THE ROLE OF PIAS PROTEINS IN THE SUMOYLATION OF 5-HT_{1A} RECEPTORS

By

Mengya Wang

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Nancy A. Muma, Ph.D., Chairperson

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Jeff Staudinger, Ph.D.

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Honglian Shi, Ph.D.

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The Dissertation Committee for Mengya Wang

certifies that this is the approved version of the following dissertation:

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Nancy A. Muma, Ph.D., Chairperson

Date approved:

ABSTRACT

Serotonin 1A receptors (5-HT_{1A} Rs) are implicated in the control of mood, cognition and memory. Dysfunction of 5-HT_{1A} R signaling results in various neuropsychiatric disorders such as depression, anxiety and schizophrenia. Thus, 5-HT_{1A} Rs are a good target for the treatment of the neuropsychiatric diseases.

Previous studies have shown that 5-HT_{1A} Rs can be SUMOylated by SUMO1 in rat brain areas such as cortex, hypothalamus and hippocampus. Studies have also shown that SUMOylated 5-HT_{1A} Rs cannot bind to agonist. In addition, it was reported that SUMOylation of 5-HT1A Rs in the hypothalamic membrane increased by treatment with the 5-HT_{1A} R agonist (+)-8-Hydroxy-2-dipropylaminotetralin ((+)8-OH-DPAT) and was further enhanced by combination of treatment of 17β-estradiol-3-benzoate (EB) and 8-OH-DPAT. Identifying the mechanism involved in SUMOylation of 5-HT_{1A} Rs will be important for understanding the regulation of 5-HT_{1A} Rs, which will inform the development of novel therapeutic approaches to the neuropsychiatric diseases. Therefore, the purpose of this thesis is to investigate the mechanisms involved in SUMOylation of 5-HT_{1A} Rs, specifically to determine whether PIAS proteins play a role in the SUMOylation of 5-HT_{1A} Rs. I hypothesize that selective PIAS proteins increase SUMOylation of 5-HT_{1A} Rs.

In the first study, I developed a cell culture model to examine the SUMOylation of 5-HT_{1A} Rs. I found that mouse Neuroblastoma 2a (N2a) cells express endogenous 5-HT_{1A} Rs and PIAS proteins. In addition, N2a cells can overexpress PIAS proteins and SENPs. The SUMOylation of 5-HT_{1A} Rs was detected around 55 kDa in N2a cells similar to that reported in rat brain tissue. Transfected PIAS constructs expressed in the membrane and cytosol fractions of

N2a cells and various PIAS constructs showed different expression levels. PIASxα significantly increased the SUMOylated 5-HT_{1A} Rs compared to other PIAS proteins.

In the second study, I tested the hypothesis that the treatment of rats with 8-OH-DPAT and/or EB increased expression of PIASxα resulting in an increased level of SUMO 1-5-HT_{1A} Rs. The results show an increase in the expression level of PIASxα in rats co-treated with EB and 8-OH-DPAT compared to the expression level of PIASxα in either the EB treated or vehicle treated groups. Interestingly, the expression level of PIASy was increased in the rats treated with EB alone compared to vehicle treated rats.

Together, these data suggest that PIASxα plays a role in increasing SUMOylation of 5-HT_{1A} Rs. Targeting PIASxα in the SUMOylation of 5-HT_{1A} Rs could have important clinical relevance for the therapy towards the neuropsychiatric disorders such as depression, anxiety and schizophrenia.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1A} Rs	serotonin 1A receptors
8-OH-DPAT	8-Hydroxy-2-dipropylaminotetralin
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BDNF	Brain-Derived Neurotrophic Factor
DEL	deSUMOylating isopeptidases
EB	17β-estradiol-3-benzoate
ER	endoplasmic reticulum
GPCR	G protein-coupled receptor
HPA	hypothalamic-Pituitary-Adrenal
MAOI	monoamine oxidase inhibitors
MDD	major depression disorder
MT	mock transfection
NDRI	norepinephrine and dopamine reuptake inhibitor
NEM	N-Ethylmaleimide

NET	norepinephrine transporter
PIAS	protein inhibitor activated STAT
PVN	hypothalamic paraventricular nucleus
RGSz1	regulator of G protein signaling Z1
SENP	sentrin-specific proteases
SERT	serotonin transporter
SIM	SUMO binding motif
SNRI	serotonin and norepinephrine reuptake inhibitor
SP-RING	Siz/PIAS RING
SSRI	selective serotonin reuptake inhibitor
STAT	activator of transcription
SUMO	small ubiquitin-like modifier
TCA	tricyclic antidepressants
Ubc9	ubiquitin carrier protein 9
USPL1	ubiquitin-specific protease-like 1

CHAPTER 1: INTRODUCTION

1.1. Depression and Anti-depressants

1.1.1. Depression

Depression is a common mental disorder, characterized by sadness, loss of interest, feelings of guilt or worthlessness, insomnia, appetite loss, feelings of tiredness and poor concentration [2]. It affects over 350 million people worldwide. It is found to be more prominent in females than males and it typically initiates at a young age with a peak age of onset in the mid 30's [3]. The annual cost for patients diagnosed with major depression disorder (MDD) is $210.5 billion in 2010 in US [4].

There are several types of depressive disorders [2] .

- Major depression: severe episodes of depression can occur only once or many times during a patient's lifetime.
- Persistent depressive disorder: this type of depression lasts more than 2 years.
- Psychotic depression: the patients feel depressed and also have some symptoms of psychosis, such as hallucinations.
- Postpartum depression: 10 to 15 percent of women after giving birth feel overwhelmed with caring for the baby and with post-partum hormonal and physical changes, will experience postpartum depression.
- Seasonal affective disorder (SAD): This type of depression occurs during winter when there is less sunlight.

- Bipolar disorder: Patients experience episodes of depression followed by mania. It is considered a separate disorder but includes the symptoms of depression.

Several brain regions are involved in the pathophysiology of depression including cingulate cortex, nucleus accumbens, amygdala and hippocampus [5]. Some hypotheses for the etiology of depression have been proposed. One of the hypotheses for the underlying cause of depression is the monoaminergic hypothesis, which suggests depletion of monoamines (e.g. norepinephrine, dopamine and serotonin) or alterations in sensitivity of post-synaptic receptors (e.g. serotonergic receptors) in the central nervous system leads to depression [6]. Drugs like Selective Serotonin Reuptake Inhibitors (SSRIs) were developed based on this hypothesis [7]. Another hypothesis is that depression is related to the Brain-Derived Neurotrophic Factor (BDNF). BDNF is expressed most widely among the neurotrophin family in the mammalian brain. It participates in the cellular proliferation, migration, differentiation as well as regulation of almost all the neuronal circuit development like neurogenesis, neuronal differentiation and neuronal polarization [8]. In the patients with depression, decreased expression of BDNF was found whereas antidepressants increased the expression level of BDNF and thus alleviated the symptoms [8-10].

1.1.2. Anti-depressants

Major antidepressants are classified into several types: monoamine oxidase inhibitors (MAOIs); tricyclic antidepressants (TCAs); selective serotonin reuptake inhibitors (SSRIs); serotonin and norepinephrine reuptake inhibitors (SNRIs); norepinephrine and dopamine reuptake inhibitors (NDRIs) [2].

Monoamine oxidase is involved in the serotonin degradation. Thus, MAOIs can reduce the degradation of serotonin, norepinephrine and dopamine. As a result, the amount release of these neurotransmitters increases. However, the side effects of MAOI such as low blood pressure, fatigue, lethal dietary and drug interactions result in MAOIs being the last line of treatment for depression.

TCAs such as amitriptyline can inhibit serotonin, norepinephrine, and dopamine transporters, which subsequently can increase the neurotransmitters in the synaptic cleft. However, non-selectivity of TCAs leads to its greater side effects including cardiotoxicity.

SSRIs like citalopram, escitalopram, fluoxetine and fluvoxamine are the most widely used anti-depressants. SSRIs are more selective and thus the side effects are less severe compared to MAOIs and TCAs. The serotonin transporter (SERT), present in the presynaptic terminals, cell bodies and dendrites of serotonergic neurons, is capable of transporting serotonin from the synaptic cleft back into the presynaptic neuron. It is thought that the SSRIs act through inhibiting serotonin re-uptake by SERT.

SNRIs such as venlafaxine act on norepinephrine transporter (NET) and serotonin transporter (SERT), but they work more efficiently on SERT than NET. In comparison to SSRIs, they treat a wider range of symptoms than SSRIs and the side effects of SNRIs are more manageable than SSRIs. However, the beneficial effects of SNRIs vary between patients.

The only NDRIs approved by FDA for clinical use is bupropion. As an atypical antidepressant, bupropion serves as an add-on medication with SSRIs. However, bupropion was withdrawn from the market and then came back but with a reduced dosage for use.

The direct actions of the anti-depressants are known. However, the mechanisms by which they improve depression have not been elucidated clearly. For example, the SSRIs can inhibit the SERT resulting in serotonin remaining in the synaptic cleft for a prolonged timed. However, the inhibition of reuptake of serotonin occurs very rapidly [11] and it usually takes an average 6–7 weeks for SSRIs to produce the anti-depressant effects. This lag in therapeutic response suggests that adaptive changes such as serotonin receptor desensitization (based on the monoamine hypothesis) and neuronal proliferation (based on the BDNF hypothesis) likely contribute to the therapeutic response. Thus, it is important to develop a novel therapy for SSRIs to reduce the therapeutic lag. Therefore, it is imperative to explore more about the adaptive mechanisms. Furthermore, more efficacious drugs are needed, considering only about 1/3 of patients experience relief from current medications [12].

1.2. Serotonin

Serotonin is a monoamine neurotransmitter, the dysregulation of which can lead to alterations of mood, anxiety, pain disorders and psychosis. Serotonin is distributed in central nervous system, enterochromaffin cells and blood platelets [13]. There are 14 known human serotonin receptors including 5-HT 1A, 1B, 1D, 1E, 1F, 2A, 2B, 2C, 3, 4, 5A, 5B, 6, 7. 5-HT 3 receptor is the only ionotropic receptor while the others are G protein coupled receptors (GPCRs). Different serotonin receptors can locate in the same cells [13].

1.2.1. Serotonin 1A Receptors

1.2.2.1. Structure, distribution and signaling

Serotonin 1A receptors (5-HT_{1A} Rs) bind to serotonin with high affinity and play an important role in the regulation of emotion. 5-HT_{1A} Rs contain 422 amino acids, with a predicted

molecular weight around 42 kDa. The identity between the sequence of 5-HT_{1A} Rs in rats and in human is 89%. 5-HT_{1A} Rs belong to the super family of GPCRs, which are expressed on the plasma membrane and possess seven membrane-spanning domains. For the GPCRs, the C-terminus is usually involved in the post-translational modifications.

5-HT_{1A} Rs are located in the both pre-synaptic and post-synaptic membrane of neurons. Presynaptic 5-HT_{1A} receptors are exclusively present in the 5-HT neurons with cell bodies located in raphe nuclei (both dorsal and median) [14-16], where central serotonergic neurons are located, mediating feedback regulation of 5-HT release. The activation of these pre-synaptic receptors causes a suppression of 5-HT synthesis. Postsynaptic 5-HT_{1A} receptors are located in the cortico-limbic regions, including the cortex (particularly prefrontal and enthorinal cortex), hippocampus, septum, amygdala as well as the hypothalamus [17]. The postsynaptic 5-HT_{1A} receptors receive serotonergic innervation from the raphe nuclei and in this way, the presynaptic 5-HT_{1A} receptors in serotonergic neurons can regulate the serotonergic activity in projection areas. In terms of cellular location, the expression of 5-HT_{1A} Rs was observed in the dendrites and soma of glutamatergic pyramidal neurons, axon terminals of GABAergic and cholinergic neurons [17] as well as in the non-neuronal cells like astrocytes [18].

5-HT_{1A} Rs couple to $G\alpha_i/G\alpha_o$ (G_o, $G_{\alpha i1/2/3}$), resulting in decreased level of cAMP, mediating inhibitory neurotransmission. 5-HT_{1A} Rs couple to $G\alpha i3$ exclusively in the anterior raphe. 5-HT_{1A} Rs couple to $G\alpha_{i3}$ and $G\alpha_o$ equally in the cerebral cortex and preferentially to $G\alpha_o$ compared to $G\alpha_{i3}$ in the hippocampus. In the hypothalamus, in addition to coupling to $G\alpha_{i/o}$, 5-HT_{1A} Rs also couple to $G\alpha_z$, a member of $G\alpha_i/G_{\alpha o}$ family [19].

