

ESTRADIOL-INDUCED DESENSITIZATION OF 5-HT_{1A} RECEPTOR SIGNALING

By

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ABSTRACT

Depression is a common psychiatric illness, affecting over 120 million people worldwide. Women are affected disproportionately compared to men, and a large body of clinical evidence suggests a role for changes in estrogen levels in the etiology of depression. Successful selective serotonin reuptake inhibitor (SSRI) antidepressant treatment is frequently correlated with normalization of HPA axis activity. It can take several weeks to begin to see therapeutic effects of SSRIs; this therapeutic lag is thought to be due in part to the time it takes for desensitization of 5-HT_{1A} receptor signaling in the paraventricular nucleus (PVN) of the hypothalamus to occur.

It takes up to seven days of chronic SSRI treatment to desensitize 5-HT_{1A}R signaling, but this effect is accelerated by estradiol (EB) treatment. Understanding estradiol modulation of 5-HT_{1A}R signaling will be important for the development of improved SSRI therapy for the treatment of depression. The purpose of this dissertation therefore was to identify the mechanisms underlying EB-induced desensitization of 5-HT_{1A}R signaling. To test the hypothesis that signaling through GPR30 is necessary for EB-induced desensitization of 5-HT_{1A}R signaling, GPR30 protein expression in the PVN was knocked down via adenoviral vector delivery of siRNA against GPR30. Reduction of GPR30 protein expression prevented EB-induced desensitization of 5-HT_{1A}R signaling. To test whether stimulation of GPR30 is sufficient for desensitization of 5-HT_{1A}R signaling, rats were treated for two days with systemic injections of the selective GPR30 agonist G-1 or EB. G-1 and EB treatment both reduced the hormone responses to 5-HT_{1A}R stimulation.

To investigate the effects of GPR30 stimulation on 5-HT_{1A}R signaling at the molecular level, changes in protein and mRNA levels of 5-HT_{1A}R, G α _z, GPR30, and RGSz1 were

examined after EB and G-1 treatment. EB treatment produced a decrease in 5-HT_{1A}R protein, while both EB and G-1 treatment increased RGSz1 mRNA and altered expression of several RGSz1 proteins, leading to the hypothesis that alteration in RGSz1 expression and posttranslational modification underlies estradiol-induced desensitization of 5-HT_{1A}R signaling. In particular, EB and G-1 treatment increased localization of sumoylated and glycosylated RGSz1 in the detergent resistant microdomain of the plasma membrane, where it could physically interact with and inactivate Gαz protein. The effects of GPR30 signaling, such as a decrease in 5-HT_{1A}R protein and increase of RGSz1 isoforms, on the 5-HT_{1A}R signaling pathway are not seen after SSRI treatment, suggesting a mechanism by which estradiol acts separately and synergistically with SSRIs to accelerate desensitization of 5-HT_{1A}R signaling. Improving the therapeutic efficacy of SSRIs through selective targeting of GPR30 and RGSz1 could have important clinical relevance for the treatment of depression.

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LIST OF ABBREVIATIONS

(+)8-OH-DPAT	(+)8-Hydroxy-2-dipropylaminotetralin
3V	third ventricle
5-HT	5- hydroxytryptamine (serotonin)
5-HT _{1A} R	serotonin _{1A} receptor
AC	adenylyl cyclase
ACTH	adrenocorticotrophic hormone
Ad	adtrack
ANOVA	analysis of variance
AP	anterior/posterior
AP-1	activator protein-1
AVP	arginine vasopressin
BCA	bicinchoninic acid
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB	cAMP-responsive element binding protein
CRF	corticotrophin releasing hormone
CTX	cortex
DEP	disheveled, Egl-10, and Pleckstrin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPN	diarylpropionitrile
DRM	detergent-resistant microdomain
DRN	dorsal raphe nucleus
DV	dorsal/ventral
E2	17 β -estradiol
EB	17 β -estradiol-3-benzoate
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	estrogen receptor

ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ERK1/2	extracellular signal-regulated kinase 1 and 2
FLX	fluoxetine
FST	forced swim test
G protein	guanine nucleotide-binding protein
G-1	4-(6-Bromo-benzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro- 3Hcyclopenta[c]quinoline
GAIP	G α interacting protein
GAP	GTP-activating protein
GFP	green fluorescent protein
GGL	G protein gamma subunit-like
GIPN	GAIP interacting protein N-terminus
GIRK	inward rectifying potassium channel
GPER	G protein coupled estrogen receptor
GPR30	G protein coupled receptor 30
GR	glucocorticoids receptor
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HEK 293	human embryonic kidney
HPA	hypothalamic pituitary adrenal
HPC	hippocampus
HRT	hormone replacement therapy
HTH	hypothalamus
IP	immunoprecipitation
ip	intraperitoneal
IRES	internal ribosome entry site
MAOI	monoamine oxidase inhibitor
MAPK	mitogen activated protein kinase
MB	midbrain
mis	missense

ML	medial/lateral
MMP	matrix metalloproteinase
MR	mineralocorticoid receptor
mRNA	messenger RNA
NEM	N-ethylmaleimide
NMDA	N-methyl-D-aspartate
OT	oxytocin
OVX	ovariectomized
PCR	polymerase chain reaction
PI3K	phosphatidylinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
POMC	pro-opiomelanocortin
PTX	pertussis toxin
PVDF	polyvinylidene fluoride
PVN	paraventricular nucleus
qPCR	quantitative PCR
RET-RGS	retinal-RGS
RFP	red fluorescent protein
RGS	regulator of G protein signaling
RNA	ribonucleic acid
Sc	subcutaneous
SD	Sprague-Dawley
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERT	serotonin transporter
SIM	SUMO interacting motif
siRNA	small interfering RNA
SNRI	serotonin norepinephrine reuptake inhibitor

SON	supraoptic nucleus
SSRI	selective serotonin reuptake inhibitor
SUMO	small ubiquitin-like modifier
TBP	TATA box binding protein
TBS	Tris-buffered saline
TCA	tricyclic antidepressant
TPH	tryptophan hydroxylase

CHAPTER ONE: INTRODUCTION

DEPRESSION

Depression, clinically known as Major Depressive Disorder, is one of the most common psychiatric illnesses, with a lifetime prevalence of greater than 17% in the general population (Anxiety and Depression Association of America). Depression is characterized by depressed mood, a loss of interest or pleasure in daily activities, impaired function in everyday life, fatigue or loss of energy, feelings of guilt and worthlessness, poor concentration, and changes in sleep, appetite, or weight (DSM-IV). Because depression can lead to substantial impairment of daily functions, it is a leading cause of disability worldwide. Fortunately, depression can be reliably diagnosed in primary care, and there are many pharmacotherapeutic treatments available.

Sex differences in depression

The prevalence of depression in women is twice as high as in men, although this higher rate is not constant across the lifespan: during childhood or advanced old age, there is little difference in rates of depression between males and females, but this difference increases towards mid-life in women without changing in men¹⁻⁴. Some studies have found that women also experience longer depressive episodes than men, although this finding is not consistent. Women are more prone to depression during times of ovarian hormone fluctuation, such as puberty, the postpartum period, the premenstrual phase of the menstrual cycle, and perimenopause^{1,5-7}. Because the increase in onset of depression in women corresponds to the female reproductive life, it has been hypothesized that alterations in ovarian sex steroids, particularly estrogens, during childbearing years may contribute to the higher rate of mood disorders in women⁸.

Treatments of depression

Serotonergic modulators

Serotonin dysfunction has been implicated in the etiology of depression⁹⁻¹⁴. Serotonin neurotransmission is a balance of several processes: synthesis, reuptake, degradation, transmitter release, and receptor activation. Synthesis is a two-step process of converting the amino acid tryptophan to 5-hydroxytryptamine (5-HT), governed by the rate-limiting enzyme tryptophan hydroxylase (TPH). Free serotonin that has not been packaged into vesicles is degraded by monoamine oxidase A (MAO-A), but most of the synaptic clearance is accomplished by the serotonin transporter (SERT, also called 5-HTT). Treatments for depression that modulate serotonin transmission target these processes.

Antidepressant drugs are classified as first or second generation pharmacotherapeutics. First generation drugs include MAO inhibitors (MAOIs) and tricyclic antidepressants (TCAs). The oldest antidepressants, MAOIs, prevent MAO degradation of serotonin, norepinephrine, and dopamine, thus increasing the amount of neurotransmitter available for release. The first MAOIs were irreversible; newer MAOIs are reversible and can be selective for MAO-A, which preferentially deaminates serotonin, norepinephrine, and epinephrine with fewer side effects than the older drugs. MAOIs often work well for patients who are treatment-resistant; however, concerns over potentially lethal dietary and drug interactions have limited clinical MAOI use. Recent research suggests that these concerns may be based largely on misinformation and outdated research, and the potential use of these drugs for treatment of depressed should be reexamined¹⁵.

TCAs, named for the characteristic three-ring structure shared by compounds in this class, are highly effective antidepressants that bind to presynaptic transporters to inhibit

neurotransmitter reuptake. Many drugs of this class are equally effective at inhibiting both norepinephrine and serotonin reuptake, though some are more effective on one transmitter than the other. In addition to blocking reuptake, many TCAs also block acetylcholine, histamine, and α -adrenergic receptors, thus contributing to their severe side effects.

Second generation antidepressants were designed to be more selective in their action and thus avoid the anticholinergic and cardiovascular effects produced by MAOIs and TCAs. Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine, and citalopram are the most commonly prescribed antidepressants. SSRIs are more selective than TCAs in blocking serotonin reuptake and although they are not much more effective, they are better tolerated. Selective norepinephrine/serotonin reuptake inhibitors (SNRIs) such as venlafaxine are several fold more selective for serotonin than norepinephrine¹⁶.

Problems with current antidepressants

One major drawback to antidepressants that modulate serotonin transmission is the risk of serotonin toxicity. Serotonin toxicity is a dangerous syndrome which can result from use of serotonergic antidepressants in combination with other serotonergic drugs or a diet high in foods that elevate tyramine levels. Onset of serotonin toxicity is rapid (within 24 hours) and features confusion, hypomania, restlessness, myoclonus, hyperreflexia, diaphoresis, shivering, tremor, diarrhea, or incoordination¹⁷.

The two major problems with all antidepressant drugs are 1) that only approximately two thirds of patients respond to treatment, and 2) therapeutic efficacy requires chronic treatment that can take up to 12 weeks to produce¹⁶. In order to improve response and remission rates, other therapies can be used as adjuvants to SSRI treatment, such as lithium, atypical antipsychotics,

electroconvulsive therapy, or deep brain stimulation¹⁸⁻²⁰. However, some of these treatments can be aggressive, and do not improve rates of remission²¹.

SEROTONIN

Serotonin receptors

Serotonin is a monoamine neurotransmitter that acts on the central nervous system (CNS) and the peripheral nervous system (PNS), as well as in non-neuronal tissues such as the gastrointestinal tract, platelets, and the cardiovascular system²². Serotonin is synthesized from the amino acid tryptophan in serotonergic neurons, which are concentrated in the medulla, pons, and the dorsal and medial raphe nuclei of the midbrain. Serotonergic neurons project to almost every area of the forebrain, including the cerebral cortex, hippocampus, limbic system, and hypothalamus. Because of its wide distribution, serotonin has important roles in many physiological functions, including sleep, feeding, sexual behavior, and thermoregulation, and has been implicated in pathological states, such as mood disorders, anxiety disorders, and psychosis²².

Serotonin produces its effects through a large and diverse family of membrane-bound receptors. The serotonin receptor family is one of the largest and most complex neurotransmitter receptor families, containing 7 different subfamilies (5-HT₁R - 5-HT₇R) and at least 14 distinct members^{23,24}. The different receptor subtypes are classified according to pharmacological characteristics, intracellular signal transduction mechanisms, and structural characteristics^{22,25}. The majority of 5-HT receptors are metabotropic G protein coupled receptors (GPCRs). GPCRs couple to heterotrimeric G proteins; upon stimulation of the receptor with a ligand or agonist, the GDP molecule bound to the G α subunit exchanges with GTP, thus activating the G protein.

Activated GTP-G α then dissociates from the G $\beta\gamma$ protein subunit, leaving both free to activate downstream effectors. Hydrolysis of GTP-G α to GDP-G α resets the signaling system.

The 5-HT₁R subfamily members preferentially couple to the Gi/o family of inhibitory G proteins to inhibit adenylyl cyclase (AC) and reduce cAMP production and protein kinase A (PKA) activation. This family includes 5 members: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} (putative), and 5-HT_{1F}. The 5-HT₂R subfamily contains 3 members, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, all coupled to the Gq/11 signaling system to stimulate the phospholipase C (PLC) signaling cascade and increase intracellular calcium signaling^{22,26}.

5-HT₃R is the only ionotropic (ligand-gated ion channel) 5-HT receptor subtype. 5-HT₃ receptors are non-selective cation channels, which produce a rapid excitatory response in neurons due to influx of Na⁺ and Ca⁺, and efflux of K⁺. Functional channels may be homopentameric, consisting of five 5-HT_{3A}R subunits, or heteropentameric, a mixture of 5-HT_{3A}R subunits plus 5-HT_{3B}, 3C, 3D, or 3E subunits^{27,28}.

5-HT₄, 5-HT₆, and 5-HT₇ receptors all preferentially couple to G α s proteins to activate AC and increase cAMP production and protein kinase A (PKA) activation. 5-HT₄Rs have a wide distribution outside of the CNS, being found in the heart, intestine, bladder, and kidney. 5-HT₇Rs are expressed in the hypothalamus, hippocampus, cerebral cortex, amygdala, and dorsal raphe^{29,30}, and play an important role in the control of circadian rhythms, thermoregulation, and learning and memory³¹.

The 5-HT₅R subfamily (A and B) remains largely undescribed, although current evidence suggests coupling to Gi/o and inhibition of AC, similar to 5-HT₁ receptors^{32,33}. Recent work has shown that 5-HT_{5A}R maintains 5-HT currents in the absence of 5-HT_{1A}R and 5-HT₂R signaling

in the prefrontal cortex, via coupling to inward rectifying K⁺ channels (GIRKs)³⁴, and thus may serve as a compensatory, biological safeguard.

5-HT_{1A} Receptor

Distribution and physiological roles

5-HT_{1A}Rs are distributed throughout the CNS, both pre- and post-synaptically. In the dorsal and medial raphe nuclei, 5-HT_{1A}Rs are somatodendritic autoreceptors; activation of autoreceptors produces a hyperpolarizing current via coupling to GIRKs to inhibit neuronal firing and reduce 5-HT release, thus providing rapid negative feedback for 5-HT signaling. Postsynaptic 5-HT_{1A}Rs are located on non-serotonergic neurons such as GABAergic^{35,36} and cholinergic³⁷ neurons, and are therefore also known as heteroreceptors. Heteroreceptors are widely distributed in the cortex and limbic systems, particularly the hippocampus, and including the entorhinal cortex, frontal cortex, amygdala, and hypothalamus²⁴.

5-HT_{1A}R has a wide variety of functions in the mammalian brain, including regulation of post-synaptic activity, synaptic plasticity, neurogenesis, neuroprotection, and learning and memory²⁴. Selective stimulation of postsynaptic 5-HT_{1A}R with (+)8-Hydroxy-2-dipropylaminotetralin ((+)8-OH-DPAT) induces the 5-HT behavioral syndrome, which includes hyperphagia, hypothermia, altered sexual behavior, and tail flick response³⁸⁻⁴⁰. 5-HT_{1A}R also plays a significant role in the regulation of mood-related behaviors, particularly depression. Administration of 5-HT_{1A}R agonists reduces depressive behaviors in the forced swim test^{41,42} and tail suspension test, and 5-HT_{1A}R knockout mice show antidepressant-like behavioral phenotypes⁴³⁻⁴⁵.

Receptor structure and signal transduction

The human 5-HT_{1A}R gene *Ht1a* is located on chromosome 5q11.2-q13²². Rat *Ht1a* is an intronless gene that codes for 422 amino acids, and shares 89% homology with the human receptor. 5-HT_{1A}R protein has a 7-transmembrane structure and is localized to the plasma membrane²⁵. Canonical 5-HT_{1A}R signaling is mediated via coupling to inhibitory G proteins (G α i1, G α i2, G α i3, G α o)⁴⁶, as inhibition of AC and PKA activity via 5-HT_{1A}R signaling has been shown to mediate 5-HT_{1A}R regulated behaviors⁴⁷.

Coupling to G proteins is brain region specific: in the dorsal raphe nucleus (DRN), 5-HT_{1A}R preferentially couples to G α i3; in the hippocampus, 5-HT_{1A}R couples to G α o⁴⁸. In the hypothalamus, 5-HT_{1A}R can couple to the G protein G α z⁴⁹. Unlike other members of the G α i protein family, G α z lacks the cysteine residue in the C terminus that serves as the substrate for bordatella pertussis toxin (PTX) catalyzed ADP ribosylation⁵⁰, making G α z the only Gi/o family member known to be insensitive to PTX inhibition.

5-HT_{1A}R signaling has been shown to activate a variety of signal transduction cascades beyond the canonical pathways. Activation of the mitogen activated protein kinase (MAPK) family member extracellular signal-regulated kinase (ERK) via phosphorylation is affected by 5-HT_{1A}Rs. Phosphorylation of proteins by ERK in neurons results in receptor and ion channel activation, gene expression, and neuroplasticity⁵¹. One target of ERK is cAMP-responsive element binding protein (CREB), a well-known transcription factor which has been linked to stress, anxiety, and depression⁵². The effect of 5-HT_{1A}R on ERK phosphorylation varies in cells of neuronal origin²⁴. In the hippocampus, 5-HT_{1A}R activation decreases ERK phosphorylation⁵³⁻⁵⁵, and differentiated raphe neurons show decreased ERK activity via G β γ subunit signaling⁵⁶. In acute prefrontal cortical slices, only simultaneous activation of both 5-HT_{1A} and N-methyl-D-

aspartate (NMDA) receptors decreases ERK phosphorylation⁵⁷. In the hypothalamus, 5-HT_{1A}R agonists rapidly but transiently increase phosphorylation of ERK^{53,55,58,59}.

There is also evidence of cross-talk between 5-HT_{1A}R signaling and growth factor signaling via tyrosine kinase receptors, phosphatidylinositol 3-kinase (PI3K), and subsequent activation of Akt. Akt is involved in mediation of neurotrophin and neurotransmitter actions^{60,61}; a major target of Akt is glycogen synthase kinase 3 (GSK3), which has been implicated in mood disorders^{24,62,63}. In neuronal cells, 5-HT_{1A}R agonists increase Akt activation in a PI3K-dependent manner, and this effect is sensitive to PTX⁶⁴.

Regulation of G protein signaling

GPCRs initiate and maintain signaling by catalyzing GDP dissociation from and GTP binding to G α subunits; signal amplitude is therefore a balance between GTP binding and hydrolysis. GTP hydrolysis can be accelerated by GTPase-activating proteins (GAPs). GAPs can thus decrease signal amplitude, terminate a signal, or suppress basal signal amplitude in the absence of stimulation. Regulators of G protein signaling (RGS) proteins are the most numerous family of GAPs, catalyzing the hydrolysis of the G α_q and G α_i/o families⁶⁵⁻⁶⁷.

RGS proteins are grouped into four distinct subfamilies based on sequence homology: the R4 family (RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16), R7 (RGS6, RGS7, RGS9, RGS11), R12 (RGS10, RGS12, RGS14), and Rz (RGSz1, RGSz2, RET-RGS1, and GAIP). RGS proteins contain a conserved domain of about 130 amino acids, called the RGS box, which binds the GTP-G α subunits and accelerates hydrolysis. In addition, most RGS proteins also contain other domains flanking the RGS box that allow for interaction with regulatory elements and localization. For example, RGS1, RGS2, RGS4, and RGS16 have an amphipathic helix at the N-terminus of the RGS box that could serve as a membrane anchor, the R7 family

possesses G protein gamma subunit like (GGL) and disheveled, Egl-10 and Pleckstrin (DEP) domains, RGS12 contains a phosphotyrosine-binding (PTB) domain, as well as a PDZ domain. A conserved cysteine string motif in RGS4, RGS16, and GAIP is known to be palmitoylated, which affects RGS targeting to the membrane and intracellular trafficking⁶⁷⁻⁷¹. These RGS box flanking regions, conserved among subfamilies, allow for physiological functions in addition to negative regulation of G protein signaling, such as kinetic scaffolds⁷², guanine nucleotide exchange factors^{73,74}, ion channel modulators⁷⁵, and cellular signal integration⁷⁶.

The *Gai/o* and *Gas* families of G proteins have an intrinsic rate of hydrolysis of bound GTP with a half-time of about 10-20 seconds. *Gaz*, although classified as belonging to the *Gai/o* family, shares only about 66% homology with this family's other members and has an extremely low rate of hydrolysis, with a half-time of around 7 minutes^{50,77}, indicating that *Gaz* signaling is difficult to switch off. *Gaz* has a limited pattern of tissue expression, being found mainly in the brain, retina, platelets, and adrenal medulla, in contrast to the more ubiquitous *Gai/o* proteins^{50,78}. RGSz1 is a selective GAP for *Gaz* that is found almost exclusively in the brain, and accelerates the rate of GTP hydrolysis by 200- to 400-fold, thus reducing the half-time to seconds and effectively regulating *Gaz* downstream signaling^{79,80}.

HYPOTHALAMIC PITUITARY ADRENAL AXIS

Physiological function

Serotonin signaling, especially 5-HT_{1A}R, is an important mediator of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is the final common pathway of the stress response. HPA axis neuroendocrine activity is governed by secretion of corticotrophin releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus, which activates release of

adrenocorticotrophic hormone (ACTH) from the anterior pituitary; ACTH in turn stimulates secretion of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex^{81,82}.

In addition to regulating peripheral functions such as metabolism and immunity, the HPA axis has significant effects on the brain. Glucocorticoids are major stress hormones that bind to two types of receptors: mineralocorticoid receptors (MR) and glucocorticoids receptors (GR). MRs and GRs are distributed throughout the brain, particularly in regions that play a role in cognitive and neuroendocrine function, such as the hippocampus, amygdala, and prefrontal cortex⁸³. MRs and GRs also serve as potent negative feedback regulators for the HPA axis at the level of CRF and ACTH synthesis and release in the PVN and pituitary, respectively⁸⁴.

Hyperactivity in depression

Although the main role of the HPA axis is to maintain homeostasis under stress, long-term activation can be harmful to the body. Abnormal HPA activity underlies many psychiatric disorders, including depression⁸⁵. The most consistent biomarkers in depressed patients show increased HPA activity, such as high levels of plasma CRF and ACTH, decreased CRF receptors in the PVN, and increased salivary cortisol⁸⁵⁻⁸⁸, as well as pituitary and adrenal hypertrophy^{89,90}. Stressful early life events (such as maternal separation or child abuse) and even prenatal stressors (such as maternal smoking) are linked to HPA axis hyperactivity and increased risk for depression and anxiety disorders⁸⁵. Successful antidepressant treatment is frequently correlated with normalization of HPA axis activity.

Mediation by serotonin

Serotonin is a major mediator of the HPA axis^{81,91}. Stimulation of the HPA axis by acute administration of an SSRI or a non-selective serotonin agonist increases plasma ACTH and

corticosterone through involvement of 5-HT_{1A}R and 5-HT_{2A/C}R⁹²⁻⁹⁶. Selective stimulation of 5-HT_{1A}R with (+)8-OH-DPAT produces an increase in the plasma levels of oxytocin, ACTH, and corticosterone, which can be prevented by treatment with the 5-HT_{1A}R antagonist WAY 100,635⁹⁷, suggesting a particular role for postsynaptic 5-HT_{1A}R in HPA axis function. Furthermore, treatment with PTX has no effect on OT and ACTH release, demonstrating that 5-HT_{1A}R mediation of these hormones is accomplished via signaling through the PTX-insensitive G α z subunit⁴⁹. Although OT is not part of the HPA axis, it is released upon stimulation with (+)8-OH-DPAT and can therefore serve as a direct marker of 5-HT_{1A}R function in the PVN.

DESENSITIZATION OF 5-HT_{1A}R SIGNALING

Therapeutic lag in SSRI treatment

Multiple lines of evidence have led to the hypothesis that improved serotonin neurotransmission underlies the therapeutic effect of antidepressants⁹⁸. To achieve clinical efficacy, chronic use of SSRIs (3-12 weeks) is required^{21,99,100}. The mechanism of action underlying SSRI therapeutic efficacy is not clearly understood, but the therapeutic lag suggests that functional, neuroadaptive changes are necessary. SSRIs target and block the presynaptic 5-HT transporter, thus preventing 5-HT clearance from synapse and prolonging 5-HT effects on both autoreceptors and postsynaptic receptors. Acutely, SSRI-induced increased stimulation of 5-HT_{1A} autoreceptors in the DRN produces a transient increase in feedback regulation, followed by desensitization of the autoreceptors and subsequent increased serotonin transmission and HPA axis activity, which may contribute to the therapeutic lag¹⁰¹⁻¹⁰³.

