ON Canada
3New York State Psychiatric Institute, and 4Department of Psychiatry, Columbia University New York, NY, USA
(under preparatiion)

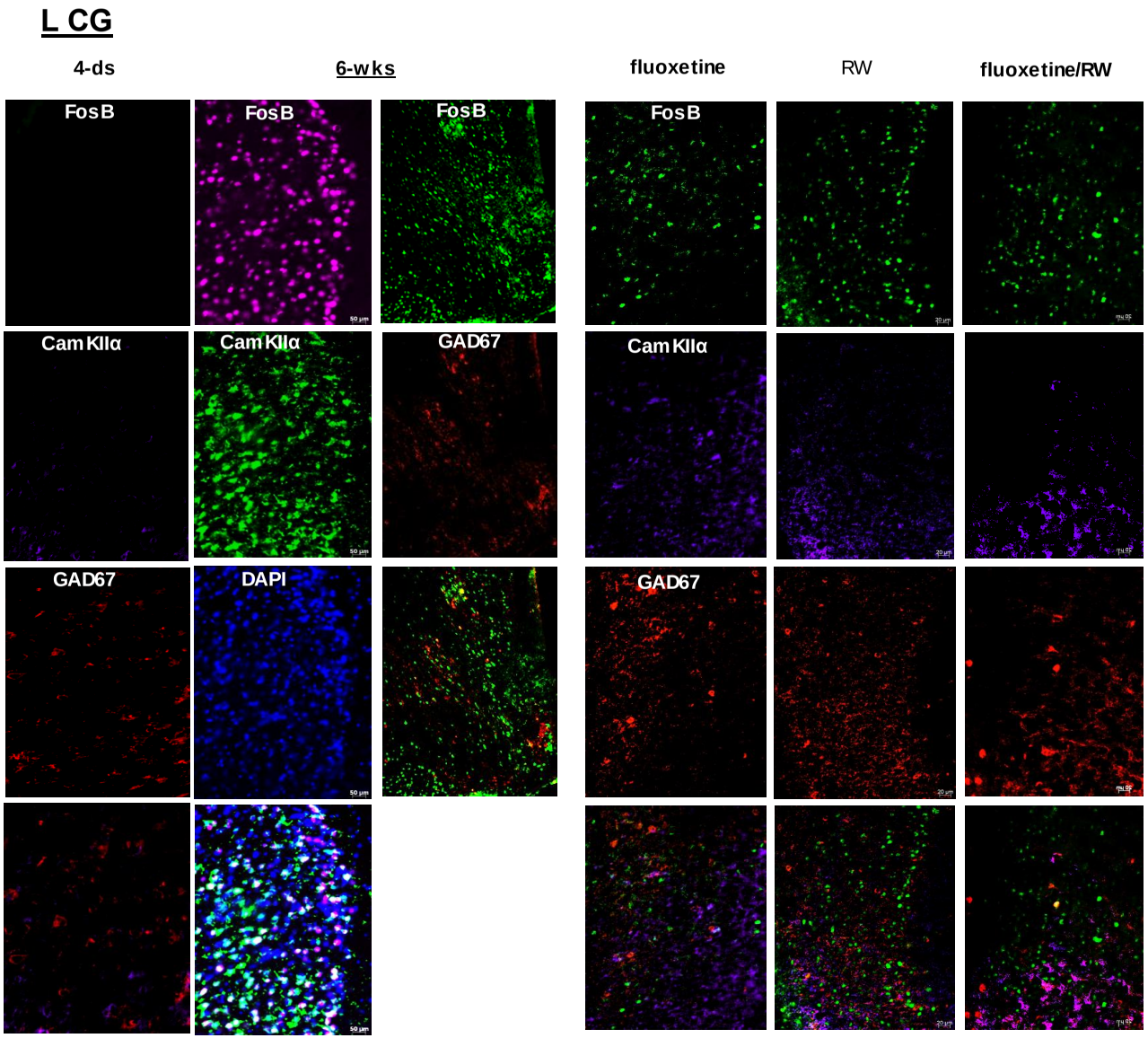
Abstract

The 5-HT1A autoreceptor mediates feedback inhibition of 5-HT activity, and is implicated in major depression. The human 5-HT1A gene (HTR1A) rs6295 G-(1019) risk allele has been associated with depression and prevents Deaf1 from binding the HTR1A promoter and regulating its transcription. Stress also contributes to depression and results in altered 5-HT1A gene methylation. We hypothesized that methyl-binding protein, MeCP2, and Deaf1 might coordinately regulate the HTR1A gene at the Deaf1 site. We used a yeast one-hybrid system to show that MeCP2 enhances Deaf1 transactivation at the human Deaf1 site, and can itself transactivate the human Deaf1 site upon methylation. Co-immunoprecipitation studies showed that MeCP2 and Deaf1 proteins interact. Chromatin immunoprecipitation assays showed that both Deaf1 and MeCP2 bind to the mouse Deaf1 site in wild-type but not Deaf1-knockout fibroblasts or brain tissues, indicating a Deaf1-dependent recruitment of MeCP2 to the 5-HT1A promoter. In reporter assays, MeCP2 modulated human and mouse HTR1A gene expression in a Deaf1-dependent fashion, promoting Deaf1-induced repression at the Deaf1 site. To address its role in vivo, mice with conditional knockout of MeCP2 in 5-HT neurons were generated. These mice exhibited increased 5-HT1A autoreceptor levels and function, consistent with the importance of MeCP2 in enhancing Deaf1 repression in serotonin neurons. These data uncover a novel Deaf1-dependent mechanism for MeCP2 and its role in repression of HTR1A transcription. Thus MeCP2 displays both Deaf1-dependent/methylation-independent and Deaf1-independent/methylation-sensitive activity that could alter stress-induced regulation of genes implicated in risk for depression.