1.2.2.2. Function

5-HT_{1A} Rs modulate serotonergic sensitivity and the imbalance of 5-HT_{1A} Rs in cortico-limbic regions, might affect the emotional state of an individual [20, 21], and impacts cognition, anxiety and depression. 5-HT_{1A} Rs can affect neuronal development and synapse formation [22]. It was shown that 5-HT_{1A} Rs receptor-deficient animals exhibited the anxiety-related behaviors [23-25] such as reduced exploration of the central area in the open field test [24]. The 5-HT_{1A} Rs knockout mice showed a decreased immobility in the forced swim test, a test for antidepressants [23]. It was also shown that the SSRI - fluoxetine elevated serotonin levels in 5-HT1A–/– mice and wild type mice, but the level of serotonin increased more dramatically in 5-HT1A–/– mice [26-28]. Taken together, these observations establish the significance of 5-HT_{1A} Rs in depression and anxiety.

1.2.2.3. Desensitization of 5-HT1A Rs

SSRIs bind serotonin transporter (SERT) and block the reuptake of serotonin (5-HT), which results in an increased level of 5-HT in the synapse. 5-HT_{1A} auto-receptors are then stimulated and mediate feedback regulation of 5-HT release, which leads to the down-regulation of 5-HT in the synapse and then the up-regulation of post-synaptic 5-HT_{1A} Rs. 5-HT_{1A}R agonists can also activate the 5-HT_{1A} auto-receptors and result in the same condition. However, with long-term exposure to 5-HT or activation of 5-HT_{1A} Rs, the auto-receptors become desensitized, leading to the increased release of 5-HT, which leads to desensitization of the post-synaptic 5-HT_{1A} receptors. Collectively, the prolonged 5-HT binding to the 5-HT_{1A} Rs causes desensitization and reduces the level of hyperpolarization [29].

Long-term administration of SSRIs also causes desensitization of post-synaptic 5-HT_{1A} Rs in the hypothalamic paraventricular nucleus (PVN) [30-32], which regulates the release of oxytocin and adrenocorticotropic hormone (ACTH). Long-term administration of SSRIs leads to reductions in 5-HT_{1A} R-stimulated release of oxytocin and ACTH as well as ACTH-stimulated release of cortisol. The hyper-responsivness of hypothalamic-Pituitary-Adrenal (HPA) Axis, which controls the release of ACTH and cortisol, is the consistent biomarker for depressed patients [33, 34]. The normalization of HPA axis function is correlated to the remission of depression. Based on these findings, we hypothesize that desensitization of 5-HT_{1A} Rs in PVN contributes to the therapeutic effects of SSRIs and is a critical step in treating depressed patients with SSRIs. If desensitization of 5-HT_{1A} receptor signaling contributes to the efficacy of anti-depressants, promoting desensitization of 5-HT_{1A} Rs may shorten the therapeutic lag time [29, 35].

Estrogens such as estradiol were shown to accelerate the desensitization of 5-HT_{1A} Rs in the PVN [29, 36]. Our previous studies have shown that administration of estradiol for 2 days results in the reduced ACTH or oxytocin responses to 5-HT1A Rs agonist, 8-OH-DPAT. Estradiol treatment causes a partial desensitization of 5-HT1A Rs, whereas treatment with fluoxetine (SSRI) for 7 days or combination of fluoxetine and estradiol for 2 days caused a full desensitization of 5-HT1A Rs [29].

However the mechanisms involved in the desensitization of 5-HT_{1A} Rs by estradiol are not clear. Previous studies in the laboratory demonstrated that 5-HT_{1A} Rs can be SUMOylated, treatment with an agonist 8-OH-DPAT increased SUMOylation of the receptors and treatment with estradiol for 2 days further increased the 8-OH-DPAT-induced SUMOylation of 5-HT_{1A} Rs in the PVN [35]. Since the administration of estradiol for 2 days induced the desensitization of 5-

HT_{1A} Rs, we hypothesize that SUMOylation could contribute to the desensitization of the 5-HT_{1A} Rs. Thus, it is important to understand role of SUMOylation in regulation of 5-HT_{1A} R and the mechanisms involved in the SUMOylation of 5-HT_{1A} Rs.

1.3. SUMOylation

1.3.1. Small Ubiquitin-like Modifier (SUMO) proteins

SUMO proteins are ubiquitin-like polypeptides that can be covalently conjugated to cellular proteins in a manner similar to ubiquitylation. There are 4 isoforms of SUMO confirmed in humans: SUMO 1, SUMO 2, SUMO 3 and SUMO 4. There exists a high degree of similarity between SUMO 2 and SUMO 3, only differing in three residues in the N-terminus [37]. The identity of SUMO 2 and SUMO3 makes them difficult to differentiate; they are usually referred to as SUMO 2/3. The similarity between SUMO 1 and SUMO 2/3 is around 45%. In addition, the subcellular distribution of SUMO 1 and SUMO 2/3 is also different. SUMO 2/3 is distributed throughout the nucleoplasm while SUMO 1 is located at the nuclear envelope and the nucleolus. There is a large amount of unconjugated SUMO 2/3 compared to SUMO 1 in cells [38]. Moreover, under various stressors, the SUMO 2/3 conjugation to substrates can be strongly enhanced whereas that of SUMO 1 cannot. Interestingly, SUMO 2/3 can be SUMOylated at Lys residues by chains of SUMO 2/3 proteins and terminated by SUMO 1, thereby resulting in the formation of poly-SUMOylation [39, 40]. Some substrates can be SUMOylated by multiple SUMO proteins at different sites on the substrates [41]. Poly-SUMOylation might create a special surface to enhance the binding of specific partners [42].

The identity between SUMO 2/3 and SUMO 4 is also high but there is a proline instead of glutamine at position 90 in SUMO 4. Native SUMO 4 can only become mature and able to conjugate to substrates under stressful conditions [43]. Another difference of SUMO 4 to the other SUMO proteins is the distribution. SUMO 4 mRNA has been only found in the kidneys, dendritic cells and macrophages while SUMO 1, SUMO 2, and SUMO 3 exist universally in all tissues in humans [37, 44]. However, the function of SUMO 4 is only understood to a limited degree.

1.3.2. Mechanism involved in SUMOylation

A comparison of sequences of the SUMO-modified proteins at the SUMOylation sites indicated that most sites contain the consensus motif Ψ-K-x-D/E (Ψ: hydrophobic residue consisting of 3–4 aliphatic residues, K: lysine conjugated to SUMO, x: any amino acid (aa), D/E: an acidic residue) [45]. The hydrophobic residue is quintessential for SUMOylation consensus motif. The preference of protein to SUMO isoforms might depend on the arrangement of hydrophobic and acidic residues in SUMOylation consensus motif [46]. The protein interaction with SUMO 1 but not with SUMO-2 can be attributed to the clusters of negatively charged amino acids at the C terminus to the hydrophobic core. SUMO 2/3 possesses Ψ-K-x-D/E sequences in their N-terminal extensions, which can explain why the SUMO2/3 can form the poly-SUMO chains [40]. In S. cerevisiae, there are three lysine residues (K11, K15 and K19) that are known SUMOylation sites at N-terminus of Smt3p, while in mammals, only one lysine residue (K11) of SUMO 2/3 is identified as a SUMOylation consensus site [42]. SUMO proteins can also bind to other proteins non-covalently through the SUMO-interaction motif (SIM). For example, one type of SUMO E3 ligase - PIAS proteins contain a SIM, allowing PIAS proteins to

interact with SUMO proteins non-covalently. Consensus sites for SIMs have been identified. However, there is little similarity between the SIMs.

SUMO proteins are conjugated to their substrates via an isopeptide bond linkage between the di-glycines at C terminus of SUMO and a lysine residue in the substrate. In mammals, the process of SUMOylation contains three steps. First, the SUMO precursor becomes mature SUMO through cleavage to expose glycine-glycine at the C-terminal by the SUMO-specific proteases (SENPs). Second, the mature SUMO is activated by an E1 activating enzyme which is a complex, formed by a heterodimer of SAE1and SAE2, and forming a thioester bond in an ATP-dependent manner. Third, the activated SUMO is then transferred from the E1 activating enzyme to E2 conjugating enzyme - ubiquitin carrier protein 9 (Ubc9), forming the thioester conjugate again. Subsequently, the SUMO protein is passed from Ubc9 to the substrate with a lysine residue (K) via an isopeptide bond. However, there are some substrates that require E3 ligases for the modification by SUMO [1]. Several SUMO E3 ligases (**Table 1**) act as an adapter between E2 enzyme and the substrates, transferring SUMO from Ubc9 to substrates, accelerating SUMOylation of substrates. Ultimately, deSUMOylating enzymes with isopeptidase activity remove SUMO from SUMO-modified proteins. In this way, SUMOylation and deSUMOylation processes provide a regulatory mechanism to tightly control the SUMO-modification of substrates [1, 47, 48] (**Figure 1**).

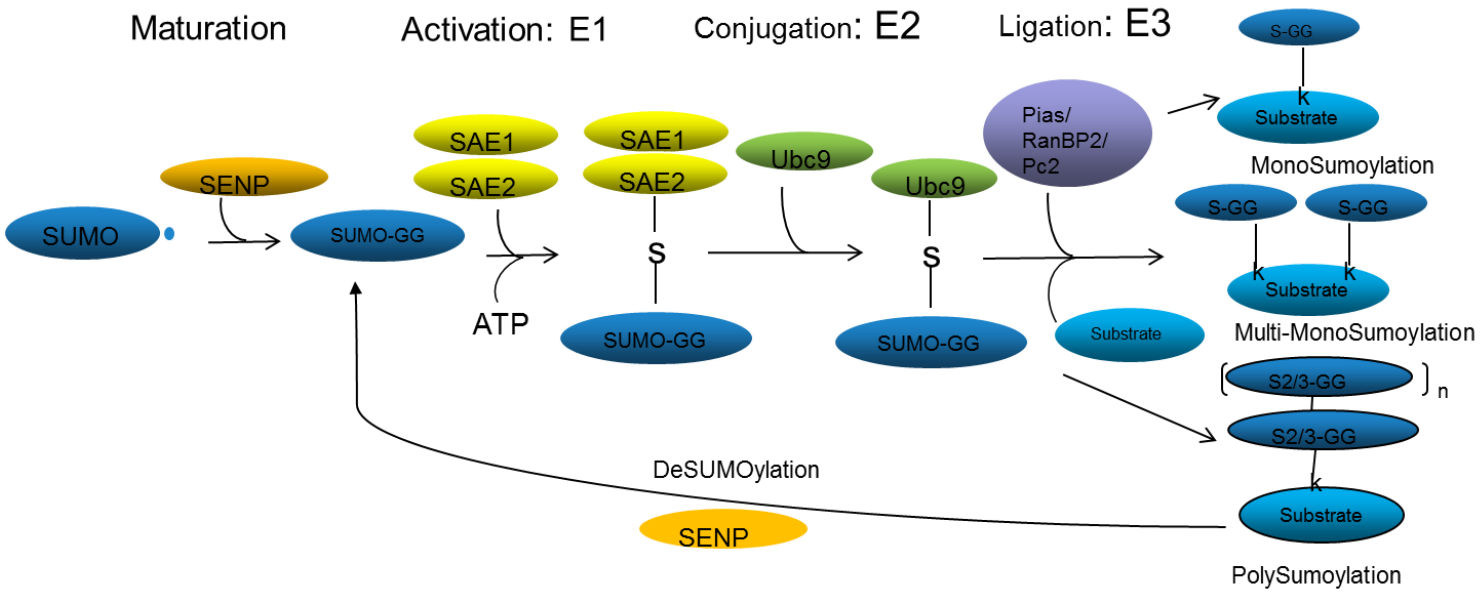


Figure 1. The mechanism of SUMOylation signaling pathway

SUMOylation is frequently associated with modulating DNA transcription. The SUMO signaling pathway is also involved in regulating mitochondrial function and morphology [49], cellular stress as well as cellular senescence and ageing [50]. Furthermore, SUMOylation can modulate synapse formation, spine morphogenesis, neurotransmission and synaptic plasticity and thus plays an important role in neurodegenerative diseases [51]. For example, SUMOylation of mutant Huntington protein is toxic in Huntington's disease [52]. Tau possesses two SIMs and can be SUMOylated at K340, which might affect the pathology of Alzheimer's disease [53].

1.3.3. SUMOylation Machinery

1.3.3.1. SUMOylation enzymes

Table 1 The enzymes involved in the SUMOylation

		Mammals	**S. cerevidise**	**S. pombe**
Small ubiquitin-related modifiers		SUMO1, SUMO2, SUMO3, SUMO4	Smt3p	Pmt3p
E1 Activating enzyme		Aos1 (SAE1), Uba2 (SAE2)	Aos1p-Uba2p	Rad31p-Fub2p
E2 Conjugating enzyme		Ubc9	Ubc9p	Hus5p
E3 ligase	SP-RING-type	PIAS1, PIAS3, PIASxα, PIASxβ, PIASγ, Mms21	Siz1p (UII1p), Siz2p (Nfi1p), Mms21p (Nse2p), Zip3p	Pli1p, Nse2p
	IR	RanBP2		
	Other	HDAC4, KAP1, Pc2, Topors		
Protease		SENP1, SENP2, SENP3, SENP5, SENP6, SENP7, DEL1, DEL2, USPL1	UIp1p (Nib1p), Ulp2p (Smt4p)	Ulp1p, Ulp2p

Table 1. The enzymes involved in the SUMOylation including Small ubiquitin-related modifiers, E1 activating enzymes, E2 conjugating enzymes, E3 ligases and deSUMOylation proteases in the mammals, S.cerevidise, and S.pombe [1].

