Characterization of SUMOylation of 5-HT1A receptors

By

Sugandha

Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Date defended: June 5th, 2019

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Characterization of SUMOylation of 5-HT1ARs

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Date Approved: June 10th, 2019

ABSTRACT

Serotonin 1A receptors (5-HT1AR) are G-protein coupled receptors involved in the control of mood, cognition and memory. Clinical and animal studies have demonstrated that abnormal levels of 5-HT1AR lead to anxiety-like and depressive-like phenotypes. Previous studies in our lab have shown that 5-HT1AR can undergo SUMOylation, a post-translational modification process analogous to ubiquitylation but involving conjugation of small ubiquitin-like modifiers (SUMOs). SUMOylated 5-HT1AR is located in the cell membrane and in the cytoplasm especially in the trans-Golgi network and endoplasmic reticulum. Differential centrifugation and receptor binding experiments suggest SUMOylation leads to inactivation of the 5-HT1AR, but further studies are needed to confirm this conclusion. Treatment with a 5-HT1AR agonist, increases the SUMOylated receptors which is further increased by treatment with estradiol, treatments that at pharmacological doses or with chronic use lead to desensitization of the 5-HT1A. Protein inhibitor of activated STAT xα (PiasXα) facilitates the SUMOylation of the 5-HT1AR, and treatment with estradiol and a 5-HT1AR agonist increases PiasXα. However, the mechanisms regulating the increase in 5-HT1AR SUMOylation with 5-HT1AR agonist treatment alone are not known. We hypothesize that sentrin proteases (SENPs) catalyze the deSUMOylation of the 5-HT1AR are reduced with agonist treatment; however, currently, there is limited knowledge regarding which of these enzymes are involved in the deSUMOylation of 5-HT1AR. Thus, the goal of this study is to determine which SENPs are involved in the deSUMOylation of 5-HT1AR. We can then determine if those enzymes are altered by treatment with 5-HT1AR agonist. In the present studies, we found that 5-HT1AR expression is maximal at 32 hours post transfection in Neuroblastoma (N2a) cells. Then, we transfected N2a cells to overexpress SENPs to determine which SENPs are involved in the deSUMOylation of the 5-HT1AR. The results suggest that SENP2 catalyzes the

deSUMOylation of the 5-HT1AR when 5-HT1AR and SUMO-1 were overexpressed in N2a cells. Further, we determined that there were two isoforms of SENP2 two SENP2 isoforms at 50 kDa and 60 kDa in the rat frontal cortex and examined whether 8-OH-DPAT, a 5-HT1AR agonist, treatment decreased expression of either isoform. Our results suggest that the levels of 60 kDa SENP2 isoform were significantly decreased with 8-OH-DPAT treatment while levels of the 50 kDa did not change. These studies suggest that increase in SUMOylation of 5-HT1ARs due to 8-OH-DPAT treatment may be mediated through a decrease in 60 kDa isoform of SENP2, but further experiments are needed to confirm this conclusion. These studies to understand the mechanism involved in the regulation and specifically SUMOylation of 5-HT1AR will inform the development of new potential targets for the treatment of anxiety and depression.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Nancy Muma. She has been a great support in the entire two years. She has made a lot of efforts to make sure that I am always going in the right direction. She has never left my hand in this entire journey. I could not have asked for a better mentor for my first research experience.

Also, I would like to thank my committee members, Dr. Liqin Zhao and Dr. Adam Smith for their guidance and insights in my project.

Next, I would like to thank my current and former lab members. They have also been there to help me. Khushboo Kapadia has always motivated me and helped me get through difficult times. Sukhmanjit Kaur has always made me laugh.

I would like to thank my parents and my friends. My parents for their constant emotional and financial support. They have helped me survive this new country. And my friends who have been nothing but supportive throughout.

Last but not the least, I would like to thank the funding source. This project was funded by JR+Inez Jay Fund, Higuchi Bioscience Centre and Protein Structure and Function by COBRE.

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LIST OF ABBREVIATIONS

5-HT1ARs	serotonin 1A receptors
8-OH-DPAT	8-Hydroxy-2-dipropylaminotetralin
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
Desi	deSUMOylating isopeptidases
DUBs	deubiquinating enzymes (DUBs)
EB	17β-estradiol-3-benzoate
ER	endoplasmic reticulum
FRET	Förster resonance energy transfer
GPCRs	G protein-coupled receptors
MT	mock transfection
NEM	N-Ethylmaleimide
NES	nuclear export signals
NLS	N-terminal localization signals
NPC	nuclear pore complex
PET	Positron emission tomography
PIAS	protein inhibitor activated STAT
PTMs	Post Translational Modifications
PVN	hypothalamic paraventricular nucleus
RanGAP	Ran GTPase activating protein
SAE	SUMO activating enzyme subunit
SENPs	sentrin-specific proteases
SERT	serotonin transporter
SIMs	SUMO binding motifs

SSRIs	selective serotonin reuptake inhibitors
SUMO	small ubiquitin-like modifier
Ubc9	ubiquitin carrier protein 9
Ulps	ubiquitin-like proteins
USPL1	ubiquitin-specific protease-like 1