1A

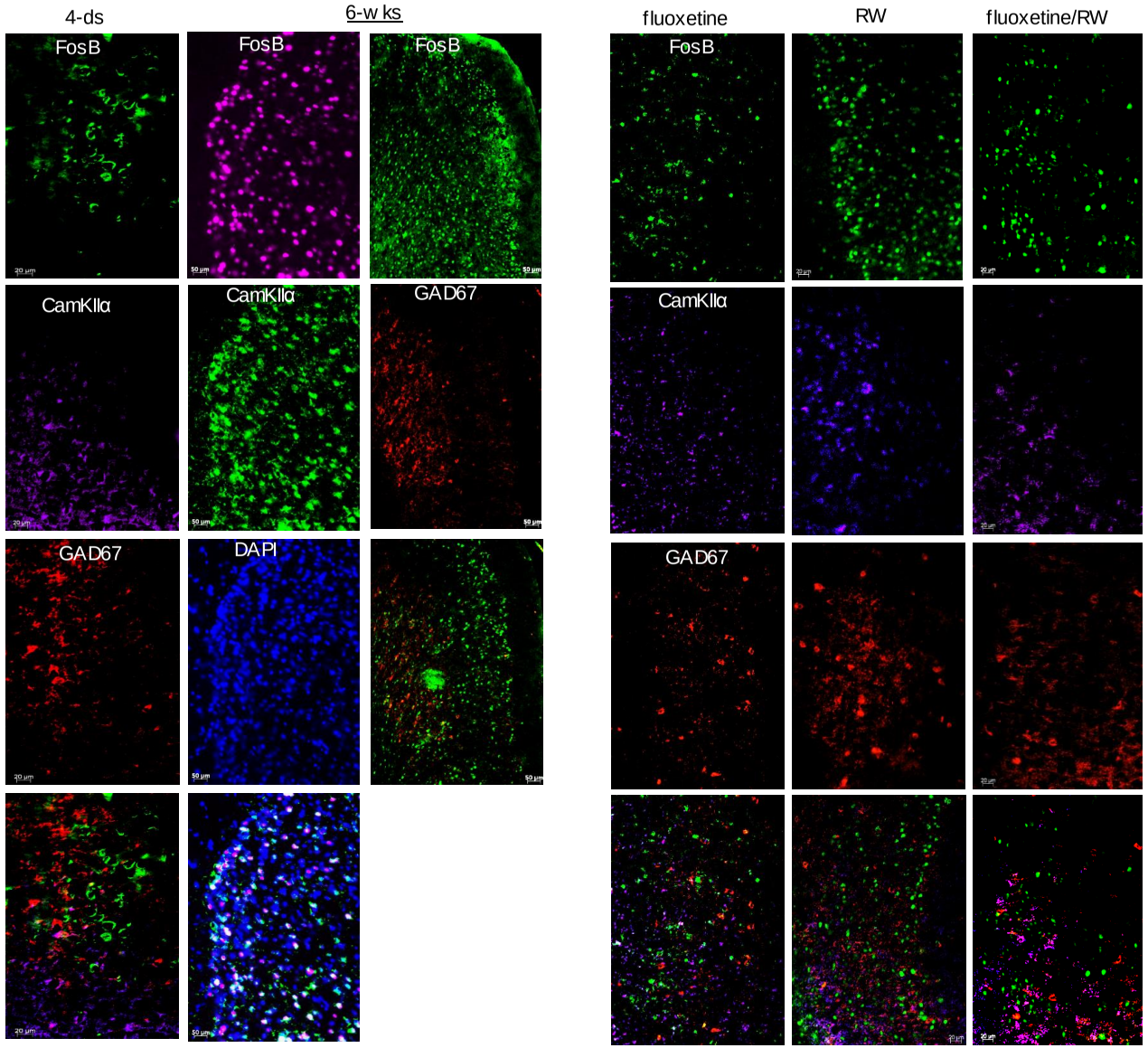

4-d post stroke
PSD/FW
PSD/RW
PSD/SSRI
PSD/SSRI-RW
L CG
R CG
L PI
R PI
L NAc
R NAc
L Hbl
R Hbl
L LSN
R LSN
L Amyg
R Amyg
L CA1
R CA1
L CA2
R CA2
L CA3
R CA3
L DG
R DG
FosB + cells
FosB
FosB/GAD67
FosB/CaMKII