E1 Activating enzymes

All the cellular SUMO E1 enzymes are found as heterodimers across species. In mammalian cells, SUMO E1 enzymes consist of SAE1 and SAE2, unlike the ubiquitin E1 activating enzymes (Ub E1s) which exist as a monomer. However, the structure of SUMO E1 enzymes is similar to Ub E1s. Specifically, in the Saccharomyces cerevisiae, Aos1p (40 kDa) resembles the N-terminus of Ub E1s, while Uba2p (71 kDa) corresponds to the C-terminus of Ub E1s and contains the active site of cysteine [54-58]. In mammals, the active site cysteine is

located at Cys-173 [56]. The thiol in the active site cysteine in SUMO E1s conjugates to the C terminus of SUMO and forms a thioester bond [56]. SAE1 and SAE2 are localized in the nucleus [59] and cytoplasm [60].

E2 Conjugating enzyme

There is only one E2 conjugating enzyme for SUMOylation – Ubc9 in yeast, invertebrates and possibly in the vertebrates [61-64], unlike ubiquitin E2 enzymes which contains multiple proteins participating in the ubiquitin pathway. But Ubc9 is homologous to ubiquitin E2 enzymes. Human Ubc9 is a 17kDa protein[37]. It is distributed throughout the mammalian cells, including the cytoplasm, nucleus, nuclear pore complex, around the nuclear envelope and the cell membrane [37, 65]. Ubc9 can recognize and catalyze the attachment of SUMO proteins to specific substrates. However, the ability of Ubc9 to bind to substrates is comparatively weak. Thus, SUMOylation does not occur for most substrates without SUMO E3 ligases [51].

SUMO E3 ligases

SUMO E3 ligases facilitate the conjugation of most substrates to SUMO under physiological conditions [66]. SUMO E3 ligases that have been identified include (Siz/PIAS RING) SP-RING-type E3 ligases, IR E3 ligase which contains two internal repeat (IR) domains and other potential SUMO ligases.

SP-RING-type E3 ligases

The SP-RING-type E3 ligases contain Siz/PIAS RING-finger-like domains (SP-RING domains). In the yeast, SUMO E3 ligases are comprised of Siz1, Siz2, Zip3p and Mms21p. The majority of conjugation of SUMO to substrates in the yeast require E3 ligases [67, 68]. In the

mammalian cells, the SP-RING-type E3 ligases include 5 types of the protein inhibitor activated STAT (PIAS) family, consisting of PIAS1, PIASxα, PIASxβ, PIAS3 and PIASy. They contain around 600 amino acid residues. PIAS1 and PIAS3 were first discovered as inhibitors of signal transducer and activator of transcription-1 (STAT1) and -3 (STAT3) and acted by blocking STAT binding to DNA in the nucleus [69-71]. In contrast, PIASy proteins inhibit the activity of STAT1 without affecting its binding. Both PIASxα and PIASxβ can repress the activity of STAT4. Later, PIAS proteins were found to function as SUMO ligases as they can enhance the interaction of SUMO with other proteins including androgen receptor (AR), p53 and glucocorticoid receptors [72-74]. Another SP-RING-type E3 ligase is Mms21, first was found as the SUMO ligase in the yeast and later was demonstrated to be a SUMO E3 ligase as well in humans [75]. Mms21 was shown to prevent apoptosis induced by prevention of DNA damage in human cells [75].

Among the 5 types of mammalian PIAS proteins, there is high similarity of amino acid sequence. The most conserved regions are the first 60 amino acids at the N terminus and the central region which is rich in cysteine residues. Five domains (**Figure 2**) [76] have been identified in PIAS proteins with different functions: an N-terminal scaffold attachment factor-A/B/acinus/PIAS (SAP) motif for direct-DNA binding or interaction with other proteins related to DNA-binding [77], the PINIT motif predicted for protein localization [78], the RING-type zinc-binding structure domain that is abundant with cysteine residues called Siz/PIAS RING(SP-RING) essential for the E3 SUMO-ligase activity [79], the SIM acting in interaction with SUMO proteins non-covalently [80], and the C-terminal serine/threonine rich region (S/T) with the least conserved sequences. There are two isoforms for PIASx - PIASxα and PIASxβ [76]. The domain

structures of these two isoforms are identical except the C-terminal serine/threonine region [76, 81].

Figure 2. The structure of PIAS Proteins. The PIAS Proteins contains 5 domains including an N-terminal scaffold attachment factor-A/B/acinus/PIAS (SAP) motif, the PINIT motif, Siz/PIAS RING (SP-RING) domain, SUMO binding motif (SIM) and the C-terminal serine/threonine rich region (S/T) domain.

PIAS1, PIASxα, PIASxβ, PIAS3, PIASy are reported to localize in nucleus and cytoplasm. However, the subcellular distribution of these proteins has not been clearly identified. In addition, the localization of other SUMOylation machinery is not completely explored as well. To date, SUMOylation has been considered to occur primarily in the nucleus. However, recently, it was reported that in cultured neurons, SUMO machinery including SUMO proteins, the E1 enzymes, E2 enzymes, E3 ligases and SENPs, are not only located in the nucleus, but also in neuronal axons and dendrites as well as synapses. Moreover, these proteins traffic to different regions of neurons during neuronal development [82-85]. These results indicate that the SUMOylation machinery in neurons is dynamic during neuronal development. However, the mechanism underlying trafficking is unclear. For the Siz1, one of the Siz/PIAS RING family of SUMO E3 ligases in the S. cerevisiae, the nuclear localization might be related to the SAP

domain. Siz1 with a deletion of SAP from bp 139 to 180 decreased nuclear localization to some extent [86, 87]. C-terminal domain of Siz1 is necessary for the distribution of Siz1 to the bud neck. More studies need to be conducted to understand the regulation of the localization of E3 ligases [87, 88].

Different PIAS proteins have preferences for different substrates. In addition, PIAS proteins have selectivity to different SUMO proteins. For example, PIASxα was capable of facilitating the interaction between SUMO1 and the tumor suppressor PTEN more than the other PIAS proteins [89]. PIASxβ acts as the ligase for SUMO1 conjugation to protein kinase C-θ (PKC-θ) in T cells [90], consequently influencing T cell activation. PIAS1, PIAS3 and PIASy interact with p53 and c-jun proto-oncogenes and repress the activity of the p53 tumor suppressor protein [91, 92]. Gocke et al., also reported that PIAS1 and PIASxβ can facilitate SUMOylation of a broader range of substrates than PIASy [93]. In terms of selectivity for SUMO proteins, PIASy prefers to facilitate the modification of lymphoid enhancer factor 1 (LEF1) by SUMO 2, but not SUMO 1, even though LEF1 can be modified by both SUMO 1 and SUMO 2 [94]. GATA-2 can be SUMOylated by SUMO 1 and SUMO 2, but PIASy prefers to enhance the conjugation of SUMO2 to GATA-2 [95].

The mechanism by which PIAS proteins facilitate the conjugation of different SUMO proteins to different substrates was elucidated to some extent in terms of structure. Yunus et al., used Siz1 to study the mechanism involved in the binding to SUMO machinery and substrates. They found the SP-CTD domain (a C-terminal domain) binds to SUMO protein, the SP-RING domain binds to Ubc9 and N-terminal PINIT domain attached to the substrate - the proliferating cell nuclear antigen (PCNA) at lysine 164, a non-consensus SUMO site [96]. They also found that E3 ligases can activate the E2~SUMO thioester [96]. This provides the information to

understand how the SUMO E3 ligases facilitate the SUMOylation and select both SUMO and substrates. This information can be used with other substrates in which E3 ligase plays an important role for SUMO modification.

IR type E3 ligase

There is only one IR type E3 ligase identified to date– RAN binding protein 2 (RanBP2). It is a member of nucleoporin family, and is around 358 kDa. RanBP2 contains an internal repeat (IR) domain, where around 50 residues are repeated twice (IR1 and IR2). Between the two repeated residues, there is a domain (M) consisting of 24 residues. Both the IR domains are responsible for the SUMO E3 ligase activity [97] . RanBP2 binds to the E2 enzyme and SUMO, changing the configuration and position of E2~SUMO complex, and thus facilitates the SUMOylation of substrates with enhanced the E2 activity [98].

Other SUMO E3 ligases

There are 4 other potential SUMO E3 ligases, including Histone deacetylase 4 (HDAC4), Pc2, KRAB-associated protein 1 (KPA1) and Topors.

HDAC4 itself is a SUMOylation substrate and also can augment the conjugation of SUMO to other proteins such as myocyte-specific enhancer factor 2 (MEF2) and LXRβ [99-101]. The evidence that HDAC4 binds to E2 enzyme also suggests that HDAC4 is analogous to the SUMO E3 ligase [102].

Pc2 is Polycomb group (PcG) protein. Pc2 can bind to the E2 enzyme. The SUMOylation of transcriptional co-repressor CtBP was enhanced by overexpression of Pc2, which can recruit the CtBP to PcG bodies [103, 104].

The human co-repressor KRAB-associated protein 1 (KAP1) contains Plant homeodomain (PHD) fingers and bromodomains. It was found that PHD domains in KAP1 can catalyze the intramolecular SUMOylation of the adjacent KAP1 bromodomains [105, 106].

Topors is a cellular RING finger protein, possessing a SIM domain which can interact with SUMO 1 or SUMO 2. It was shown that Topors enhanced the conjugation of SUMO 1 to p53 in vivo [107, 108].

1.3.3.2. The SUMO De-conjugation System

In mammals, there are 9 types of SUMO proteases including 6 types of sentrin-specific proteases (SENPs) (SENP1, 2, 3, 5, 6 and 7), 2 types of deSUMOylating isopeptidases (DESl1 and DESl2), and ubiquitin-specific protease-like 1 (USPL1). Some SUMO proteases can process the full-length SUMO proteins to become mature by exposing double glycine at C-terminal and de-conjugate SUMO proteins from substrates. The detailed information about the isoforms of SUMO proteases is listed in the **Table 2** [51, 109].

Table 2. The characteristics of SUMO proteases in mammals

Isoforms	SUMO preference	Main subcellular distribution	Cleavage of ProSUMO	Removal of SUMO from substrates
SENP1	SUMO1 & SUMO 2/3	Nucleus and cytoplasm	SUMO 1	Yes
SENP2	SUMO 2/3 > SUMO1	Nucleus and cytoplasm	SUMO 1 and SUMO 2	Yes
SENP3	SUMO 2/3	Nucleus	Unknown	Yes
SENP5	SUMO 2/3	Nucleus	SUMO 3	Yes
SENP6	SUMO 2/3 chains	Nucleoplasm	No	Yes, SUMO2/3 chains
SENP7	SUMO 2/3 chains	Nucleoplasm	No	Yes, SUMO2/3 chains
DESl1	SUMO 1 & SUMO 2/3	Nucleus and cytoplasm	Possibly	Yes
DESl2	unknown	Nucleus and cytoplasm	No	Unknown
USPL1	SUMO 2/3 > SUMO1	Cajal bodies in nucleus	Possibly	Yes

1.4. SUMOylation of G Protein coupled receptors (GPCRs)

To date, there are only four GPCRs that are identified as the substrates for SUMOylation, including metabotropic glutamate receptors (mGluRs), a type of glutamate receptor. There are 8 subtypes (mGluR1–8) in the family of mGluRs. It was reported that metabotropic glutamate

receptor 8b C-terminus has affinity for Pias1 and Pias3L in vitro. Pias1 enhanced the SUMOylation of mGluR8b at K882 and K903 in HEK 293 cells [110, 111].

mGluR7, a presynaptic GPCR, was also studied to examine whether it is indeed the substrate for SUMOylation. mGluR7 was found to interact with SUMO1 and SUMO2 in vitro, and SUMOylation was abolished by mutation of the receptor at lysine 889 [112]. Recently, Choi et al., demonstrated that mGluR7 can be SUMOylated at Lys889 both in brain and primary cortical neurons. L-AP4, an mGluR7 agonist and SENP1 reduced the SUMOylation of mGluR7. They also found that SENP 1 promotes the internalization of mGluR7, which indicates that deSUMOylation stimulates the internalization of mGluR7 [113].

The third GPCR that is known to be SUMOylated is cannabinoid receptor 1 (CB_1). Gowran et al., found that SUMOylated CB_1 was observed in control neurons while there is no detectable SUMO-CB_1 in neurons treated with ∆9-tetrahydrocannabinol (∆9-THC), the CB_1 agonist, which suggests that ∆9-THC induces the deSUMOylation of CB_1 [114].