CHAPTER 1: INTRODUCTION

1.1 Serotonin 1A Receptors (5-HT1ARs)

Serotonin 1A receptors (5-HT1ARs) are one of the most abundant and widely studied receptor subtype. It is present in brain areas that are involved in controlling mood, emotion and memory [1]. 5-HT1ARs belong to the family of inhibitory G-protein coupled receptors (GPCRs) and function by coupling to Gi/Go family of proteins and control numerous intracellular signaling cascades [2, 3]. In a mammalian brain, 5-HT1AR exist as two populations-autoreceptors that mediate negative feedback or as heteroreceptors that mediate post-synaptic inhibitory responses to serotonin. Autoreceptors are present on the soma and dendrites in the raphe nuclei [4]. Autoreceptor activation reduces neuronal excitability by hyperpolarizing the membrane, therefore suppressing neuronal transmission [5]. Post-synaptic heteroreceptors are expressed in limbic and cortical regions of the brain such as hippocampus, amygdala, hypothalamus, frontal cortex, which modulates mood, learning and memory [6, 7]. Therefore, 5-HT1ARs expressed in these brain regions are important targets in various psychological disorders.

Moreover, impairment in the function of 5-HT1ARs may lead to anxiety, depression and other mood related disorders [8-10]. Several postmortem studies in suicide and depressed patients suggest decreased binding of 5-HT1ARs as compared to healthy individuals [11]. Some postmortem studies have also shown increased 5-HT1AR levels in prefrontal cortex in schizophrenia [12]. Positron emission tomography (PET) studies have shown a reduction in both pre and post- synaptic 5-HT1ARs in depressed patients [13]. Also, reduced 5-HT1AR levels were found in amygdala, cortex and raphe nuclei in patients with social anxiety disorders and panic disorder [14]. 5-HT1AR knockout mice display increased stress responsiveness [15]. Genetic

knockout of the 5-HT1AR led to the development of an anxiety-like phenotype in mice while early overexpression of the receptor reduced the phenotype [16]. 5-HT1AR knockout mice display decreased exploratory activity and increased fear of open fields [17]. Specific deletion of 5-HT1A heteroreceptors in the dentate gyrus eliminated the effects of SSRIs in several behavioral tests [18]. Together these studies demonstrate that 5-HT1ARs are important targets in anxiety and depression.

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, and citalopram are the most commonly prescribed anti-depressant drugs [19]. SSRIs prevent the reuptake of serotonin by blocking the serotonin transporter (SERT) thereby, increasing the levels of serotonin in the synapse. However, 3-4 weeks of treatment with SSRIs is required to see the clinical effects of these drugs. Initial rise in the levels of serotonin in the synapse activates the 5-HT1A autoreceptors and they exert a negative feedback inhibition, resulting in decreased serotonin release[20]. Sustained use of the anti-depressants leads to the desensitization of the somatodendritic 5-HT1A autoreceptor [21]. This results in an increased serotonergic neuron firing rate and serotonin neurotransmission [22]. The specific blockade of 5-HT1A autoreceptors by pindolol, an anti-hypertensive drug which is also a partial antagonist of 5-HT1ARs, escalates the clinical effects of SSRIs [23, 24]. Several animal studies suggest that the desensitization of the presynaptic 5-HT1A autoreceptors accelerates the onset of action of many anti-depressant drugs [25]. Studies show that mice with higher 5-HT1A autoreceptor levels were not able to respond to acute stress, and showed no behavioral response to anti-depressants [15]. Several studies suggest that preferential activation of the post-synaptic 5-HT1A heteroreceptors leads to anti-depressant like effect [26]. Overall, these studies suggest that activation of post-synaptic 5-HT1ARs or blockade/desensitization of presynaptic 5-HT1ARs is required for anti-depressant effect [27].

Since, 5-HT1ARs are involved in these affective disorders, it is important to understand how they are regulated. Several studies have demonstrated that 5-HT1ARs undergo various post translational modifications (PTMs).

1.2 Post Translational Modifications (PTMs)

Post translational modification is a process which involves the attachment of chemical moieties such as sugars, lipids or polypeptides to a protein after its translation. PTMs of proteins may affect the enzymatic activity, localization and may alter its interactions with other proteins. PTMs may also alter the signaling cascades downstream of their target proteins [28, 29]. There are many post translational modifications such as ubiquitination, palmitoylation, phosphorylation, acetylation, glycosylation etc.

1.2.1 PTMs of 5-HT1ARs:

5-HT1ARs are known to undergo the following PTMs- palmitoylation [30], phosphorylation [31] and SUMOylation [32]. Studies suggest that palmitoylation is an irreversible modification. The 5-HT1AR is modified by palmitic acid, where the fatty acid gets attached to the cysteine residues 417 and 420 of the 5-HT1AR. Palmitoylation may either be required for the interaction of the 5-HT1AR with G-proteins or it could help in the trafficking of the receptor to the detergent resistant membrane (DRM) of the plasma membrane. Phosphorylation of 5-HT1ARs mediated by protein kinase C is involved in the desensitization of the receptor [33]. Our lab has recently shown that 5-HT1ARs undergo SUMOylation.