1B-1

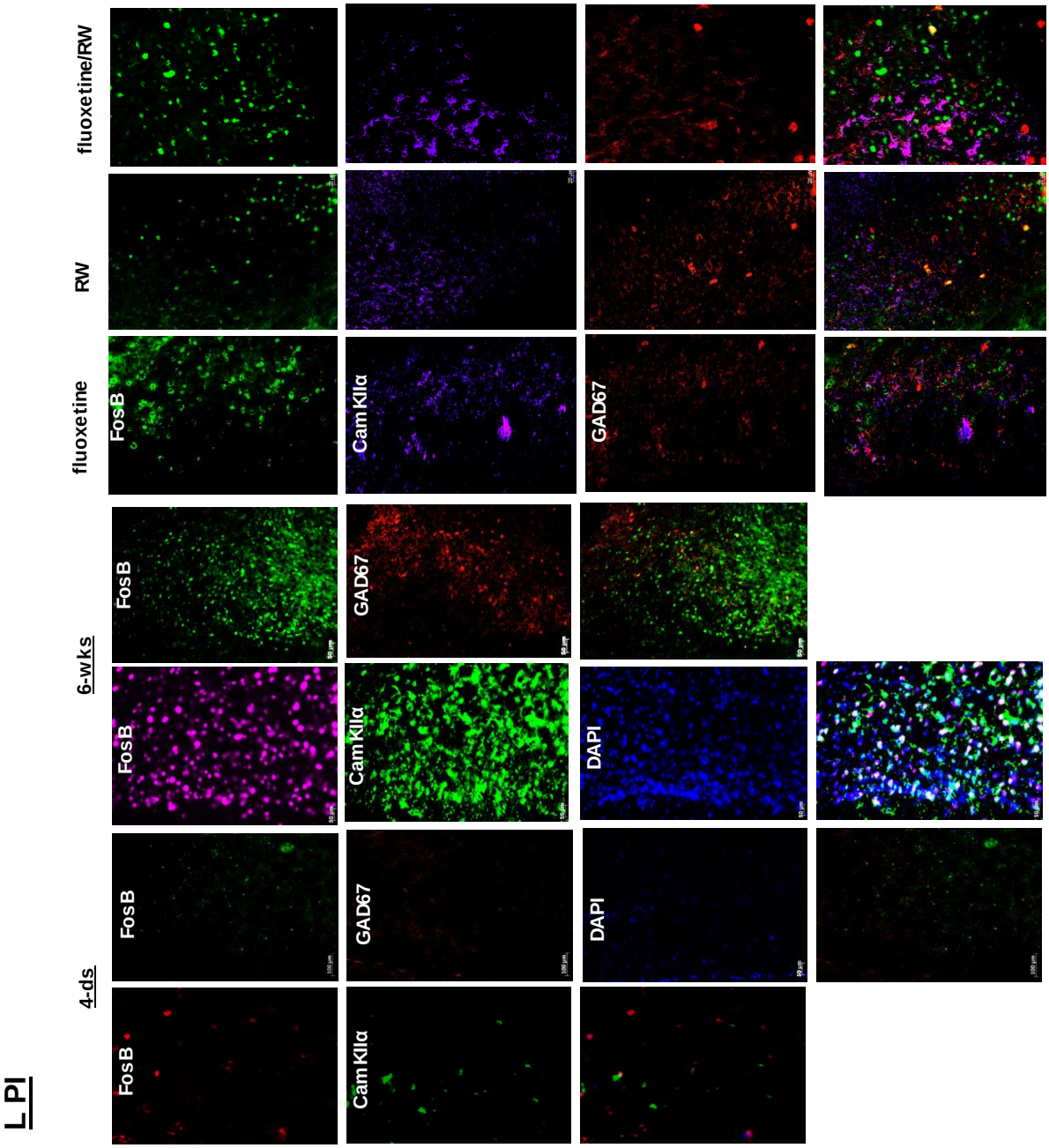

L CG
4-ds
6-wks
fluoxetine
RW
fluoxetine/RW
FosB
FosB
FosB
FosB
CamKIIα
CamKIIα
GAD67
CamKIIα
GAD67
DAPI
GAD67