1.4.1. SUMOylation of 5-HT1A Rs

Research [35] in our laboratory, demonstrated that 5-HT_{1A} Rs can be SUMOylated. Studies showed that SUMO 1 conjugated to 5-HT_{1A} Rs in the membrane fraction but not cytosolic fraction of rat cortex, resulting in a protein band around 55kD on western blots. The SUMOylated 5-HT_{1A} Rs were not only detected in the cortex, but also observed abundantly in hypothalamus, amygdala, hippocampus, dorsal raphe but less SUMO 1-5-HT_{1A} Rs in midbrain. In terms of the subcellular distribution, active 5-HT_{1A} Rs are located in the detergent-resistant microdomain (DRM), while just small portion of SUMO 1-5-HT_{1A} Rs were observed to be co-localized with flotillin-1, a DRM marker. SUMO 1-5-HT_{1A} Rs predominantly co-localized with

TGN38, a marker for trans-Golgi network (TGN), and calreticulin, a marker for the endoplasmic reticulum (ER). In order to determine whether the SUMO 1-5-HT_{1A} Rs are functional, Li et al., used 8-OH-DPAT, a 5-HT_{1A} R agonist, in an agonist binding assay. They found that the distribution of 5-HT_{1A} Rs that bound 8-OH-DPAT did not overlap with the SUMO 1-5-HT_{1A} Rs. These results suggests that SUMO1-5-HT_{1A} Rs have low affinity to the 5-HT_{1A} Rs agonist [35]. Taken together, these results suggest that SUMO 1-5-HT1A Rs are not active receptors. Based on these results, we speculate that after SUMOylation, 5-HT1A Rs may traffic from the DRM to the ER and TGN.

Previous studies [35] also showed that the SUMOylated 5-HT_{1A} Rs were increased in the hypothalamic membrane fraction and DRM of cortex of rats following the treatment with 8-OH-DPAT for 15 min compared with the rats treated with saline. Moreover, administration of estradiol for 2 days can increase the 8-OH-DPAT-induced SUMOylated 5-HT_{1A} Rs. Taken together, these results suggest that SUMOylation inactivates the 5-HT_{1A} Rs, which may possibly accelerate the internalization or desensitization of 5-HT_{1A} Rs in response to agonist stimulation.

1.5. Statement of Purpose

Depression is a common mental disorder, affecting over 350 million people worldwide. Although the mechanisms causing depression have been extensively studied and there are many types of anti-depressants on the market, there is still no effective cure for depression. Only 30-40% of patients can be effectively treated with the current medications [12] and the response time to anti-depressants usually last an average 6-7 weeks [12, 115]. SSRIs inhibit SERT activity and thus increase the level of 5-HT in the synapse. Increased level of 5-HT in the synapse for a prolonged time desensitizes both pre-synaptic and post-synaptic 5-HT_{1A} Rs in PVN [29, 31, 116]. Considering that chronic treatment of SSRIs decreased the sensitivity of 5-HT_{1A} Rs, we

hypothesize that desensitization of 5-HT_{1A} receptor signaling may contribute to the delay of therapeutic effects of SSRIs. Previous work in our laboratory showed that 5-HT_{1A} Rs can be SUMOylated and are predominantly located in the endoplasmic reticulum and golgi. Based on this, we hypothesized that SUMOylation of 5-HT_{1A} Rs may be related to desensitization of 5-HT_{1A} Rs. We also reported that SUMOylation of 5-HT_{1A} Rs were up-regulated by acute treatment with a 5-HT_{1A} receptor agonist, 8-OH-DPAT. In addition, SUMO 1- 5-HT_{1A} Rs increased more when rats were treated with estradiol prior to injection of 8-OH-DPAT, suggesting that 8-OH-DPAT and estradiol can accelerate SUMOylation of 5-HT_{1A} Rs. However, the mechanism underlying the SUMOylation of 5-HT_{1A} Rs is not clear. Specifically, the mechanism underlying the increase of SUMO1-5-HT_{1A} Rs by 8-OH-DPAT or the increase of 8-OH-DPAT-induced SUMO1-5-HT_{1A} Rs by estradiol are still unknown. Exploring the mechanism involved in SUMOylation of 5-HT_{1A} Rs will improve our understanding of desensitization of 5-HT_{1A} receptors, and inform the development of an adjunct therapy to reduce the therapeutic lag of SSRIs.

Therefore, the purpose of this study is to determine (1) the mechanism responsible for SUMOylation of 5-HT_{1A} Rs and (2) the mechanism responsible for 8-OH-DPAT-induced SUMO1-5-HT_{1A} Rs and the increase of 8-OH-DPAT-induced SUMO1-5-HT_{1A} Rs by estradiol.

To date, there are only four GPCRs reported to be the substrates of SUMO proteins. mGluR 8b was reported to interact with PIAS 1 and PIAS3L in vitro. The SUMOylation of mGluR8b was enhanced by PIAS1 in HEK 293 cells [110, 111]. Thus, we proposed the hypothesis that **PIAS proteins are involved in increasing the SUMOylation of 5-HT_{1A} receptors.**

To test this hypothesis, we first set up a cell model for the detection of SUMOylation of 5-HT_{1A} Rs. We examined the expression of 5-HT_{1A} Rs and the SUMOylation machinery including SUMO proteins, Ubc9 and PIAS proteins in a mouse neuroblastoma 2a (N2a) cells. Further, we transfected N2a cells with plasmid constructs of 5-HT_{1A} receptors and SUMO1 to detect the expression of SUMO 1- 5-HT_{1A} Rs in N2a cells. Next, we transfected N2a cells with plasmid constructs of different members of PIAS family to determine the effects of different PIAS proteins on SUMO 1- 5-HT_{1A} Rs.

Based on the results that 8-OH-DPAT increased SUMO 1- 5-HT_{1A} Rs and estradiol accelerated 8-OH-DPAT-induced SUMO 1- 5-HT_{1A} Rs, we asked the question whether PIAS proteins increase in the PVN membrane fraction and participate in the SUMOylation of 5-HT_{1A} Rs following the treatment by 8-OH-DPAT or co-treatment of estradiol and 8-OH-DPAT.

To answer this question, we proposed our second hypothesis that **treatment of the 5-HT_{1A} receptor agonist and estradiol can enhance the expression of specific PIAS proteins in the membrane fraction of PVN in hypothalamus**.

To test this hypothesis, we used the PVN of the rats treated with 8-OH-DPAT and combined treatment with 8-OH-DPAT and estradiol to examine the expression levels of specific PIAS proteins in the membrane fraction of the PVN.

CHAPTER 2: MATERIALS AND METHODS

2.1. Plasmid constructs

The plasmid constructs used in the experiments are listed in **Table 3**. For plasmid isolation and purification, a QIAGEN®Plasmid Midi Kit (25) (Cat#12143, QIAGEN, USA) was used.

Table 3. Summary of the plasmid constructs used

Plasmid constructs	**Vector**	**Tag**	**Source**
pcDNA4 HisMax C-5-HT_{1A} Receptor	pcDNA4 HisMax C	His	
SRa-HA-SUMO 1	pcDNA3/HA	HA	Addgene, Plasmid# 17359
Flag-mPIAS1	pFLAG-CMV-(3800bp)	Flag	Plasmid# 15206, Addgene
Flag-hPIASx alpha	pFLAG-CMV-(3800bp)	Flag	Plasmid# 15209, Addgene
Flag-hPIASx beta	pFLAG-CMV-(3800bp)	Flag	Plasmid# 15210, Addgene
Flag-mPIAS3	pFLAG-CMV-(3800bp)	Flag	Plasmid# 15207, Addgene
Flag-hPIASy	pFLAG-CMV(3800bp)	Flag	Plasmid# 15208, Addgene
Flag-SENP1	pFLAG-CMV(4700bp)	Flag	Plasmid# 17357, Addgene

Flag-SENP2	pFLAG-CMV(4700bp)	Flag	Plasmid# 18047, Addgene
Flag-SENP6	pFLAG-CMV(4700bp)	Flag	Plasmid# 18065, Addgene

2.2. Cell culture and harvesting

Mouse Neuroblastoma 2a cells (N2a cells) were maintained in 50% Dulbecco's Modified Eagle Medium (1X DMEM, high glucose, pyruvate, Cat# 11995-073, Thermo Fisher, USA) and 50% Opti MEM (Cat# 31985088, Thermo Fisher, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Cat# S11150, Atlanta Biologicals, USA) and 1% Penicillin-Streptomycin solution (Cat# P0781-100ML, Sigma, USA). For transfection assays, N2a cells were plated in 10cm dish at a density of 2.2 x 10^6 per dish. After 16-24 hours, cells were transfected with the mammalian expression plasmids constructs using Lipofectamine 3000 (Cat# L3000015, Thermo Fisher, USA). Medium was changed 6 hours and 24 hours after transfection.

48 hours after transfection, cells were washed with phosphate-buffered saline and hypotonic buffer (0.25M sucrose 50 mM Tris, pH 7.5, 5mM EDTA, 100 mM NaCl. 20mM NEM was added before use). The cells were harvested with hypotonic buffer (20mM NEM, 1/100 dilution of phosphatase inhibitors and protease inhibitors were added before use). After sonication, the homogenate was transferred to a centrifuge tube and spun at 25,000xg (16,500 rpm) for 1 hr at 4^0C. The supernatant was collected as cytosol fraction.

The pellet was reconstituted with solubolization buffer (20 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl. 1% sodium cholate, 20mM NEM, 1/100 dilution of phosphatase inhibitors and protease inhibitors were added before use). After sonication, the vials were shaken horizontally at high speed, at 4^oC for at least 1 hour. The homogenate was centrifuged at 25,000xg, at 4^oC for

1 hour. Supernatant was collected as the membrane fraction. The solution was then aliquoted and stored in -80^{o}C. BCA assay (Cat# 23228, Cat# 1859078, BCA Protein Assay, Thermo Fisher, USA) was used to measure the concentration of protein.

2.3. Treatment of rats and preparation of membrane fraction of rats PVN

The PVN tissue from rats used in this study was from rats used in a previously published study to determine the effects of 17β-estradiol-3-benzoate (EB) on oxytocin and ACTH responses [117]. The rats were treated with drugs as described below. 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.)). (+)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.

Rats were given unilateral intra-PVN injections of GPR30-mis-Ads as previously described [117]. Five days after injection, rats were treated with either estradiol (10ug/kg, 0.4 ml/kg, sc) or sesame oil for 2 days. 20 hours after last injection, rats were treated with 8-OH-DPAT (200ug/kg, sc) or saline and then were decapitated 15 min after the treatment. Brains were removed. The PVN was removed and stored at -80°C.

PVN tissue was homogenized using 100ul homogenization buffer (50 mM Tris, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100. 20mM NEM, 1/100 dilution of phosphatase inhibitors and protease inhibitors were added before use) using motorized homogenizer (Powergene 1000 with 5mm probe) at speed 5, 4^{o}C for 10 sec or until all of the tissue was

homogenized. The homogenate was spun at 25,000xg (16,500 rpm) for 1 hr at 4^0C. The supernatant was collected as the cytosol fraction. The membrane fraction was reconstituted with 50 µl solubilization buffer described above for cell lysate. The following procedure for membrane fraction of PVN was the same as that for cell lysates.

2.4. Immunoprecipitation (IP)

Immunoprecipitation was conducted using 250ug protein of membrane fraction of N2a cells or hypothalamic region of rat brain, prepared as described above. The sample was added to 50 ul prewashed rprotein G agarose (Cat# 15920-010, Invitrogen, USA) in total volume of 250ul IP buffer (50 mM Tris, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100, containing 20 mM N-ethymaleimide, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail I and II, respectively) and then was rotated at 4^oC for 1h. After centrifugation at 10,000rpm at 4^oC for 10min, the supernatant was incubated with 2.5ug anti-SUMO1 antibody (Cat# sc-5308, Santa Cruz, USA) or mouse IgG control (Cat# sc-2025, Santa Cruz, USA) on a rotator at 4^oC overnight. The solution were added to 100ul pre-washed beads and then rotated at 4^oC for 2 hours. The protein G beads-immune complex was centrifuged at 1000rpm for 3 minutes at 4^oC. The pellet was washed with 0.5ml ice-cold IP buffer 3 times. After washing, the proteins were eluted from the immune complexes using 50ul 2X sample buffer with β-mercaptoethanol and incubated at 95^oC for 5 min following by centrifugation at 12,000rpm for 5 min at room temperature. The eluate was stored in -80^oC or was loaded onto 10% SDS-PAGE gels.