ESTROGEN

Physiological roles

Estrogens are involved in the regulation of affective states, and both clinical and experimental evidence implicates estrogen levels with the etiology of depression. Naturally-occurring estrogens are a group of biologically-active steroidal hormones¹⁰⁴. Estrogens in mammals regulate a number of physiological processes, such as reproduction, cardiovascular protection, bone integrity, and cellular homeostasis¹⁰⁵. Estrogens are both neuroactive steroids, in that they are synthesized by an endocrine gland and secreted into the blood, and neurosteroids, in that they can be synthesized locally in the brain via aromatization of androgen precursors or *de novo* from cholesterol. It has been hypothesized that neuroactive estrogens are responsible for the classical genomic effects of estradiol, while brain-synthesized estrogens are the source of rapid estrogen effects in the brain¹⁰⁶⁻¹⁰⁸.

Both clinical and experimental studies have shown that 17 β -estradiol (E2, the major circulating estrogen) has numerous effects on the brain throughout the lifespan, including sexual differentiation during development, mood, locomotor activity, pain sensitivity, memory and attentional mechanisms, and is protective against neurodegeneration and brain injury^{109,110}. Estradiol has been found to have neurotrophic, anti-apoptotic, and anti-inflammatory actions, all of which may contribute to its neuroprotective effects¹⁰⁹.

Estrogen receptors

Nuclear receptors

The hydrophobic steroid structure of estradiol allows it to easily pass through the plasma membrane, and historically most of the focus on estradiol signaling has been on intracellular estrogen receptors (ERs). There are two intracellular ERs, ER α and ER β . These receptors are

encoded by two distinct genes, *ESR1* and *ESR2*, respectively. ER α and ER β share significant sequence homology, especially in their DNA and ligand binding domains (96% and 56%, respectively)¹¹¹. Both are classical steroid nuclear receptors which, upon ligand binding, dimerize and localize in the nucleus where they bind to estrogen response elements (EREs) on DNA to regulate transcription¹¹². ER α and ER β have similar affinity for a number of estrogens and estrogen antagonists¹¹³ as well as a similar ability to regulate genes containing the ERE¹¹⁴. In addition, both ERs can interact with other transcription factors, such as Fos and Jun, which bind DNA at the AP-1 site to regulate transcription independently of EREs¹¹⁵.

ER α and ER β are differentially distributed throughout the body: ER α shows high expression in the pituitary, kidney, and adrenal tissue, while ER β shows moderate to high expression in prostate, lung, bladder, and brain. Both ERs are expressed highly in the ovary, testis, and uterus¹¹³. In the brain, ER α and ER β expression overlap significantly, although there are regions where expression of one is higher than the other. ER α is highly expressed in areas that regulate energy homeostasis and reproductive function, such as the preoptic area and arcuate and ventromedial nuclei of the hypothalamus. ER β , on the other hand, shows high mRNA and protein expression in regions involved in the regulation of mood and HPA axis activity, including the hippocampus, DRN, amygdala, cerebral cortex, hypothalamic PVN, and hypothalamic preoptic area^{109,116-118}. ER α and ER β immunoreactivity has been shown to vary across the estrous cycle¹¹⁹, and ER expression on synapses is regulated by estrogen in an age-dependent manner: ER α labeling is decreased in young animals and unaffected in older animals¹²⁰, while ER β immunoreactivity is increased in both young and aged animals compared to midlife¹²¹.

Membrane receptors

Nongenomic, rapid estrogen actions at the plasma membrane have been known for a number of years¹²²; however, it wasn't until more recently that this aspect of estrogen activity began to be investigated. Nongenomic estrogen effects include rapid modulation of intracellular second messengers, such as nitric monoxide, cAMP and cGMP, activation of MAPK and PLC signaling pathways, activation of transcription factors such as CREB, and modulation of G protein coupling^{109,123,124}, which are important in neuroprotection, among other processes. Although rapid estrogen signaling is commonly referred to as nongenomic, these effects can also involve local protein synthesis¹²⁵ and the activation of signaling cascades that lead to transcriptional changes independent of nuclear ERs¹⁰⁸.

While emerging evidence suggests that palmitoylation of ER α may contribute to localization of the receptor to the plasma membrane¹²⁶ and thus mediate nongenomic signaling, the majority of intracellular ERs accumulate in the nucleus¹²³. Several splice variants of the genes encoding ER α and ER β (*ESR1* and 2, respectively) have been identified and proposed as membrane ERs¹²⁷. Additionally, novel membrane ER proteins have been identified, namely Gq-mER, ER-X, and G protein coupled receptor 30 (GPR30/GPER1).

Gq-mER is a GPCR which preferentially couples to G α q proteins, signaling via PLC-PKC-PKC pathways to attenuate GABA_B receptors and μ -opioid receptor activation of GIRKs in hypothalamic POMC neurons¹²⁸. Gq-mER is selectively activated by STX, an analogue of 4-OH-tamoxifen; treatment with STX has demonstrated that Gq-mER is important in the control of homeostatic functions, such as feeding, temperature regulation, and ovulation^{127,128} independent of ER α / β activation.

ER-X is highly expressed in the brain, uterus, and lung, and appears to be a seven-transmembrane receptor associated with caveolar-like microdomains which signals via the MAPK/ERK pathway. Interestingly, 17 α -estradiol (generally considered an inactive natural enantiomer of 17 β -estradiol) is even more potent than 17 β -estradiol in activating MAPK/ERK through ER-X^{129,130}, which sets ER-X apart from other estrogen receptors pharmacologically. The physiological functions of ER-X are not yet clear. ER-X expression is upregulated during development and declines dramatically in the adult, but is upregulated following ischemic stroke, suggesting that the receptor may be important for brain injury and aging in addition to development¹²⁹.

GPR30 was originally cloned as an orphan GPCR¹³¹ and subsequently identified as a membrane ER with high and selective affinity for estrogens¹³²⁻¹³⁵. Human GPR30, also known as GPER, is located on chromosome 7p22.3 and is composed of 3 exons. An activator protein-1 (AP-1) site exists in the second intron of the gene, which may be a transcriptional regulatory region that is activated in response to epidermal growth factor (EGF)¹³⁶. The protein is a seven transmembrane receptor comprised of 375 amino acids, with an apparent molecular weight of about 41kD. GPR30 has been reported to localize to the plasma membrane, the endoplasmic reticulum and the Golgi apparatus^{134,137,138}.

GPR30 distribution and signal transduction

Studies in GPR30 knockout mice have demonstrated that GPR30 has important roles in the immunological system, circulatory system, and glucose homeostasis¹³⁹. In the brain, high GPR30 immunoreactivity has been observed in the isocortex, piriform and entorhinal cortex, hippocampus, and hypothalamus with no apparent gender differences^{140,141}. GPR30 distribution is distinct from ER α or ER β ¹⁴¹, and colocalizes with oxytocin (OT) and CRF in the PVN and

supraoptic nucleus (SON) of the rat hypothalamus^{134,141-143}, suggesting a role for estradiol and GPR30 in the release of these hormones.

In human ER-negative SKBR3 and MCF-7 breast cancer cells, stimulation of GPR30 with estradiol increases production of cAMP and activation of PKA via coupling to G α s. The G β γ subunit activates Src tyrosine kinase which, via matrix metalloproteinase (MMP), releases HB-EGF into the extracellular space to stimulate EGF receptors and activation of PI3K and ERK¹⁴⁴⁻¹⁴⁶. Interestingly, the ER antagonists tamoxifen and ICI 182, 780 act as agonists for GPR30; stimulation of GPR30 with tamoxifen has been shown to activate PI3K signaling cascade¹³⁴. Furthermore, estradiol signaling through GPR30 can also lead to transcriptional effects. GPR30 signaling upregulates nerve growth factor production by inducing c-fos expression via cAMP in an ERE-independent manner^{136,147,148}, and induces cyclin D2 and Bcl-2 expression via PKA-mediated CREB phosphorylation^{147,149}.

Regulation of mood

Depression and anxiety scores have been shown to improve with administration of estradiol in naturally and surgically menopausal women^{150,151}, and hormone replacement therapy (HRT, both estrogen and progesterone) improves mood in menopausal women¹⁵². In addition, augmentation of SSRI treatment with estradiol improved mood compared to those taking SSRIs alone^{153,154}, and women taking estrogen therapy for the management of menopausal symptoms show a better response to SSRI treatment of depression than those taking HRT alone^{155,156}. However, other studies have found no effect of combining estrogen therapy with SSRIs, and therefore estrogen therapy is not considered a first-line treatment for major depression¹⁵⁷⁻¹⁶⁰.

This lack of effect could be due to the particular SSRI or estrogen compound used. In preclinical trials, the antidepressant effect of estrogenic compounds in animal models of

depression this depends on the type of estrogen tested. 17 β -estradiol, ethinylestradiol, and estradiol benzoate all produce an antidepressant-like action in the forced swim test¹⁶¹⁻¹⁶³, while diethyl-stilbestrol has no effect¹⁶¹, and combination of suboptimal doses of fluoxetine and 17 β -estradiol produced synergistic effects in the forced swim test and chronic mild stress behavioral assays¹⁶⁴.

Estrogen modulation of serotonin

Ovarian steroids are known to regulate the serotonin system of rodents and primates^{6,165,166} by increasing gene and protein expression of the rate-limiting enzyme in serotonin synthesis (TPH2), increasing transport and binding of SERT, decreasing gene and protein expression of 5-HT_{1A} autoreceptors and 5-HT_{1A}R binding and coupling to G α_i , and by decreasing MAO-A gene and protein expression¹⁶⁷⁻¹⁷¹.

In non-human primates, chronic treatment with estradiol decreases 5-HT_{1A}R mRNA and agonist binding in the raphe^{169,172}, as well as decreasing G protein coupling. In addition, estradiol treatment significantly decreases MAO-A and MAO-B expression within serotonin and hypothalamic target neurons^{6,173}, thereby increasing availability of serotonin at the postsynaptic receptor. Interestingly, a study in the Atlantic croaker hypothalamus found that inhibition of aromatase activity reduced TPH and serotonin expression, which was corrected with estradiol treatment, demonstrating a role for locally produced estradiol in modulation of the serotonin system¹⁷⁴.

Sex differences have been observed in the serotonin system, such as decreased SERT binding in female rodents and humans and greater stress-induced increase in female amygdalar serotonin levels^{12,95,175}. Gonadal hormones have been implicated as modulators of serotonin transmission in raphe projection regions^{176,177} and therefore can affect response to stress.

Females in both rodent and human studies exhibit higher levels of glucocorticoids in response to various stressors, possibly due to gonadal hormones: androgen administration decreases indices of HPA axis activity (secretion of ACTH, glucocorticoids, and stress-induced c-fos in the PVN), while estradiol treatment has the opposite effect¹⁰³. Furthermore, estradiol has been shown to increase TPH2 expression in ovariectomized rats¹⁷⁸, possibly leading to increased serotonergic transmission.

Consistent findings show that both basal and stress-induced adrenal steroid secretion is greater in females than in males¹⁷⁹⁻¹⁸¹, and estrogen replacement in ovariectomized female rodents can inhibit responses to stress¹⁸²⁻¹⁸⁴. Administration of 17 β -estradiol to postmenopausal women increases circulating levels of OT and arginine vasopressin (AVP)¹⁸⁵, and rapidly stimulates intrahypothalamic and peripheral OT release^{142,186}, demonstrating direct modulation of the HP axes.

Neuroendocrine challenge: a model for HPA axis hyperactivity and therapeutic lag

HPA axis hyperactivity is an important biological marker for depression. Neuroendocrine challenge studies in both humans and rodents have shown that chronic antidepressant treatment produces desensitization of postsynaptic 5-HT_{1A}R signaling, as evidenced by decreased OT and ACTH release in response to 5-HT_{1A}R agonists¹⁸⁷⁻¹⁸⁹. In rats, neuroendocrine challenge with the 5-HT_{1A}R selective agonist 8-OH-DPAT increases OT and ACTH release; the increase in ACTH levels is similar to the HPA axis hyperactivity seen in depression. Desensitization of the hormone response to 8-OH-DPAT by SSRIs takes 7-14 days¹⁸⁹⁻¹⁹¹, thus modeling the antidepressant therapeutic lag and demonstrating that desensitization of postsynaptic 5-HT_{1A}R signaling in the PVN is required for SSRI efficacy. We can therefore use OT and ACTH levels after 5-HT_{1A}R stimulation as peripheral markers for HPA activity and to examine the functional

impact of GPR30-mediated estradiol signaling on 5-HT_{1A}R signaling. Understanding the interaction between GPR30-mediated estradiol signaling and 5-HT_{1A}R signaling will be important for the development of novel targets for the acceleration of treatment of depression.

CHAPTER TWO

STATEMENT OF PURPOSE

Mood disorders are more prevalent in women^{2,192}, suggesting a gender-dependent risk factor for mood disorder development. Estrogen treatment has been shown to alleviate symptoms of mood disorders in women, especially when used in conjunction with SSRI treatment¹⁹³⁻¹⁹⁶. Serotonin signaling influences mood disorders, such as depression, anxiety, premenstrual dysphoric disorder, and post-menopausal and postpartum depression¹⁹⁷⁻¹⁹⁹. 5-HT_{1A}Rs in the PVN are involved in HPA axis function, which has been found to be abnormal in mood disorders such as depression⁹⁰; successful antidepressant treatment is associated with normalization of HPA axis function⁸⁴. SSRIs are the most widely prescribed class of drug for the treatment of mood disorders. It takes 3-12 weeks to achieve clinical efficacy with SSRIs²⁰⁰; this therapeutic lag is thought to be in part due to the time it takes for desensitization of the 5-HT_{1A}R signaling in the PVN of the hypothalamus to occur²⁰¹⁻²⁰³. Thus, acceleration of the desensitization of 5-HT_{1A}R signaling in the PVN may reduce the therapeutic lag of SSRIs.

In the rat PVN, it takes seven days of SSRI treatment to show full desensitization of 5-HT_{1A}R signaling^{189,204}, as demonstrated by a lack of OT and ACTH hormone release in response to 5-HT_{1A}R stimulation. Our laboratory has demonstrated that a physiologically active estrogen, 17 β -estradiol 3-benzoate (EB), alone is capable of inducing a partial desensitization of 5-HT_{1A}R signaling in the PVN of rats within two days^{204,205}. Furthermore, we have shown that combining EB with the SSRI fluoxetine results in full desensitization of 5-HT_{1A}R signaling in just two days²⁰⁶. Therefore, combining EB treatment with SSRIs to accelerate desensitization of 5-HT_{1A}R signaling may have clinical relevance to decrease therapeutic lag. However, because estrogen

therapy is associated with increased risk of breast cancer, stroke, and heart disease²⁰⁷⁻²⁰⁹, identifying the estrogen receptor and associated changes in 5-HT_{1A}R signaling components that contribute to desensitization will suggest new targets to accelerate the onset of SSRI therapeutic effects.

Previous work in our laboratory has shown that one day of EB treatment is not enough to induce 5-HT_{1A}R signaling desensitization, suggesting that genomic effects are involved²⁰⁵. There are two known nuclear estrogen receptors, which upon binding estradiol, dimerize and translocate to the nucleus where they bind EREs on DNA to regulate transcription. In the PVN, ER α density is low, while density of ER β is much higher, especially in OT neurons^{165,210-212}. However, recent work in our laboratory has shown that an ER β selective agonist DPN did not mimic the effect of EB on 5-HT_{1A}R signaling, and siRNA knockdown of ER β did not prevent EB-induced desensitization of 5-HT_{1A}R signaling. It is therefore unlikely that ER β is involved in this mechanism²¹³.

The non-classical membrane estrogen receptor, GPR30, is expressed in the PVN and colocalizes with 5-HT_{1A}R, OT, and CRF in the PVN^{140,143,214}. Intra-PVN treatment with the selective GPR30 agonist G-1 decreased 5-HT_{1A}R signaling similar to EB, and inhibition of GPR30 $\beta\gamma$ signaling by PTX treatment abolished the EB-induced desensitization response¹⁴³. These data suggest that signaling through GPR30 is involved in the EB-induced desensitization of 5-HT_{1A}R signaling.

The purpose of this study is to identify the mechanisms responsible for estradiol-induced desensitization of 5-HT_{1A}R signaling, and to answer two questions. First, *which estrogen receptor is responsible for the effects of EB on 5-HT_{1A}R signaling?* And second, *what are the*

estradiol-induced changes in the 5-HT_{1A}R signaling pathway that lead to the desensitization response?

To answer the first question, we proposed the hypothesis that **signaling through GPR30 is necessary and sufficient for EB-induced desensitization of 5-HT_{1A}R signaling**. To test whether GPR30 is sufficient, we pretreated ovariectomized (OVX) Sprague-Dawley rats with the selective GPR30 agonist G-1, with systemic s.c. injections of 2.5 and 5mg/kg for two days, then challenged with the selective 5-HT_{1A}R agonist (+)8-OH-DPAT and examined the hormone response. To test whether GPR30 signaling is necessary, we developed a recombinant adenovirus containing siRNA against GPR30, injected it directly into the PVN of OVX rats, and then evaluated the effects of EB treatment on hormone response

5-HT_{1A}R is a G-protein-coupled receptor that couples to the Gi/o family of inhibitory G proteins^{215,216}. In the PVN, OT and ACTH release is mediated via 5-HT_{1A}R coupling to Gαz⁴⁹. Gαz is active in the GTP-bound form and has a very slow intrinsic rate of GTP hydrolysis. RGSz1, a regulator of G protein signaling, has a high affinity for Gαz and increases the rate of GTP hydrolysis by over 400-fold, effectively down-regulating Gαz downstream signaling^{79,80,217}. The predicted molecular weight of RGSz1 is 27-29kD. However, in the brain RGSz1 displays protein isoforms of many different sizes, which may represent different posttranslational modifications^{73,218}. We have shown previously that two day treatment with EB resulted in an increase in RGSz1 mRNA, as well as RGSz1 proteins^{219,220}. Changes in posttranslational modification of RGSz1 could affect RGSz1 interaction with and ability to regulate Gαz, thus contributing to desensitization of 5-HT_{1A}R signaling.

To address our second question (what are the estradiol-induced changes in the 5-HT_{1A}R signaling pathway that lead to the desensitization response?) we used tissue from the above

experiments and investigated EB and G-1 induced changes in mRNA and protein levels of components of the 5-HT_{1A}R signaling pathway, including 5-HT_{1A}R, *Gαz*, and RGSz1, which led to our second hypothesis: that **alteration in RGSz1 expression and posttranslational modification underlies estradiol-induced desensitization of 5-HT_{1A}R signaling.**

To explore this hypothesis, we examined and characterized the different RGSz1 protein isoforms in the brain, including identifying the different posttranslational modifications and subcellular localization. Finally, we propose a mechanism by which EB signaling through GPR30 impacts the 5-HT_{1A}R signaling pathway to produce desensitization of the hormone response to 5-HT_{1A}R stimulation. Understanding the estrogen-receptor-mediated mechanisms that result in rapid desensitization of 5-HT_{1A}R signaling will suggest novel targets for the improved treatment of depression and other mood disorders.

CHAPTER THREE

GPR30 IS NECESSARY FOR ESTRADIOL-INDUCED DESENSITIZATION OF 5-HT_{1A} RECEPTOR SIGNALING IN THE PARAVENTRICULAR NUCLEUS OF THE RAT HYPOTHALAMUS

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ABSTRACT

Estrogen therapy used in combination with SSRI treatment improves SSRI efficacy for the treatment of mood disorder. Desensitization of 5-HT_{1A} receptors takes one to two weeks to develop in animals and is necessary for SSRI therapeutic efficacy. Estradiol modifies 5-HT_{1A} receptor signaling and induces a partial desensitization in the paraventricular nucleus (PVN) of the rat within two days, but the mechanisms underlying this effect are currently unknown. The purpose of this study was to identify the estrogen receptor necessary for estradiol-induced 5-HT_{1A} receptor desensitization. We previously showed that estrogen receptor β is not necessary for 5-HT_{1A} receptor desensitization and that selective activation of estrogen receptor GPR30 mimics the effects of estradiol in rat PVN. Here, we used a recombinant adenovirus containing GPR30 siRNAs to decrease GPR30 expression in the PVN. Reduction of GPR30 prevented estradiol-induced desensitization of 5-HT_{1A} receptor as measured by hormonal responses to the selective 5-HT_{1A} receptor agonist, (+)8-OH-DPAT. To determine the possible mechanisms underlying these effects, we investigated protein and mRNA levels of 5-HT_{1A} receptor signaling components including 5-HT_{1A} receptor, *Gaz*, and *RGSz1*. We found that two days of estradiol increased protein and mRNA expression of *RGSz1*, and decreased 5-HT_{1A} receptor protein but increased 5-HT_{1A} mRNA; GPR30 knockdown prevented the estradiol-induced changes in 5-

HT_{1A} receptor protein in the PVN. Taken together, these data demonstrate that GPR30 is necessary for estradiol-induced changes in the 5-HT_{1A} receptor signaling pathway and desensitization of 5-HT_{1A} receptor signaling.

INTRODUCTION

Women in peri- to post-menopausal states experience a fluctuation, then decline, in estrogen levels²²¹. The greatest reduction of estrogen levels is observed during the late menopausal transition and the first year post-menopause²²²⁻²²⁴. Decreased levels of estrogens are associated with various neuropsychiatric disorders such as depression, anxiety, and panic disorders in women²²⁵. During peri-menopause, there is a higher incidence of first onset of mood disturbances^{226,227}. Change in serotonergic function, particularly 5-HT_{1A} receptor function^{6,228,229}, is a hallmark of such disorders^{177,230-233}.

SSRIs are commonly used to treat mood disorders. Desensitization (attenuation) of both somatodendritic 5-HT_{1A} autoreceptor signaling in the midbrain and postsynaptic 5-HT_{1A} receptor signaling in the PVN region of the hypothalamus are thought to contribute to the therapeutic efficacy of SSRIs^{101,188,201,234,235}, which can take three to 12 weeks to achieve²¹. Desensitization of 5-HT_{1A} receptor signaling in the PVN can be measured by neuroendocrine challenge tests that detect changes in OT and ACTH levels in response to 5-HT_{1A} receptor agonists^{190,236}. In humans, chronic treatment with SSRIs reduces the release of OT and ACTH in response to 5-HT_{1A} receptor stimulation, thus demonstrating desensitization of the receptor signaling¹⁸⁸. Estrogens have been shown to enhance the efficacy of SSRIs for the treatment of mood disorders and hot flashes in women^{237,238}. In rats, SSRIs produce full desensitization of 5-HT_{1A} receptor signaling

in PVN in seven to 14 days^{189,191}, while estradiol alone can produce a partial desensitization with two days of treatment²⁰⁴.

In order to improve current therapies for mood disorders, it is important to understand which estrogen receptor is involved in the regulation of 5-HT_{1A} receptors in the PVN. Of the two classical nuclear estrogen receptors (ERs) α and β , density of ER β is higher than ER α in the PVN, especially in OT neurons^{165,210,211,239}. Recent work in our laboratory has shown that ER β is not involved in 5-HT_{1A} receptor desensitization²¹³. This result, together with the low expression of ER α in the PVN, led us to investigate the non-classical, membrane estrogen receptor GPR30. GPR30 binds estrogen with high affinity^{134,135}, is expressed in the PVN, and colocalizes with 5-HT_{1A} receptors, OT, and CRF in the PVN^{140,143,214}. Previous work in our laboratory showed that treatment with the GPR30-selective agonist G-1 decreases 5-HT_{1A} receptor signaling, similar to estradiol¹⁴³. These data suggest a role for GPR30 in the estradiol-mediated desensitization of 5-HT_{1A} receptor signaling in the PVN.

The 5-HT_{1A} receptor is known to couple to the pertussis toxin-insensitive G protein subunit *G α z*^{215,216}. Stimulation of 5-HT_{1A} receptors in the hypothalamus stimulates release of OT and ACTH via coupling to *G α z*⁴⁹. RGSz1, one of six splice variants of RGS20, is a highly selective GAP for *G α z*. It is exclusively found in the brain, where it accelerates hydrolysis of *G α z*-bound GTP over 400-fold, with a K_m of 2nM^{79,80,217}. Two day treatment with estradiol produces a dose-dependent upregulation of RGSz1 in the PVN, without changing *G α z* levels, that parallels the decreased hormone response induced by estradiol²¹⁹, suggesting that RGSz1 is important in estradiol-induced desensitization of 5-HT_{1A} signaling.