1B-2

R CG

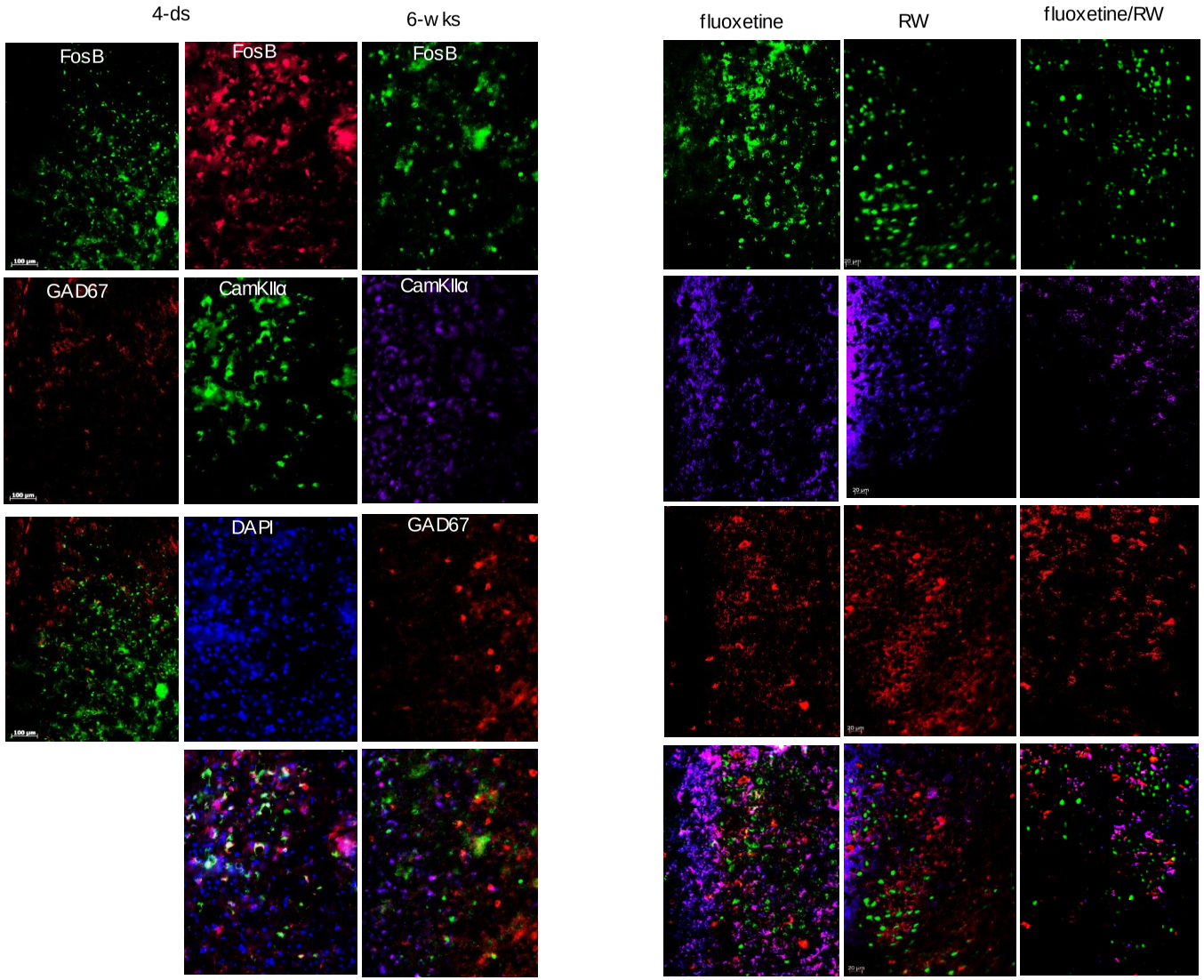

4-ds
6-w ks
fluoxetine
RW
fluoxetine/RW
FosB
FosB
FosB
FosB
CamKIIα
CamKIIα
GAD67
CamKIIα
GAD67
DAPI
GAD67