1.2.2 SUMOylation:

SUMOylation is a reversible and transient process where a member of the Small Ubiquitin-like Modifier (SUMO) protein family covalently attaches to the lysine residues of the target

protein. This process is governed by an enzymatic pathway which is very similar to the ubiquitination pathway[34]. The SUMO family is highly conserved in all eukaryotes. SUMO proteins belong to the family of Ubiquitin-like proteins (Ulps). There are four SUMO isoforms present in mammals: SUMO1 to SUMO4[35, 36]. SUMO1 is 101 amino acid long and approximately 11.5 kDa [37, 38]. It is 18% similar to ubiquitin. SUMO2 and SUMO3 are nearly identical to each other and are often referred to as SUMO2/3. They differ only by 3 amino acids residues at the N-terminal. SUMO1 and SUMO2/3 share around 47% homology. SUMO4 is the least characterized of all, and no modified substrate has been reported [39]. Some studies suggest that SUMO4 may be unable to SUMOylate any substrate protein because of a proline residue that is present in its sequence which hinders its maturation to an active SUMO form [40].

SUMOylation has a variety of effects on the cells. It is involved in changing the interaction with DNA, RNA or other proteins [41]. Several studies suggest that SUMOylation changes the conformations and the enzymatic activities of its target proteins. SUMOylation also modulates other protein modifications [42]. Various studies have demonstrated that deleting or knocking down the SUMO genes or any components involved in the SUMO pathway is fatal for mammalian cells [43]. Many studies suggest that SUMOylation dysfunction may lead to various neuropathological problems [34]. Thus, studying the SUMO pathway and the enzymes involved in SUMOylation is important to understand the role of SUMOylation in the affective disorders.

1.2.3 The SUMO Pathway:

SUMOylation is a dynamic pathway that leads to the formation of an isopeptide bond between the carboxy terminal of the SUMO protein to the ε-amino group of a substrate [44]. It usually occurs on a consensus motif of the substrate protein (Ψ-K-X- [D/E]), where Ψ is any large hydrophobic residue (such as I, V or L), K is the target lysine, X can be any residue and D/E is

aspartate or glutamate) [29]. The SUMO isoforms are translated as inactive precursor proteins which are cleaved by the family of enzymes called sentrin proteases [34]. These enzymes expose the diglycine site on the C-terminal of the SUMO protein which is required for conjugation. SUMO proteins are then adenylated by the ATP-dependent formation of a thioester bond with the active site of the E1 enzyme (heterodimer of SUMO activating enzyme subunit 1 and subunit 2, SAE1 and SAE2). The activated SUMO is transferred to the substrate protein with the help of E2 conjugating enzyme called Ubiquitin conjugating enzyme 9 (Ubc9) [45, 46]. Ubc9 often works in conjunction with E3 ligases to catalyze the transfer of SUMO to the target protein. Since, SUMOylation is a reversible process, the SUMO proteins are deconjugated from the substrate protein by sentrin proteases.

1.3 Sentrin Proteases:

Despite being a covalent interaction, SUMOylation can be easily reversed by isopeptidase activity of deSUMOylation enzymes. These deSUMOylation enzymes are referred to as SUMO specific proteases or SUMO isopeptidases. The known SUMO-specific isopeptidases and proteases are cysteine proteases which are classified into three families: the Ulp/SENP (ubiquitin-like protease/ sentrin-specific protease) family, the Desi (deSUMOylating isopeptidase) family and USPL1 (ubiquitin-specific peptidase-like protein 1).

Ulp/SENP proteins belong to the C48 family subgroup of the Cysteine E (CE) superfamily of thiol proteases. Ulp1 was the first protease to be discovered in the yeast, *Saccharomyces cerevisiae*, while Ulp2 (Smt3 specific proteases) was the second [47]. Initially in humans, there were seven proteases classified into Ulp family members which were known as SENPs [48]. Later on it was found that SENP8 does not act on SUMO paralogs, instead acts on Nedd8, a ubiquitin-family member [49, 50]. There are no sequence similarities in SENP enzymes with their

mechanistic relatives, deubiquinating enzymes (DUBs). Finally, there are six SENPs that are involved in deSUMOylation, SENP 1, SENP2, SENP3, SENP5, SENP6 and SENP7. Sequence analyses studies demonstrate that SENP1, SENP2, SENP3 and SENP5 are evolutionary related to Ulp1 branch whereas SENP6 and SENP7 are more similar to Ulp2 branch. These studies further reveal the pairwise similarities between SENP1 and SENP2, SENP3 and SENP5, SENP6 and SENP7. Ulp/SENPs are cysteine proteases and possess a conserved C-terminal domain with His-Asp-Cys catalytic triad and a distinct N-terminal region. The non-conserved N-terminal domain of the Ulp/SENPs determines the subcellular localization and substrate specificity of these proteases. These enzymes have characteristic subcellular locations which also determines their activity towards specific substrates. Most SENPs also contain several SUMO interacting motif (SIMs) modules in their N-terminal domains, which may contribute to the selection of substrates or facilitate the targeting of specific SENPs to poly-SUMO chains. Ulp1 is found at the nucleoplasmic face of the nuclear pore complex (NPC), and Ulp2 is found in the nucleoplasm. SENP1 contains both N-terminal localization signals (NLS) and nuclear export signals (NES), but is primarily present in the nucleus [51-53]. SENP2 being closely related to SENP1, is also found in nuclear membrane along with NPC [54, 55]. SENP3 and 5 reside in nucleolus and cytoplasm [56] while SENP6 and 7 are present in nucleoplasm.

The deSUMOylating isopeptidases (Desi) family consists of two enzymes, Desi 1 and Desi 2. They belong to C97 family of cysteine proteases [57]. These proteases are found in plants and metazoan but are absent in lower eukaryotes such as yeast. Studies have shown that Desi 1 forms a homodimer, in which the groove between the two subunits forms the active site. This region consists of two conserved cysteine and histidine residues that form a catalytic dyad [57, 58]. Desi family proteins are primarily found in the cytoplasm.