In this study, we investigated the hypothesis that GPR30 expression in the PVN is necessary for the estradiol-induced desensitization of 5-HT_{1A} receptor signaling. To test this

hypothesis, we injected a recombinant adenovirus containing a small interference RNA (siRNA) against GPR30 into the rat PVN and then evaluated the effects of estradiol on 5-HT_{1A} receptor signaling, as well as changes in mRNA and proteins involved in 5-HT_{1A} receptor signaling, including the 5-HT_{1A} receptor, Gα_z, and RGSz1.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley (SD) rats (225-250g) from Harlan (Haslett, MI) were housed two per cage in a temperature-, humidity-, and light-controlled room (12h light/dark cycle). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and as approved by the University of Kansas Institutional Animal Care and Use Committee. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

Drugs

17β-Estradiol-3-benzoate (EB) was purchased from Sigma-Aldrich (St. Louis, MO). EB was first dissolved in 100% ethanol to a concentration of 1mg/ml and then diluted with sesame oil to a concentration of 25μg/ml. The EB solution and sesame oil were administered at 0.4 ml/kg (EB dose 10μg/kg, subcutaneous (s.c.)). (+)8-Hydroxy-2-dipropylaminotetralin (+)8-OH-DPAT) was purchased from Tocris (Ellisville, MO). (+)8-OH-DPAT was dissolved in 0.85% NaCl (saline) at a concentration of 0.2mg/ml and administered at a dose of 0.2mg/kg, s.c. Solutions were made fresh before injection.

Procedures

Experiment 1: effect of estradiol on 5-HT_{1A} receptor function

Prior to surgery, rats were anesthetized by an intraperitoneal (i.p.) injection of a cocktail of ketamine hydrochloride (100mg/kg) plus xylazine hydrochloride (7mg/kg). Rats were ovariectomized (OVX) by removing both ovaries via a single ventral midline incision. Five days after OVX, rats were given s.c. injections of either EB (10µg/kg, 0.25ml/kg, s.c.) or vehicle (sesame oil) once a day for two days. 18h following the last injection of EB or vehicle, rats were injected with the selective 5-HT_{1A} receptor agonist, (+)8-OH-DPAT (0.2mg/kg, s.c.) or vehicle (saline). 15 minutes later, animals were sacrificed by decapitation. Trunk blood was collected in centrifuge tubes containing 0.5ml 0.3M EDTA (pH 7.4). Brains were removed and snap-frozen in isopentane and dry ice. Plasma and brains were stored at -80°C until use.

Experiment 2: effect of recombinant adenovirus containing GPR30 siRNA on EB-induced desensitization of 5-HT_{1A} receptors

Generation and evaluation of recombinant adenoviruses

Recombinant adenoviruses were generated as described previously^{213,240}. Briefly, potential GPR30 siRNA sequences were designed using Block It™RNAi Designer program provided by Invitrogen. Four potential siRNAs and two mismatch sequences (Table 1) were converted to DNA sequences to further test and generate recombinant adenovirus. The potential GPR30 siRNAs were evaluated using a pSOS-HUS vector, which contains an siRNA site and a target gene site that allows transfection of siRNAs and the target gene into the same cells. A sequence encoding green fluorescent protein (GFP) is adjacent to an internal ribosome entry site (IRES), followed by the target gene cloning site, so that observed GFP expression can be used as a

marker for GPR30 expression. When an siRNA inhibits the transcription of GPR30, the expression of GFP is also reduced. Therefore, the expression of GFP observed can be used as a marker for GPR30 expression.

A full sequence of GPR30 (Accession No: U92802) (SOS-GPR30-HUS) was cloned into the target gene cloning site. The siRNA or mismatch siRNA sequences were inserted into *sfi*I sites of SOS-GPR30-HUS (SOS-GPR30-siRNA-HUSs and SOS-GPR30-mis-HUSs, respectively) as described by Luo et al²⁴⁰, followed by *Not*I digestion after ligation. The clones containing siRNA sequences were identified by PCR with U6 forward and siRNA antisense primers. The SOS-GPR30-siRNA-HUSs or SOS-GPR30-mis-HUSs were transfected into HEK293 cells using lipofectamine reagent (Invitrogen, Carlsbad, CA) to evaluate the knockdown efficiency of the GPR30 siRNAs. The number and density of GFP-expressing cells were observed for five consecutive days after the transfection.

The sequences of siRNAs in the SOS-GPR30-siRNA-HUS that significantly reduced the number and brightness of GFP-containing cells as well as SOS-GPR30-mis-HUSs that had no effect on GFP were selected and inserted into pSES-HUS vector. pSES-HUS is a shuttle vector for adenovirus and contains a red fluorescent protein (RFP), as described above and Luo et al²⁴⁰. The SES-GPR30-siRNA-HUSs and SES-GPR30-mis-HUSs were further recombined into Ad-Easy-1 vector to generate high titer adenoviruses containing the GPR30 siRNA or mismatch sequences (GPR30-siRNA-Ads and GPR30-mis-Ads, respectively)²⁴¹. The high titer GPR30-siRNA-Ads and GPR30-mis-Ads ($\sim 10^{11-12}$ active viral particles/ml) were stored at -80°C. Before use, the high titer adenoviruses were dialyzed with saline for at least 40min at 4°C followed by 1:1 dilution with saline to reduce tissue damage caused by the high-salt storage solution.

To test the GPR30-siRNA-Ads *in vivo*, we conducted two studies. First, we tested the time-course of GPR30 knockdown by GPR30-siRNA-Ads. Then, we confirmed that knockdown of GPR30 was due to the GPR30-siRNA-Ads and not due to the viral injection or infection. Rats were anesthetized and OVX as described in experiment 1, then given unilateral intra-PVN injections of GPR30-siRNA-Ads (402, 737, and 1135 combined) using stereotaxic technique at a rate of 0.5 μ l/min, 1 or 2 μ l/side at the coordinates of AP= -1.8, ML= 0.5 and DV= -8.3 mm with respect to the bregma. The needle (31 gage) was left in the injection site for an additional 20min to reduce movement of the viral solution into the needle track. Three, five, or 10 days after injection, rats were sacrificed and the brains were removed. Brains were sectioned into 300 μ m sections using a cryostat. Regions with viral injection as indicated by RFP and the contralateral regions (as controls) were punched out for immunoblot analysis of GPR30. The percent inhibition of GPR30-siRNA-Ads was calculated by comparing the ratio of GPR30: β -actin between the injected side (I) and the contralateral side (C) of each animal (% inhibition = $(1-I/C) \times 100\%$).

To confirm that the observed knockdown of GPR30 protein was due to the GPR30-siRNA-Ads and not a result of the virus itself or tissue damage caused by the injection, OVX rats were given unilateral intra-PVN injections of saline, control-Ad (Adtrack, empty recombinant shuttle vector), GPR30-mis-Ads (737 and 1135 combined), or GPR30-siRNA-Ads (402, 737, and 1135 combined) using stereotaxic technique as described above. Five days after injection, rats were sacrificed and the brains were collected to evaluate the efficiency of knockdown of GPR30 expression by the siRNAs, compared to the various controls, as described above.

Table 3.1: Primers for generation of siRNA and mismatch siRNA constructs. S = sense; AS = antisense. Numbers refer to starting base pair.

siRNA

GPR30-737-S	AGCCTGTGCTATTCCCTCATTTTT
GPR30-737-AS	AATGAGGGAATAGCACAGGCTTTT
GPR30-1135-S	AACGGAGCAGTCAGATGTCAAGTTCATTTTT
GPR30-1135-AS	ATGAACTTGACATCTGACTGCTCCGTTTTT
GPR30-402-S	AGGACGAGCAGTATTACGATTTTTT
GPR30-402-AS	AATCGTAATACTGCTCGTCCTTTT
GPR30-272-S	AGCAACATCCTCATCTTGGTGGTGAATTTTT
GPR30-272-AS	ATTCACCACCAAGATGAGGATGTTGCTTTTT

Mismatch

GPR30-1135mis-S	AACGGACGGACTTGTAGAACTAGTCATTTTT
GPR30-1135mis-AS	ATGACTAGTTCTACAAGTCCGTCCTTTTTT
GPR30-402mis-S	AGGAACGATATGCATGCGATTTTTT
GPR30-402mis-AS	AATCGCATGCATATCGTTCCTTTTT

Effect of GPR30-siRNA-Ad on EB-induced desensitization of 5-HT_{1A} receptors in the PVN

Rats were anesthetized and OVX as described above, then given bilateral intra-PVN injections of control-Ad or GPR30-siRNA-Ads (402, 737, 1135 combined) using stereotaxic technique at a rate of 0.5µl/min, 1µl/side at the coordinates of AP= -1.8, ML= ±0.5, and DV= -8.3 mm with respect to bregma. The needle was left in the injection site for an additional 20min to reduce movement of the viral solution into the needle track. Five days after OVX and viral injection, rats were treated with either 10µg/kg/day EB or oil (s.c.) once a day for two days. 18h after the last EB or oil treatment, rats were injected with 0.2mg/kg (+)8-OH-DPAT or saline (s.c.) and sacrificed via decapitation 15 minutes later. Brains were removed and snap-frozen in dry-ice-cooled isopentane and then on dry ice. Trunk blood was collected in tubes containing 0.5ml 3M EDTA (pH 7.4). Brains and plasma were stored at -80°C until use.

Radioimmunoassay of plasma OT and ACTH

Plasma OT was determined by a radioimmunoassay as previously described with minor modifications¹⁹⁰. Briefly, OT was extracted from 0.5 ml plasma with 1ml cold acetone followed by 2.5ml petroleum ether. The ether layer was aspirated and the samples were dried in a Centrivap vacuum concentrator at 4°C. The dried OT residue was resuspended in 1ml of cold assay buffer (0.05M phosphate buffer pH 7.4 containing 0.125% bovine serum albumin and 0.001M EDTA). The plasma extracts were used for the radioimmunoassay as previously described. The radioactive ¹²⁵I oxytocin (specific activity: 2200 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA). Several standard recovery samples containing 0.5ml pooled plasma and 8 and 16 pg OT were included throughout the extraction and assay procedure. The plasma OT concentrations were calculated based on the recovery and dilution factors. Plasma ACTH concentrations were determined by radioimmunoassay as previously described²⁴². ¹²⁵I ACTH (0.00102mCi) was obtained from DiaSorin (Stillwater, MN).

Quantitative Real-Time PCR

RNA was isolated from the PVN using TRI-Reagent according to the manufacturer's instruction (Sigma-Aldrich, St. Louis, MO). Briefly, 5µg RNA was treated with 5µl DNase I in DNase I buffer (total reaction volume 15µl) for 15min at room temperature; the reaction was stopped with 5µl 25mM EDTA followed by incubation at 65°C for 10min. 10µl of the DNase-treated RNA reaction mixture was incubated with 1µl 0.3M oligo(dT)₂₀ and 1µl 10mM dNTPs at 65°C for 5 minutes. A master mix containing 5x reverse transcriptase buffer, 0.1M DTT, RNase Out, and 200 units Superscript II enzyme (Invitrogen, Carlsbad, CA) was added to each sample according to the manufacturer's protocol. The reverse transcription was performed by incubation at 42°C for 50min and then 70°C for 15min. 1µl RNase H was then added to the reaction which was then

incubated at 37°C for 20 min. The complimentary DNA (cDNA) generated was used for real-time PCR.

Real-time quantitative PCR (qPCR) was performed in 96-well plates using SYBR Green Plus master mix (Invitrogen, Carlsbad, CA) and the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). mRNA levels of GPR30, 5-HT_{1A} receptor, and RGSz1 were examined using the primers listed in Table 2. The mRNA levels were normalized to TATA-box binding protein (TBP) mRNA. Each qPCR reaction was conducted in a total volume of 20µl, containing 2µl cDNA, 10µM primers, and 10µl SYBR Green Plus master mix. PCR was performed at 50°C for 2min, 95°C for 10min, then 40 cycles of 95°C for 15s and 60°C for 1min per cycle. All samples were run in triplicate. ΔCt was calculated as the target gene – TBP mRNA for each sample; ΔΔCt was calculated as ΔCt for the experimental condition – ΔCt for the control condition, for each target gene.

Table 3.2: Forward and reverse primers for qPCR

Gene	Forward (5'---3')	Reverse (5'---3')
TBP	CAGGAGCCAAGAGTGAAGAACA	GCTTCTGCACAACCTAGCGTATT
GPR30	CCACGCTCAAGGCAGTCATA	GCACTGCTGAACTTGACATCTGA
5-HT _{1A}	GATCTCGCTCACTTGGCTCAT	GCGCCAGCCCAGCAT
RGSz1	AGACATTCCAGCGTGTGAAGAA	GGCCCCAGGCACAGACTT

Immunoblot assays

The PVN was punched out from 300µm-thick sections prepared using a cryostat microtome. PVN tissue was homogenized in 100µl homogenization buffer (10mM Tris pH 7.6, 100mM NaCl, 1mM EDTA, 0.1% sodium cholate, 1% phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), 0.1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)) by brief sonication followed by shaking for 1hr at 4°C. Samples were then centrifuged at 25,000xg for

1hr; the supernatant was collected and stored at -80°C. Protein concentration was measured using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Protein (10µg/lane) was resolved on a 12% SDS-PAGE gel followed by transfer to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated in blocking buffer (5% non-fat milk in Tris-buffered saline with 0.1% Tween-20) to reduce non-specific binding and probed overnight using the following primary antibodies: rabbit anti-GPR30 (1:1000, Novus Biologicals, Littleton, CO); rabbit anti-5-HT_{1A} receptor (1:1000, Abcam Cat.#85615, San Francisco, CA); rabbit anti-Gαz (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-RGSz1 (1:5000); rabbit anti-ERβ (1:1000, Alexis Biochemicals/Enzo Life Sciences, Plymouth Meeting, PA). After washing, membranes were incubated with the appropriate secondary antibody conjugated with horse radish peroxidase. Bands were detected with ECL substrate solution (GE Healthcare Biosciences, Piscataway, NJ) using BioRad ChemiDoc XRS+ molecular imager (BioRad, Hercules, CA). Due to the limited amount of PVN protein, all primary antibodies were used on the same blots, with washing in between uses. Monoclonal mouse actin antibody (1:10 000, MP Biomedicals, Solon, OH) was used as a loading control. Bands were analyzed densitometrically using ImageLab software (BioRad, Hercules, CA). Each band was normalized to actin and calculated as percent of the control group in each blot. All samples were run in duplicate or triplicate.

Characterization of anti-RGSz1

RGSz1 antiserum was raised in rabbits against the last 15 amino acids of the C-terminal of RGSz1 (YKDLLTSLAEKTVEA) and affinity purified by Biosynthesis (Lewisville, TX). To characterize the antibody, the full sequence of RGSz1 was inserted into a pcDNA vector

(Invitrogen, Carlsbad, CA) which was then transfected into HEK293 cells. Cell lysates were collected, protein was separated by SDS-PAGE, and Western blotting was performed as described above. Representative Western blot is shown in **Figure 3.2A** (left).

Microscopy

18 μ m-Thick tissue sections were collected from the beginning, middle, and end of each 300 μ l PVN section as described above to evaluate RFP expression. Images were captured using a Nikon Eclipse Ti and Metamorph Software (Molecular Devices, Sunnyvale, CA) at 4x magnification.

Statistical analysis

All data are expressed as means \pm SEM. One- or two-way analysis of variance (ANOVA) and Student-Newman-Keuls post hoc tests were conducted using a statistical program (Statview version 5.0 software, SAS Institute Inc., Cary, NC).

RESULTS

Experiment 1: Effects of EB on 5-HT_{1A} receptor signaling in the PVN

EB induces desensitization of 5-HT_{1A} receptor signaling

In our first experiment, female SD rats were OVX and treated with EB (10 μ g/kg/day) for two days. To confirm that two-day EB treatment resulted in desensitization of 5-HT_{1A} receptor signaling in our rat model, 18 hours after the second EB injection, the rats were challenged with the selective 5-HT_{1A} receptor agonist (+)8-OH-DPAT (0.2mg/kg) and plasma hormone levels were examined. EB treatment did not alter the baseline levels of plasma OT in comparison to

vehicle treatment (**Figure 3.1A**). Activation of 5-HT_{1A} receptors by (+)8-OH-DPAT increased plasma OT levels in oil-treated rats as expected. The magnitude of the OT response to (+)8-OH-DPAT was significantly reduced in EB-treated rats (two-way ANOVA: main effect of (+)8-OH-DPAT: $F_{(1,25)}=299.649$, $p<0.0001$; main effect of EB: $F_{(1,25)}=11.231$, $p=0.0026$; interaction between EB and (+)8-OH-DPAT: $F_{(1,25)}=12.197$, $p=0.0018$).

Baseline levels of plasma ACTH were unchanged by two-day EB treatment (**Figure 3.1B**), and stimulation of 5-HT_{1A} receptors by (+)8-OH-DPAT increased plasma ACTH levels significantly. The ACTH response to (+)8-OH-DPAT was significantly reduced in EB-treated animals compared to oil treatment (two-way ANOVA: main effect of (+)8-OH-DPAT: $F_{(1,27)}=532.827$, $p<0.0001$; main effect of EB: ($F_{(1,27)}=9.470$, $p=0.0047$; interaction between EB and (+)8-OH-DPAT: $F_{(1,27)}=11.041$, $p=0.0026$). Thus, both oxytocin and ACTH plasma concentration results suggest the desensitization of 5-HT_{1A} receptor signaling in the PVN.

EB treatment alters expression of components of the 5-HT_{1A} receptor signaling pathway

To determine the cellular mechanisms mediating desensitization of 5-HT_{1A} receptor signaling, we examined effects of EB treatment on the 5-HT_{1A} receptor system in the PVN at the molecular level. EB reduced the protein levels of 5-HT_{1A} receptors by 32% as measured on Western blots (**Figure 3.2A**, right) ($F_{(1,5)}=9.083$, $p=0.0296$). Two RGSz1 bands (55kD, 45kD) were measured. The protein levels in both bands were significantly increased by 79% ($F_{(1,5)}=21.753$, $p=0.0035$) and 25% ($F_{(1,5)}=6.75$, $p=0.0484$), respectively, in the EB group compared to the oil-treated group. There were no significant differences in the protein levels of either Gαz protein ($F_{(1,5)}=2.256$, $p=0.2075$) or GPR30 protein ($F_{(1,5)}=0.323$, $p=0.5943$) (**Figure 3.2B**).

Quantitative real-time PCR was conducted to further examine the expression of components of the 5HT_{1A} receptor signaling pathway. Our results showed that EB treatment increased mRNA levels of 5-HT_{1A} receptor by 299% (EB vs. oil, $F_{(1,6)}=6.129$, $p=0.0481$). RGSz1 mRNA levels increased by 308% (EB vs. oil, $F_{(1,6)}=10.161$, $p=0.0189$), while GPR30 mRNA did not change significantly ($F_{(1,6)}=0.000132$, $p=0.99$) (**Figure 3.2C**).

Experiment 2: effects of reduction in the GPR30 expression on EB-induced desensitization of 5-HT_{1A} receptor in the PVN

Generation and evaluation of GPR30-siRNA-Ads

To select efficient GPR30 siRNA sequences, SOS-GPR30-siRNA-HUSs and SOS-GPR30-mis-HUSs were transfected into HEK293 cells. The cells transfected with GPR30-siRNA-HUS 402, 737 and 1135, but not 272 (numbers refer to first base pairs of each siRNA sequence), displayed reductions in the number of GFP-containing cells and the intensity of GFP compared to SOS-GPR30-mis-HUS and SOS-GPR30-HUS plasmids, beginning two days after transfection. Therefore, we selected the 402, 737, and 1135 siRNA constructs for production of GPR30 siRNA recombinant adenovirus (GPR30-siRNA-Ad). **Figure 3.3A** shows examples from 3 days after transfection.

To test the time course of GPR30 knockdown by GPR30-siRNA-Ads, we unilaterally injected 1 or 2 μ l GPR30-siRNA-Ads into the PVN of rats (**Figure 3.3B**) and collected the brains 3, 5 and 10 days after the injection. By comparing GPR30 expression in the viral infected sides to their contralateral sides, we observed that the GPR30-siRNA-Ads significantly inhibited the expression of GPR30 in a dose-dependent manner (% inhibition: 59.0 ± 8.12 (n=3) for 1 μ l vs. 77.87 ± 4.77 (n=6) for 2 μ l viral injection). This inhibition of GPR30 expression was observed

3 days after the injections. There was no difference between 3 and 5 days, but there was a slight reduction in inhibition 10 days after the injection (89.47% inhibition for 3 days, 85.93% for 5 days and 66.35% for 10 days after 2 μ l viral injection). These data demonstrated that the GPR30-siRNA-Ads are able to efficiently knock down GPR30 expression 3 days after injection. Although no visible tissue damage was observed, to avoid possible toxicity and to restrict the knockdown of GPR30 to the PVN, we used 1 μ l of GPR30-siRNA-Ads and the 5 day time point in further studies.

To compare the effects of the GPR30-siRNA-Ads with the GPR30-mis-Ads *in vivo*, we unilaterally injected 1 μ l of GPR30-siRNA-Ads into the PVN, with saline, empty viral vector (Adtrack, control-Ad), or GPR30-mis-Ads as controls. In the GPR30-siRNA-Ads construct, red fluorescent protein (RFP) is expressed independently of the siRNAs, making the presence of RFP useful for tracking the virus infection (**Figure 3.3C**). Five days after the injection of the virus, GPR30 protein level in the viral-infected side was measured and percent inhibition was calculated relative to the contralateral (non-injected) side of each animal (representative Western blots shown in **Figure 3.3D**). The GPR30-siRNA-Ads were successful in reducing GPR30 protein expression by about 42%, compared to the contralateral side ($F_{(3,10)}=6.225$, $p=0.0118$). None of the three controls had a significant effect on GPR30 protein levels (**Figure 3.3E**). However, in a separate experiment (not shown), GPR30-mis-Ads interfered with the neuroendocrine response. Therefore, we used control-Ad as the control for the following experiment.

Effect of GPR30-siRNA-Ads on GPR30 in the PVN

To determine the role of GPR30 in the EB-induced desensitization of 5-HT_{1A} receptor signaling in the PVN, we performed bilateral injections of control-Ad or GPR30-siRNA-Ad constructs in the PVN of OVX rats. Five days after viral injection, rats were treated with 10µg/kg/day EB or oil (s.c.) for two days and then challenged with 0.2mg/kg (+)8-OH-DPAT (s.c.) before being sacrificed, as described above. Knockdown of GPR30 was verified by Western blotting (**Figure 3.4A**). We used both reduction in GPR30 levels and the location of RFP expression as exclusion criteria for incorrect injections of GPR30-siRNA-Ads; rats with no RFP or RFP that was not in the PVN were excluded from subsequent analyses. GPR30 protein was successfully reduced to about 68% of control ($F_{(1,12)}=7.856$, $p=0.0160$) (**Figure 3.4B**). This 32% inhibition is a little lower than the reduction in the previous test. This could be due to the way tissue was collected and GPR30 levels compared: we measured GPR30 in the entire PVN if there was viral infection in at least part of the PVN, whereas previously we only injected one side of the PVN and only used tissue that had RFP expression. ERβ expression can be influenced by treatment with estradiol²¹³; to ensure that effects of EB on 5-HT_{1A} receptor signaling in the neuroendocrine challenge tests were due to the reduction of GPR30 and not a change in ERβ levels, we measured ERβ protein in the PVN following infection with GPR30-siRNA-Ads and found no significant change ($F_{(1,15)}=1.913$, $p=0.1773$) (**Figure 3.4**).

Effect of GPR30-siRNA-Ads on EB-induced desensitization of 5-HT_{1A} receptor signaling

Desensitization of 5-HT_{1A} receptor signaling was examined by measuring the plasma levels of OT (**Figure 3.5A**) and ACTH (**Figure 3.5B**). The baseline level of plasma OT was not altered by EB treatment or GPR30-siRNA-injection. In animals injected with the control-Ad, activation of 5-HT_{1A} receptors by (+)8-OH-DPAT increased plasma OT levels. In the control-Ad-infected

group, there was a significant reduction in OT response to (+)8-OH-DPAT after EB treatment compared to oil treatment, demonstrating desensitization of 5-HT_{1A} receptor signaling. In the GPR30-siRNA-Ads-injected group, OT levels in response to (+)8-OH-DPAT were not significantly different from the control-Ad-injected, oil-treated group, and EB treatment did not induce 5-HT_{1A} receptor signaling desensitization (three-way ANOVA: main effect of viral injection, $F_{(1,56)}=2.193$, $p=0.1442$; main effect of (+)8-OH-DPAT, $F_{(1,56)}=1219.852$, $p<0.0001$; main effect of EB, $F_{(1,56)}=4.208$, $p=0.0449$; interaction between EB and (+)8-OH-DPAT, $F_{(1,56)}=2.162$, $p=0.1471$; interaction between EB and viral injection, $F_{(1,56)}=0.719$, $p=0.4002$; interaction between (+)8-OH-DPAT and viral injection, $F_{(1,56)}=2.393$, $p=0.1275$; interaction between EB, (+)8-OH-DPAT, $F_{(1,56)}=0.3866$, $p=0.761$).