2.5. Immunoblot assays

Protein samples were resolved in the 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. After transferring, the PVDF membranes were

incubated in 5% non-fat milk in Tris-buffered saline, pH 7.6, with 0.1% Tween-20. The membranes were incubated overnight with primary antibodies listed in Table 4. After washing 4 times, the membranes were incubated with the appropriate following secondary antibodies listed in **Table 4.**

Table 4. Antibodies used for immunoprecipitation and immunoblots

Antibodies	**Dilution**	**Source**
Rabbit anti-5-HT1A Receptors	1:1000	Cat# PA5-28090, Thermo Fisher, USA
Rat-anti-Flag	1:2000	Cat# 200474-21, Agilent, USA
Mouse-anti-Na+, K+, ATPase	1:1000	Cat# 05-369, Millipore, USA
Rabbit-anti-PIAS 1	1:2000	Gift from Dr. Yoshiaki Azuma
Rabbit-anti-PIASx alpha	1:2000	Gift from Dr. Yoshiaki Azuma
Rabbit-anti-PIAS 3	1:2000	Gift from Dr. Yoshiaki Azuma
Rabbit-anti-PIASy	1:2000	Gift from Dr. Yoshiaki Azuma[118]
Mouse-anti-βactin	1:20000	Cat# 691001, MP Biomedicals, LLC
Goat-Anti-Mouse IgG	1:10000	Cat# 119380, Jackson ImmunoResearch, USA
Goat Anti-Rabbit IgG	1:10000	Cat# 120745, JacksonImmunoResearch, USA

Immunodetection was performed using an ECL kit (Cat# WBLUR0500, Millipore, USA, Cat# 10026384, Cat# 10026385, BioRad, USA) and ImageLab 3.0 software (BioRad, Hercules, CA). To normalize the protein levels, the intensity of bands were first normalized to the mean

intensity of the bands of mock transfection samples following by normalized to its beta-actin band.

2.5. Immunocytochemistry

The N2a cells were plated on coverslips, which had been coated with 500ul poly-D-lysine and then put back into incubator. After 30 min, the media containing the 50% Dulbecco's Modified Eagle Medium (1X DMEM, high glucose, pyruvate, Cat# 11995-073, Thermo Fisher, USA) and 50% Opti MEM (Cat# 31985088, Thermo Fisher, USA) supplemented with 10% charcoal Fetal Bovine Serum (FBS) (Cat# S11150, Atlanta Biologicals, USA) and 1% Penicillin-Streptomycin solution (Cat# P0781-100ML, Sigma, USA) was added into plates.

After 16-24 hours, the cells were transfected with plasmid constructs of pcDNA4 HisMax C-5-HT_{1A} R or pmCherry-C1- 5-HT_{1A} R to the N2a cells using Lipofectamine 3000 (Cat# L3000015, Thermo Fisher, USA). Medium was changed 24 hours after transfection.

48 hours after transfection, the cells were washed with 2ml cold phosphate-buffered saline, pH 7.4 (PBS) and then fixed and permeabilized with ice-cold methanol for 2 min in -20˚C. Next, the cells were washed with PBS and incubated with blocking buffer (10% Normal Goat Serum) at room temperature for 30 min. After washing, cells were then incubated overnight with the mouse-anti-Na+, K+, ATPase antibody (1:500) (Cat# 05-369, Millipore, USA) and rabbit anti-5-HT1A R antibody (1:200) (Cat# PA5-28090, Thermo Fisher, USA) at 4°C. Next, cells were washed three times with PBS and incubated with Alexa Fluor 488 conjugated goat-anti-mouse (1:500) (Cat# A-11001, Thermo Fisher, USA) and Alexa Fluor 568 conjugated goat anti-rabbit (1:500) (Cat# A-11011, Thermo Fisher, USA) diluted in the blocking buffer for 2 hrs. Cells were then washed with PBS and then with autoclaved ddH_2O. Finally, the cells were mounted onto

microscope slides using Prolong Gold antifade regent with DAPI. The slides were stored at 4˚C and imaged using a confocal microscope.

2.6. Data analysis and statistics

All data were analyzed by one-way or two-way analysis of variance (ANOVA) using a statistical program (GraphPad Prism, version 6.02) followed by post-hoc tests including Dunnett's multiple comparisons test (only comparisons with one control) or Tukey's multiple comparisons test. The data are presented as mean ± SEM.

CHAPTER 3: RESULTS

3.1. Overexpression of 5-HT_{1A} Rs

To determine whether the N2a cells express endogenous 5-HT_{1A} Rs and can express exogenous 5-HT_{1A} Rs constructs, we first transfected N2a cells with plasmid constructs of pcDNA4 HisMax C- 5-HT_{1A} R. Immunolabeling was used to identify the endogenous expression and overexpression of 5-HT_{1A} Rs. Our results (**Figure 1**) show that there was an increase of expression level of 5-HT_{1A} Rs in the cells transfected with pcDNA4 HisMax C- 5-HT_{1A} R constructs, indicating that N2a cells can overexpress 5-HT_{1A} Rs. Furthermore, the overexpressed 5-HT_{1A} Rs are located not only in the plasma membrane, but also in the cytosol of N2a cells.

3.2. Overexpression of SUMO machinery

In order to investigate which PIAS proteins and SENPs are involved in the SUMOylation of 5-HT_{1A} Rs, we transfected various Flag-PIAS and Flag-SENP constructs into N2a cells. The expression level of transfected PIAS and SENPs constructs was examined by immunoblotting with an anti-Flag antibody. In the whole cell lysate, among the various PIAS constructs transfected, the PIASy construct expressed most abundantly followed by the PIAS1 construct. The expression level of transfected PIAS3 construct is less abundant than PIAS1 construct. Transfected constructs PIASxα and PIASxβ expressed the least amount of proteins. Interestingly, PIASxβ expressed so little that in the whole lysate, PIASxβ can hardly be detected (**Figure 2A**). In the membrane fraction, the expression of transfected PIASy is the most abundant. The expression of transfected PIAS1 construct is much lower than PIASy but more than PIAS3 and PIASxα, which show similar expression levels. In contrast, the expression of PIASxβ is the lowest among the transfected PIAS constructs. Comparing the expression level of

each PIAS protein in the whole lysate and membrane indicates that the ratio of PIASxα and PIASxβ in the membrane compared to that in the whole lysate is higher than that of other PIAS proteins (**Figure 2B**), indicating that PIASxα and PIASxβ may traffic to membrane of cells and could be involved in the SUMOylation of membrane proteins such as 5-HT_{1A} Rs. The expression level of endogenous and transfected PIAS proteins in the membrane fraction were also detected using specific anti-PIAS antibodies. Results showed that N2a cells express endogenous PIAS proteins but not robustly (**Figure 2C**). In the cells transfected with PIAS constructs, the total expression level of each PIAS protein in the membrane fraction of cells was also detected to verify the expression of transfected PIAS constructs (**Figure 2C**).

We also examined the expression levels of SENP1, SENP2, SENP6 in N2a cells. Transfected constructs of SENP1, SENP2, SENP6 were expressed in a similar level in the whole lysate (**Figure 2D)**. Altogether, these data indicates that N2a cells are a good model to express SUMO machinery.

Na+, K+ ATPase, the plasma membrane marker was used to verify separation of membrane and cytosolic fractions (**Figure 2E**). The results show that there was abundant Na+, K+ ATPase in the membrane fraction while there was little in the cytosolic fraction, indicating successful separation of membrane and cytosol fraction.

3.3. SUMOylation of 5-HT_{1A} Rs and the role of different PIAS proteins in SUMOylation of 5-HT_{1A} Rs

Since we have already examined the expression level of SUMO machinery, we sought to examine if SUMO 1 protein can covalently bind with 5-HT_{1A} Rs. N2a cells were co-transfected with constructs of pcDNA4 His MaxC-5-HT_{1A} R and HA-SUMO 1. Cell lysates were

immunoprecipitated with mouse monoclonal anti-SUMO 1 antibody and immunoblotted with rabbit polyclonal anti-5-HT_{1A} R antibody to detect the SUMO 1- 5-HT_{1A} R complex. We observed bands around 55kDa (**Figure 3A**), which is the predicted molecular size for SUMO 1-5-HT_{1A} R complex as previously detected in different regions of rat brain tissue. In addition, when an irreversible cysteine peptidase inhibitor that inhibits sentrin/SUMO-specific proteases (SENPs), the protease responsible for deSUMOylation of proteins i.e. N-ethylmaleimide (NEM) was not used to harvest N2a cells and conduct immunoprecipitation experiments, the intensity of SUMOylated 5-HT_{1A} Rs was dramatically decreased, thus confirming that the band around 55 kDa represents the SUMOylated 5-HT_{1A} Rs.

Next, we examined the effect of different PIAS proteins on the SUMOylation of 5-HT_{1A} Rs. N2a cells were co-transfected with pcDNA4 His MaxC-5-HT_{1A} R and HA-SUMO 1 with or without different constructs expressing PIAS proteins. SUMO 1-5-HT_{1A} Rs were immunoprecipitated with mouse monoclonal anti-SUMO 1 antibody and incubated with rabbit polyclonal anti-5-HT_{1A} R antibody. One-way ANOVA shows that there is significant effect of transfection ($F_{(5, 12)} = 4.612$, $p = 0.0140$). Dunnett's multiple comparisons post-hoc test shows that PIASxα significantly increased the SUMOylated 5-HT_{1A} Rs compared to the cells transfected with 5-HT_{1A} Rs and SUMO 1 (**Figure 3B**). PIAS1, PIAS3 and PIASy constructs overexpressed abundantly, but did not significantly alter SUMOylation of 5-HT_{1A} Rs. PIASxβ expressed the least and there was no significant effect on SUMOylation of 5-HT_{1A} Rs.

Considering the difference in expression of different PIAS constructs, especially PIASxβ, more PIASxβ construct was transfected to N2a cells in order to obtain higher levels of protein expression. However, the expression level of PIASxβ did not increase much (**Figure 3C**).

Interestingly, when N2a cells were just transfected with PIASxβ constructs, there was a significant decrease on the SUMO1-5-HT_{1A} Rs compared to mock transfected cells (**Figure 3D**).

3.4. The effect of treatment of EB and 8-OH-DPAT on the PIAS proteins in the PVN

Previous experiments in our laboratory demonstrated that acute treatment with 8-OH-DPAT, a 5-HT_{1A} R agonist for 15 min increased the level of SUMO1-5-HT_{1A} Rs in the hypothalamic membrane. In addition, treatment with EB for 2 days followed by acute treatment with 8-OH-DPAT for 15 min further enhanced the level of SUMO1-5-HT1A Rs. In order to understand the mechanisms underlying the effect of treatment on SUMOylation of 5-HT1A Rs, I examined the expression level of different PIAS proteins in the membrane fraction of the PVN of rats following identical treatment with 8-OH-DPAT and EB. Specific anti-PIAS1, anti-PIASxα, anti-PIAS3 and anti-PIAS4 antibodies were used to detect the expression level of corresponding PIAS protein.

For PIAS1, two bands around its predicted molecular weight were observed using immunoblotting (**Figure 4A**). Since the right band for PIAS1 cannot be identified among these two bands, both the two bands were measured to quantify the effect of treatments on the expression of PIAS1. Two-way ANOVA shows there was no significant effect of pretreatment, treatment and interaction on the expression level of PIAS1 (Upper bands: Two-way ANOVA: main effect of pretreatment (EB): $F_{(1,8)=}3.041$, p=0.1193; main effect of treatment (8-OH-DPAT): $F_{(1,8)}=0.006659$, p=0.9370; interaction between pre-treatment and treatment: $F_{(1,8)}=0.01447$, p=0.9072; Lower bands: Two-way ANOVA: main effect of pretreatment (EB): $F_{(1,8)=}3.551$, p=0.0963; main effect of treatment (8-OH-DPAT): $F_{(1,8)}=1.498$, p=0.2558; interaction between pre-treatment and treatment: $F_{(1,8)}=0.2503$, p=0.6303).

For the PIASxα expression level, two-way ANOVA showed that there was significant effect of EB treatment but no significant effect of 8-OH-DPAT treatment or the interaction between EB and 8-OH-DPAT. Tukey's multiple comparisons post-hoc test shows that the expression of PIASxα was unchanged with the activation of 5-HT1A Rs by acute treatment with 8-OH-DPAT alone compared to the vehicle treatment (**Figure 4B**). In addition, treatment with EB alone also had no significant effect on the PIASxα expression level in comparison to vehicle treated rats. Moreover, there was no alteration of the expression level of PIASxα when combined treatment of EB and 8-OH-DPAT is compared to treatment of EB alone. However, rats treated with EB followed by 8-OH-DPAT showed a significant increase in the PIASxα level in the rat PVN membrane fraction by about 40% compared to the 8-OH-DPAT-treated rats. (Two-way ANOVA: main effect of pre-treatment (EB): $F_{(1,8)}$= 7.446, p=0.0259; main effect of treatment (8-OH-DPAT): $F_{(1,8)}$ =3.822, p=0.0863; interaction between pre-treatment and treatment: $F_{(1,8)}$ = 4.086, p=0.0779).