1B-3


L PI
4-ds
6-wks
fluoxetine
RW
fluoxetine/RW
FosB
CamKIIα
GAD67
DAPI

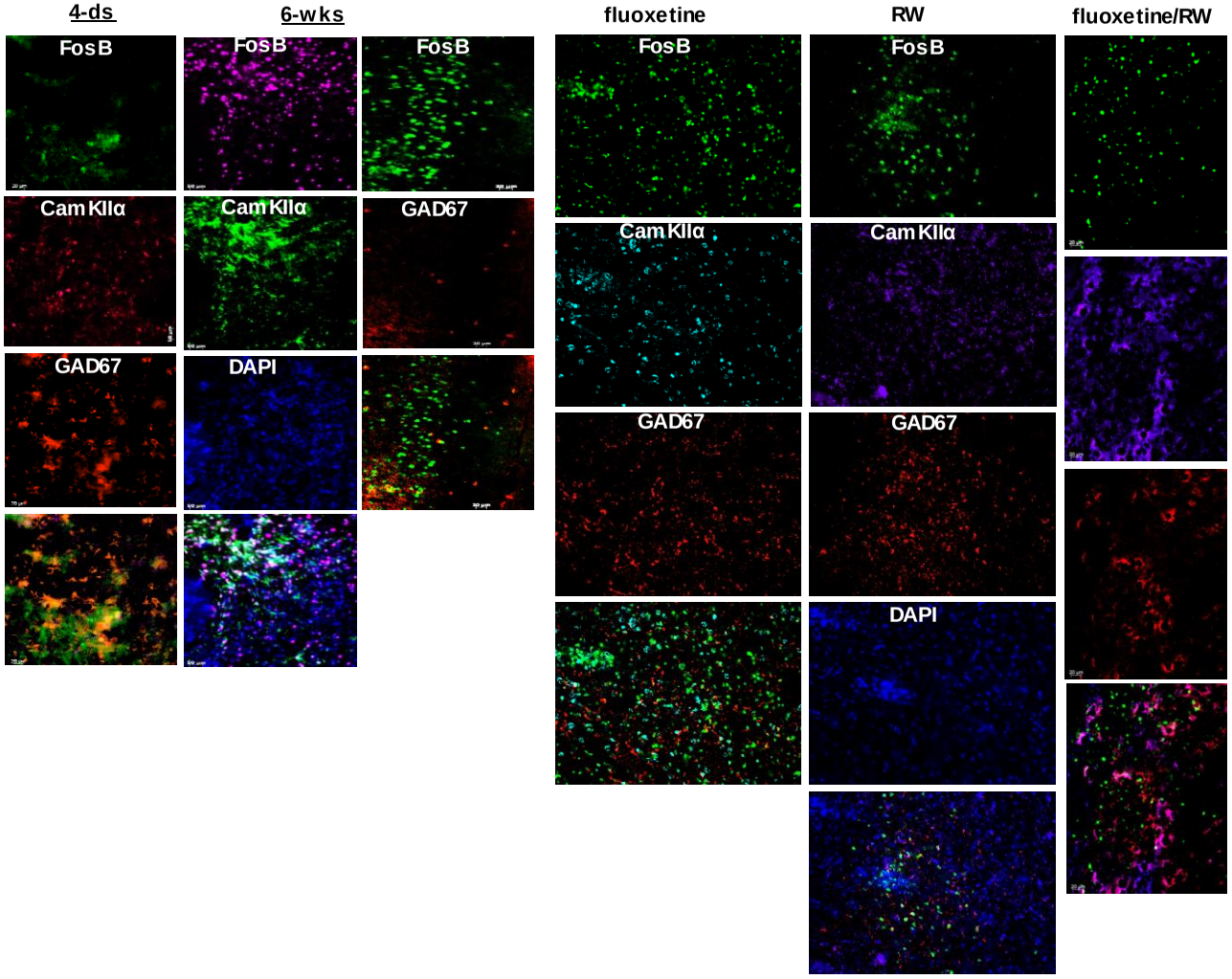
1B-4

R PI


4-ds
6-w ks
fluoxetine
RW
fluoxetine/RW
FosB
FosB
FosB
GAD67
CamKIIα
CamKIIα
DAPI
GAD67