Plasma ACTH levels in response to (+)8-OH-DPAT showed a similar pattern. Neither EB treatment nor GPR30-siRNA-Ad injection had an effect on baseline levels of plasma ACTH. In animals injected with the control-Ad, activation of 5-HT_{1A} receptors by (+)8-OH-DPAT increased plasma ACTH levels, while EB treatment produced a significant reduction in ACTH response to (+)8-OH-DPAT, demonstrating desensitization of 5-HT_{1A} receptor signaling. In the GPR30-siRNA-Ad-injected group, ACTH levels in response to (+)8-OH-DPAT were not significantly different from the control-Ad-injected, oil-treated group, and EB treatment did not have an effect on plasma ACTH levels (three-way ANOVA: main effect of viral injection, $F_{(1,62)}=1.939$, $p=0.1688$; main effect of (+)8-OH-DPAT, $F_{(1,62)}=802.128$, $p<0.0001$; main effect of EB, $F_{(1,62)}=0.688$, $p=0.4100$; interaction between (+)8-OH-DPAT and EB, $F_{(1,62)}=1.930$, $p=0.1697$; interaction between (+)8-OH-DPAT and viral injection, $F_{(1,62)}=0.3849$, $p=0.766$; interaction between EB and viral injection, $F_{(1,62)}=5.122$, $p=0.271$; interaction between (+)8-OH-

DPAT, EB, and viral injection, $F_{(1,62)}=5.043$, $p=0.0283$). Together, these results suggest that GPR30 is necessary for desensitization of 5-HT_{1A} receptor signaling.

To further investigate the effects of EB treatment on 5-HT_{1A} receptor signaling, we next examined 5-HT_{1A} receptor protein and the G protein mediating the hormone responses, *Gαz*, using Western blotting (**Figure 3.6A**). EB treatment resulted in reduced levels of 5-HT_{1A} receptor protein in the control-Ad-injected group ($F_{(1,35)}=6.694$, $p=0.0011$); this effect was not seen in the GPR30-siRNA-Ad-injected group, further suggesting that GPR30 may be involved in the reduction of 5-HT_{1A} receptor protein. The GPR30-siRNA-Ad injection itself had no effect on 5-HT_{1A} receptor protein levels. Levels of *Gαz* protein were unchanged, regardless of treatment ($F_{(1,35)}=1.051$, $p=0.3875$) (**Figure 3.6B**).

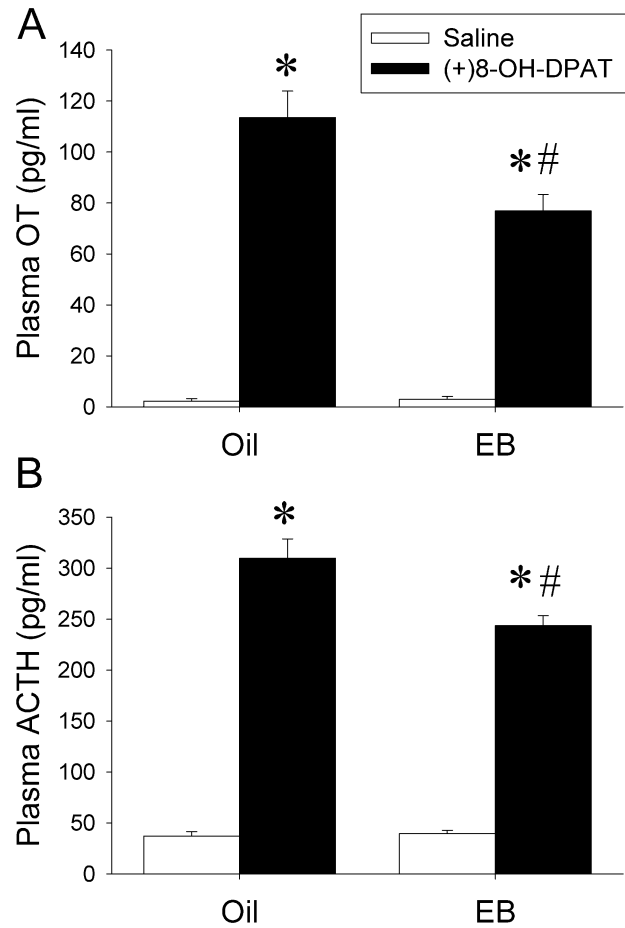


Figure 3.1. EB treatment induces 5-HT_{1A} desensitization. Plasma OT (**A**) and ACTH (**B**) levels in response to saline or (+)8-OH-DPAT challenge in rats treated with oil or EB. Data are presented as mean \pm SEM (n=6-8/group). *Significantly different from saline-challenged animals with same treatment, $p < 0.0001$; #significantly different from oil/(+)8-OH-DPAT-treated animals, $p < 0.005$ by Student-Newman-Keuls post hoc test.

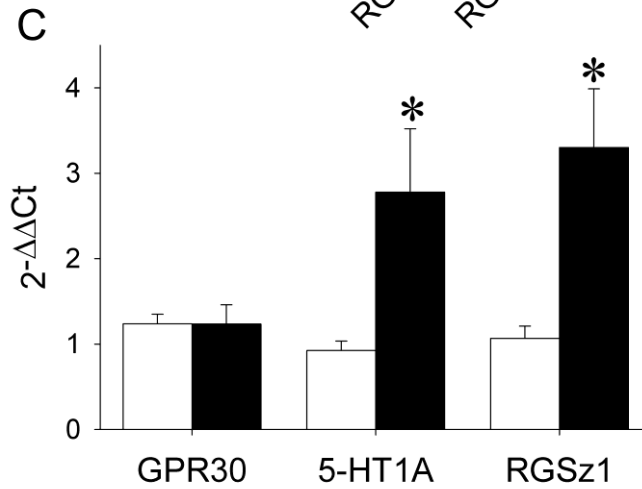
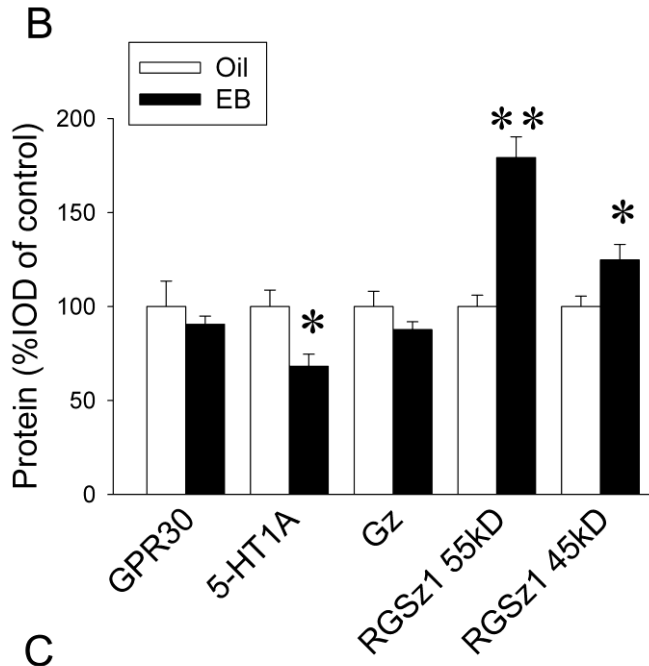
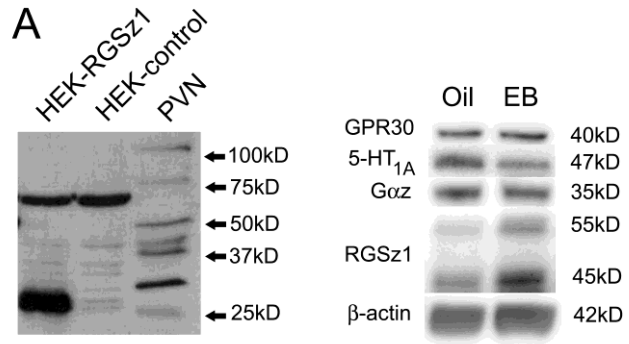


Figure 3.2. EB-induced changes in 5-HT_{1A} signaling pathway. (A) Left, characterization of RGSz1 antibody. Lane 1, HEK293 cells transfected with RGSz1. Lane 2, HEK293 cells

transfected with empty vector (pcDNA). Lane 3, PVN tissue lysate. Right, Western blot of PVN protein after oil or EB treatment with antibodies against GPR30, 5-HT_{1A}, Gα_z, and RGSz1, with β-actin as loading control. **(B)** Quantitation of protein levels after EB pretreatment, normalized to β-actin and expressed as percent of control (oil). Data are expressed as mean ± SEM (n=6), **p*<0.05, ***p*<0.001 by Student-Newman-Keuls post hoc test. **(C)** qPCR of mRNA isolated from PVN tissue, normalized to control (oil). Changes in mRNA levels are expressed as mean 2^{-ΔΔCt} ± SEM (n=4-6), **p*<0.05 by Student-Newman-Keuls post hoc test.

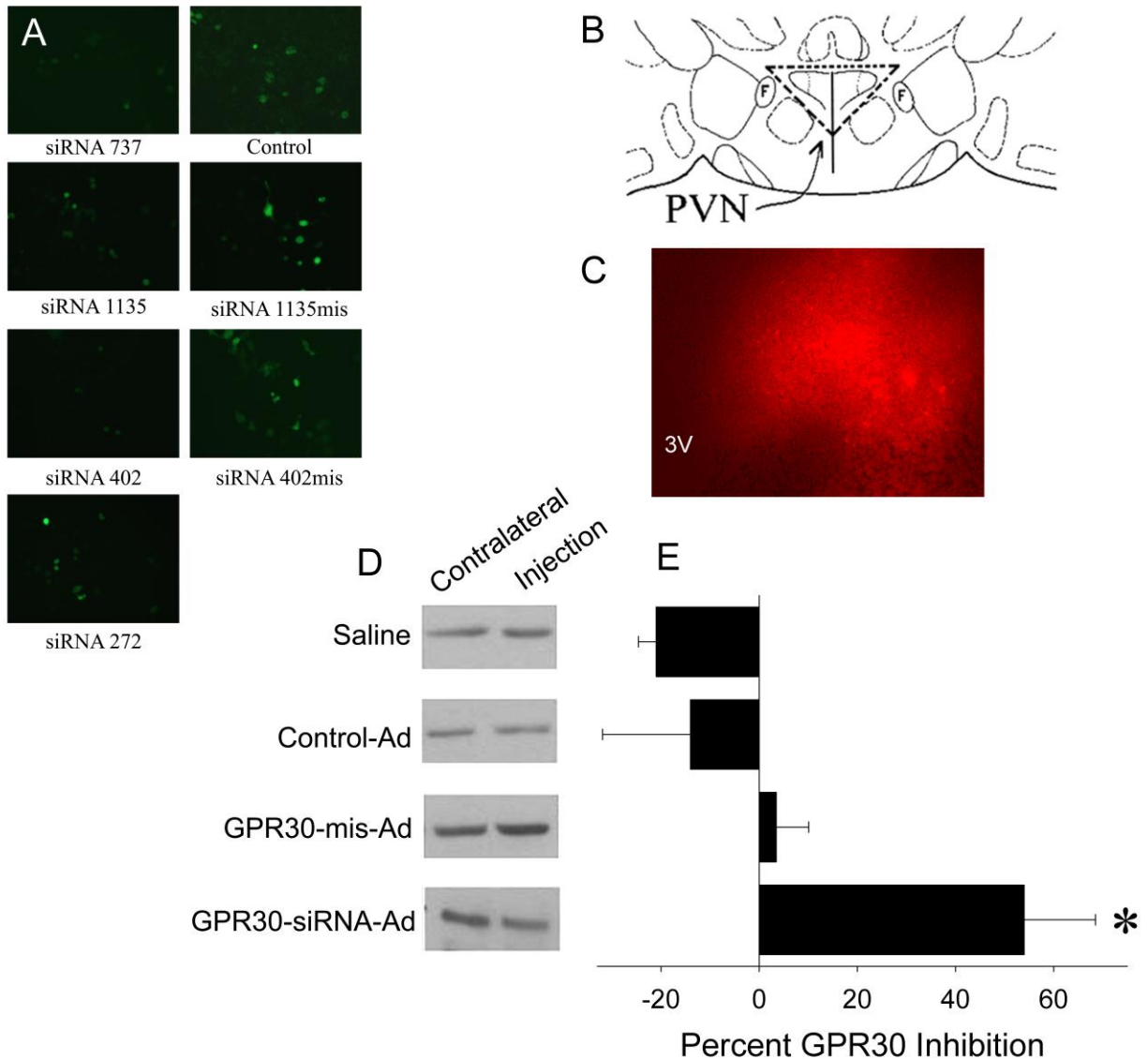


Figure 3.3. Generation and evaluation of recombinant adenoviruses containing GPR30-siRNAs. (A) Selection of GPR30-siRNAs: SOS-GPR30-siRNA-HUS (left panels), SOS-GPR30-mis-siRNA- HUS (right panels), and SOS-GPR30-HUS (control) (right panels) constructs were transfected into HEK293 cells. The number and brightness of GFP-containing cells were observed three days after the transfection. (B) Diagram of PVN injection site. F = fornix. Vertical line represents the 3rd ventricle. PVN is within the dashed triangle. (C) An example of RFP expression in the PVN five days after unilateral injection of GPR30-siRNA-Ads. 3V = 3rd ventricle. (D) Examples of Western blot for GPR30 protein from PVN tissue with unilateral injection of saline, control-Ad, GPR30-mis-Ads, and GPR30-siRNA-Ads five days after infection. (E) Quantitation of GPR30 knockdown, presented as mean percent inhibition (injected side compared to contralateral) \pm SEM (n=4-5), * p <0.05 * p <0.05 by Student-Newman-Keuls post hoc test.

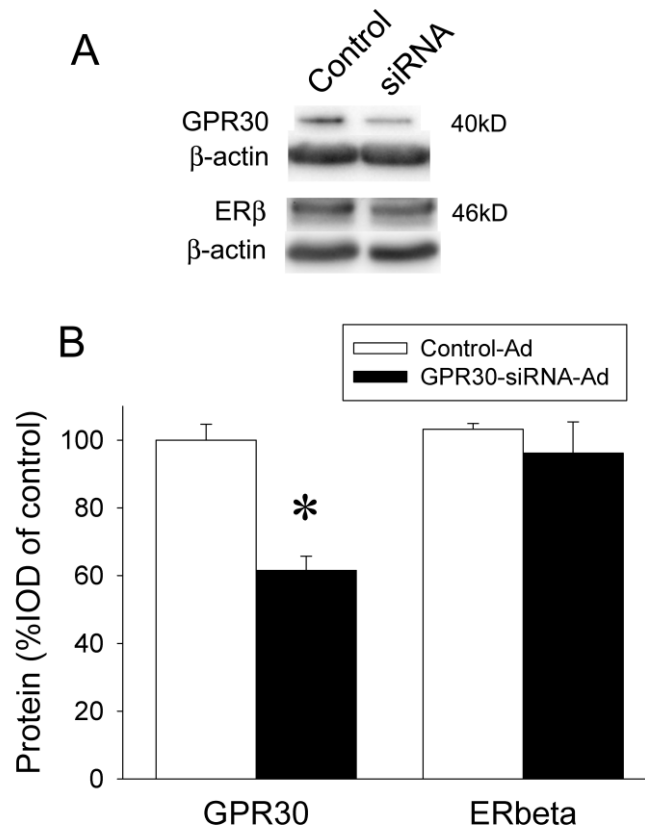


Figure 3.4. Confirmation of GPR30 knockdown. **(A)** Examples of Western blots of GPR30 and ER β with β -actin as loading control in GPR30-siRNA-Ad- and control-Ad-injected PVN. **(B)** Quantitation of GPR30 knockdown and ER β protein levels in the PVN of GPR30-siRNA-Ad- and control-Ad-injected rats. Data are expressed as mean percent of control-Ad \pm SEM (n=4-7), * p <0.01 by Student-Newman-Keuls post hoc test.

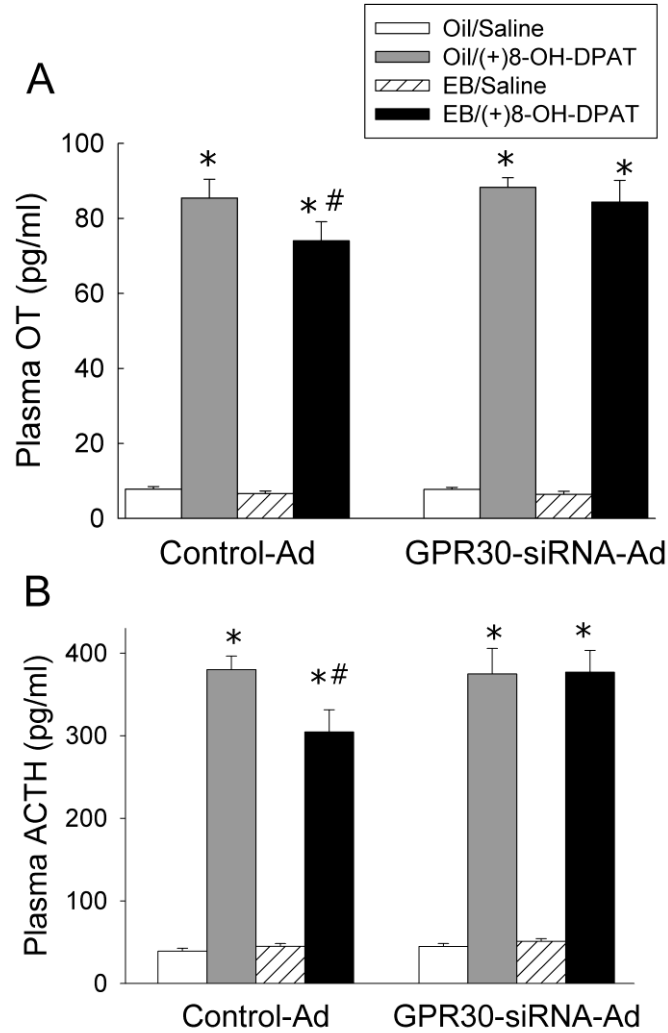


Figure 3.5. Effects of GPR30-siRNA-Ads on plasma OT (**A**) and ACTH (**B**) responses to (+)8-OH-DPAT in EB-treated rats. Data are expressed as mean \pm SEM, (n=7-10). *Indicates significantly different from saline-challenged animals with same treatment, $p < 0.0001$, #indicates significantly different from control-Ad/oil-treated rats with same challenge, $p < 0.05$ by Student-Newman-Keuls post hoc test.

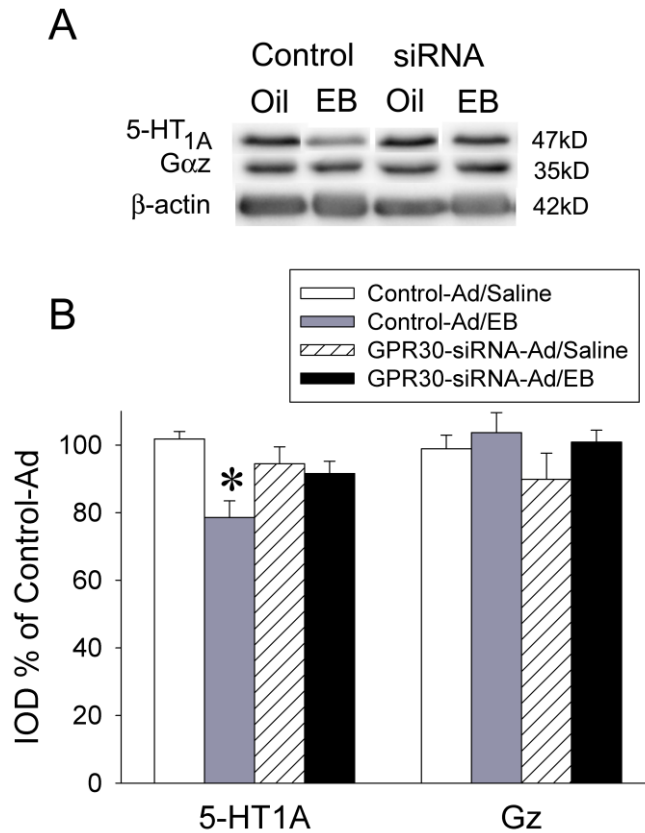


Figure 3.6. GPR30-siRNA-Ads prevented EB-induced reductions of 5-HT_{1A} receptor protein in the PVN. **(A)** Representative Western blots of 5-HT_{1A} receptor and Gα_Z protein levels with β-actin loading control in the PVN of rats injected with control-Ad or GPR30-siRNA-Ad followed by treatment with oil or EB. **(B)** Quantitation of Western blots. Data are expressed as mean percent of control-Ad/oil, ± SEM (n=7-12) after normalization to β-actin. **p*<0.001 by Student-Newman-Keuls post hoc test.

DISCUSSION

We previously reported that 2-day peripheral administration of EB resulted in the desensitization of 5-HT_{1A} receptor signaling in the rat PVN^{204,243}. Our previous study demonstrated that this desensitization is not mediated by ERβ, as treatment with a selective ERβ

agonist, DPN, did not mimic the results of EB treatment. Furthermore, siRNA knockdown of ER β in the PVN did not prevent EB-induced desensitization of 5-HT $_{1A}$ receptor signaling²¹³. However, 2-day intra-PVN injections of the selective GPR30 agonist, G-1, did result in desensitization of 5-HT $_{1A}$ receptor signaling¹⁴³. Therefore, in this study we chose to focus on the role of GPR30. We show for the first time that GPR30 expression is necessary for the EB-induced desensitization of 5-HT $_{1A}$ receptor signaling, and that this mechanism involves a decrease in 5-HT $_{1A}$ receptor protein expression and possibly an increase in the rate of *Gaz*-GTP hydrolysis, as suggested by an increase in RGSz1 protein expression.

To identify the role of GPR30 in EB-induced changes in 5-HT $_{1A}$ receptor signaling, we injected an adenovirus containing siRNA sequences against GPR30 directly into the PVN. Treatment with GPR30-siRNA-Ads produced a modest reduction of GPR30 protein, by about 32%. Despite such a modest reduction in GPR30 protein, GPR30-siRNA-Ads infection was successful in completely preventing the reduction of both the OT and ACTH hormone responses to (+)8-OH-DPAT produced by EB treatment, reinforcing the hypothesis that GPR30 is necessary for estradiol-induced 5-HT $_{1A}$ receptor desensitization.

To further elucidate the GPR30-mediated mechanism through which EB impacts 5-HT $_{1A}$ receptor desensitization, we examined changes in the 5-HT $_{1A}$ receptor signaling pathway. Two-day treatment with EB resulted in a decrease in 5-HT $_{1A}$ receptor protein in the PVN, which is consistent with previous studies that have reported a decrease in 5-HT $_{1A}$ receptor binding sites in the hypothalamus and DRN of nonhuman primates¹⁶⁹ and reduced 5-HT $_{1A}$ receptor protein levels in the DRN²⁴⁴ after chronic estradiol treatment. In contrast, we found that 2-day EB treatment increased 5-HT $_{1A}$ receptor mRNA. This upregulation of 5-HT $_{1A}$ receptor mRNA in the PVN may be a feedback response to the decrease in protein levels induced by estradiol, or estradiol

signaling may be affecting 5-HT_{1A} receptor translational control. Other studies have demonstrated that acute estradiol administration decreased 5-HT_{1A} receptor gene expression in the rat limbic system²⁴⁵, while chronic estradiol decreased 5-HT_{1A} receptor mRNA in the rat DRN²⁴⁶ but increased 5-HT_{1A} receptor mRNA in the DRN in nonhuman primates²⁴⁷. These discrepancies could be due to tissue-specific regulatory mechanisms, differences in chronic versus short-term estradiol administration, or species differences. Notably, the 32% reduction in GPR30 induced by infection with the GPR30-siRNA-Ads prevented the EB-induced changes in 5-HT_{1A} receptor protein, thus reinforcing an important role for GPR30 in this mechanism.