USPL1 is the only known mammalian SUMO-specific protease which belongs to the C98 family [59]. USPL1 is found in invertebrates and metazoan vertebrates. Unlike Ulp/SENPs, USPL1 shows sequence homology to ubiquitin-deconjugating enzyme USP1. USPL1 contains a catalytic triad composed of Cys-His-Asp residues [59, 60]. USPL1 is primarily found in nucleus.

In humans, SENPs act as deconjugating as well as maturation and processing enzymes. SENPs help in the maturation of SUMO proteins by exposing the C-terminal diglycine moiety on inactive SUMO proteins and help initiate the SUMOylation process. Different SENPs have different activities in either deconjugation or processing depending on the substrate specificity of the SENPs. The substrate specificity of SENPs is determined by the amino acids that are found on the C-terminal to the di-glycine motif. For example, SENP1 displays little specificity for deconjugation of SUMO2 and SUMO3 while it is essential for the deconjugation of SUMO1-modified proteins[61, 62]. SENP2 catalyzes deconjugation better than processing and deconjugates Ran GTPase Activating Protein 1 SUMO-1 (RanGAP1-SUMO-1), RanGAP1-SUMO2 and Ran-GAP1-SUMO3 with similar efficiency. SENP2 possesses different substrate specificities during processing, for example, it processes SUMO2 more efficiently than SUMO1 and SUMO3 [62]. SENP3 and SENP5 show both processing and deconjugating activity for SUMO2 and SUMO3 but possess neither for SUMO1. SENP6 and SENP7 have eminent deconjugating activity for poly SUMO2/3 chains while exert very weak processing or deconjugating activity on monomeric SUMO modified substrates.

SENP enzymes play an important role in maintaining normal cellular functions by keeping a balance between SUMOylated and un-SUMOylated proteins. These enzymes are required for proper cell cycle progression. Several studies have shown that knockout of SENP1 or SENP2 in mice is lethal in the embryogenic stage, showing increased levels of SUMOylated proteins [63,

64]. Studies have depicted the role of SENP enzymes in the development and progression of cancer especially in prostate cancer [65, 66]. Many studies have demonstrated that SENP1 activates oncogenic signaling pathways such as c-Jun and androgen-receptor mediated transcription.

In this study, we have chosen to use SENP1, SENP2 and SENP6 based on their substrate specificities. As described before, these 3 SENPs show substrate specificity towards SUMO1 while SENP3 and SENP5 do not possess any activity for SUMO1.

1.4 SUMOylation of 5-HT1ARs:

My thesis project focuses on the SUMOylation of 5-HT1ARs. Previous studies in our lab have shown that 5-HT1ARs are SUMOylated by SUMO1 in the rat brain tissue. SUMOylated 5-HT1ARs were detected in the membrane fractions of cortex, amygdala, hippocampus, hypothalamus, dorsal raphe and midbrain but not in the cytosolic fractions, as demonstrated by immunoprecipitation and immunoblotting [32]. Using differential centrifugation, receptor binding assays and western blotting, Li et al [32] observed that 5-HT1ARs that were bound to (+)8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) did not overlap with SUMOylated 5-HT1ARs suggesting that SUMOylated 5-HT1ARs have a low affinity for 8-OH-DPAT. Also, it was observed that SUMOylated 5-HT1ARs did not exist in the same fractions as Gαz proteins which couple to the receptors. These results suggest that SUMOylation of 5-HT1ARs inactivates the 5-HT1ARs, but more studies are needed to verify this interpretation.

To determine the location of the SUMOylated 5-HT1ARs, Li et al [32] performed a cold tritonX-100 treatment followed by sucrose gradient centrifugation in order to isolate the detergent resistant microdomain (DRM). They found that majority of the SUMOylated receptors were present in the tritonX-100 soluble fraction and much less is present in the DRM. To further explore

the subcellular distribution of the SUMOylated 5-HT1ARs, Li et al conducted a discontinuous gradient centrifugation using 7.5-30% iodixanol. It was observed that the SUMOylated receptor predominantly co-localized with TGN-38, a marker for trans golgi network (TGN) and calreticulin, a marker for the endoplasmic reticulum (ER). A small fraction of the SUMOylated receptors were found in the plasma membrane, determined by the Na^+/K^+ ATPase. Imaging-based approaches were used to confirm the subcellular location of SUMOylated 5-HT1AR. Robust labelling and co-localization of SUMO1 and 5-HT1ARs antibodies were demonstrated in the plasma membrane of SH-SY5Y cells. SUMO1 and 5-HT1ARs antibodies were also found to FRET in the peri-nuclear region as well as the plasma membrane. This further supports the previous data that SUMOylated receptors are located in the TGN and ER of the cell.

Acceptor photobleach Förster resonance energy transfer (FRET) was performed to determine the primary site of SUMOylation on 5-HT1AR. SUMO prediction programs were used to predict lysine residues on 5-HT1ARs that could get SUMOylated. Mutant 5-HT1ARs were constructed wherein the identified lysine (K) residues were mutated to arginine (R). Either the wild-type 5-HT1AR or the mutant 5-HT1AR were transfected in the SHSY5Y cells and it was seen that there was a small but significant increase in the FRET distance with K324R, K232.235R and K232.235.324R mutants as compared to the wild-type 5-HT1A receptor suggesting that lysine 232, 235 and 324 of the amino acid sequence of the 5-HT1AR could be SUMOylated. However, further studies are needed to confirm the exact site of SUMOylation [77]. Next, we will be using mass spectrometry analysis to identify the primary site of SUMOylation on 5-HT1ARs.