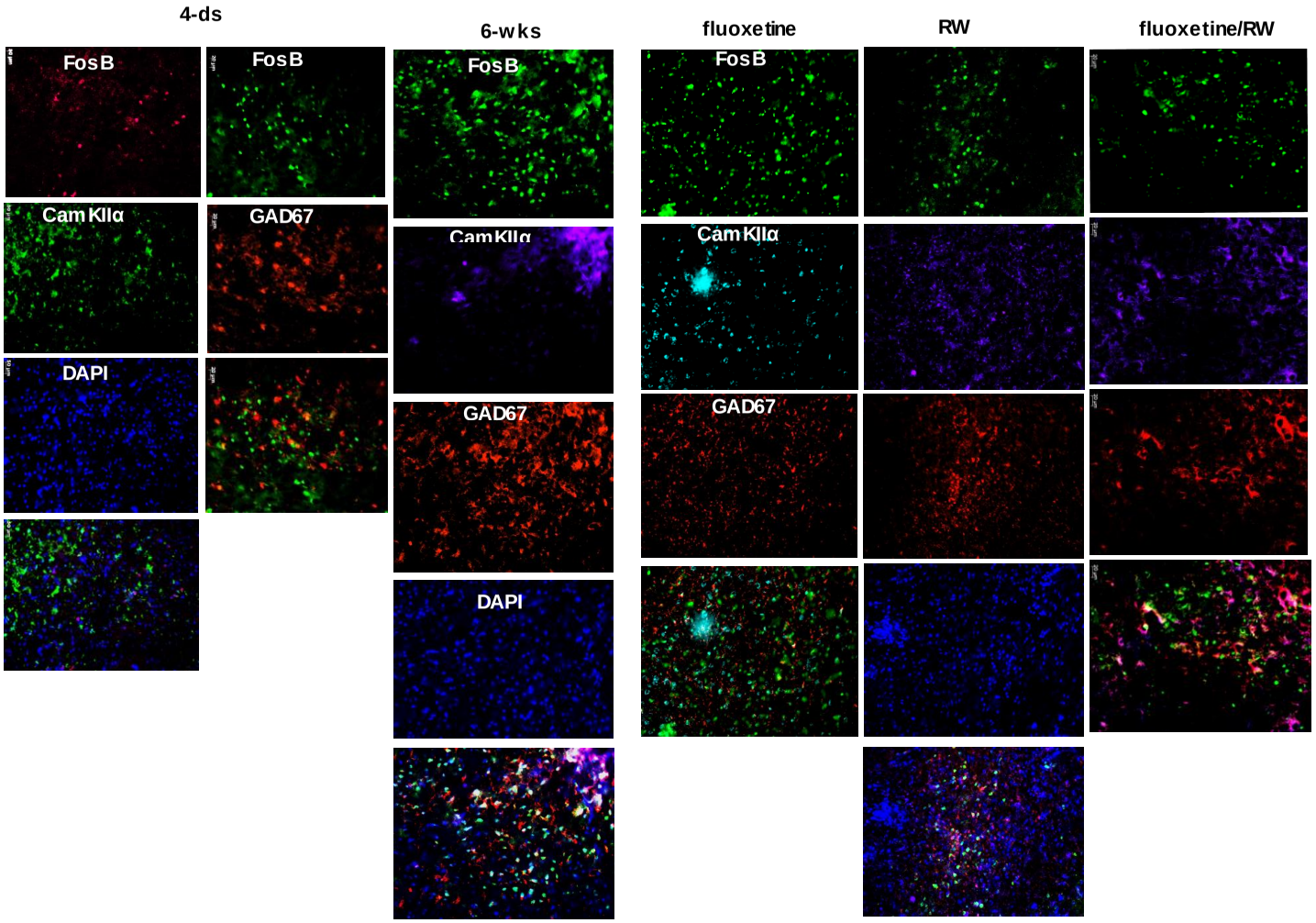
1B-5

L NAc


4-ds
6-wks
fluoxetine
RW
fluoxetine/RW
FosB
CamKIIα
GAD67
DAPI

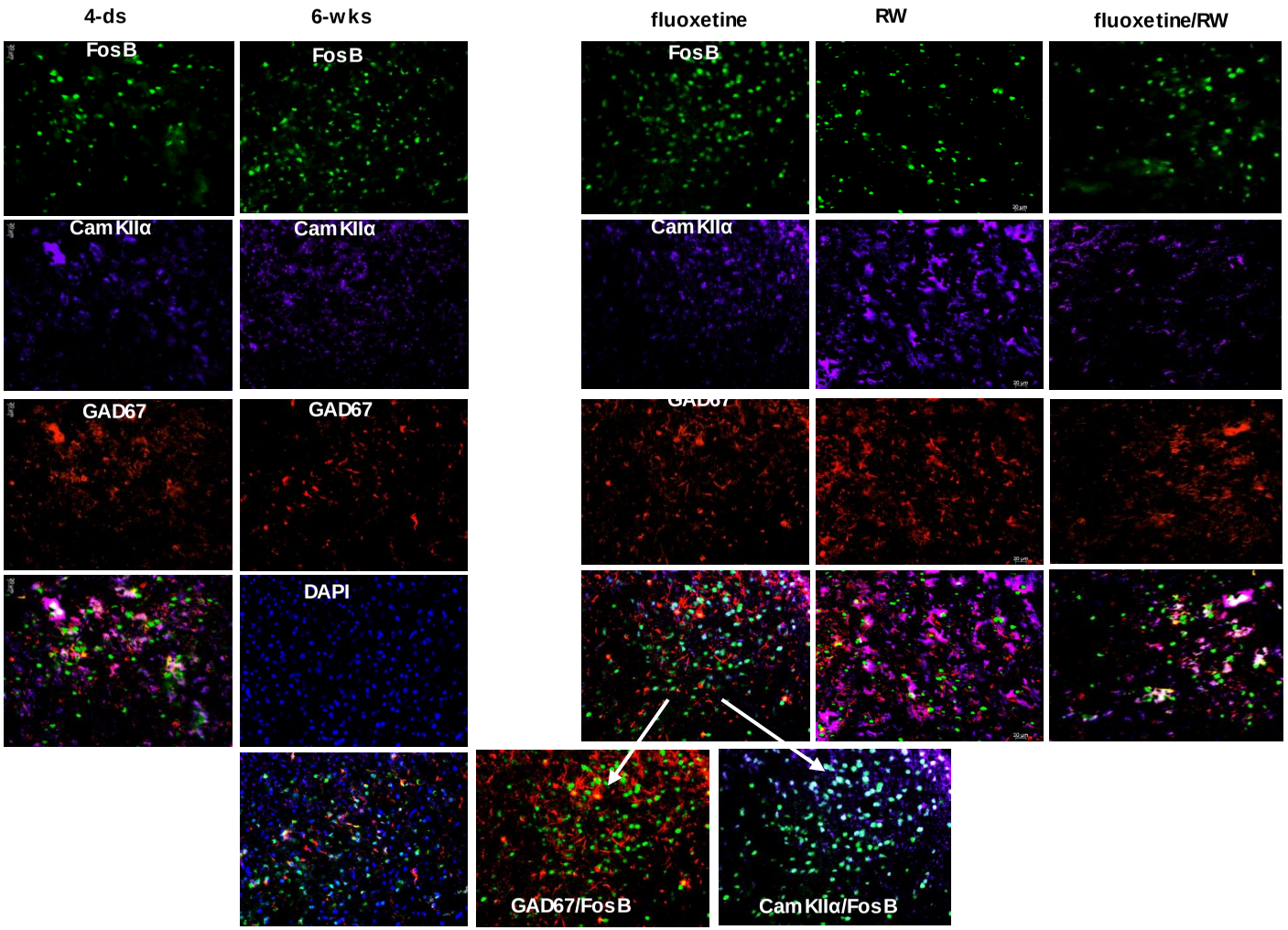
1B-6

R NAc


4-ds
6-wks
fluoxetine
RW
fluoxetine/RW
FosB
FosB
FosB
FosB
CamKIIα
GAD67
CamKIIα
CamKIIα
DAPI
GAD67
GAD67
DAPI