There are some studies linking changes in 5-HT_{1A} receptor expression to depression in human female patients²⁴⁸, which would indicate a role for estradiol in mediating 5-HT_{1A} receptor expression as well as function. Pet-1, which is necessary for development of the serotonin system²⁴⁹⁻²⁵², directly binds to Pet-1 elements in the human 5-HT_{1A} receptor promoter region, and is critical for 5-HT_{1A} receptor expression in the midbrain²⁵³. In macaques, long-term OVX reduced expression of Pet-1 in the DRN²⁵⁴, while in rats, two-day administration of estradiol after OVX increased Pet-1 mRNA levels in the DRN²⁵⁵, indicating a clear role for regulation of midbrain 5-HT_{1A} receptor expression by estradiol. A reduction in inhibitory DRN 5-HT_{1A} autoreceptors, induced by estradiol via Pet-1, may play a role in desensitizing post-synaptic 5-HT_{1A} receptors in the PVN by increasing serotonergic tone, similar to chronic SSRI treatment.

Though GPR30 is a membrane receptor that signals through heterotrimeric G proteins, studies have demonstrated that signaling through GPR30 has effects on gene transcription, independent of classical estrogen response elements^{136,148}. GPR30 has been shown to act independently of ER α and ER β to stimulate the MAPK/ERK1/2 signaling system via a PTX, G $\beta\gamma$ pathway^{132,256}. We showed previously that treatment with PTX inhibits modulation of 5-

HT_{1A} receptor signaling by estradiol¹⁴³, which again suggests that GPR30 signaling plays a role in this mechanism. In the current study, we saw an estradiol-induced decrease in 5-HT_{1A} receptor expression that was precluded by GPR30 protein reduction. This suggests that estradiol could alter 5-HT_{1A} receptor expression levels through the GPR30-Gβγ, MAPK/ERK1/2 pathway to transiently decrease 5-HT_{1A} receptor mRNA in the PVN. The subsequent decrease in 5-HT_{1A} receptor protein levels could then feedback to lead to upregulation of mRNA expression, though the mechanisms remain unclear

5-HT_{1A} receptors in the PVN are coupled to the G protein *Gαz* to stimulate OT and ACTH (via CRF) release^{49,215,216}. Previous studies have shown that chronic estradiol treatment results in decreased DRN protein levels of *Gai₃*, but not *Gai₁*, *Gai/o*, or *Gαz* in nonhuman primates¹⁶⁹. Similarly, in the present study we found that 2-day EB administration had no effect on *Gαz* protein or mRNA levels in the PVN. These results are consistent with previous reports showing that EB treatment does not affect levels of *Gαz* protein in the rat PVN^{219,243}. Knockdown of GPR30 by GPR30-siRNA-Ads also had no effect on *Gαz* levels, indicating that GPR30 signaling does not regulate *Gαz* protein levels, and that alterations of *Gαz* levels are not responsible for estradiol-mediated 5-HT_{1A} receptor desensitization.

While a decrease in 5-HT_{1A} receptor protein expression may play a role in EB-induced desensitization, it is interesting that estradiol treatment also reduces 5-HT_{1A} receptor function^{204,243,257,258} and coupling to G proteins²⁵⁹, though we show that *Gαz* protein expression is not affected by EB. We therefore examined protein and mRNA expression of RGSz1, the GAP that is highly selective for *Gαz*^{79,80,217}. Consistent with a previous report²¹⁹, we found that 2-day EB treatment increased RGSz1 protein and now have found an increase in RGSz1 mRNA in the PVN. By increasing the hydrolysis of *Gαz*-GTP by over 400-fold⁸⁰, high levels of RGSz1 could

effectively reduce the ability of Gα_z to activate downstream effectors and hormone release upon 5-HT_{1A} receptor stimulation, thus contributing to the desensitized response. Work is currently being done in our laboratory to investigate the potential interactions between RGSz1, Gα_z, and 5-HT_{1A} receptors.

Since OT-containing neurons express very little ERα²³⁹, we have not yet directly addressed ERα as a potential candidate for desensitization of 5-HT_{1A} receptor signaling. However, some studies have shown that hypothalamic neurons, as well as other cell types, have a number of ERα splice variants that are located in the plasma membrane and could mediate estradiol signaling^{126,260-265}. Recent studies have found a novel, membrane-targeted, 36kD splice variant (ERα36) with a unique C-terminal sequence in human tissues and human cell lines^{266,267}, and suggest that ERα36 may be involved in responses heretofore believed to be mediated by GPR30. In ER-negative breast cancer cell lines that express ERα36 (e.g. MDA-MB-231 and SK-BR-3), the selective estrogen receptor modulator tamoxifen and ER antagonist ICI 182, 780 were unable to block nongenomic estrogen signaling^{268,269}, similar to effects seen when GPR30 is expressed¹³⁷. A recent study by Kang et al.²⁷⁰ demonstrated that GPR30 expression and signaling induced ERα36 expression, that G-1 specifically binds and activates ERα36, and that ERα36 mediates nongenomic estrogen signaling independent of GPR30 in full-length-ERα-negative but ERα36-positive breast cancer cells. They suggest that GPR30 signals through the Src/MAPK/AP-1 pathway to activate ERα36 promoter activity and induce ERα36 expression, and that G-1 may be an agonist for ERα36, not GPR30. Other studies have reported ERα36 effects or immunoreactivity in rodent tissues; however, they failed to directly demonstrate expression of this splice variant^{271,272}. Indeed, we performed immunohistochemical staining of rat brain tissue using the antibody kindly provided by Wang's group²⁶⁹, raised against the unique

C-terminal sequence of ER α 36, and found strong immunoreactivity in neurons throughout the rat brain. However, we conducted a BLAST search for the unique 88 base pair sequence of human ER α 36 in the rat genome and no comparable sequence was detected. These data suggest that while it is possible that the rat brain may contain an ER α splice variant that plays a role in estradiol-GPR30 signaling, this putative splice variant is not the same as the ER α 36 described in humans.

The results of this study demonstrate that GPR30 expression is necessary for estradiol-induced desensitization of 5-HT_{1A} receptor signaling in the PVN, and that the mechanism likely involves a decrease in 5-HT_{1A} receptor protein levels and an increase in RGSz1 expression. Further studies are needed to clarify whether ER α or any of its splice variants play a role in this mechanism. Because the effects of SSRIs occur slowly and are thought to involve desensitization of pre-synaptic and post-synaptic 5-HT_{1A} receptors^{21,101,201,234,235}, elucidating the estrogen-receptor-mediated mechanisms that result in rapid desensitization of 5-HT_{1A} signaling will be important in the design of novel treatments that activate specific estrogen receptors in conjunction with SSRI therapy, for the improved treatment of depression and other mood disorders.

CHAPTER FOUR

GPR30 STIMULATION ALTERS POSTTRANSLATIONAL MODIFICATION OF RGSZ1 AND INDUCES DESENSITIZATION OF 5-HT_{1A} RECEPTOR SIGNALING IN THE RAT HYPOTHALAMUS

ABSTRACT

Desensitization of 5-HT_{1A} receptor (5-HT_{1A}R) signaling in the PVN is associated with effective SSRI antidepressant treatment, which in rats takes seven days to develop. Estradiol alone produces a partial desensitization of 5-HT_{1A}R signaling, and synergizes with SSRIs to result in a rapid and complete desensitization with two days of treatment as measured by a decrease in the oxytocin and ACTH responses to selective stimulation of 5-HT_{1A}R. GPR30 is necessary for estradiol-induced desensitization of 5-HT_{1A}R signaling, although the mechanisms underlying this effect are still unclear. The purpose of this study was to determine whether peripheral delivery of the GPR30 selective agonist G-1 could induce desensitization of 5-HT_{1A}R signaling, and to investigate the effects of treatment on 5-HT_{1A}R signaling components. Two-day systemic G-1 treatment produced partial desensitization of oxytocin and ACTH responses to 5-HT_{1A}R stimulation. Estradiol and G-1 treatment produced dramatic alterations to a GTPase activating protein selective for G α _z, RGSz1, which likely represent changes in posttranslational modification. RGSz1 isoforms are differentially glycosylated, sumoylated, and phosphorylated, and are differentially distributed in subcellular organelles. Sumoylated and glycosylated RGSz1 localized to the detergent resistant microdomain (DRM) of the plasma membrane, and was increased by estradiol and G-1 treatment. Because active G α _z also localizes to the DRM, increased DRM-localized RGSz1 could functionally reduce G α _z downstream signaling, and thus

reduce oxytocin and ACTH release. Together, these results identify GPR30 and RGSz1 as targets for the development of new adjuvants for the treatment of depression.

INTRODUCTION

Mood disorders are more prevalent in women^{192,273,274} and depression is associated with periods of hormone fluctuation and reduction in estrogen levels^{226,227,275-277}, suggesting a gender-dependent risk factor for the development of mood disorders. In rodents, females exhibit higher sensitivity to serotonergic stimulation and increased hypothalamic-pituitary-adrenal (HPA) axis activity compared to males¹⁰³. Serotonin is a major mediator of the HPA axis, and change in serotonergic function is a hallmark of many neuropsychiatric disorders. Dysregulation of the HPA stress axis is the most common and predictive physiological symptom in depression^{87,278,279}, and normalization of the stress response is associated with successful antidepressant treatment⁸⁴.

Recent studies have shown that depression-like behavior in rodents is modulated by specific serotonin receptors, in particular 5-HT_{1A}R^{280,281}. Chronic treatment with SSRIs produces desensitization (attenuation) of 5-HT_{1A}R signaling in the PVN, as measured by a reduction in the OT and ACTH response to 5-HT_{1A}R stimulation^{187,282,283}. It takes 3-12 weeks to achieve clinical efficacy with SSRIs; this therapeutic lag is thought to be partly due to the time it takes for desensitization of 5-HT_{1A}R signaling in the PVN to occur²⁰¹⁻²⁰³.

Neuroendocrine challenge tests that detect peripheral changes in hormone responses to 5-HT_{1A}R agonists can be used to measure desensitization of 5-HT_{1A}R signaling in the PVN^{190,236}. Recently, we have shown that 2-day estradiol treatment accelerates SSRI-induced 5-HT_{1A}R desensitization²⁰⁶, which may have clinical relevance to decrease therapeutic lag. However,

because estrogen therapy in peri- and post-menopausal women is associated with increased risk of breast cancer, stroke, and heart disease^{207,209,284}, identifying the estrogen receptor and associated changes in 5-HT_{1A}R signaling components that contribute to desensitization will suggest new targets for the treatment of depression.

Previous data in our laboratory have demonstrated that estradiol signaling through the membrane estrogen receptor GPR30 is both necessary and sufficient for estradiol-induced desensitization of 5-HT_{1A}R signaling^{143,220}, but the effect of GPR30 signaling on 5-HT_{1A}R signaling components to produce desensitization is still unclear. In the PVN, 5-HT_{1A}R couples to Gα_Z to mediate the release of OT and ACTH⁴⁹. Gα_Z is active in the GTP-bound form and has a very slow intrinsic rate of GTP hydrolysis. RGSz1, a regulator of G protein signaling, has a high affinity for Gα_Z and increases the rate of GTP hydrolysis by over 400-fold, effectively down-regulating Gα_Z downstream signaling^{79,80,217}. Increased RGSz1 activity could therefore contribute to desensitization of 5-HT_{1A}R signaling.

RGSz1 is part of the RGS-Rz subfamily of RGS proteins⁶⁷ and is an alternative splice variant of the RGS20 gene, which is expressed almost exclusively in nervous tissue⁷⁹. RGSz1 in the rat is a 242 amino acid protein containing the RGS-Rz conserved domain (RGS box) that binds the GαGTP subunit to accelerate hydrolysis. The predicted molecular weight of RGSz1, based on its amino acid sequence, is 27-29kD. However, RGSz1 in the brain displays protein isoforms of many different sizes, which may represent different posttranslational modifications. We have shown previously that 2-day estradiol treatment resulted in an increase in RGSz1 mRNA, as well as RGSz1 proteins^{219,220}. RGSz1 has been shown to undergo posttranslational modification in isolated mouse synaptosomal membranes, including glycosylation,

SUMOylation, and phosphorylation^{73,218}, which may affect its interaction with and ability to regulate *Gαz* and thus contribute to desensitization of 5-HT_{1A}R signaling.

In this study, we investigated the hypothesis that alterations in RGSz1 expression and posttranslational modification after GPR30 stimulation underlie estradiol-induced desensitization of 5-HT_{1A}R signaling. To test this hypothesis, we pretreated ovariectomized rats with estradiol or the GPR30 selective agonist G-1 to induce desensitization of 5-HT_{1A}R signaling, and found that G-1 and estradiol pretreatment significantly altered specific RGSz1 isoforms. To further investigate how these changes could underlie desensitization of 5-HT_{1A}R signaling, we examined and characterized these isoforms, including identifying the different posttranslational modifications and subcellular localization of the different RGSz1 isoforms.

MATERIALS AND METHODS

Animals

Female OVX Sprague-Dawley rats (225-250g) purchased from Harlan (Haslett, MI) were housed two per cage in a temperature-, humidity-, and light-controlled room (12 h light/dark cycle). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and as approved by the University of Kansas Institutional Animal Care and Use Committee. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

Drugs

17β-Estradiol-3-benzoate (EB) was purchased from Sigma-Aldrich (St. Louis, MO). EB was first dissolved in 100% ethanol to a concentration of 25μg/ml and then diluted to the final

concentration with sesame oil. The EB solution and sesame oil were administered at 0.4ml/kg (EB dose 10µg/kg subcutaneous (s.c.)). G-1 (1-(4-(6-Bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-ethanone)) was purchased from EMD Chemicals (Newark, NJ). G-1 was dissolved in 100% DMSO to a concentration of 5mg/ml and 10mg/ml and administered at 0.5ml/kg (G-1 dose 2.5mg/kg and 5mg/kg s.c.). Fluoxetine-HCl (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.85% NaCl (saline) at a concentration of 5mg/ml and administered at 2ml/kg (fluoxetine dose 10mg/kg s.c.). (+)8-Hydroxy-2-dipropylaminotetralin ((+)8-OH-DPAT) was purchased from Tocris (Ellisville, MO). (+)8-OH-DPAT was dissolved in 0.85% NaCl (saline) at a concentration of 0.2mg/ml and administered at a dose of 0.2mg/kg s.c. Solutions were made fresh before injection.

Experimental Procedure

Effects of G-1 treatment on desensitization 5-HT_{1A}R signaling in the PVN

5 days after OVX, rats were treated with either EB (10µg/kg/day), 2.5 or 5 mg/kg/day G-1, or vehicle (DMSO) once a day for 2 days. 18h after the last G-1 or vehicle treatment, rats were injected with 0.2mg/kg (+)8-OH-DPAT or saline and sacrificed via decapitation 15 minutes later. Brains were removed and snap-frozen in dry-ice-cooled isopentane and then on dry ice. Trunk blood was collected in tubes containing 0.5ml 3M EDTA (pH 7.4). Brains and plasma were stored at -80°C until use.

Effects of G-1 and fluoxetine combined on desensitization 5-HT_{1A}R signaling in the PVN

5 days after OVX, rats were treated with either EB (10µg/kg/day), 5 mg/kg/day G-1, fluoxetine (10 mg/kg/day), EB/fluoxetine combined, G-1/fluoxetine combined, or vehicle (DMSO) once a day for 2 days. 18h after the last treatment, rats were injected with 0.2mg/kg (+)8-OH-DPAT or

saline and sacrificed via decapitation 15 minutes later. Brains were removed and snap-frozen in dry-ice-cooled isopentane and then on dry ice. Trunk blood was collected in tubes containing 0.5ml 3M EDTA (pH 7.4). Brains and plasma were stored at -80°C until use.

Biochemical assays

Radioimmunoassay of plasma oxytocin and ACTH

Plasma oxytocin and ACTH were determined by radioimmunoassay as previously described with minor modifications^{190,242}. The radioactive 125I oxytocin and 125I ACTH (specific activity of each: 2200 Ci/mmol) were obtained from Perkin Elmer (Waltham, MA).

Subcellular fractionation of rat cortex

Subcellular fractionation was performed as described previously²⁰⁶. Briefly, the cortex from four rats was homogenized in 4 volumes of homogenate buffer (0.32M sucrose, 1mM EDTA 10mM Tris-HCl pH 7.4, 20 mM *N*-ethylmaleimide (NEM), 1:100 dilution of protease inhibitor (Sigma) and phosphatase inhibitors II and III (Sigma)) using a Powergen 1000 homogenizer at speed 5, 4°C for ~10 sec. After a centrifugation at 1500x g, 4°C for 15 min, the supernatant was used for discontinuous gradient centrifugation. Iodixanol (Optiprep, Sigma) was diluted into 7.5, 10, 12.5, 15, 20, 25 and 30% with Diluent (0.25mM sucrose, 6mM EDTA 60mM Tris-HCl pH 7.4, 20 mM NEM, 1:100 dilution of protease and phosphatase inhibitors). 1 ml of each solution from 7.5% to 30% was underlaid sequentially in a 13 ml SW41 centrifuge tube. 1 ml brain homogenate supernatant was layered on the 5% iodixanol solution and centrifuged at 200,000x g at 4°C for 3 hours in a SW41 rotor. After the centrifugation, 0.5 ml fractions were collected from the top to the bottom of the tube. The membrane proteins were then solubilized with 50 µl 10%

sodium cholate, then sonicated and shaken for one hour at 4°C. The fractions were aliquoted and stored at -80°C until use. The protein concentration of the fractions was measured using Pierce BCA protein assay kit (Thermo Scientific Inc, Rockford IL). 10 µg of protein were used for immunoblots. All experiments were performed in triplicate.

Isolation of DRM from cortex tissue

Isolation of the detergent-resistant microdomain (DRM) was performed as described previously²⁰⁶. Briefly, rat cortex was homogenized with a homogenizer driven by an overhead motor at 500 rpm for 25 strokes in 10 volumes of homogenate buffer (10mM Tris-HCl pH 7.4, 5mM EDTA, 320 mM sucrose, 20mM NEM, 1:100 dilution of protease and phosphatase inhibitor cocktails). The homogenate was centrifuged at 800xg for 15 min at 4°C to remove the nucleus and cell debris. The supernatant was then centrifuged at 30,000xg for 30 min at 4°C, and the resulting pellet was resuspended in 2.2ml of extracting buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA, 0.5% Triton X-100 (v/v), 20mM NEM, 1:100 dilution of protease and phosphatase inhibitor cocktails) and then incubated for 10min on ice. 2ml of the Triton X-100-treated homogenate was adjusted to a final concentration of 40% sucrose with an 80% sucrose solution in extracting buffer. The 40% sucrose Triton X-100-treated homogenate was placed on the bottom of a 13ml centrifuge tube and overlaid with 6ml of 30% sucrose. Finally, 2ml of 5% sucrose buffer was placed on the top of the 30% sucrose. The sucrose gradient was then centrifuged at 230,000xg, 4°C for 16h. After the centrifugation, 1ml fractions were collected from top to bottom. 0.1ml of 10% sodium cholate was added to the fractions to solubilized membrane proteins, which were then sonicated and shaken for 1h at 4°C. The

fractions were aliquoted and stored at -80°C until use. The protein concentration of the fractions was measured using Pierce BCA protein assay kit. $10\ \mu\text{g}$ of protein were used for immunoblots.

Immunoblot assays

The PVN was punched out from $300\ \mu\text{m}$ -thick sections prepared using a cryostat microtome. PVN tissue was homogenized in $100\ \mu\text{l}$ homogenization buffer (50 mM Tris, 150 mM NaCl, and 10% sucrose, pH 7.4, 20 mM NEM, 1:100 protease and phosphatase inhibitor cocktails) using a Bullet Blender at speed 10, 4°C for 8 min with 1mm beads. The cortex was homogenized in 10 volumes of homogenate buffer using a Powergen 1000 homogenizer at speed 5, 4°C for ~ 10 sec. After centrifugation at $25,000\times g$, 60min, at 4°C , the supernatant was reserved as the cytosol fraction. The membrane proteins, located in the pellet, were solubilized in 3 volumes of solubilization buffer (20mM Tris pH 8, 1mM EDTA, 100mM NaCl, 1% sodium cholate containing 20mM NEM and 1:100 protease and phosphatase inhibitor cocktails) via sonication followed by shaking for 1 hour at high speed, 4°C . The membrane fraction was collected after centrifugation at $25,000\times g$, 60min, at 4°C . Protein ($10\ \mu\text{g}/\text{lane}$) was resolved on a 12% SDS-PAGE gel followed by transfer to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated in blocking buffer (5% non-fat milk in Tris-buffered saline with 0.1% Tween-20) to reduce non-specific binding and probed overnight using the following primary antibodies: goat anti-SUMO2/3 1:200 (#sc-5231, Santa Cruz Biotechnology, Dallas, TX), mouse anti-phospho(S/T/Y) 1:100 (#E3074, Spring Biosciences, Pleasanton, CA), affinity purified rabbit anti-RGSz1 1:100 (Biosynthesis, Lewisville, TX), mouse anti-flotillin-1 1:2000 (#610820, BD Biosciences, San Jose, CA), mouse anti- Na^+/K^+ ATPase $\alpha 1$ 1:2000 (#sc-21712, Santa Cruz Biotechnology), rabbit anti-calreticulin 1:2000 (#Ab4, Abcam, Cambridge, MA), goat anti-EEA1

(C-15) 1:1000 (#sc-6414, Santa Cruz Biotechnology), mouse anti- β -actin (C4) 1:20,000 (#69100, MP Biomedicals, Solon, OH). After washing, membranes were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase at a dilution of 1:10,000. Bands were detected with ECL substrate solution (GE Healthcare Biosciences, Piscataway, NJ) using BioRad ChemiDoc XRS+ molecular imager (BioRad, Hercules, CA). Bands were analyzed densitometrically using ImageLab software (BioRad, Hercules, CA). Each band was normalized to actin and calculated as percent of the control group within each blot. All samples were run in triplicate and the average for each was used for the final quantification.

Glycoprotein isolation

Glycoproteins from the membrane fraction of rat cortex were purified using a Pierce glycoprotein isolation kit (Thermo Scientific Inc). Glycoproteins were eluted from the column by heating at 95°C in 200ul SDS-PAGE buffer for 5 minutes. Glycoproteins were separated on 12% polyacrylamide gels and transferred to PVDF membrane, which was probed with affinity-purified anti-RGSz1 (1:100).

Immunoprecipitation

The membrane and cytosol fractions of the cortex containing 500-1000 mg of protein were pre-cleared with 25 ml pre-washed protein G agarose beads (Invitrogen Carlsbad, CA) in total volume of 500 ml of IP buffer (50 mM Tris, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100, 20 mM NEM 1:100 protease inhibitor cocktail and 1:100 phosphatase inhibitor cocktail I and III) with rotation at 4°C for 1h. After centrifugation at 3000x g at 4°C for 5 min, the supernatant was incubated with primary antibody (4µg mouse anti-SUMO-1 (D-11), #sc-5308,

Santa Cruz Biotechnology; 1:50 rabbit anti-RGSz1, Biosynthesis) or 4µg IgG control and rotated at 4°C overnight. 50-100 ml of pre-washed protein G beads were added to each tube and rotated at 4°C for 2 h. Protein G beads were pelleted by centrifugation at 1000x g, at 4°C for 3 min and then resuspended in 0.5 ml ice cold IP buffer. After washing 3 times, the protein complexes were eluted in 25 ml 2X sample buffer without β-mercaptoethanol by heating at 95°C for 5 min, then centrifuging at 3000xg for 5 min. The supernatant was collected and stored at -80°C. Western blotting was performed as described above.

Statistical analysis

All data are expressed as means ± SEM. One- or two-way analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* tests were conducted using a statistical program (Statview version 5.0 software, SAS Institute Inc., Cary, NC).

RESULTS

GPR30 stimulation desensitizes 5-HT_{1A}R signaling

Our previous work has demonstrated that intra-PVN injection of EB and the GPR30 selective agonist G-1 results in partial desensitization of 5-HT_{1A}R signaling, as determined by reduced OT and ACTH release in response to 8-OH-DPAT challenge similar to EB¹⁴³. To determine whether peripheral delivery of G-1 could reproduce the partial desensitization of OT and ACTH response, female SD rats were OVX and treated with vehicle, EB (10 µg/kg/day), and G-1 (2.5 and 5 mg/kg/day) for two days. 18 hours after the second injection, rats were challenged with (+)8-OH-DPAT (0.2 mg/kg) and plasma was collected to examine hormone levels. Baseline levels of plasma OT were unchanged in the treatment groups compared to

vehicle (**Figure 4.1A**). Activation of 5-HT_{1A}R by (+)8-OH-DPAT increased plasma OT levels in vehicle-treated rats. The OT response was significantly reduced in the EB-treated group, indicating partial desensitization of the 5-HT_{1A}R signaling pathway. The high dose of G-1 (5 mg/kg) produced a desensitization response comparable to EB; the low dose of G-1 (2.5 mg/kg) also reduced the OT response, although the effect was not as robust (two-way ANOVA: main effect of (+)8-OH-DPAT: $F_{(1,37)} = 493.6$, $p < .0001$; main effect of pretreatment: $F_{(3,37)} = 8.541$, $p = .0002$; interaction between pretreatment and challenge: $F_{(3,37)} = 5.840$, $p = .0023$).