Estrogens are involved in regulation of emotional states. Administration of estrogens such as estradiol were shown to accelerate the desensitization of the 5-HT1ARs in the paraventricular nucleus of the hypothalamus (PVN) suggesting the role of 5-HT1ARs in the regulation of mood

mediated by estrogens [14, 15]. Previous studies in our lab have shown that treatment with estradiol in conjunction with a SSRI, fluoxetine accelerates fluoxetine-induced desensitization of 5-HT1ARs in the PVN [16]. However, further studies are needed to confirm the mechanism involved in estradiol mediated desensitization of 5-HT1ARs.

Previous studies in our lab have shown that 5-HT1ARs are SUMOylated and treatment with 8-OH-DPAT, an agonist of 5-HT1AR, increased the SUMOylated receptors and treatment with 17β-estradiol-3-benzoate (EB) for 2 days further increased the SUMOylation of 5-HT1ARs. Hence, we hypothesize that SUMOylation could be involved in the desensitization of the 5-HT1ARs mediated by estradiol. Therefore, it is important to understand the role of SUMOylation in 5-HT1AR functioning and the mechanisms underlying the SUMOylation of 5-HT1ARs.

To understand the mechanism of SUMOylation of 5-HT1ARs, one of the previous students in our lab performed immunoprecipitation and immunoblotting studies to identify which PIAS proteins facilitate the SUMOylation of the 5-HT1ARs. Different PIAS constructs were transfected in N2a cells and it was observed that PiasXα significantly increased the SUMOylation of 5-HT1ARs. Also, PiasXα expression significantly increased in the membrane fraction of the rat PVN when treated with EB and 8-OH-DPAT as compared to rats treated with 8-OH-DPAT alone.

1.5 Statement of Purpose:

The purpose of this study is to determine the enzymes that regulate deSUMOylation of 5-HT1ARs and determine if treatment with a 5-HT1AR agonist decreases the expression of that SENP. Previous studies demonstrated that treatment with a 5-HT1AR agonist increases the SUMOylation of the 5-HT1ARs but the mechanism by which this occurs is not known. The

enzyme PiasXα increases the SUMOylation of 5-HT1ARs however treatment with the 5-HT1AR agonist 8-OH-DPAT did not result in an increase in PiasXα or any other Pias protein.

The second goal of this study focuses on finding the primary site of SUMOylation of 5-HT1ARs. Identifying the SUMO site would help us better understand the 5-HT1AR structure and function. Mutating the site where 5-HT1AR gets SUMOylated will help us understand how the 5-HT1AR function is modulated in the absence of SUMOylation.

Overall, these goals would help us gain more insight into the regulation of 5-HT1AR signaling and the identification of novel targets for disorders involving 5-HT1AR signaling.

CHAPTER 2: MATERIALS AND METHODS

2.1. Plasmid constructs:

The plasmid constructs that were used in the experiments are listed in the table below. The QIAGEN plasmid MIDI prep kit (Cat#12143, QIAGEN, USA) was used to isolate the plasmids.

Plasmid	Vector	Tag	Source
pcDNA4 HisMax C- 5-HT1A Receptor	pcDNA4 HisMax C	His, Xpress	-
pEGFP-N1	pEGFP-N1	EGFP	-
pcDNA3+SUMO1	pcDNA3	His	-
pEFIFRES-P-6His SUMO1	pSCAI80	His	A gift from Dr. Ronald T Hay (University of Dundee, Dundee, UK)
pcDNA3.1^{+}/C-(K)DYK 5-HTR1A	pcDNA3.1^{+}/C-(K)DYK	Flag	GenScript (#OHu27647D)
Flag-SENP1	pFLAG-CMV	Flag	Plasmid# 17357, Addgene
Flag-SENP2	pFLAG-CMV	Flag	Plasmid# 18047, Addgene
Flag-SENP6	pFLAG-CMV	Flag	Plasmid# 18065, Addgene

2.2 Cell culture and transfections:

Mouse Neuroblastoma cells (N2a cells) were cultured in 50% Dulbecco's modified eagle medium (1X DMEM, high glucose, pyruvate, Thermo Fisher, USA) and 50% Opti-MEM (Thermo Fisher, USA) along with 10% Foetal Bovine Serum (Atlanta Biologicals, USA) and 1% Penicillin-Streptomycin (Sigma Aldrich, USA). For transfections, cells were transfected with the mammalian expression vectors (Table 1) in the 10 cm plates after 20-24 hours of plating with Lipofectamine 3000 (Thermo Fisher, USA). Media was changed after 4 hours of transfection. After 32 hours of transfection, cells were harvested.