1B-7

L amygdala



1B-8

R amygdala

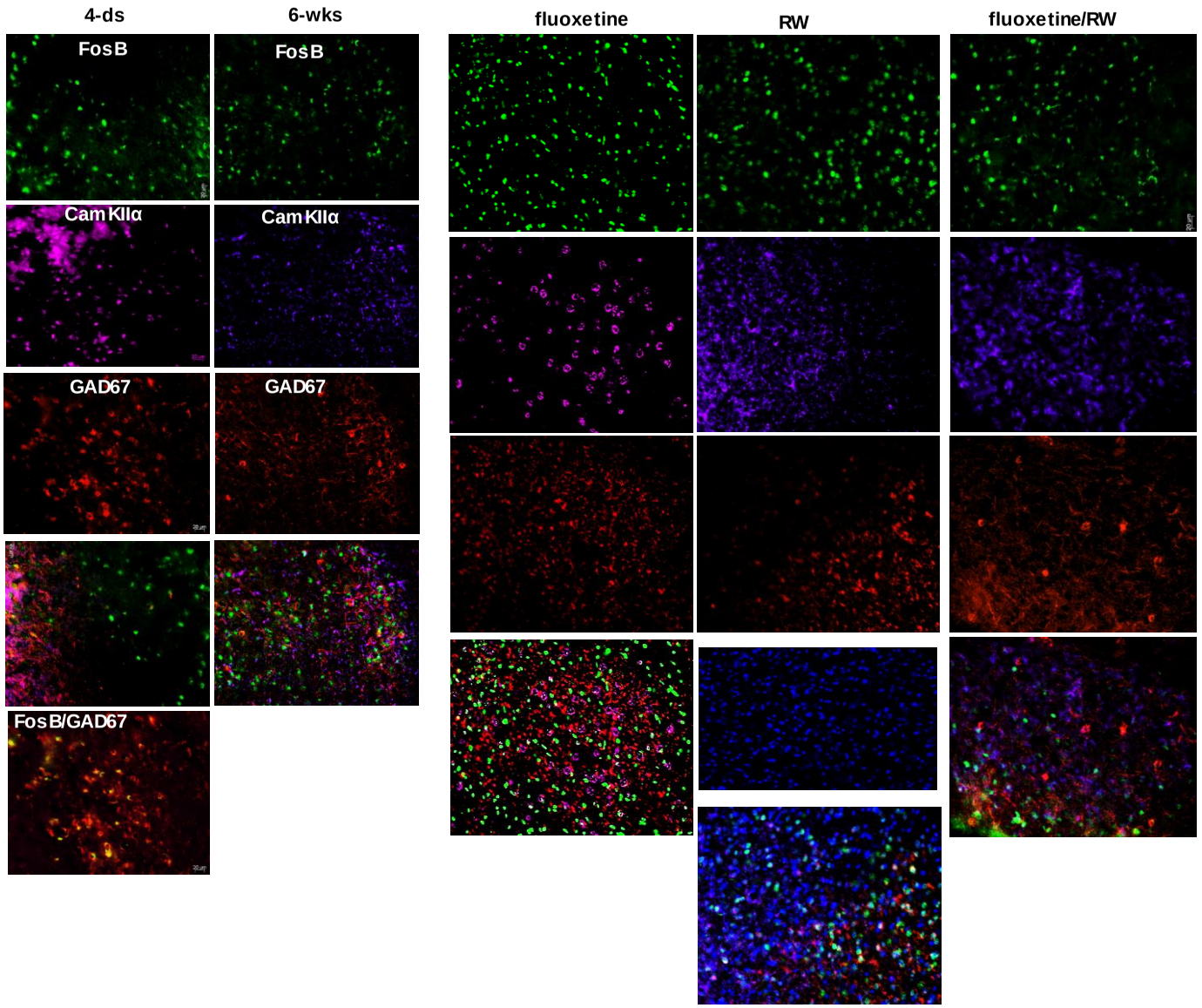


Figure 1. Comparisons of co-localization of mean number (±SEM) of FosB-immunoreactive nuclei with GAD67or CaMKIIα-vGLUT3 positive cells per mm^2 in brain areas of PSD and treated PSD.

Fig. 1A presents the quantifications of FosB+ cells in different brain regions of 4 days post stroke, 6 weeks post stroke (PSD) and treated PSD (RW, voluntary exercise vs fluoxetine vs fluoxetine/RW) groups.