ACTH baseline response was not affected by any pre-treatment (**Figure 4.1B**). Stimulation of 5-HT_{1A}R by (+)8-OH-DPAT increased ACTH levels in vehicle-treated rats. The magnitude of the ACTH response to (+)8-OH-DPAT was significantly reduced in EB-treated rats. Both doses of G-1 reduced ACTH significantly compared to vehicle and EB (two-way ANOVA: main effect of (+)8-OH-DPAT: $F_{(1,44)} = 842.6$, $p < .0001$; main effect of pretreatment: $F_{(3,44)} = 7.707$, $p = .0003$; interaction between pretreatment and challenge: $F_{(3,44)} = 7.180$, $p = .0005$). Together, these data demonstrate that signaling through GPR30 is sufficient to desensitize the OT response to 5-HT_{1A}R stimulation, and that control of ACTH release is even more sensitive to this mechanism.

Combined fluoxetine and G-1 treatment

Our previous study demonstrated that combining EB treatment with the SSRI fluoxetine accelerated full desensitization of 5-HT_{1A}R signaling²⁰⁶. Having shown that peripheral injection of G-1 for two days produced a partial desensitization response comparable to EB, we next investigated whether G-1 treatment would accelerate fluoxetine-induced desensitization of 5-HT_{1A}R signaling. OVX female rats were treated with vehicle, EB (10µg/kg/day), G-1 (5mg/kg/day), and fluoxetine (10mg/kg/day) for two days. 18 hours after the second injection,

rats were challenged with (+)8-OH-DPAT (0.2mg/kg) and plasma was collected to examine hormone levels. Basal levels of OT were unchanged in the treatment groups compared to vehicle (**Figure 4.2A**). Activation of 5-HT_{1A}R by (+)8-OH-DPAT significantly increased OT levels in the vehicle group compared to saline challenge. Two days of fluoxetine alone and EB alone produced comparable partial desensitization to (+)8-OH-DPAT challenge. EB and fluoxetine combined partially desensitized the OT response, although OT levels were significantly reduced only compared to fluoxetine alone and were no different from EB alone. G-1 treatment and G-1/fluoxetine combined reduced OT levels significantly compared to vehicle treatment, but not compared to any other treatment (three-way ANOVA: main effect of (+)8-OH-DPAT: $F_{(1,67)} = 888.0$, $p < .0001$; main effect of treatment 1 (EB, G-1): $F_{(2,67)} = 8.731$, $p = .0004$; main effect of treatment 2 (FLX): $F_{(1,67)} = 6.517$, $p = .013$; interaction between treatment 1 and 2: $F_{(2,67)} = .190$, $p = .83$; interaction between treatment 1 and challenge: $F_{(2,67)} = 6.896$, $p = .0019$; interaction between treatment 2 and challenge: $F_{(1,67)} = 5.669$, $p = .0201$; interaction between treatment 1, treatment 2, and challenge: $F_{(2,67)} = .172$, $p = .84$).

ACTH baseline levels were not affected by any treatment (**Figure 4.2B**). Activation of 5-HT_{1A}R by (+)8-OH-DPAT significantly increased ACTH levels in the vehicle group compared to saline challenge. Two days of fluoxetine alone and EB alone produced comparable partial desensitization to (+)8-OH-DPAT challenge. EB and fluoxetine combined reduced ACTH release significantly further than either treatment alone, although as with the OT response, this was still only a partial desensitization response. G-1 treatment alone reduced ACTH significantly compared to fluoxetine alone and EB alone, and this effect was just as robust as the EB/FLX effect. G-1 and fluoxetine combined reduced ACTH release by the same magnitude as G-1 alone (three-way ANOVA: main effect of (+)8-OH-DPAT: $F_{(1,75)} = 520.6$, $p < .0001$; main effect of

treatment 1 (EB, G-1): $F_{(2,75)} = 19.80$, $p < .0001$; main effect of treatment 2 (FLX): $F_{(1,75)} = 3.652$, $p = .06$; interaction between treatment 1 and 2: $F_{(2,75)} = 1.967$, $p = .15$; interaction between treatment 1 and challenge: $F_{(2,75)} = 19.24$, $p < .0001$; interaction between treatment 2 and challenge: $F_{(1,75)} = 3.691$, $p = .06$; interaction between treatment 1, treatment 2, and challenge: $F_{(2,75)} = 2.695$, $p = .07$).

GPR30 stimulation alters RGSz1 isoforms in the PVN

Our previous studies have suggested a role for RGSz1 in estradiol-induced desensitization of 5-HT_{1A}R signaling^{205,219,220}. The predicted molecular weight of RGSz1 is 27-29kD; however, western blotting with an affinity-purified RGSz1 antibody reveals a ladder of bands with differential distribution in the cytosol and membrane fractions, and distinct patterns in different brain regions, which could represent different posttranslational modifications or alternative splice variants (**Figure 4.3A**). To test the hypothesis that alterations in RGSz1 expression and posttranslational modification after GPR30 stimulation underlie estradiol-induced desensitization of 5-HT_{1A}R signaling, we first examined the effect of 5mg/kg G-1 and EB treatment on the expression of these different RGSz1 isoforms in the PVN.

In the cytosol fraction, both G-1 and EB treatment had dramatic effects on RGSz1 expression. Both pretreatments increased expression of the 80kD ($F_{(2,15)} = 28$, $p < .0001$) and 50kD bands ($F_{(2,17)} = 5.3$, $p = .017$) and decreased expression of the 135kD ($F_{(2,20)} = 7.7$, $p = .0033$) and 40kD bands ($F_{(2,17)} = 20$, $p < .0001$), with no change in the 35kD ($F_{(2,17)} = 1.2$, $p = .29$) relative to control. G-1 pretreatment increased expression of the 90kD band relative to both vehicle and EB ($F_{(2,17)} = 12$, $p = .0006$). (+)8-OH-DPAT had no effect on any of the RGSz1 isoforms (**Figure 4.3B**). Because the unmodified 29kD RGSz1 band is in such low abundance

relative to the other isoforms, it is not easily detected with the affinity-purified antibody and thus was not measured in these experiments.

In the membrane fraction, the predominant bands were at 145kD and 40kD. G-1 treatment produced a robust increase in the 145kD band ($F_{(2,15)} = 4.4$, $p = .03$), while EB treatment had no effect relative to control. Interestingly, both EB and G-1 treatment increased the 135kD isoform ($F_{(2,14)} = 6.074$, $p = .01$) and decreased the 80kD isoform ($F_{(2,16)} = 28$, $p < .0001$). These results correspond to the changes in the cytosol, in which EB and G-1 treatment had the opposite effect on the respective bands. As in the cytosol, the abundance of the 40kD band was not affected by pretreatment ($F_{(2,14)} = .25$, $p = .79$), and (+)8-OH-DPAT challenge had no effect (**Figure 4.3C**).

GPR30 stimulation alters RGSz1 isoforms in the frontal cortex

The PVN does not contain enough protein to perform IP or subcellular fractionation. To determine whether the frontal cortex would be suitable to examine EB- and G-1-induced changes in RGSz1 isoforms via these methods, we first examined the RGSz1 expression pattern. Western blotting demonstrated that the frontal cortex expresses a different pattern of RGSz1 than the PVN. The 90kD and 80kD isoforms were abundantly expressed in frontal cortex cytosol (**Figure 4.4A**) as well as membrane (**Figure 4.4D**). The frontal cortex did not express a 50kD band, but rather one of lesser molecular weight, about 45kD, and the 40kD was less relatively abundant than in the PVN. There was no 145kD band in the membrane fraction, but rather a more abundant 135kD band. In the cytosol, EB and G-1 increased the 135kD ($F_{(2,42)} = 7.071$, $p = .002$), 90kD ($F_{(2,37)} = 4.913$, $p = .01$), 80kD ($F_{(2,38)} = 6.749$, $p = .003$), 45kD ($F_{(2,38)} = 3.460$, $p = .04$), and 40kD ($F_{(2,38)} = 5.783$, $p = .006$) isoforms, but had no effect on the 35kD isoform ($F_{(2,39)} =$

2.302, $p = .11$) (**Figure 4.4B**). Challenge with (+)8-OH-DPAT had no effect on any band (**Figure 4.4C**).

In the membrane, EB and G-1 treatment had no effect on the 135kD isoform ($F_{(2,33)} = 2.640$, $p = .11$). G-1 treatment alone increased the 90kD isoform compared to vehicle and EB treatments ($F_{(2,33)} = 6.605$, $p = .004$). The 80kD band was increased by G-1, but only compared to EB treatment ($F_{(2,35)} = 3.918$, $p = .03$). G-1 increased the 45kD band relative to vehicle treatment, but not EB treatment ($F_{(2,33)} = 4.302$, $p = .03$). Both EB and G-1 produced an increase in the 40kD RGSz1 band compared to vehicle treatment ($F_{(2,36)} = 5.067$, $p = .01$) (**Figure 4.4E**). Challenge with (+)8-OH-DPAT had no effects (**Figure 4.4F**).

RGSz1 isoforms have selective subcellular localization

To understand the functional importance of the difference RGSz1 isoforms, we examined their subcellular localization via gradient centrifugation to separate the subcellular organelles based on their density (**Figure 4.5A**). We used cortical tissue for these experiments because this region expresses the same bands as the hypothalamus (**Figure 4.5A**), and the PVN does not contain enough protein for this method. The 135kD isoform colocalized with Na^+/K^+ ATPase, a plasma membrane marker. Interestingly, while the 90kD and 50kD bands did not distinctly colocalize with any marker, they did localize with each other and showed some overlap with the plasma membrane marker (**Figure 4.5B**). The 80kD and 35kD bands were located in the early endosome, as marked by EEA1 (**Figure 4.5C**); however, as the fractions containing the early endosome also contain cytosolic markers²⁰⁶, these isoforms could be located in the cytosol as well. Finally, the 45kD and 40kD bands were predominantly located in the ER, as determined by colocalization with calreticulin (**Figure 4.5D**).

Because activated *Gaz* proteins are localized in DRMs of the plasma membrane²⁰⁶, the RGSz1 isoform that facilitates GTP-*Gaz* hydrolysis would need to be located in the DRM. To this end, we isolated DRM with Triton X-100 treatment followed by sucrose gradient centrifugation. DRM fractions were identified by flotillin 1 (**Figure 4.6A**). Interestingly, the 135kD (**Figure 4.6B**) and 90kD (**Figure 4.6C**) proteins distributed in two distinct populations: one which was located in the DRM as marked by flotillin, and one which was located outside the DRM. The 50kD RGSz1 isoform localized entirely in the DRM (**Figure 4.6C**), while the 80kD and 40kD were not colocalized with the DRM, consistent with their location outside the plasma membrane (**Figure 4.5**).

Posttranslational modification of RGSz1

To determine whether the increased molecular weights of the different RGSz1 isoforms could be due to posttranslational modifications, we first used computer-assisted analysis of the RGSz1 amino acid sequence to predict potential modification sites. We found two possible sites for *N*-linked glycosylation (Center for Biological Sequence Analysis, <http://www.cbs.dtu.dk/services/NetOGlyc/>), as well as potential phosphorylation sites on several Ser, Thr, and Try residues (Center for Biological Sequence Analysis, <http://www.cbs.dtu.dk/services/NetPhos/>). In addition, we identified one SUMO consensus site via SUMOsp2.0 GPS program (<http://sumosp.biocuckoo.org>), and a cysteine string motif that is likely to be palmitoylated (<http://csspalm.biocuckoo.org>) (**Figure 4.7A**).

To examine glycosylation of RGSz1, 1000 μ g of protein from the membrane fraction of rat cortical tissue was bound to a column containing WGA resin, then eluted from the column using elution buffer. Purified glycoproteins were separated using SDS-PAGE and Western blotting with affinity-purified anti-RGSz1 detected glycosylated RGSz1 protein. This procedure

identified the 135kD band as the major glycosylated isoform (**Figure 4.7B**); however, the yield was very low. Boiling the sample in SDS-PAGE buffer increased the amount of glycoproteins released from the WGA column, and allowed us to detect significant bands at 135kD, 80kD, and 40kD, as well as several others (**Figure 4.7C**), suggesting that there are three major glycosylated RGSz1 protein isoforms, and the lesser bands are present in less abundant amounts or represent degradation products.

To determine sumoylation of RGSz1, immunoprecipitation (IP) was performed. First, SUMO-1 modified proteins were immunoprecipitated from 500ug of cytosol and membrane fractions of rat cortical tissue and the proteins were examined via western blotting with affinity purified RGSz1 antibody. In the cytosol fraction, three bands were detected at 90kD, 50kD, and 45kD. In the membrane fraction, a 135kD band was detected, as well as a major band at 90 and 40kD (**Figure 4.7D**).

Next, anti-RGSz1 antibody was used to IP RGSz1 proteins from 1000ug of cytosol and membrane fractions of rat cortical tissue. Western blotting with anti-SUMO2/3 revealed a ladder of bands, with the clearest being at 55kD, 45kD, and 35kD in the cytosol and membrane fractions. Although the 55kD and 45kD bands overlap with the IgG nonspecific control, the 45kD density is much higher, indicating that it may be a real band that is the same size as the IgG (**Figure 4.7F**). Western blotting with an antibody against serine/threonine/tyrosine phosphorylation detected a strong band at 35kD and a lighter band at 40kD in both the cytosol and membrane fractions after RGSz1 IP (**Figure 4.7E**).

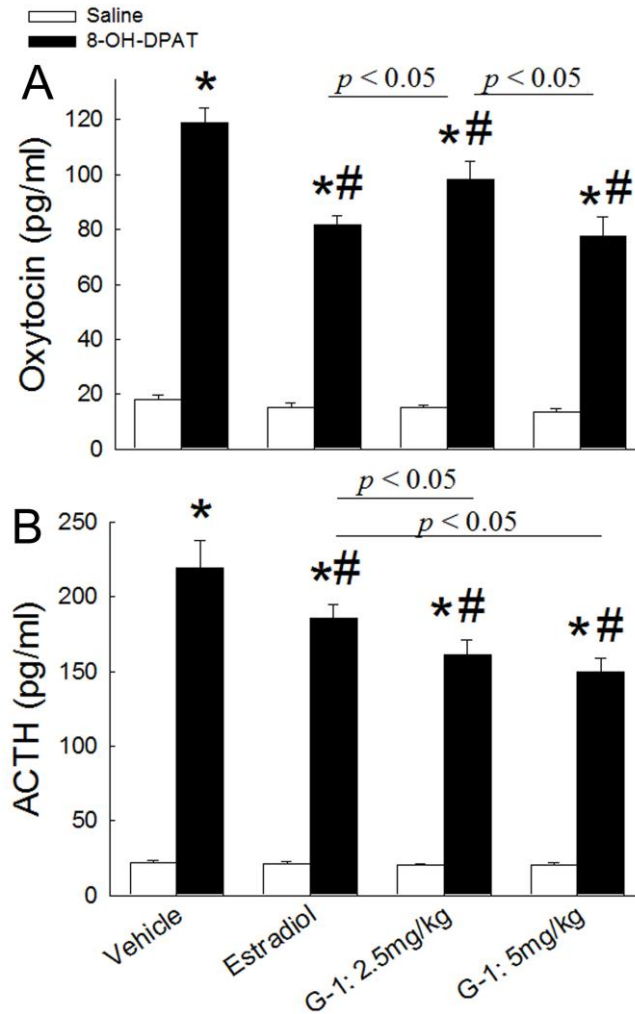


Figure 4.1: Effects of 10 μ g/kg EB, 2.5mg/kg G-1, or 5mg/kg G-1 treatment for 2 days on plasma OT (A) and ACTH (B) levels in response to saline or (+)8-OH-DPAT challenge in OVX rats. The data are presented as the mean \pm SEM ($n = 7-8$). (*)Significantly different from saline-challenged group with same treatment, $p < .0001$; (#)significantly different from (+)8-OH-DPAT-challenged vehicle group, $p < .005$ by Student-Newman-Keuls *post hoc* test.

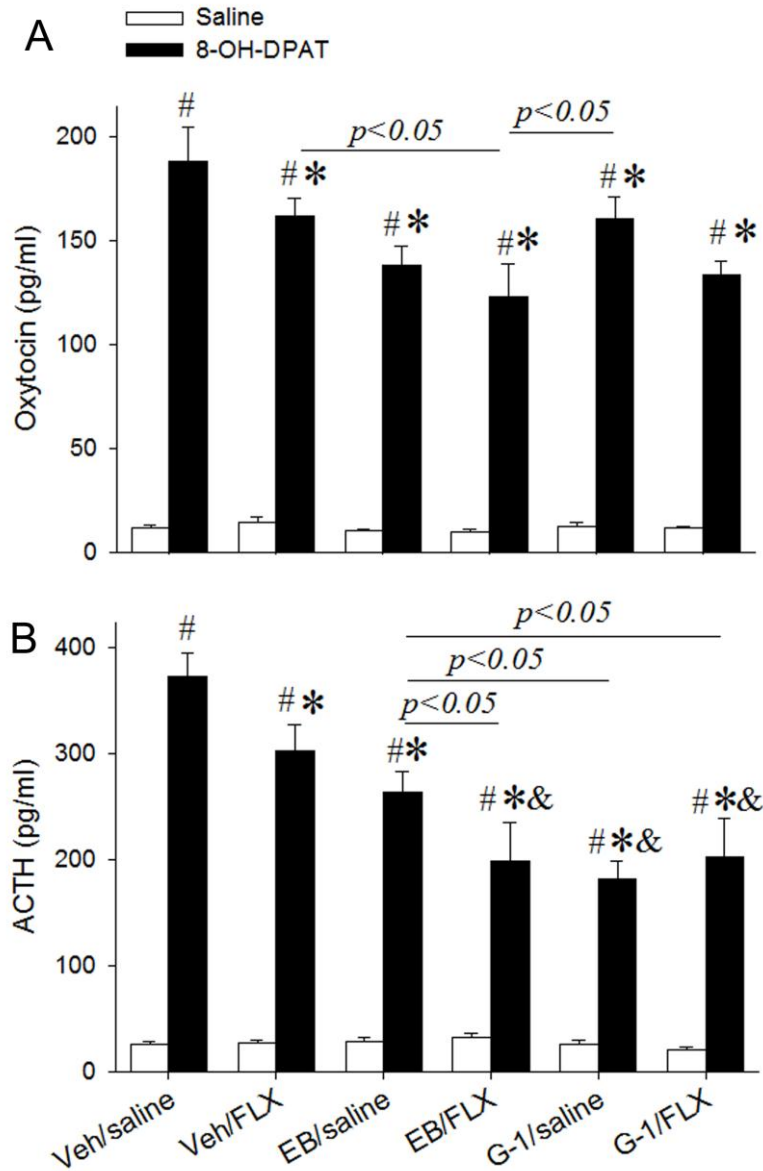
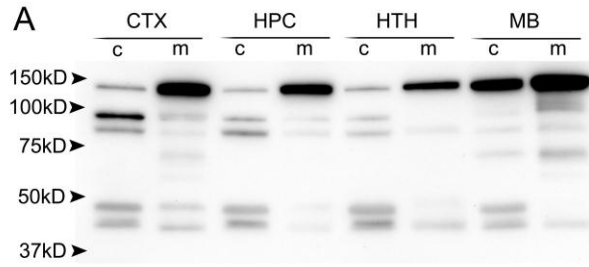


Figure 4.2: Effects of vehicle (Veh), 10 μ g/kg EB, 5mg/kg G-1, and/or 10mg/kg fluoxetine (FLX) treatment for 2 days on plasma OT (A) and ACTH (B) levels in response to saline or (+)8-OH-DPAT challenge in OVX rats. The data are presented as the mean \pm SEM ($n = 7-8$). (*)Significantly different from saline-challenged group with same treatment, $p < .0001$; (#)significantly different from (+)8-OH-DPAT-challenged vehicle group, $p < .005$; (&)significantly different from Veh/FLX group $p < .05$ by Student-Newman-Keuls *post hoc* test.



PVN Cytosol RGSz1

PVN Membrane RGSz1

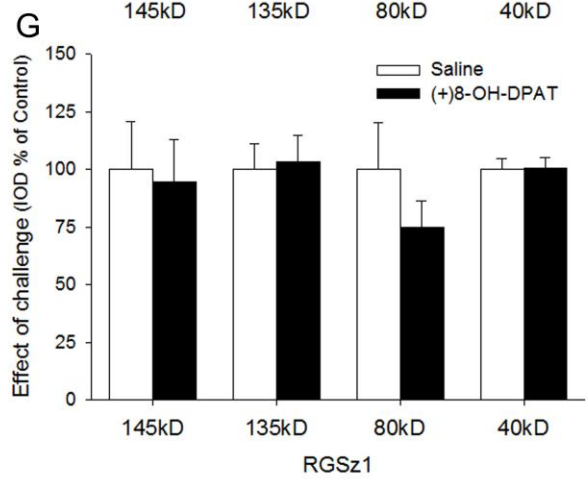
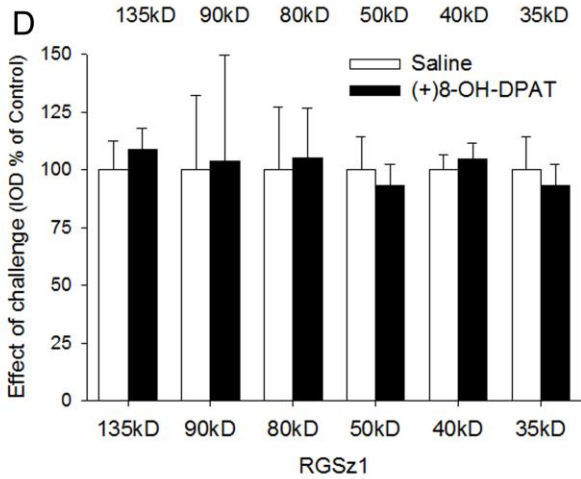
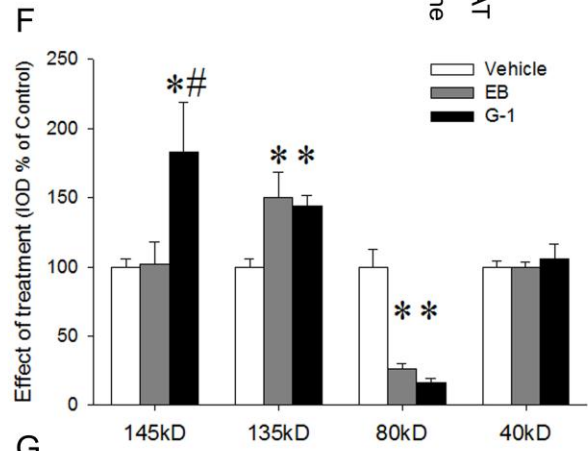
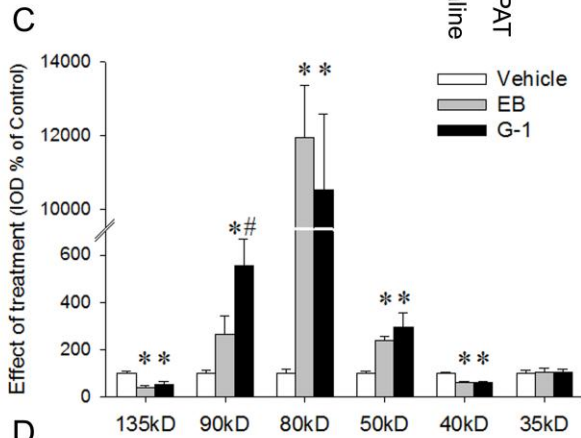
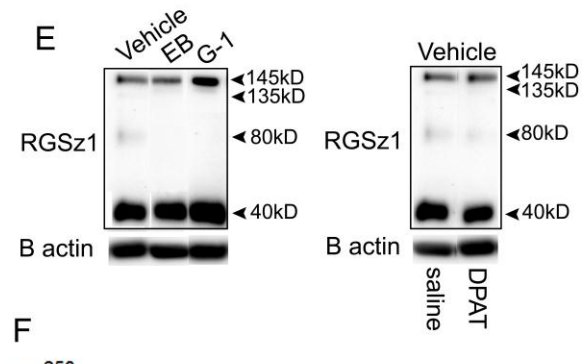
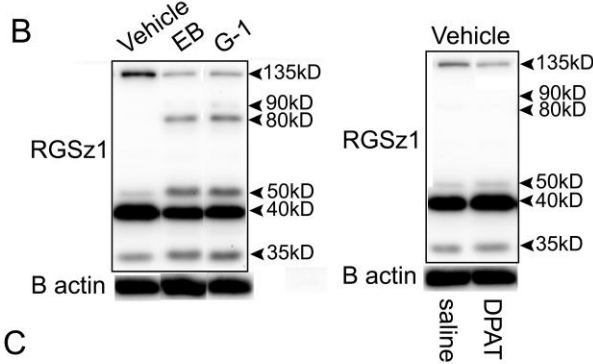


Figure 4.3: RGSz1 protein in the rat brain. (A) Immunoblot detection of RGSz1 expression in the cytosol (c) and membrane (m) fractions of the cortex (CTX), hippocampus (HPC), hypothalamus (HTH), and midbrain (MB) using affinity-purified anti-RGSz1 antibody. (B) Representative immunoblot of cytosolic RGSz1 from the PVN of rats treated with 10 μ g/kg EB or 5mg/kg G-1, with β -actin as loading control. Effect of treatment (C) and challenge (D) is quantified and combined from three separate immunoblots. (E) Representative immunoblot of RGSz1 from the membrane fraction of PVN of rats treated with 10 μ k/kg EB or 5mg/kg G-1, with β -actin as loading control. Effect of treatment (F) or challenge (G) is quantified and combined from three separate immunoblots. Bands were analyzed densitometrically (integrated optical density, IOD). Each band was normalized to β -actin and expressed as percent of control (vehicle or saline). Data are expressed as mean \pm SEM ($n = 4$). (*) Significantly different from vehicle control; (#) significantly different from EB, $p < .05$ by Student-Newman-Keuls *post hoc* test.