2.3 Harvesting:

Harvesting was done either 32 or 48 hours post transfection. For harvesting, cells were first washed with 1x Phosphate buffered saline (1xPBS) and then with hypotonic buffer (0.25M sucrose 50 mM Tris, pH 7.5, 5mM EDTA, 100 mM NaCl). Cells were harvested with hypotonic buffer containing 20mM N-ethylmaleimide (NEM), 1/100 dilution of phosphatase inhibitors (Sigma Aldrich, USA) and protease inhibitors (Sigma Aldrich, USA) were added fresh) and centrifuged for 1 hour. Then the supernatant was removed as the cytosolic fraction and the pellet was resuspended in the solubilization 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 fresh). After sonication, the vials were shaken horizontally at 4^{o}C for at least 1 hour. After this, vials were centrifuged at 25000xg for 1 hour. The supernatant was removed as the membrane fractions and aliquoted in 30 µl volumes and stored at -80^{o}C. BCA assay (Cat# 23228, Cat# 23224,

BCA Protein Assay, Cat#23209, Albumin Standard, Thermo Fisher, USA) was performed to determine the protein concentrations.

2.4 Tissue Preparation:

Ovariectomized female rats were injected with either saline or 8-OH-DPAT (200 µg/kg) subcutaneously and then decapitated 5 minutes later. Brains were removed, frontal cortex was dissected, frozen on dry ice and stored at -80°C until use.

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 fresh) using motorized homogenizer (Powergene 1000 with 5mm probe) at speed 5, 4°C for 3 minutes or until all of the tissue was homogenized. The procedure for isolation of the membrane fraction of frontal cortex was the same as that for cell lysates.

2.5 Immunoprecipitation (IP):

After harvesting, immunoprecipitation was done using 450 µg of protein from the membrane fractions of the cell lysates. The samples were added to pre-washed 50 µl of protein G Dynabeads (Cat #1003D, Invitrogen, Thermo Fisher, USA) and volume was made up to 500 µl with ice-cold IP buffer (50 mM Tris, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100. 20 mM NEM, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail I and II were added fresh) and then were rotated for 1 hour at 4ºC. Then the beads were removed, and either the 10 µg of SUMO1 antibody (D11) (Cat# sc-5308, Santa Cruz, USA) or mouse IgG were added to the

samples and shaken at 4°C overnight. After 12-16 hours of incubation, 75µl of pre-washed beads were added to the samples and shaken for 2 hours. Then the beads were washed three times and the proteins were eluted using 2x sample buffer with 2-mercaptoehanol by incubating at 95°C for 5 minutes. The protein samples were then stored at -80°C or resolved on a gel.

2.6 His-Tag Purification:

For his-tag purification, 300 µg protein from the membrane fractions of the cell lysates was used. A polyHis Pull-down assay kit (Cat#21277, Invitrogen, Thermo Fisher, USA) was used. PolyHis proteins were pulled down using cobalt affinity beads from the kit. Sample was added to 50 µl of pre-washed beads and incubated overnight at 4°C. After 12-16 hours of incubation, beads were washed several times with a lysis buffer from the kit. The his-tagged samples were eluted using 100 mM imidazole solution. Then the eluted sample was filtered through a 30-kDa-cutoff filter device (Cat# VN01H22, Sartorius, USA) to eliminate imidazole before it was run on an SDS-PAGE gel.

2.7 Western blotting (WB):

Protein samples were resolved on a 10% polyacrylamide SDS gel at 150 V for 60-70 minutes and then transferred to a Polyvinylidene difluoride (PVDF) membrane at 100 V for 2 hours. Then the membrane was incubated in 5% non-fat dry milk in 1x Tris buffered saline and Tween 20(1xTBST) for 1 hour at room temperature. After this, the membrane was incubated in one of the primary antibodies listed below (Table 2), overnight at 4°C. Then the membrane was

washed and incubated with the appropriate secondary antibodies listed below (Table 2), for 1 hour at room temperature.

Immunodetection was performed using an ECL kit (GE Healthcare, USA and BioRad, USA) and ImageLab 3.0 software (BioRad, Hercules, CA). The protein levels were normalized to β-actin bands.

Table 2. Antibodies used for immunoblotting and immunoprecipitation:

Antibodies	**Dilution (WB)/ Amount (IP)**	**Source**
Rabbit anti- 5-HT1AR	1:1000	Cat# PAS-28090, Invitrogen, Thermo Fisher, USA
Mouse anti- 6xHis	1:500	Cat#Y1011, UBPBio, USA
Rat anti-Flag	1:500	Cat# 200474-21, Agilent, USA
Mouse anti-Flag	3 µg	Cat# MA1-91878, Invitrogen, Thermo Fisher, USA
Mouse anti-SUMO-1 (D-11)	10 µg	Cat# sc-5308, Santa Cruz Biotechnologies, USA
Rabbit anti-SENP2	1:500	Cat# PA5-86255, Invitrogen, Thermo Fisher, USA
Mouse IgG isotype control	10 µg	Cat# 31903, Invitrogen, Thermo Fisher, USA
Goat anti-Mouse IgG	1:20000	Cat# 119380, Jackson ImmunoResearch, USA
Goat anti-Rabbit IgG	1:20000	Cat# 120745, Jackson ImmunoResearch, USA
Goat anti-Rat IgG	1:20000	Cat#112-035-003, Jackson ImmunoResearch, USA
Mouse anti β-actin	1:20000	Cat# 691001, MP Biomedicals, LLC

2.8 Statistical Analysis

All data were analyzed by one-way ANOVA or unpaired t-tests using a GraphPad Prism software (Version 6.02). Data analyzed by one-way ANOVA were followed by post hoc tests such as Dunnett's multiple comparison test (comparison with a control). All data are presented as mean ± SEM of at least three different experiments.