With respect to PIAS3, two-way ANOVA analysis showed that there was no significant effect of pre-treatment, treatment and interaction on the expression level of PIAS3 (Two-way ANOVA: main effect of pre-treatment(EB): $F_{(1, 8)}$ =0.02531, p=0.8775 ; main effect of treatment (8-OH-DPAT): $F_{(1,8)}$=0.006120, p=0.9396; interaction between pre-treatment and treatment: $F_{(1,8)}$= 0.009228, p=0.9258)

In terms of expression level of PIASy, two-way ANOVA showed there was significant effect of EB treatment but not 8-OH-DPAT treatment or interaction between EB and 8-OH-DPAT treatment. Tukey's multiple comparisons post hoc test shows that the expression level of PIASy was increased significantly in the rats treated with EB alone compared to vehicle treated rats. However, no effect on PIASy expression was seen in 8-OH-DPAT-treated group compared

to vehicle treated rats or the rats treated with EB and 8-OH-DPAT together. Intriguingly, there was no significant change between EB treated rats and rats co-treated with EB and 8-OH-DPAT. (Two-way ANOVA: main effect of pre-treatment(EB): $F_{(1,8)}$=8.463, p=0.0196; main effect of treatment (8-OH-DPAT): $F_{(1,8)}$=0.1524, p=0.7064; interaction between pre-treatment and treatment: $F_{(1,8)}$=3.760, p=0.0885).

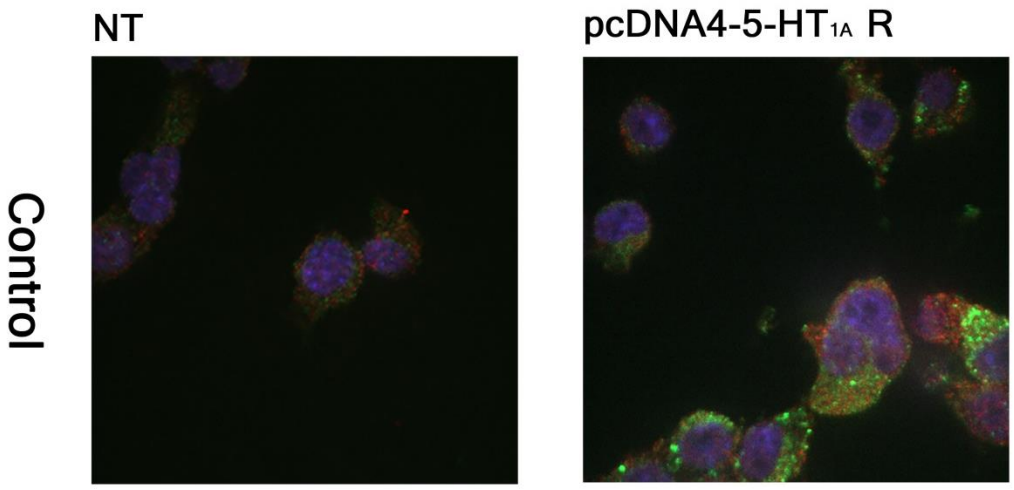


Figure 1. Overexpression of 5-HT1A Rs

Figure 1. Overexpression of 5-HT_{1A} Rs. The N2a cells were transfected with plasmid construct of pcDNA4 His MaxC-5-HT_{1A} R. 48 hrs after transfection, the cells were fixed in methanol. Cells were incubated overnight with the mouse-anti-Na^+, K^+, ATPase antibody following by Alexa Fluor 488 conjugated goat-anti-mouse and with rabbit anti-5-HT1A R antibody following by Alexa Fluor 568 conjugated goat anti-rabbit. The Na^+, K^+, ATPase (right panel, green) were shown in the plasma membrane of N2a cells. The expression of pcDNA4 His MaxC-5-HT_{1A} R was located both in the plasma membrane and the cytosol of N2a cells (right panel, red).

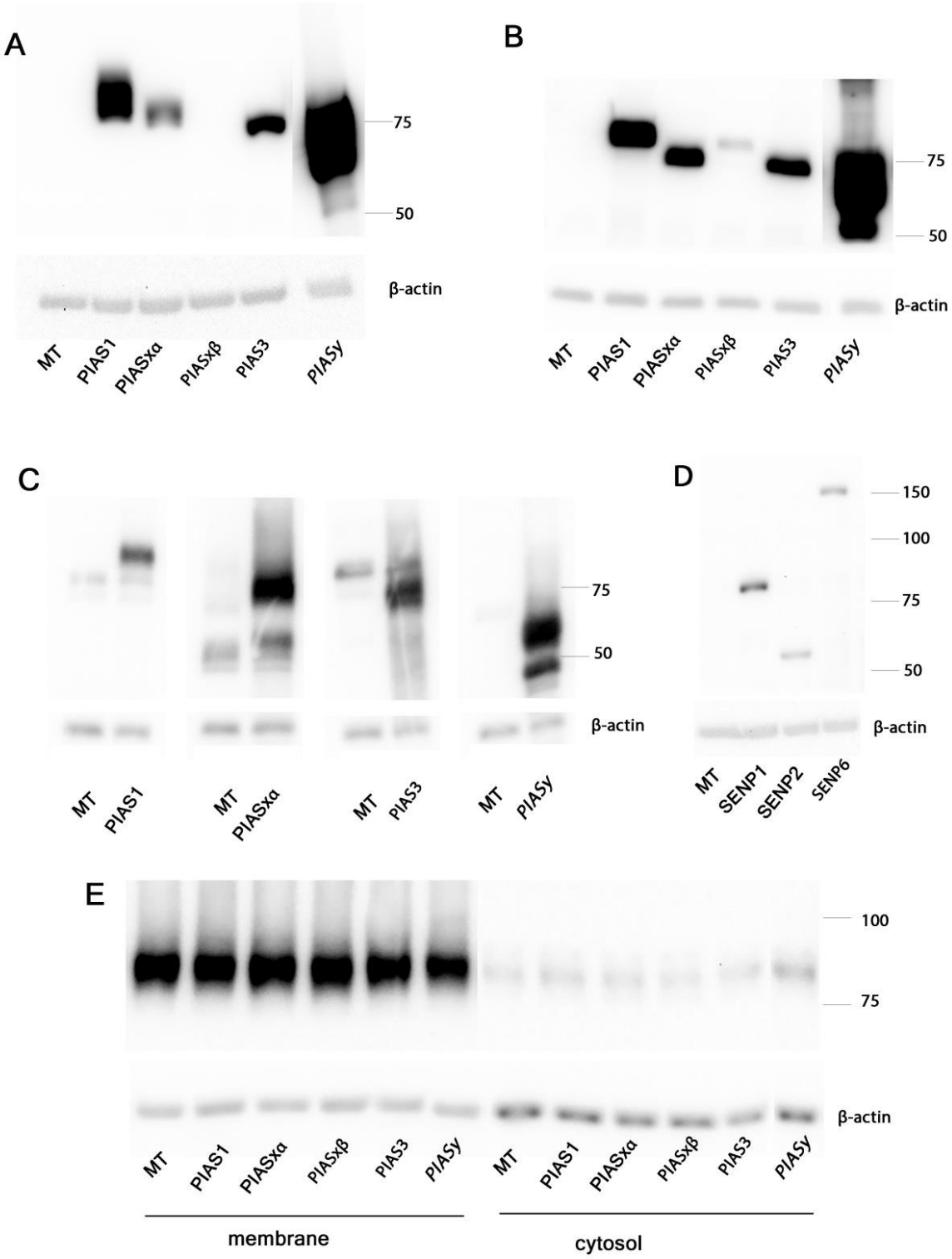
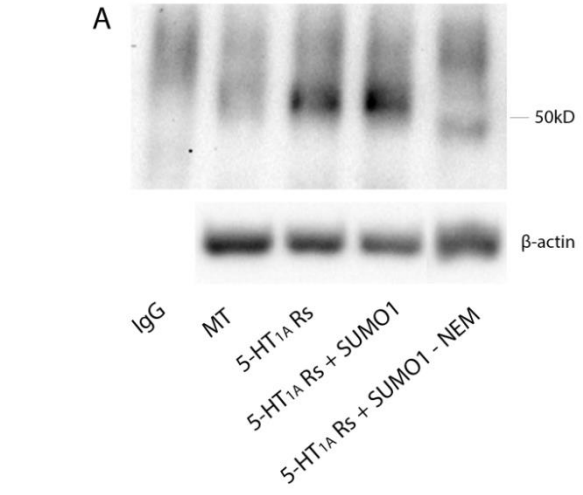
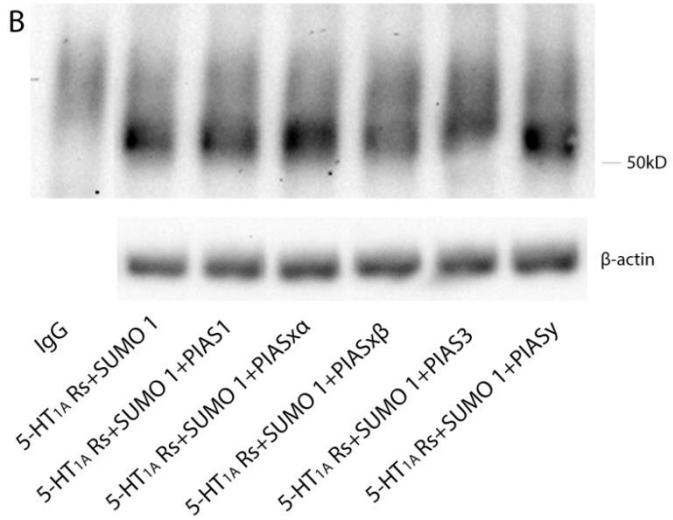
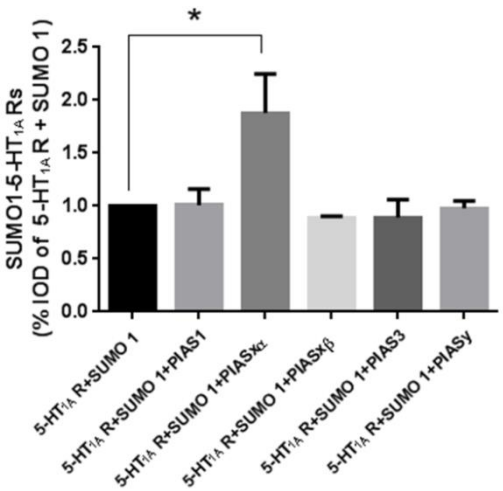


Figure 2. The expression of PIAS proteins in N2a cells.

Figure 2. The expression of PIAS proteins in N2a cells. N2a cells were transfected with Flag-tagged PIAS plasmid constructs. The whole cell lysates **(A)** and the membrane fraction of cells **(B)** were resolved on 10% SDS-PAGE gels, proteins were transferred to PDVF membranes and the blot was incubated with anti-flag antibody. **(C)** The expression of each PIAS protein in the membrane fraction was detected using specific anti-PIAS antibodies. **(D)** The expression of transfected SENPs in the whole lysate. **(E)** The separation of membrane fractions was verified by immunoblotting using Na^+/K^+ ATPase as plasma membrane marker. MT: Mock transfection


A
50kD
β-actin
IgG
MT
5-HT1A Rs
5-HT1A Rs + SUMO1
5-HT1A Rs + SUMO1 - NEM


B
50kD
β-actin
IgG
5-HT1A Rs+SUMO 1
5-HT1A Rs+SUMO 1+PIAS1
5-HT1A Rs+SUMO 1+PIASxα
5-HT1A Rs+SUMO 1+PIASxβ
5-HT1A Rs+SUMO 1+PIAS3
5-HT1A Rs+SUMO 1+PIASy


*
SUMO1-5-HT1A Rs
(% IOD of 5-HT1A R + SUMO 1)
2.5
2.0
1.5
1.0
0.5
0.0
5-HT1A R+SUMO 1
5-HT1A R+SUMO 1+PIAS1
5-HT1A R+SUMO 1+PIASxα
5-HT1A R+SUMO 1+PIASxβ
5-HT1A R+SUMO 1+PIAS3
5-HT1A R+SUMO 1+PIASy