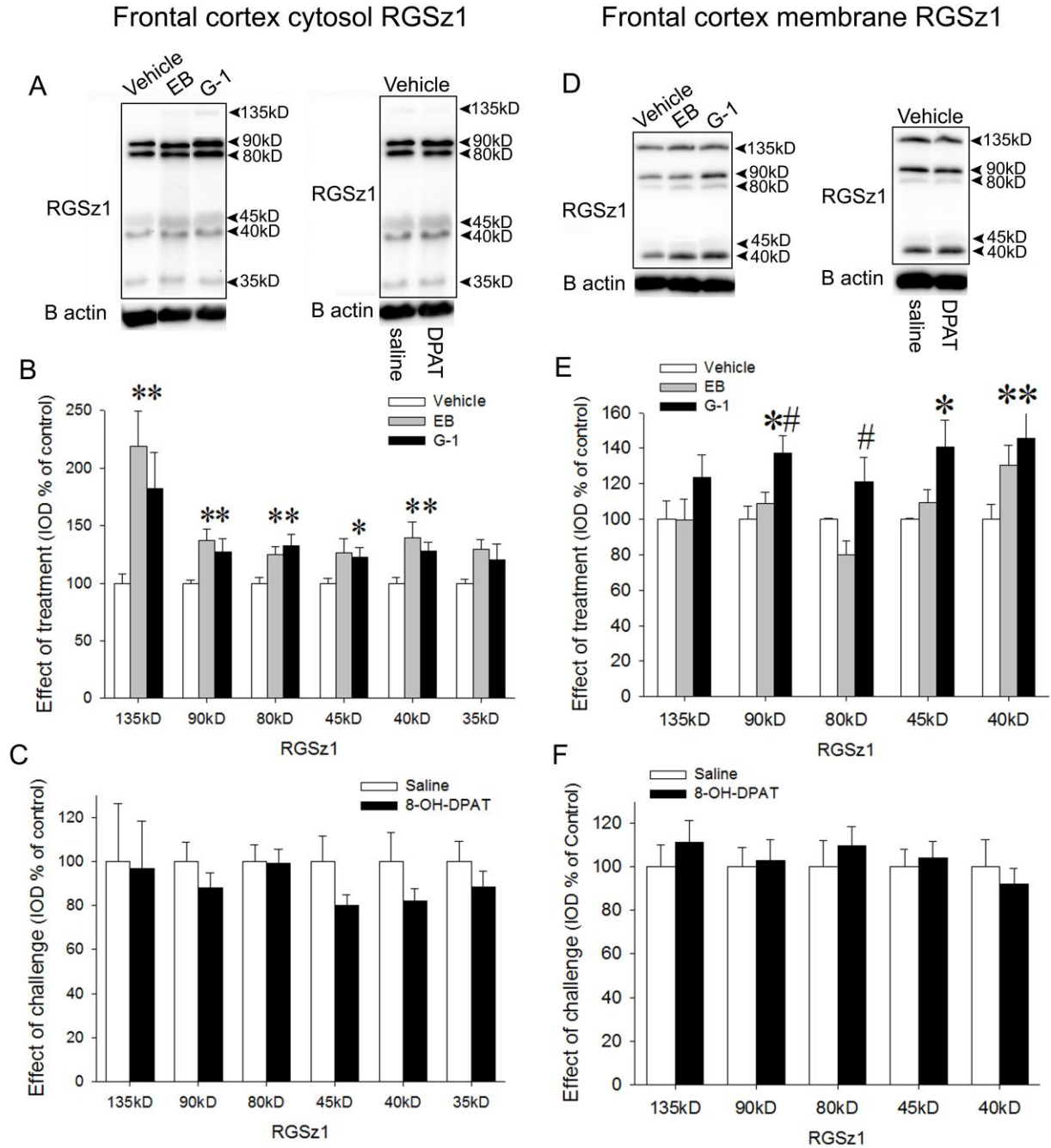


Figure 4.4: RGSz1 protein in the frontal cortex. (A) Representative immunoblot of cytosolic RGSz1 from the frontal cortex of rats treated with 10µg/kg EB or 5mg/kg G-1, with β-actin as loading control. Effect of treatment (B) and challenge (C) is quantified and combined from three separate immunoblots. (D) Representative immunoblot of RGSz1 from the membrane fraction of frontal cortex of rats treated with 10 µg/kg EB or 5mg/kg G-1, with β-actin as loading control.

Effect of treatment (E) or challenge (F) is quantified and combined from three separate immunoblots. Bands were analyzed densitometrically (integrated optical density, IOD). Each band was normalized to β -actin and expressed as percent of control (vehicle or saline). Data are expressed as mean \pm SEM ($n = 8$). (*) Significantly different from vehicle control; (#) significantly different from EB, $p < .05$ by Student-Newman-Keuls *post hoc* test.

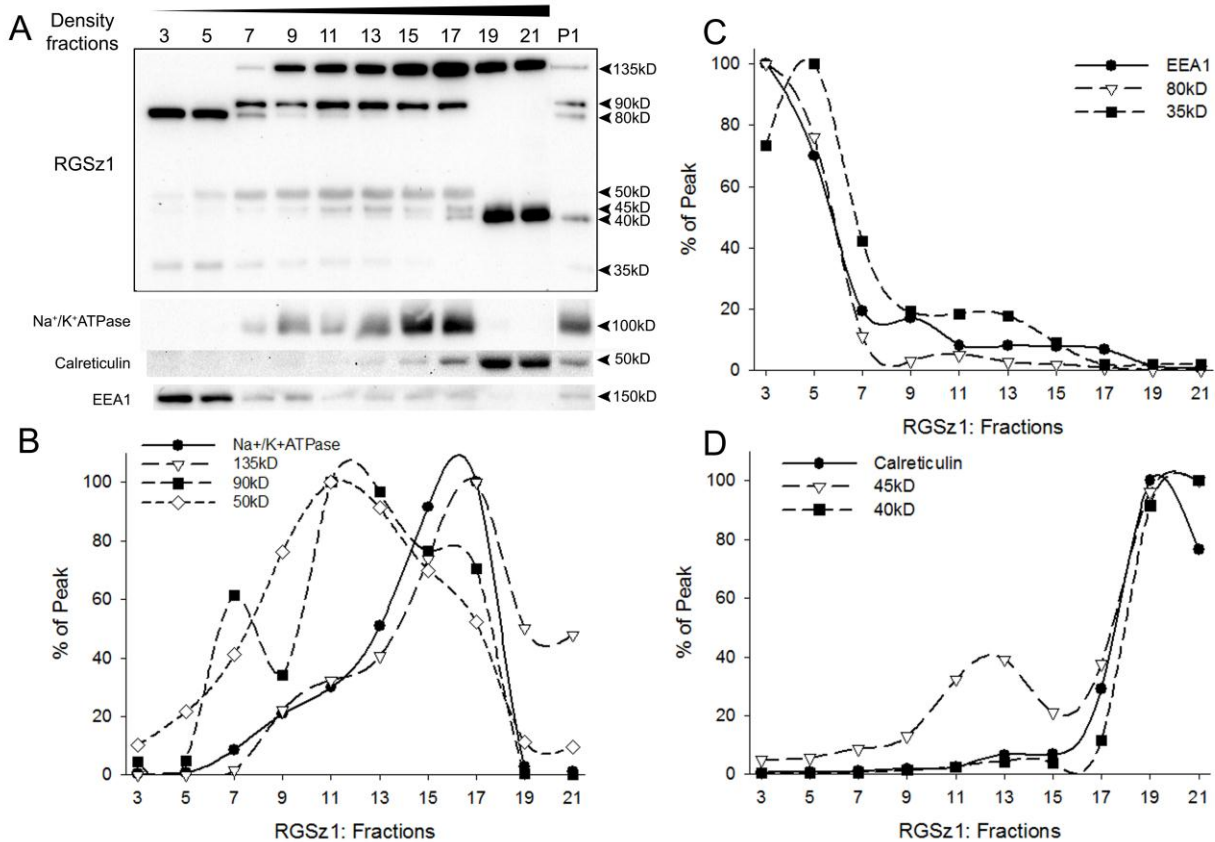


Figure 4.5: Subcellular distribution of RGSz1 protein in subcellular fractions from discontinuous iodixanol gradient centrifugation prepared with rat cortex. Representative immunoblot is shown in (A). Graphical representations show the colocalization of RGSz1 with plasma membrane marker, Na⁺/K⁺ATPase (B); early endosome marker EEA1 (C); and ER

marker calreticulin (D). Data are expressed as % of peak across fractions, and represent the average of three experiments.

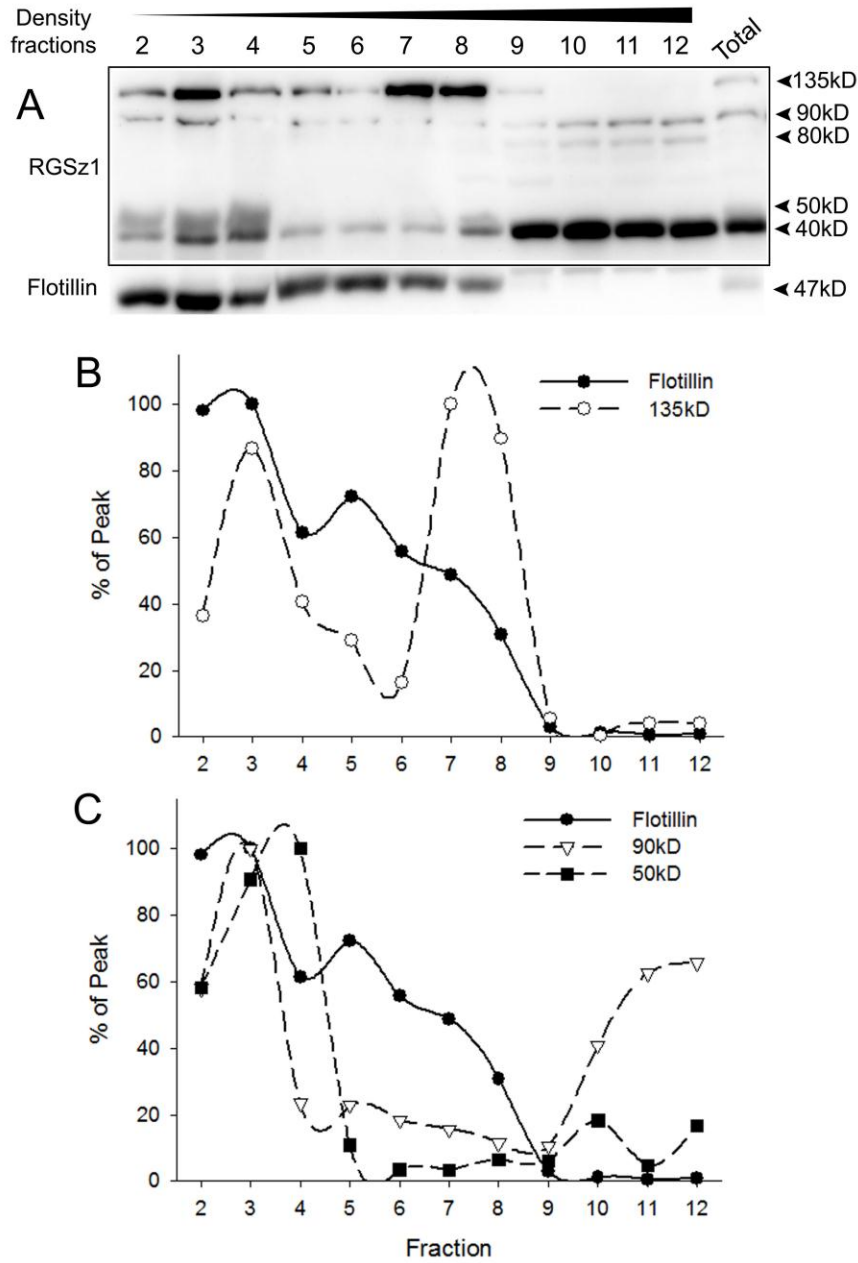
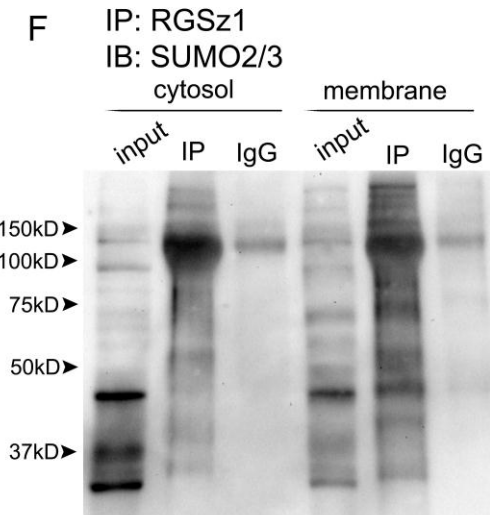
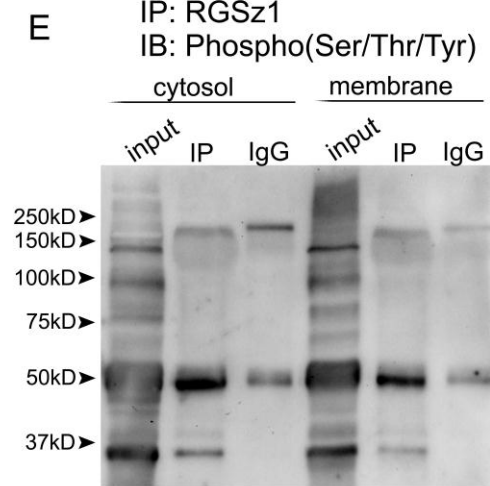
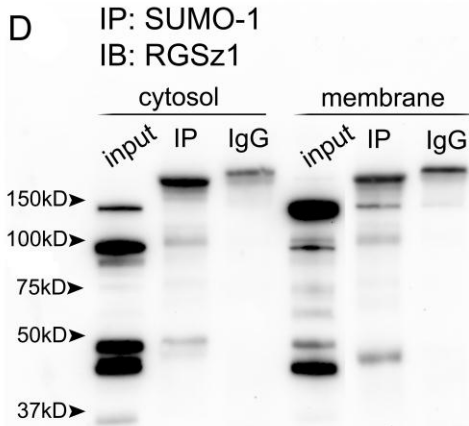
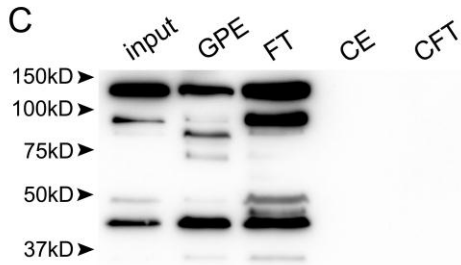
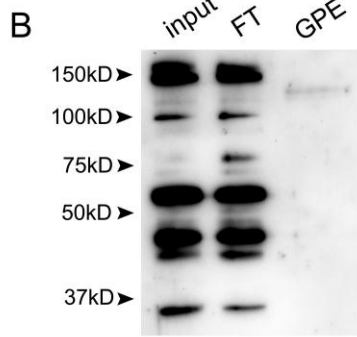


Figure 4.6: Distribution of RGSz1 protein in sucrose gradient fractions of Triton X-100 treated cortex homogenates. (A) A representative immunoblot is shown. Fractions containing the DRM were identified by the DRM marker flotillin. Total: Triton X-100 treatment without sucrose gradient centrifugation. Graphical representations show the colocalization of RGSz1 with

flotillin (**B** and **C**). Data are expressed as % of peak across fractions, and represent the average of three experiments.

A 1 MRTANGGPRAGASPSASPEDNLPVESEQTEMRRMQMCSGSETQGSVPSQ
 51 QQGVGGRGSNACCFWCCCCSCSCLTVRNQEDQRQPQRAAHELRTDIPACE
 101 ESPMPZLEEVCAWAQSFNLMVTPAGRNAFREFLRTEFSSEENMLFWMAE
 151 ELKREANKSSTIEEKARIYEDYISILSPKEVSLDSRVREVINRNMVDPSQ
 201 HIFDDAQLQIYTLMHRDSYPRFMNSTVYKDLLES LAEKAVEA



G

Band	Subcellular Location	Modifications	Effect of EB Cytosol	Effect of EB Membrane	Effect of G-1 Cytosol	Effect of G-1 Membrane
145	Plasma membrane			X		↑
135	Plasma membrane	Glycosylation SUMO-1	↓	↑	↓	↑
90		Glycosylation SUMO-1	X		↑	
80	Endosome	Glycosylation	↑	↓	↑	↓
50		SUMO-1	↑		↑	
40	ER	Glycosylation	↓	X	↓	X
35	Endosome	Phosphorylation	X		X	

Figure 4.7: Posttranslational modifications of RGSz1. **(A)** Rat RGSz1 primary amino acid sequence. Predicted glycosylation sites are underlined and bolded; predicted sumoylation site is bolded; predicted palmitoylation motif is underlined and italicized; predicted phosphorylation sites are shaded in gray. **(B)** RGSz1 immunoblot of glycoprotein isolated from cortical membrane fraction. Input: sample before isolation. FT: column flow-through. GPE: eluted glycoproteins. **(C)** RGSz1 immunoblot of isolated glycoprotein eluted from column by boiling in SDS-PAGE sample buffer. CE: control eluate (lectin column without protein added). CFT: control flow-through. **(D)** Immunoprecipitation (IP) of SUMO-1 and immunoblot detection of RGSz1 in cytosol and membrane fractions of cortex. Input: sample before IP. IgG: mouse immunoglobulin G control. IB: immunoblot. **(E)** IP of phosphorylated RGSz1 in cytosol and membrane fractions of cortex. IgG: rabbit immunoglobulin G control. **(F)** IP of RGSz1 and immunoblot detection of SUMO2/3 in cytosol and membrane fractions of cortex. IgG: rabbit immunoglobulin G control. **(G)** Summary of RGSz1 isoform characterization. ↑: increased expression after treatment. ↓: decreased expression after treatment. X: no change in expression after treatment. Bands that were unmeasured in the PVN are left unmarked.

DISCUSSION

The most consistent biomarker in depressed patients is increased HPA axis activity⁸⁵, and normalization of HPA axis function is associated with successful antidepressant treatment⁸⁴. We can model HPA axis activity in rats through neuroendocrine challenge, in which the HPA axis is activated by acute injection of the selective 5-HT_{1A}R agonist (+)8-OH-DPAT. Reduction in the magnitude of OT and ACTH hormone response to (+)8-OH-DPAT demonstrates desensitization of 5-HT_{1A}R signaling in the PVN^{190,236}, which is related to the therapeutic effects of SSRIs^{187,188}.

Two days of EB treatment produces a partial desensitization of 5-HT_{1A}R signaling. We previously reported that this effect is mediated by GPR30, as knockdown of GPR30 expression prevents EB-induced desensitization, and intra-PVN injection of G-1 produces a partial desensitization in the hormone response to (+)8-OH-DPAT^{143,220}. However, the ability of systemically-delivered G-1 to affect central 5-HT_{1A}R signaling in rats had not yet been tested. In the present study, we administered 10µg/kg EB, 2.5mg/kg G-1, and 5mg/kg G-1 via subcutaneous injection for two days, then measured the OT and ACTH response to (+)8-OH-DPAT challenge. The results demonstrated that systemic G-1 treatment had a dose-dependent effect on OT release, with the higher dose reducing OT release by the same magnitude as EB. This dose-dependent effect of G-1 on OT release is consistent with our previous study using injections of G-1 directly into the PVN¹⁴³. The ACTH response did not show a dose-dependent effect with systemic injections; consistent with our previous intra-PVN results, both doses of G-1 produced a similar reduction of plasma ACTH¹⁴³, which was even more robust than EB treatment. Increasing the dose of EB or extending the length of treatment does not further reduce the hormone response to (+)8-OH-DPAT^{205,206}. The results here suggest that G-1 is more effective than EB in desensitizing 5-HT_{1A}R signaling, and it is possible that higher doses may attenuate the OT response even further.

In this experiment, we did not see the same full desensitization in the hormone responses after combined EB and fluoxetine treatment as we saw previously²⁰⁶. However, EB alone and G-1 alone had the same effects on reduced hormone release as in the first experiment in this study (**Figure 4.1**): EB produced a partial desensitization in both OT and ACTH responses, 5mg/kg G-1 produced a partial desensitization in the OT response that was not significantly different than EB, and 5mg/kg G-1 reduced the ACTH response to (+)8-OH-DPAT significantly further than

EB alone. This suggests that the effects of fluoxetine to desensitize 5-HT_{1A}R signaling did not have as robust an effect as previously. While two days of fluoxetine did produce a reduction in the hormone response to (+)8-OH-DPAT, it was very slight. A control group of seven-day fluoxetine treatment should be included to determine whether full desensitization with fluoxetine alone could be achieved, or if there was some problem with the drug preparation. However, these results confirm the differential effects of EB and G-1 on OT and ACTH release, and suggest that G-1 treatment is more potent than EB in accelerating fluoxetine-induced desensitization of 5-HT_{1A}R signaling..

The difference in the OT and ACTH responses to 5-HT_{1A}R stimulation after G-1 and EB treatment is notable. There is a partial estrogen response element in the promoter region of the CRF gene, and estradiol treatment has been shown to increase CRF mRNA and protein expression, as well as AVP mRNA, likely through an ER β -mediated mechanism. In addition, data suggest that estradiol may interact directly, rather than through a receptor, with CRF and AVP to regulate the HPA axis¹¹⁷. This could explain the inconsistent data regarding EB-induced attenuation of ACTH response to (+)8-OH-DPAT. Selective stimulation of GPR30 with G-1 avoids an EB-induced increase in CRF, thus blunting the ACTH response. CRF action is regulated by CRF binding protein, which is higher in females than males and is positively regulated by ovarian hormones²⁸⁶. Increased expression of CRF binding protein via GPR30 could therefore serve to suppress ACTH release. In addition, circulating AVP from the SON may induce ACTH release from the pituitary²⁸⁷. GPR30 is highly expressed in the SON, and stimulation by G-1 may regulate AVP in these cells. Further work is needed to pursue these possibilities.

It is clear from the present study that signaling through GPR30 produces dramatic changes in expression and posttranslational modification of RGSz1. Not only is expression of RGSz1 isoforms brain-region-specific, with the hypothalamus expressing fewer bands than the cortex or midbrain, but changes in RGSz1 protein after EB or G-1 treatment are also different depending on the region. Previous work in our laboratory found that EB treatment produced a decrease in the 40kD RGSz1 isoform in the hippocampus but no change in the amygdala. Furthermore, a decrease of the 40kD band was observed in the PVN after EB treatment but this band was increased in the rest of the hypothalamus²⁰⁵. The disparity between RGSz1 expression in the frontal cortex versus PVN again suggests that addition or subtraction of posttranslational modification is controlled locally, most likely due to the types of estrogen receptors expressed in each region. Unlike in the PVN, G-1 treatment and not EB altered RGSz1 isoform expression in the membrane fraction. This reinforces the evidence from the PVN that RGSz1 modification is regulated by GPR30 signaling. EB signaling through ER α and ER β often has opposing effects, so selectively activating GPR30 would preclude any contrary signaling through other estrogen receptors.