Fig. 1B shows the images obtained from the regions with FosB+ cells. B1-2, L and R cingulate gyrus (CG); B3-4, L and R pre/infra limbic (PI); B5-6, L and R nucleus accumbence (NAc); B7-8, L and R amygdala.

Data represent mean ± SEM in n=4/group, *$p<0.05$, **$p<0.01$, two way ANOVA vs other group as lines show.

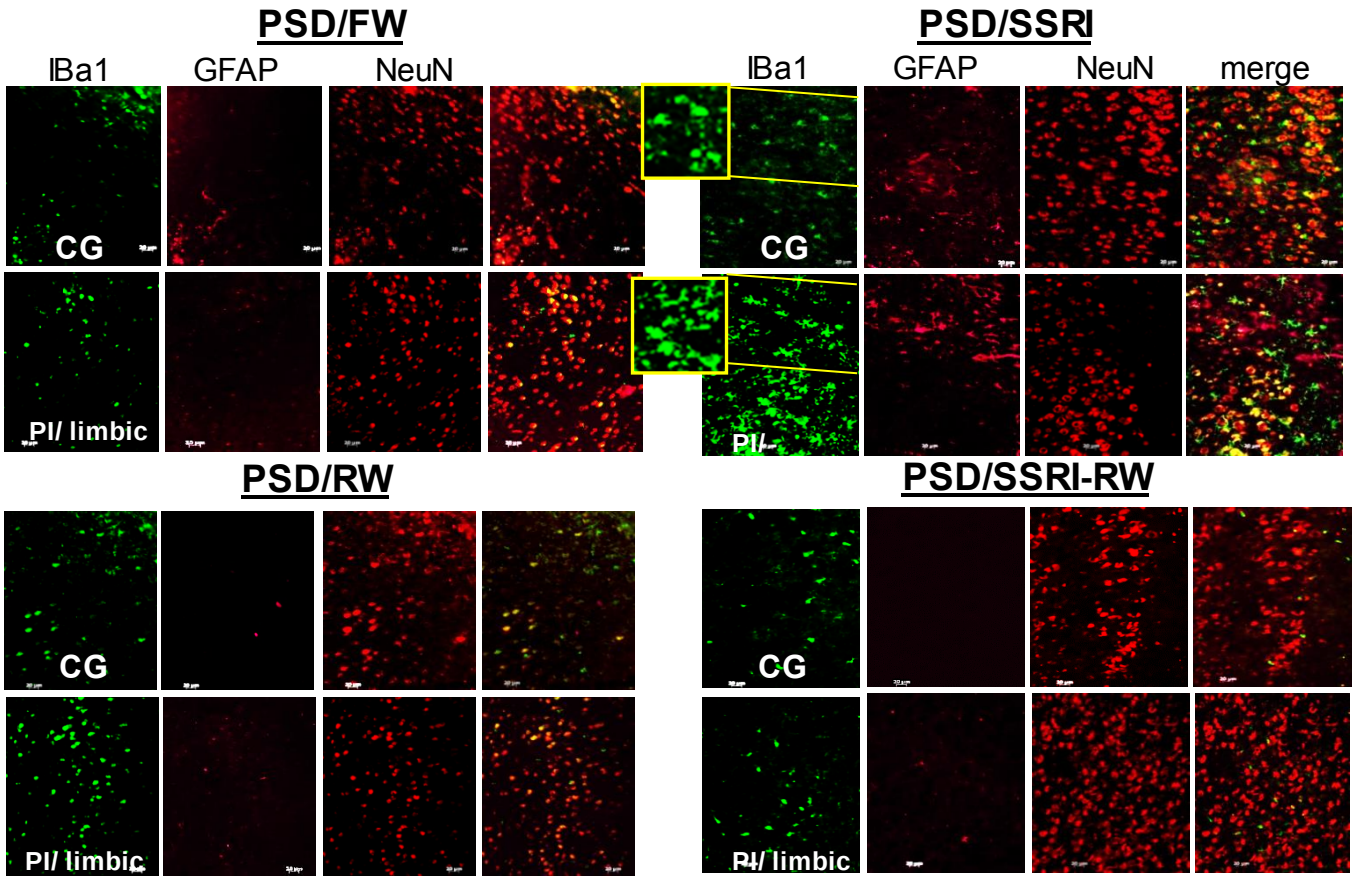


Figure 2. Chronic treatment-induced higher glial activation at the lesion site. Sections of the left mPFC were examined by immunofluorescence at the cingulate gyrus (CG) and pre- and infra-limbic (P/I limbic) cortex from PSD mice chronically treated with FW, RW, SSRI and SSRI-RW. Representative images of sections stained with anti-IBa1 (microglia), GFAP (astrocyte) and NeuN (neurons). In all groups, the lesion site had NeuN-expressing cells. In PSD/FW and PSD/RW and PSD/SSRI-RW some scattered IBa1 (yellow square, 40X) and GFAP positive cells were also observed. In contrast, in PSD/SSRI, IBa1 and GFAP positive cells are morphologically presented in active state and located closely to NeuN positive cells (merge image, yellow colored cells).
Images were taken under 20X magnification, Scale bar, 20 µm.