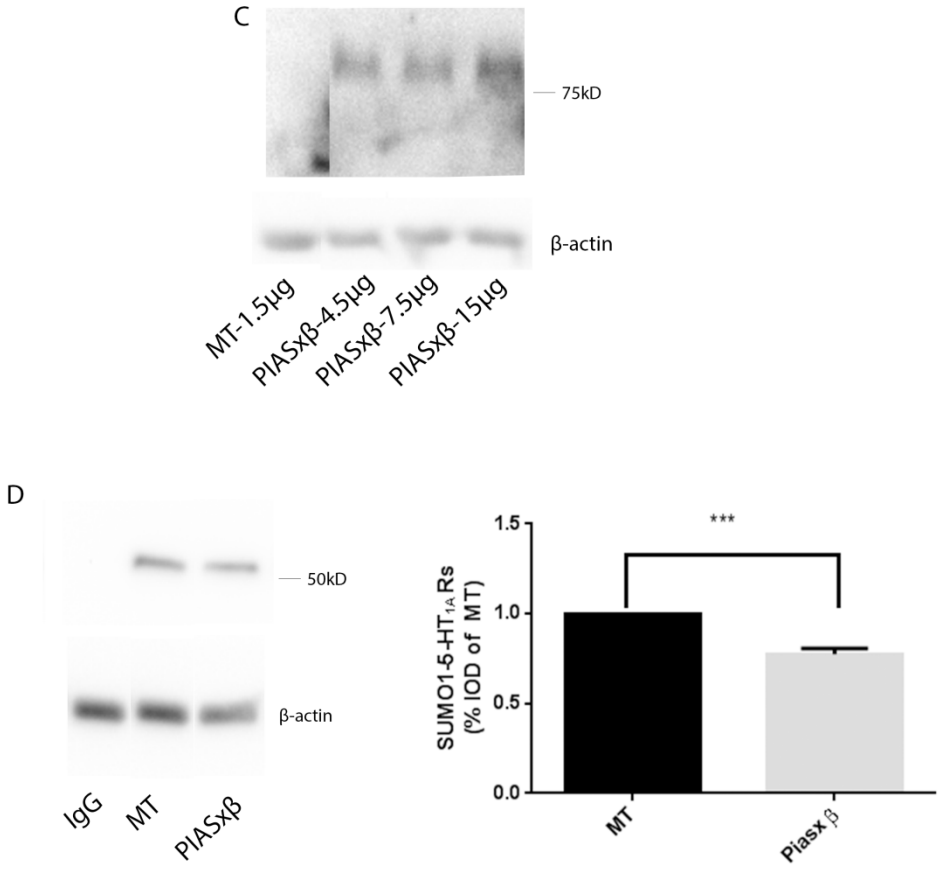
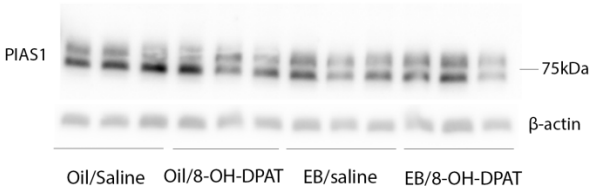


Figure 3. The role of PIAS proteins in the SUMOylation of 5-HT1A Rs.

Figure 3. The role of PIAS proteins in the SUMOylation of 5-HT$_{1A}$ Rs. The N2a cells were transfected with pcDNA4 His MaxC-5-HT$_{1A}$ R, HA-SUMO 1 and various plasmid constructs of PIAS as indicated in the figures. Membrane fractions were isolated 48 hours after transfection. The SUMOylated 5-HT$_{1A}$ Rs were immunoprecipitated using a mouse anti-SUMO 1 antibody and immunoblotted using a rabbit anti-5-HT$_{1A}$ Rs antibody. **(A)** 5-HT$_{1A}$ Rs were SUMOylated by SUMO1 in N2a cells. IgG: immunoprecipitation with the same amount of mouse IgG instead of a mouse anti-SUMO1 antibody. MT: Mock transfected, cells were transfected with empty

vector. Various amounts of empty vector were added to get the same amount of plasmid constructs in total per dish. **(B)** The effect of PIAS proteins on SUMOylation of 5-HT_{1A} Rs. Data are presented as mean ± SEM (n=3). * One-way ANOVA shows there is significant effect of transfection of PIAS on SUMOylation of 5-HT 1ARs ($F(5, 12) = 4.612$, $P = 0.0140$). Dunnett's multiple comparisons post-hoc test shows that SUMOylation of 5-HT1A R significantly increased in the group transfected with pcDNA4 His MaxC-5-HT_{1A} R, HA-SUMO 1 and PIASxα compared to the group transfected with the pcDNA4 His MaxC-5-HT_{1A} R and HA-SUMO 1 ($p<0.05$). **(C)** N2a cells were transfected with 1.5 µg empty vector (MT), 1.5 µg, 7.5 µg and 15µg plasmid construct of Flag-PIASxβ respectively. The expression level of PIASxβ was detected using anti-Flag antibody. **(D)** N2a cells were transfected with same amount of empty vector and construct of PIASxβ. SUMO1-5-HT_{1A} Rs were immunoprecipitated by anti-SUMO1 antibody and detected by anti-5-HT_{1A} R antibody. *** unpaired t test shows there is significant difference between mock transfected group and PIASxβ transfected group, $p=0.0002$) MT: Mock transfection.

A



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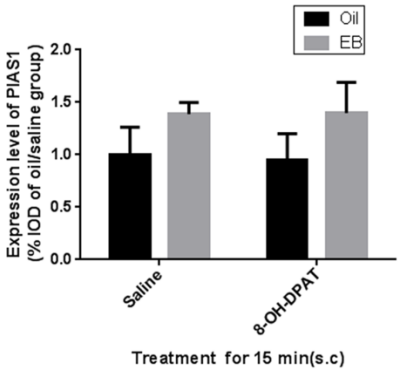

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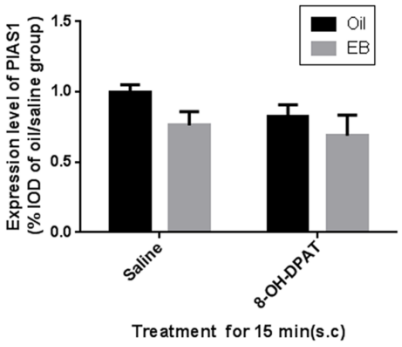
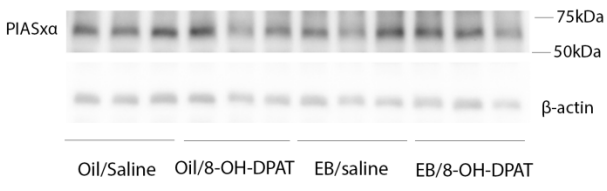
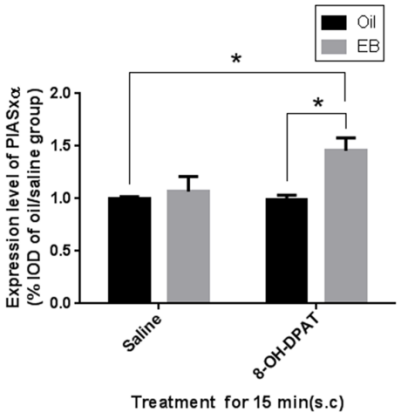
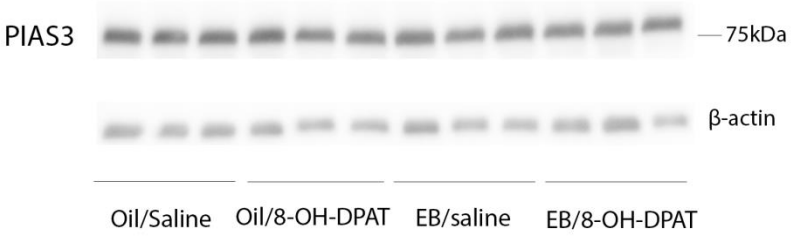


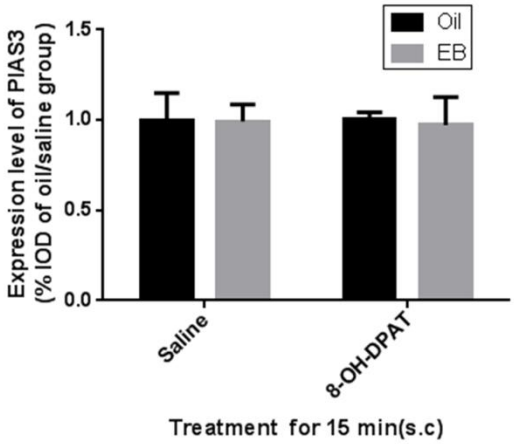
B



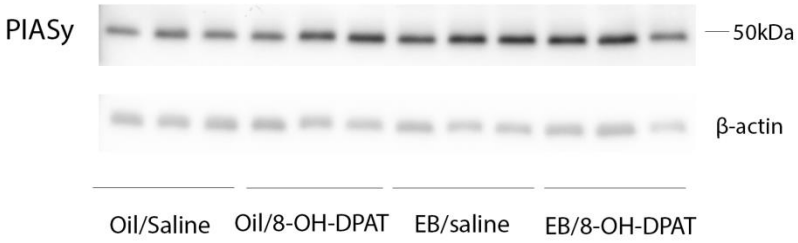


C


PIAS3
75kDa
β-actin
Oil/Saline
Oil/8-OH-DPAT
EB/saline
EB/8-OH-DPAT


Oil
EB
Expression level of PIAS3
(% IOD of oil/saline group)
1.5
1.0
0.5
0.0
Saline
8-OH-DPAT
Treatment for 15 min(s.c)

D


PIASy
50kDa
β-actin
Oil/Saline
Oil/8-OH-DPAT
EB/saline
EB/8-OH-DPAT

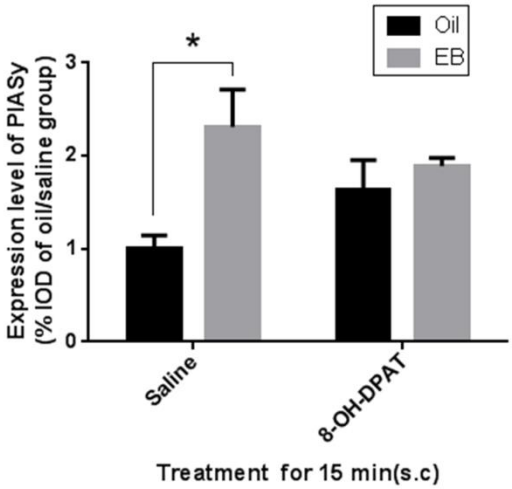

Oil
EB
*
Expression level of PIASy
(% IOD of oil/saline group)
3
2
1
0
Saline
8-OH-DPAT
Treatment for 15 min(s.c)

Figure 4. The effect of EB and 8-OH-DPAT on the PIAS proteins in the rat PVN.

Figure 4. The effect of EB and 8-OH-DPAT on the PIAS proteins in the rat PVN. Rats were given unilateral intra-PVN injections of GPR30-mis-Ads. 5 days after injection, Rats were treated with either estradiol (10ug/kg, 0.4 ml/kg, s.c) or oil for 2 days. 20 hours after the last injection, rats were treated with 8-OH-DPAT (200ug/kg, s.c) or saline and then were decapitated 15 min after the treatment. **(A), (C)** The effect of EB and 8-OH-DPAT on the PIAS1 and PIAS3 respectively. **(B)** The effect of EB and 8-OH-DPAT on PIASxα. * Two-way ANOVA shows there is significant effect of EB on the expression of PIASxα. Post hoc by Tukey's multiple comparisons test shows that treatment of EB and 8-OH-DPAT together induced PIASxα to significantly increase in rats PVN membrane fraction compared to the group treated with 8-OH-DPAT alone ($p<0.05$). **(D)** The effect of EB and 8-OH-DPAT on PIASy. * Two-way ANOVA shows there is significant effect of EB on the expression of PIASy. Post hoc by Tukey's multiple comparisons test shows that PIASy was increased significantly in the group treated with EB alone compared to vehicle-treated group ($p<0.05$).

CHAPTER 4: DISCUSSION

Previous studies have reported SUMOylation of 5-HT_{1A} Rs by SUMO 1 in rat brain areas such as hypothalamus, hippocampus and cortex. However, the mechanisms regulating SUMOylation of 5-HT_{1A} Rs were not elucidated. In this study, we focused on the mechanisms underlying the SUMOylation of 5-HT_{1A} Rs by SUMO1. Our studies have demonstrated for the first time that 1) N2a cells express endogenous SUMO machinery and SUMOylation machinery can be over-expressed in N2a cells via transfection. 2) The SUMOylation of 5-HT_{1A} Rs was detected around 55 kDa in N2a cells similar to that seen in the rat brain. 3) The PIAS proteins were found to be distributed in the membrane fraction of N2a cells, although previous stuides reported to their location only in the nucleus and cytosol of cells. 4) Although transfected PIASxα construct was expressed at low levels in the whole lysate, a higher proportion of PIASxα was expressed in the cell membrane fraction as compared to the cytosolic fraction, suggesting that PIASxα is positioned to participate in the SUMOylation of 5-HT_{1A} Rs. 5) Even though the expression level of transfected PIASxα was low, PIASxα facilitated the covalent conjugation between SUMO 1 and 5-HT_{1A} Rs. Although there was a much higher amount of PIAS1 and PIASy proteins in the whole lysate preparation as well as the membrane fraction of N2a cells after transfection, PIAS1 and PIASy did not affect SUMOylation of 5-HT_{1A} Rs. 6) The expression level of PIASxα in the membrane fraction of rat PVN is increased significantly by treatment with EB and 8-OH-DPAT compared to the rats treated with 8-OH-DPAT alone. EB treatment alone increase PIASy level in the membrane fraction of rat PVN in comparison to vehicle treated rats.