Investigation of RGSz1 posttranslational modification identified sumoylation, glycosylation, and phosphorylation of RGSz1 isoforms. Sumoylation is the covalent addition of a small ubiquitin-related modifier (SUMO) to a protein. Sumoylation affects a variety of cellular processes including subcellular localization and transcriptional regulation, but it is thought that its primary function is to control protein-protein interactions. Vertebrates express three SUMO isoforms (SUMO1 and the nearly identical SUMO2/3). SUMO2/3 contains a SUMO consensus sequence, and can form poly-SUMO chains via isopeptide linkages; SUMO1 does not contain a consensus site and sumoylation of a poly-SUMO chain with SUMO1 can thus serve to terminate

the chain^{288,289}. RGSz1 contains a SUMO consensus sequence, as well as a SUMO-interacting motif (SIM – amino acids 173-176) which allows for the non-covalent interaction of a protein with SUMO. The present study demonstrated that the 135kD, 90kD, and 50kD RGSz1 isoforms are sumoylated with SUMO1 and located in the DRM. Furthermore, treatment with EB and G-1 resulted in an increase in the 135kD RGSz1 in the membrane, with a corresponding decrease in the non-sumoylated 80kD isoform. We observed a ladder pattern of SUMO2/3 immunoreactivity after RGSz1 IP, which suggests chains of SUMO2/3. Addition of a SUMO1 molecule as a cap to a poly-SUMO2/3 chain could account for the increase in apparent molecular weight from 80kD to 135kD; thus sumoylation may be acting as a molecular switch to increase RGSz1 in the DRM of the plasma membrane to regulate *Gaz* signaling. Our previous study found that sumoylated *Gaz* is located in the DRM, and is decreased by EB treatment. Since the DRM is the location of active GPCR signaling proteins, the increase in RGSz1 in the DRM would reduce the activity of *Gaz* signaling by hydrolyzing the activated GTP-bound *Gaz* to inactive GDP-bound *Gaz*. The EB and G-1 induced increase of DRM-localized RGSz1 together with the EB-induced decrease of DRM-localized *Gaz* would reduce 5-HT_{1A}R signaling.

We found that the 35kD, and possibly the 50kD, RGSz1 isoforms are phosphorylated. Phosphorylation is critical in the control of many cellular pathways, and may be a regulator of non-nuclear sumoylation as well²⁸⁹. Depending on the substrate, the negative charge of a phosphate group can enhance or inhibit sumoylation^{290,291}. Additionally, phosphorylation of a serine residue adjacent to the SUMO consensus sequence acts as a switch to favor sumoylation over acetylation²⁹². While RGSz1 does not have the precise motif that serves as a SUMO-acetyl switch, there is a serine residue (S159) adjacent to the SUMO consensus site that, if

phosphorylated, could facilitate sumoylation. S159 is located within a predicted *N*-linked glycosylation site, and could thus also function to inhibit or facilitate RGSz1 glycosylation.

Protein glycosylation plays an important role in protein structure, signal transduction, cell-cell interactions, and hormone action²⁹³⁻²⁹⁵. Addition and editing of carbohydrate units to proteins occurs in the ER and Golgi apparatus. Here, we found that a major glycosylated form of RGSz1, at 40kD, localized to the ER. The RGSz1 proteins of greater molecular weights than 40kD are also glycosylated, suggesting that the 40kD band, which is the most abundant of the RGSz1 isoforms, may serve as a pool from which, once glycosylated, other modifications such as sumoylation or palmitoylation can be added or removed, thus regulating RGSz1 localization and function.

The RGSz1 primary amino acid sequence contains a conserved cysteine string motif which is predicted to be palmitoylated. Palmitoylation occurs at the membrane and has a functional effect on membrane attachment and targeting, microdomain localization, and protein orientation at the membrane. Several RGS proteins are known to undergo palmitoylation, including RGSZ family member GAIP²⁹⁶. Palmitoylation of RGS4 and RGS16 is necessary for their targeting to DRM and ability to regulate G protein signaling, as well as endosomal trafficking^{69,297}. We found that the 80kD RGSz1 isoform colocalized with the early endosome marker EEA1, and that EB and G-1 treatment greatly increased its expression in the cytosol. This suggests that endosomal trafficking of RGSz1 could occur from the Golgi to the plasma membrane, where palmitoylation could then stabilize the 90kD and 135kD RGSz1 isoforms in the DRM.

Interestingly, we found that while EB and G-1 treatment produced comparable changes in most of the RGSz1 bands measured in the PVN, only G-1 affected the 145kD and 90kD bands in

the membrane and cytosol, respectively, producing a dramatic increase relative to control and EB treatment. That their expression was so markedly affected by G-1 treatment suggests that these isoforms may be of particular importance to GPR30-mediated desensitization of 5-HT_{1A}R signaling, and could contribute to the apparent sensitivity of the ACTH response to G-1 over EB treatment; ACTH release is under the control of CRF, and while the mechanism by which *Gaz* mediates CRF release is still unclear, it could be particularly susceptible to regulation by the 145kD RGSz1 isoform. Localization of the 90kD isoform in the DRM supports a significant role for this isoform in the regulation of *Gaz*. The 145kD band appears to be specific to the membrane fraction of the PVN: it is not seen in the cortex, hippocampus, amygdala, or even the other regions of the hypothalamus. The PVN does not contain enough protein to perform immunoprecipitation of RGSz1, so characterization of this isoform is difficult; however, its localization to the membrane suggests a role for palmitoylation, glycosylation, or acylation.

RGSz1 is a product of the RGS20 gene (NM_001127495.1), which also codes for Ret-RGS. RGS20 spans seven exons, five of which code for RGSz1. Mouse and human RGSz1 mRNA contains multiple possible start codons, which may contribute to the heterogeneity of protein size in these species²⁹⁸. Rat mRNA, however, only contains one start codon upstream of the known transcription start site, and there is an in-frame stop codon 55 base pairs later. Therefore, alternate transcription of RGSz1 is not likely to produce the higher molecular weight proteins that we see in the rat brain. Little is known of potential splice variants of RGSz1; there are two introns in the RGS domain, and alternative splicing could give rise to different protein isoforms with different levels of GAP activity or affinities for *Gaz*. Further investigation into this possibility is required.

In conclusion, the present results demonstrate for the first time that two-day treatment via peripheral delivery of the selective GPR30 agonist G-1 produces desensitization of 5-HT_{1A}R signaling. This is significant for potential translation to preclinical and clinical studies, as peripheral injections are more feasible than intra-PVN delivery. We also showed that RGSz1 in the rat brain is posttranslationally modified, and that EB and G-1 treatment altered these modifications in the PVN. RGSz1 posttranslational modification therefore likely has a significant functional impact on RGSz1 activity and regulation of *Gaz* signaling in the PVN. Of particular importance is the stabilization of RGSz1 in the DRM of the plasma membrane, via sumoylation and glycosylation, where it can physically interact with activated *Gaz* and shut off 5-HT_{1A}R downstream signaling, thus reducing HPA axis activity. This suggests a new mechanism by which EB accelerates SSRI-induced desensitization of 5-HT_{1A}R signaling. Because estrogen therapy is associated with increased risk for breast cancer, heart disease, and stroke^{207,209,284}, it is important to identify more selective therapeutic adjuvants for SSRI treatment of depression. Our results provide evidence that targeting GPR30 and RGSz1 may provide a selective mechanism for the acceleration of SSRI therapeutic effects.

CHAPTER 5: GENERAL CONCLUSIONS

REVIEW OF RESULTS AND SIGNIFICANCE

Acute treatment with an SSRI such as fluoxetine blocks serotonin transporters and increases serotonergic transmission at both pre- and post-synaptic sites. Stimulation of pre-synaptic, inhibitory 5-HT_{1A} autoreceptors decreases serotonergic tone transiently; however, continued stimulation of post-synaptic 5-HT_{1A}R would potentiate HPA axis activity. After chronic SSRI treatment, the autoreceptors in the DRN desensitize, which increases serotonergic tone in target regions such as the PVN. This increase in serotonin release, combined with continued SSRI-induced prevention of synaptic clearance, would increase post-synaptic 5-HT_{1A}R signaling until these receptors desensitize. The time that it takes for desensitization of both autoreceptor and post-synaptic receptor signaling to occur likely underlies the therapeutic lag associated with SSRI treatment^{201-203,299}. The results presented here, when compared with the findings of other studies investigating the molecular effects SSRIs and estradiol treatment, suggest three general ways in which these two treatments promote desensitization of 5-HT_{1A}R signaling: 1) decreased 5-HT_{1A}R protein, 2) decreased active Gα_z, and 3) altered RGSz1 expression.

In chapter three, we identified two important EB-induced changes in the hypothalamic PVN 5-HT_{1A}R signaling pathway: decreased levels of 5-HT_{1A}R protein, and increased levels of two isoforms of RGSz1 protein and RGSz1 mRNA. These molecular changes accompanied EB-induced partial desensitization of 5-HT_{1A}R signaling as measured by reduced OT and ACTH release after challenge with the selective 5-HT_{1A}R agonist (+)8-OH-DPAT. We next used a recombinant adenovirus containing siRNAs against GPR30 to knockdown GPR30 protein

expression in the PVN by about 32%. This reduction was enough to prevent the reduction of the hormone response to (+)8-OH-DPAT, demonstrating that GPR30 is necessary for EB-induced desensitization of 5-HT_{1A}R signaling.

The results that only a partial reduction in GPR30 protein levels (<40%) was successful in preventing EB-induced desensitization of 5-HT_{1A}R signaling suggests that a high level of PVN GPR30 expression is necessary for this effect of EB. This is most likely due to EB activation of ER α and ER β in addition to GPR30. EB stimulation may produce a counterbalancing effect through ER α expressed on GABAergic neurons in the periventricular regions of the hypothalamus that project to the PVN. 17 β -estradiol treatment increases CRF mRNA and protein expression, as well as AVP mRNA expression, compared to controls¹¹⁷; if these effects are mediated through ER α/β and not GPR30, G-1 treatment would have a stronger effect than EB treatment. The findings in chapter four that G-1 treatment reduces ACTH release even further than EB supports this idea. Additionally, 5-HT_{2A/C}R in the PVN colocalize with CRF, and regulate CRF and AVP, and thus ACTH, release^{93,300}. There is evidence for estradiol modulation of 5-HT_{2A}R: ovariectomy decreased 5-HT_{2A}R mRNA in the PVN, and one month treatment of 17 β -estradiol increased 5-HT_{2A}R binding sites while decreasing 5-HT_{1A}R binding sites in the frontal cortex of ovariectomized monkeys³⁰¹. Added to the finding that ACTH release is more sensitive to G-1 treatment than OT, these results suggest a complex system of receptor interactions by which EB attenuates the hypothalamic-pituitary endocrine systems.

In chapter four, we demonstrated that two day EB and G-1 treatment produced robust alterations in RGSz1 protein expression in the PVN that accompanied EB- and G-1-induced desensitization of 5-HT_{1A}R signaling. We also characterized the posttranslational modifications and subcellular localization of these RGSz1 isoforms. A 145kD RGSz1 band, which was

detectable only in the membrane fraction of the PVN, was increased by G-1, and not EB treatment, as well as a 90kD band in the cytosol; these changes may be important for the difference in EB versus G-1 treatment effects on ACTH release after 5-HT_{1A}R stimulation. Significantly, the 135kD isoform was glycosylated and sumoylated and localized to the DRM of the plasma membrane. EB and G-1 treatment decreased the 135kD protein in the cytosol and increased it in the membrane. Increased RGSz1 in the DRM would function to increase GAP-accelerated hydrolysis of GTP-bound Gα_z, thus reducing Gα_z downstream effector activation and producing desensitization of 5-HT_{1A}R signaling.

Previous results demonstrated that EB and fluoxetine have a synergistic effect on desensitization of 5-HT_{1A}R signaling²⁰⁶. Fluoxetine has been shown to decrease circulating estradiol in intact animals^{302,303}; this could in part contribute to the therapeutic lag of fluoxetine treatment. Additionally, short-term (two day) treatment with fluoxetine increased 5-HT_{1A}R protein in the PVN, an effect that was blocked by EB treatment²⁰⁶. Indeed, in chapter three we found a decrease in 5-HT_{1A}R protein after EB treatment, and further evidence shows that EB treatment decreases density of 5-HT_{1A}R binding sites^{169,301}, likely by increasing sumoylation-mediated internalization of 5-HT_{1A}R³⁰⁴. Two-day EB treatment also increases 5-HT_{1A}R mRNA, which could explain why longer than two days or higher dose does not further desensitize 5-HT_{1A}R signaling^{205,220}.

In addition to decreased 5-HT_{1A}R availability, a reduction in Gα_z protein levels would have the functional effect of reducing hormone release after 5-HT_{1A}R stimulation. While some studies report decreased Gα_z after EB treatment²⁰⁴ and some report no change^{219,220}, levels of Gα_z protein have been shown to be decreased following chronic fluoxetine treatment¹⁹¹. The RGS-Rz subfamily binds with the dileucine-rich region of GAIP interacting protein N terminus

(GIPN), a putative E3 ubiquitin ligase that links RGS-Rz proteins with Gai3 degradation; GAIP functions as an adaptor that binds to $G\alpha$ subunits through the RGS domain and to GIPN through a cysteine string motif³⁰⁵. While this has not been demonstrated for RGSz1 and *Gaz*, RGSz1 possesses the same cysteine string motif and could potentially interact with GIPN to the effect of promoting ubiquitination and degradation of *Gaz*. In chapter four, we found that 80kD and 50kD RGSz1 isoforms were increased in the cytosol after EB and G-1 treatment, and that the 80kD isoform was associated with the early endosome; depalmitoylation of membrane-associated RGSz1 could promote trafficking of *Gaz*-bound RGSz1 from the plasma membrane to the early endosome and subsequent ubiquitination and degradation of *Gaz*. This could explain the decrease in *Gaz* sometimes seen after EB treatment²⁰⁴, and contribute to EB-induced acceleration of fluoxetine-induced 5-HT_{1A}R signaling.

Another important factor in desensitization 5-HT_{1A}R signaling is the regulation of *Gaz* signaling, apart from its protein expression. Phosphorylation and palmitoylation of *Gaz* inhibits RGSz1 GAP activity and potentiates *Gaz* downstream signaling^{79,80,306,307}. *Gaz* is phosphorylated at two sites by PKC and at one of those sites by PAK, which could allow regulation by several signaling pathways⁶⁷. One such pathway is the activation of and subsequent phosphorylation by GSK3 β . GSK3 β is inhibited by phosphorylation and is a target of the mood stabilizer lithium. Acute and chronic treatment with fluoxetine and imipramine, respectively, increased the phosphorylated (inactive) form of GSK3 β , an effect which was associated with antidepressant-like behavior in the forced swim task (FST)^{308,309}. Inhibition or deficiency of GSK3 β also decreases immobility in the FST, suggesting that inactivation of GSK3 β , and therefore decreased phosphorylation of *Gaz* and increased RGSz1-*Gaz* interaction, is important in the antidepressant effects of fluoxetine³¹⁰.

Additionally, stimulation of 5-HT_{1A}R by 8-OH-DPAT increased phosphorylation of GSK3 β , an effect mediated by Gai and Akt signaling that was prevented by 5-HT_{1A}R antagonist WAY 100,635³¹¹. This suggests a negative feedback mechanism whereby 5-HT_{1A}R signaling through Gai has the effect of decreasing signaling through Gaz by preventing GSK3 β -mediated phosphorylation of Gaz. Stimulation of 5-HT_{2A}R had the opposite effect of activating GSK3 β ³⁰⁸, indicating that serotonergic regulation of GSK3 β depends on the balance between the two receptor subtypes. 5-HT_{2A}R activation by fluoxetine could therefore counterbalance a 5-HT_{1A}R-mediated increase in GSK3 β phosphorylation, thus delaying the onset of therapeutic action.

GPR30 signaling through the $\beta\gamma$ subunit transactivates epidermal growth factor receptor (EGFR) signal transduction cascades³¹², including activation of PKC. While PKC phosphorylates Gaz, inhibiting GAP activity, it also phosphorylates GSK3 β . Increased GSK3 β phosphorylation via GPR30-EGFR activation in addition to EB-induced increases in RGSz1 could tip the balance in favor of inactivated Gaz, thus contributing to desensitization of 5-HT_{1A}R signaling. GPR30-induced phosphorylation of GSK3 β has not been investigated directly, but EGFR can promote GSK3 β inactivation through an Akt-mediated pathway. Interestingly, ICI182780, which is an antagonist for ER α/β but an agonist for GPR30³¹², induced GSK3 β phosphorylation in the mouse uterus³¹³ and 17 β -estradiol increased Akt phosphorylation of GSK3 β in the caudate nucleus and putamen of ovariectomized monkeys³¹⁴. These results suggest a role for GPR30 mediation of GSK3 β , and thus regulation of Gaz activity.

Activation of 5-HT_{1A}R by 8-OH-DPAT in transfected COS cells promoted proteosomal degradation of recombinant RGSz1 via a Gai/o-mediated pathway³¹⁵. Fluoxetine-induced increase in serotonin transmission could thus reduce RGSz1 levels and potentiate Gaz signaling. A previous study reported that chronic fluoxetine treatment had no effect on unmodified RGSz1

levels *in vivo*, but two days of EB treatment increased expression²¹⁹. In the present studies, we found that the 35kD, and possibly 50kD, RGSz1 isoforms are phosphorylated, and the 50kD isoform was increased by EB and G-1 treatment. Phosphorylation by a MAP kinase Fus3p increased the half life of Sst2p, a yeast RGS protein³¹⁶, and ubiquitin-directed proteolysis of RGS7 was prevented by activation of MAP kinase p38³¹⁷. GPR30 activation of MAP kinase signaling cascades could thus promote the phosphorylation of RGSz1, leading to increased stability and protection against fluoxetine-induced degradation.

Fluoxetine	Estradiol
Decreases Gαz	Decreases or no change in Gαz
Increases 5-HT _{1A} R protein	Decreases 5-HT _{1A} R protein/increases sumoylation and internalization
	Decreases density of 5-HT _{1A} R binding sites
Decreases or no change in unmodified RGSz1	Increases unmodified RGSz1
	Increases DRM-localized RGSz1
Increases inactivation of GSK3β	Increases inactivation of GSK3β

Table 5.1: Comparison of fluoxetine and estradiol effects on 5-HT_{1A}R signaling.

LIMITATIONS OF THE PRESENT STUDIES

One major limitation of the present studies is the small size, and thus low protein yield, of the PVN. Both fluoxetine and EB treatments have differential effects throughout the brain; although we were able to use cortical protein to characterize RGSz1 isoforms, we could not use these same methods with protein from the PVN, and so must assume that the posttranslational

modifications seen in the cortex are the same in PVN RGSz1 proteins of the same size. For the same reason, we were unable to directly establish a causal relationship between alteration of RGSz1 posttranslational modification and G-1-induced desensitization of 5-HT_{1A}R signaling. Furthermore, cells transfected with RGSz1 cloned from the rat PVN only express the unmodified 27kD RGSz1 protein, and further work is required to directly demonstrate posttranslational modification of RGSz1.

Another limitation is the RGSz1 antibody used. Rabbit-anti-RGSz1 was produced and affinity purified by Biosynthesis, and it was this antibody that was used in the earlier experiments²⁰⁵ (chapter three). Using this antibody, we were able to detect a 55kD RGSz1 isoform in the PVN that was affected by EB treatment. However, this antibody also showed a large amount of nonspecific binding. To reduce the background noise, we affinity-purified the anti-RGSz1 antibody again, and while this method substantially reduced nonspecific binding, it also decreased the antibody binding to the 55kD RGSz1 isoform. This isoform is more abundant in other brain regions, such as the cortex, and can thus still be detected on western blots, but not in the PVN. Similarly, the 27kD unmodified RGSz1 protein is much harder to detect with the purified antibody, and could not be measured in the experiments in chapter four.

Also lacking in these studies is a behavioral model for depression. The FST is one of the most common behavioral tests for depressive-like drug effects in animal models; however, this test and others (such as novelty-suppressed feeding test, tail suspension test, learned helplessness, and inescapable stress models) produce a generalized stress in which the HPA axis is activated via 5-HT_{1A}R, 5-HT_{1B}, 5-HT_{2A/C}R, and 5-HT₇R, making it difficult to tease out receptor-specific effects. EB acts as an antidepressant in the FST, as does acute fluoxetine³¹⁸ and 5-HT_{1A}R agonists such as azapirones and 8-OH-DPAT²⁴; the increased stress hormone levels

induced by acute fluoxetine and 5-HT_{1A}R agonists is likely what produces the animals' increased activity in these behavioral measures. In our model, we are using decreased stress hormone levels as the measure of effective antidepressant treatment. In the clinic, increased stress hormone levels, namely ACTH and cortisol, is an important biomarker in clinical depression, and normalization of HPA axis activity correlates with successful antidepressant treatment. Therefore our neuroendocrine model can be used to demonstrate functional changes in PVN 5-HT_{1A}R and HPA axis activity after EB and G-1 treatment and represent what can be measured in a clinical setting.

FUTURE STUDIES

To directly show that RGSz1 is posttranslationally modified, and that the modifications are altered by EB and G-1 treatment, further work should be done to express RGSz1 in cells along with the appropriate cellular machinery for sumoylation, glycosylation, phosphorylation, and palmitoylation. Once RGSz1 posttranslational modifications can be induced in cell culture, site-directed mutagenesis of the posttranslational modification sites predicted in this study could be performed to provide further evidence for important roles for RGSz1 posttranslational modification. Additionally, mass spectrometry analysis of the RGSz1 isoforms detected via western blotting could confirm that the proteins detected were indeed RGSz1, especially given the nonspecific binding demonstrated by the RGSz1 antibody. Further, genetic approaches of knocking down or overexpressing RGSz1 in the PVN *in vivo* will be important to directly demonstrate the necessity of RGSz1 in EB-induced desensitization of 5-HT_{1A}R signaling. Selectively altering specific RGSz1 posttranslational modifications, such as sumoylation or glycosylation, *in vivo* will be more complicated, as these modifications are vital in a large

number of cellular processes. However, administration of MAP kinase inhibitors, for example, to target GPR30-EGFR signaling pathways could help identify the specific mechanisms involved in the regulation of RGSz1 posttranslational modification.

One of the most translationally-significant findings in these studies is that systemic delivery of G-1 produced desensitization of 5-HT_{1A}R signaling. Using peripheral, rather than intra-PVN, injections make G-1 treatment feasible for use in clinical trials. Selectively activating GPR30 over ER α and ER β produces a more robust desensitization of the ACTH response to (+)8-OH-DPAT, which may translate directly to clinical findings that depressed patients demonstrate elevated CRF and cortisol levels. Using G-1, rather than estrogens, as an adjunctive to SSRI treatment of depression may decrease the side effects and increased risk for disease that are associated with estrogen therapy. EB treatment requires at least two days to induce desensitization of 5-HT_{1A}R signaling, but increasing the dose from 10 μ g/kg or extending the length of treatment does not reduced hormone release any further. In these experiments, we only examined the effects of G-1 at one time point. One of the most important molecular changes induced by G-1 was the alteration of RGSz1 posttranslational modification and changes in subcellular localization; these types of changes may not necessarily require changes in transcriptional activity, and so could possibly be produced in a much shorter time frame than two days. Therefore, it will be important to establish a time course for G-1 treatment. Because we saw a dose-dependent effect of G-1 treatment on ACTH release in both neuroendocrine experiments, a dose response curve should be established as well. Lower doses of G-1 may be just as effective as EB to attenuate the ACTH response to (+)8-OH-DPAT, and doses higher than 5 mg/kg may attenuate the response even further, leading to increased acceleration of fluoxetine-induced desensitization of 5-HT_{1A}R signaling or possibly even full desensitization due to G-1

alone. That G-1, not EB, had such a distinct effect on ACTH and not OT levels suggests a mechanism in which GPR30 signaling is effecting expression or release of CRF and AVP, as discussed in chapter four. It will therefore be important to examine possible changes in these compounds after G-1 treatment. Finally, repeating the G-1 and fluoxetine combined treatment experiment from chapter four with the best time course and dose of G-1, and better controls for fluoxetine, will be important to directly demonstrate whether G-1 treatment works synergistically with fluoxetine to improve the therapeutic efficacy of SSRIs.

CONCLUSIONS

The data presented here support the hypotheses that GPR30 is both necessary and sufficient for estradiol-induced desensitization of 5-HT_{1A}R signaling, and that GPR30 stimulation mediates alterations in RGSz1 expression and posttranslational modification. Alterations in RGSz1, particularly in DRM-localized sumoylated and glycosylated RGSz1, could increase RGSz1 acceleration of G α Z-GTP hydrolysis, thus reducing G α Z signaling and 5-HT_{1A}R-mediated hormone release. These results suggest a mechanism by which estradiol accelerates fluoxetine-induced desensitization of 5-HT_{1A}R signaling, as well as identify GPR30 and RGSz1 as potential novel targets for the improved treatment of depression.

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