CHAPTER 3: RESULTS

3.1 Overexpression of 5-HT1A receptors

To determine the optimal time of 5-HT1A receptor overexpression, a time-course experiment with the N2a cells was performed. N2a cells were transfected with the flag tagged 5-HT1AR (DYK-5-HT1AR) and harvested 32, 40 and 48 hours after transfection. Immunoblotting was performed using the anti-flag antibody. Our results show that the expression of the 5-HT1A receptor is maximum 32 hours after transfection (Figure 1). The first time-point we tested was 32 hours although there could be higher expression of the 5-HT1AR before 32 hours.

3.2 Identification of SUMOylated 5-HT1A receptor

To determine if there is any difference in the size of SUMOylated 5-HT1A receptor of different species, rat and human constructs of 5-HT1A receptor were transfected in N2a cells. Rat and mouse cortex tissue were also examined. As N2a cells also express 5-HT1A receptor endogenously, a non-transfected control was also included in this experiment. Membrane fraction of the cell lysates and tissue homogenates was immunoprecipitated using mouse monoclonal SUMO1 antibody and examined on immunoblots with rabbit polyclonal 5-HT1A receptor antibody. We observed the SUMOylated 5-HT1A receptor band around 50 kDa in all the samples demonstrating that there is no difference in the size of the SUMOylated receptor among different species and the polyclonal antibody was able to detect SUMO1-5-HT1AR from all species examined (Figure 2).

3.3 Effect of different SENPs on the SUMOylation of 5-HT1A receptors

To determine which SENP enzyme is involved in the deSUMOylation of 5-HT1A receptors, the N2a cells were transfected with flag tagged SENP1, SENP2 and SENP6. The expression of the SENP enzymes in the membrane fractions were detected using an anti-flag antibody (Figure 3A). Membrane fraction of the cell lysates was immunoprecipitated using mouse monoclonal SUMO1 antibody and immunoblotted with rabbit polyclonal 5-HT1A receptor antibody to detect the SUMOylated 5-HT1A receptor (Figure 3B). The experiment was repeated three times. One-way ANOVA followed by Dunnet's multiple comparison post hoc tests shows no significant difference in the amount of SUMOylated 5-HT1AR in the presence of any of the SENPs ($F_{(3.8)}$=1.511, p=0.28) (Figure 3C).

3.4 Effect of different SENPs on the SUMOylation of 5-HT1A receptors with overexpression of 5-HT1ARs and SUMO1

To determine which SENP enzyme is involved in the SUMOylation of 5-HT1ARs, we used another approach, in which we overexpressed DYK-5-HT1AR and SUMO1 along with SENP1, SENP2 and SENP6. Cells were harvested 32 hours after transfection. Membrane fractions of the cell lysates were immunoprecipitated with mouse anti-SUMO1 antibody and immunoblotted with either rat anti-flag antibody (Figure 4A) or rabbit anti-5-HT1AR antibody (Figure 4C). Each experiment was repeated three times and four times respectively. The flag antibody labeled the SUMOylated 5-HT1AR at 42 kDa, which is lower than the previously observed molecular weight of SUMOylated 5-HT1ARs i.e. at 55 kDa. However, the 5-HT1AR antibody labeled the SUMOylated 5-HT1AR at 52 kDa. Results show that SENP2 significantly decreases the amount

of SUMOylated 5-HT1AR as compared to the control when one-way ANOVA was performed followed by Dunnett's multiple comparison test both in case of anti-flag antibody ($F_{(3,8)}$= 4.907, $p < 0.05$; Figure 4B) and anti-5-HT1AR antibody ($F_{(3,12)} = 4.907$, $p<0.0001$; Figure 4D).

3.5 Effect of 8-OH-DPAT treatment on the expression of SENP2 in rat frontal cortex

Since, SENP2 catalyzes the deSUMOylation of 5-HT1ARs, we hypothesize that SENP2 decreases with 8-OH-DPAT treatment resulting in increase in the SUMOylation of 5-HT1ARs. To determine if the SENP2 expression decreases with 8-OH-DPAT treatment, SENP2 protein levels were examined on immunoblots. Membrane fraction of the frontal cortex samples from rats treated with 8-OH-DPAT or saline was examined on immunoblots with an anti-SENP2 antibody (Figure 5A). We observed two isoforms of SENP2 at 50 kDa and 60 kDa molecular weight. Unpaired t-test results show that there is no significance difference in the expression of SENP2 with 8-OH-DPAT treatment and the control for the lower bands i.e. 50 kDa molecular weight isoform of SENP2 (Figure 5B). However, unpaired t-test results for the upper bands i.e. 60 kDa molecular weight isoform show a significant decrease in the expression of SENP2 with 8-OH-DPAT treatment as compared to the saline treated group (Figure 5C).

3.6 Purification of 5-HT1ARs and SUMOylated 5-HT1ARs

To identify the primary site of SUMOylation on 5-HT1ARs, mass spectrometry studies will be performed. In order to examine the samples on a mass spectrometer, a purified

SUMOylated 5-HT1AR is required. The following procedure is used to purify the 5-HT1AR and SUMOylated 5-HT1AR. N2a cells are either transfected with 5-HT1AR and/or 6His-SUMO1$^{(T95K)}$. Cells were harvested 32 hours after transfection. Membrane fractions of the cell lysates were immunoprecipitated using anti-flag antibody in order to purify the 5-HT1AR. The samples were run on a 10% SDS-PAGE gel and the gel was stained with Coomassie blue stain (Figure 6A). Next, his-tagged pull down was performed on the membrane fractions to purify the his-tagged SUMO1 (6His-SUMO1$^{(T95K)}$) along with the 5-HT1AR i.e. SUMOylated 5-HT1AR (Figure 6B).