In the current study, we used N2a cell line, a mouse neuroblastoma cell line, which I found endogenously expresses 5-HT_{1A} Rs. The predicted molecular weight of 5-HT_{1A} Rs is around 42kDa. However anti-5-HT_{1A} R antibody detected bands of very strong intensity at 55kDa. Initial experiments conducted by our laboratory using rat brain tissue found that the anti-5-HT_{1A} R antibody detected several protein bands including a ~75kDa, 55kDa and other small bands but not a 42 kDa. The highest intensity band detected by the 5-HT1A R antibody was the 55kDa band, which was verified as SUMO1-5-HT_{1A} Rs band later using immunoprecipitation. These results suggest that monoSUMOylation of 5-HT_{1A} Rs is an abundant post-translational modification (PTM) in rat brain tissue. Our laboratory also showed that in the HEK 293 cells, bands around 42 kDa and 55 kDa were detected. This different pattern of 5-HT1A Rs in the rat brain tissue suggests that there are differences in PTM of 5-HT_{1A} Rs between brain tissue and cell lines. However, the bands for 5-HT_{1A} Rs in N2a cells are similar to what we observed in the rat brain tissue indicating that N2a cells mimic the SUMOylated 5-HT_{1A} Rs identified in rat brain. Thus, N2a cells are a better model to study the SUMOylation of 5-HT_{1A} Rs.

Once we established the cell model, we looked at the distribution of PIAS proteins in N2a cells. Studies have shown that SUMO machinery traffics to different regions of neurons during the neuronal development [84, 85]. Our laboratory has previously reported that SUMO1-5-HT_{1A} Rs are distributed in the ER and Golgi, while a small proportion of SUMO1-5-HT_{1A} Rs are located in DRM where the active 5-HT_{1A} Rs are located. Based on this background, we hypothesized that PIAS proteins facilitate SUMOylation of 5-HT_{1A} Rs in N2a cells by distributing in the plasma membrane (**Figure 5**).

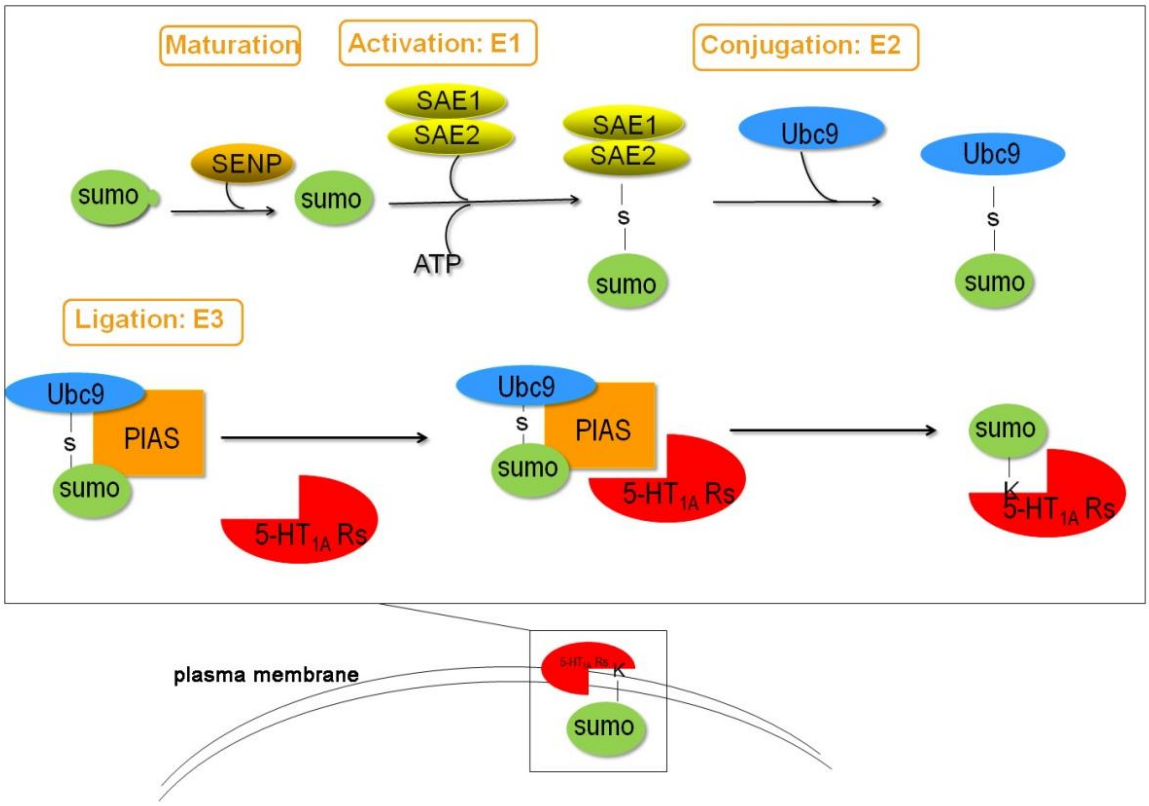


Figure 5. The schematic representation of mechanisms underlying the SUMOylation of 5-HT_{1A} Rs in N2a cells. The PIAS protein might need to be located to the plasma membrane to accelerate the transfer of SUMO 1 protein to 5-HT_{1A} Rs.

In the present study, cells were transfected with PIAS constructs and PIAS proteins were found to be expressed in the membrane fraction of N2a cells. However, previous studies reported that the PIAS proteins are located in the cytosol and nucleus. The disparity between the distribution of PIAS proteins might be because of the differences in cell lines or tissues. Moreover, the distribution of PIAS proteins in the membrane fraction of N2a cells is consistent with our hypothesis that PIAS proteins increase SUMOylation of 5-HT_{1A} Rs in the plasma membrane. However, in my experiment, I used sodium cholate to extract the hydrophobic

proteins located in the plasma membrane including DRM as well as Golgi and ER. In order to study the distribution of PIAS proteins and SUMO1-5-HT_{1A} Rs and to examine whether they co-localize, separation of different sub-cellular fractions of N2a cells by ultracentrifugation or immunocytochemistry needs to be done.

There was a surprisingly large difference in the expression of PIAS proteins in whole lysates and the membrane fraction of N2a cells following transfection. This apparent difference between the expression levels of each transfected PIAS constructs appeared even though the contructs have the same vector backbone. The difference in expression level of transfected PIAS in the plasma membrane may be associated to the selectivity of each PIAS protein to different substrates. Interestingly, the PIASxβ construct expressed the least among the PIAS constructs and transfection of higher amounts of PIASxβ still resulted in lower expression of PIASxβ. Transfection with PIASxβ alone led to a decrease in SUMOylation of 5-HT_{1A} Rs compared to the mock transfected cells. In contrast, transfection of PIASxβ together with constructs of 5-HT_{1A} R and SUMO 1 showed no significant effect on the SUMOylation of 5-HT_{1A} Rs. These results suggest that PIASxβ may require more 5-HT_{1A} Rs, SUMO and Ubc9 to be involved in the SUMOylation of 5-HT_{1A} Rs.

I also examined SUMOylation of 5-HT_{1A} Rs by transfection of various SUMO-1 and 5-HT_{1A} R constructs during initial studies of the N2a cells as a cell model. pmchcerry-C1-5-HT_{1A} R, pAcGFP- C1-5-HT_{1A} R, pmchcerry-C1-SUMO1 and pAcGFP-SUMO1 constructs were transfected into N2a cells and immunoprecipitation was conducted using anti-SUMO antibody. However, the appropriate molecular weight protein bands were not observed (data not shown). It is likely that the mcherry tag and GFP tag which are around 28 kDa, interferes with the binding or trafficking of SUMO machinery for 5-HT_{1A} Rs.

In the N2a cell model, it was found that PIASxα was able to enhance the covalent conjugation between SUMO1 and 5-HT_{1A} Rs, which leads to the question if there is an increase in PIASxα expression when the SUMOylation of 5-HT_{1A} Rs increased. In our current experiments, we found that combination of EB and 8-OH-DPAT significantly increased the expression levels of PIASxα in PVN membrane fraction compared to rats treated with 8-OH-DPAT alone. Previous studies in our laboratory showed that co-treatment of EB and 8-OH-DPAT significantly up-regulated the SUMOylation of 5-HT_{1A} Rs in the hypothalamic membrane compared to the rats treated with 8-OH-DPAT alone. Taken together, these previous and current results suggest that combined treatment of EB and 8-OH-DPAT facilitate SUMOylation of 5-HT_{1A} Rs via increasing PIASxα in the PVN membrane. Further experiments to determine the expression level of PIASxα in the whole homogenate of PVN need to be conducted to identify whether the total expression level of PIASxα was increased or whether PIASxα traffics from cytosol to membrane to participate in the SUMOylation of 5-HT_{1A} Rs.

In the present study, administration of EB alone did not have significant effect on the expression level of PIASxα in comparison to vehicle treatment, indicating that EB alone cannot increase the expression level of PIASxα, which is consistent to our previous finding that treatment of EB alone did not increase SUMOylation of 5-HT_{1A} Rs.

The unchanged expression level of PIASxα in the PVN membrane fraction by acute treatment of 8-OH-DPAT compared to vehicle treated rats was observed in the present study, indicating that 8-OH-DPAT alone did not alter the expression level of PIASxα. However, previous studies in our laboratory demonstrated that 8-OH-DPAT alone increased the level of SUMO1-5-HT_{1A} Rs in the hypothalamic membrane and DRM. The reason why 8-OH-DPAT did not increase PIASxα might be explained by the different tissue fractions used. In the

hypothalamus, 5-HT_{1A} Rs are not only located in PVN of hypothalamus, but also in other parts of hypothalamus, such as dorsomedial hypothalamus (DMH) [116, 119]. 8-OH-DPAT might regulate the SUMOylation of 5-HT_{1A} Rs in other parts of hypothalamus. It is also likely that treatment of 8-OH-DPAT increases SUMOylation of 5-HT_{1A} Rs through other mechanisms, e.g. the increased activity of PIASxα, increased activity or expression level of other E3 ligases, the decreased activity or expression of SENPs by the acute treatment of 8-OH-DPAT. Further experiments are needed to be done to examine the activity or expression of these enzymes after administration of 8-OH-DPAT.

In the membrane fraction of rat PVN, it was found that treatment of EB alone significantly increased the expression level of PIASy , indicating that PIASy may be involved in EB-induced SUMOylation of other proteins like RGSz1 [120]. It was reported that RGSz1 can be SUMOylated and isoforms of SUMOylated- RGSz1 were around 35 kDa, 45kDa, 50kDa, 90 kDa and 135kDa in the rat cortex. EB treatment increased the putative SUMOylated 135-kDa RGSz1 in PVN membrane fraction while 8-OH-DPAT had no effect on the putative SUMOylated 135-kDa RGSz1. Gαz, which couples to 5-HT_{1A} Rs in hypothalamus, was also identified to be modulated by treatment with EB and to be the substrate of SUMO proteins [29]. However, previous study in our laboratory demonstrated that acute treatment with 8-OH-DPAT significantly increased SUMOylated-Gαz while treatment with EB alone for 2 days significantly decreased SUMOylated-Gαz, which suggests that PIASy is not likely involved in SUMOylation of Gαz. Interestingly, there is no effect for combined treatment of EB and 8-OH-DPAT on the expression level of PIASy in comparison to rats treated with 8-OH-DPAT alone, indicating 8-OH-DPAT might interfere with the EB-induced SUMOylation of other proteins.

To date, they are only four GPCRs including mGluR8b, mGluR7, CB_1 and 5-HT_{1A} Rs are identified as the substrates of SUMO proteins [35, 110-114]. Choi et al.,[113] found that SUMOylation stabilizes mGluR7 on the neuronal surface. DeSUMOylation of mGluR7 by L-AP4, an mGluR7 agonist leads to a profound increase in the internalization of mGluR7 indicating that deSUMOylation enhances endocytosis of mGluR7. Our laboratory demonstrated that acute treatment of 8-OH-DPAT promoted the SUMOylation of 5-HT_{1A} Rs and majority of SUMO 1-5-HT_{1A} Rs are located in the ER and Golgi. Further experiments need to be done to detect the trafficking of SUMO 1-5-HT_{1A} Rs, PIAS proteins and deSUMOylation proteases, which could provide a deeper insight into the mechanisms involved in regulation of 5-HT_{1A} Rs.

My experiments found that PIASxα facilitates the SUMOylation of 5-HT_{1A} Rs, which could become a target for regulation of 5-HT_{1A} Rs. In the future, PIASxα can be knocked down in N2a cells or animal model to verify that PIASxα involves in increasing SUMOylation of 5-HT_{1A} Rs. In addition, experiments will be conducted to identify the binding site of PIASxα on 5-HT_{1A} Rs and the binding site of SUMO1 on 5-HT_{1A} Rs. Further, the function of deSUMOylation proteases such as SENPs leading to the significant deSUMOylation of 5-HT_{1A} receptors should also be investigated.

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