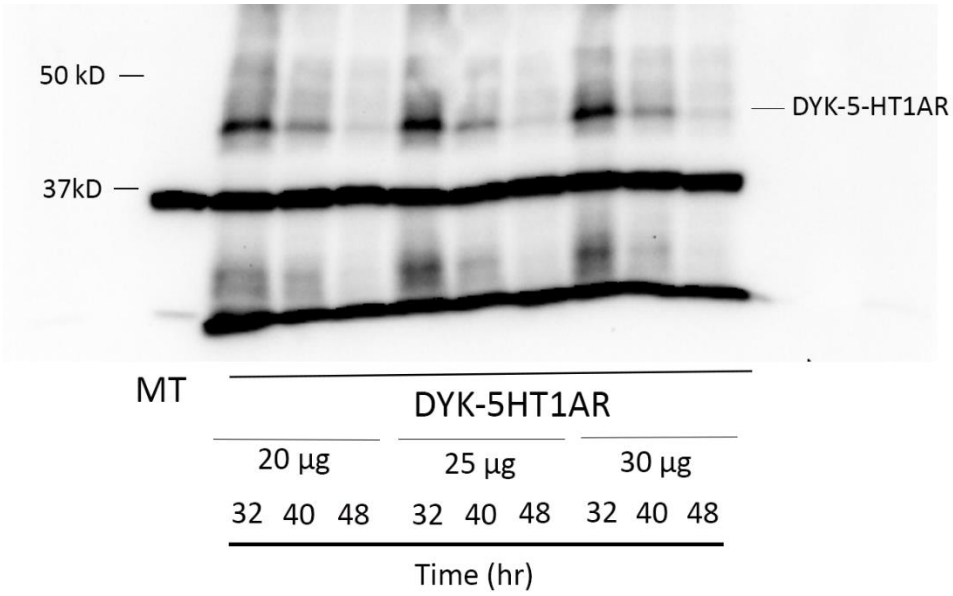

Figure 1. Optimization of transfection time for overexpression of 5-HT1ARs.

N2a cells were transfected with pcDNA3.1^{+}/C-(K)DYK 5-HT1AR plasmid and cells were harvested 32, 40 and 48 hours after transfection. 20, 25 and 30 µg of proteins from membrane fractions of the lysates were examined on immunoblots with an anti-flag antibody. 5-HT1AR expression was maximum when harvested 32 hours after transfection. The bands at 37 kDa are non-specific bands from the anti-flag antibody. MT: Mock Transfection.

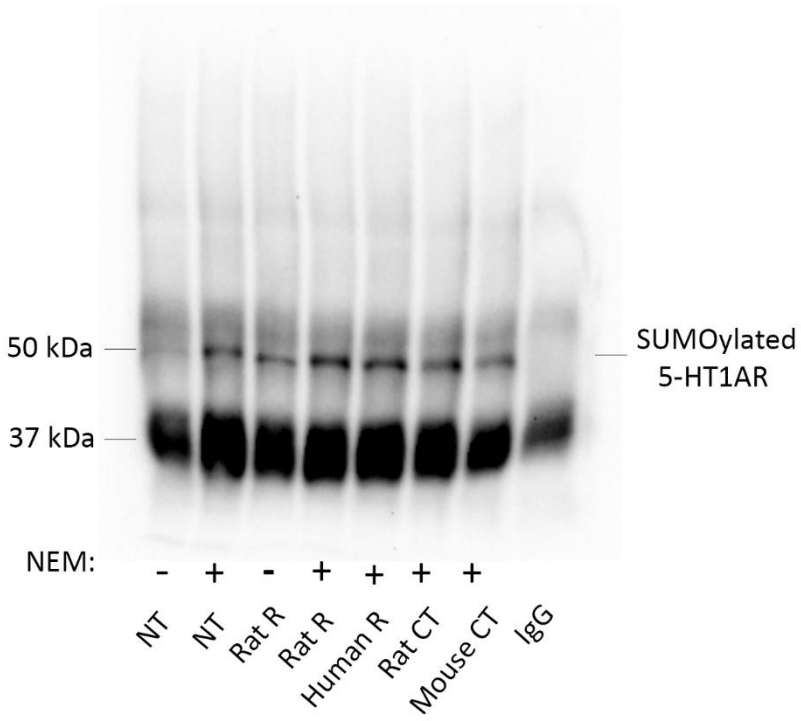

Figure 2. Comparison of the SUMOylated 5-HT1ARs across different species.

Membrane fractions of either N2a cell lysates or tissue homogenates were immunoprecipitated with mouse anti-SUMO1 antibody and examined on immunoblots with rabbit anti-5-HT1AR antibody. Blot shows that the molecular weight of SUMOylated 5-HT1AR is similar in human, rat and mouse tissues and transfected cells. NT: Non- transfected, Rat R: Rat 5-HT1AR construct, Human R: Human 5-HT1AR construct, Rat CT: rat cortex tissue, Mouse CT: mouse cortex tissue, IgG: Immunoglobulin G, cells were immunoprecipitated with the same amount of mouse IgG. NEM: N-ethylmaleimide.

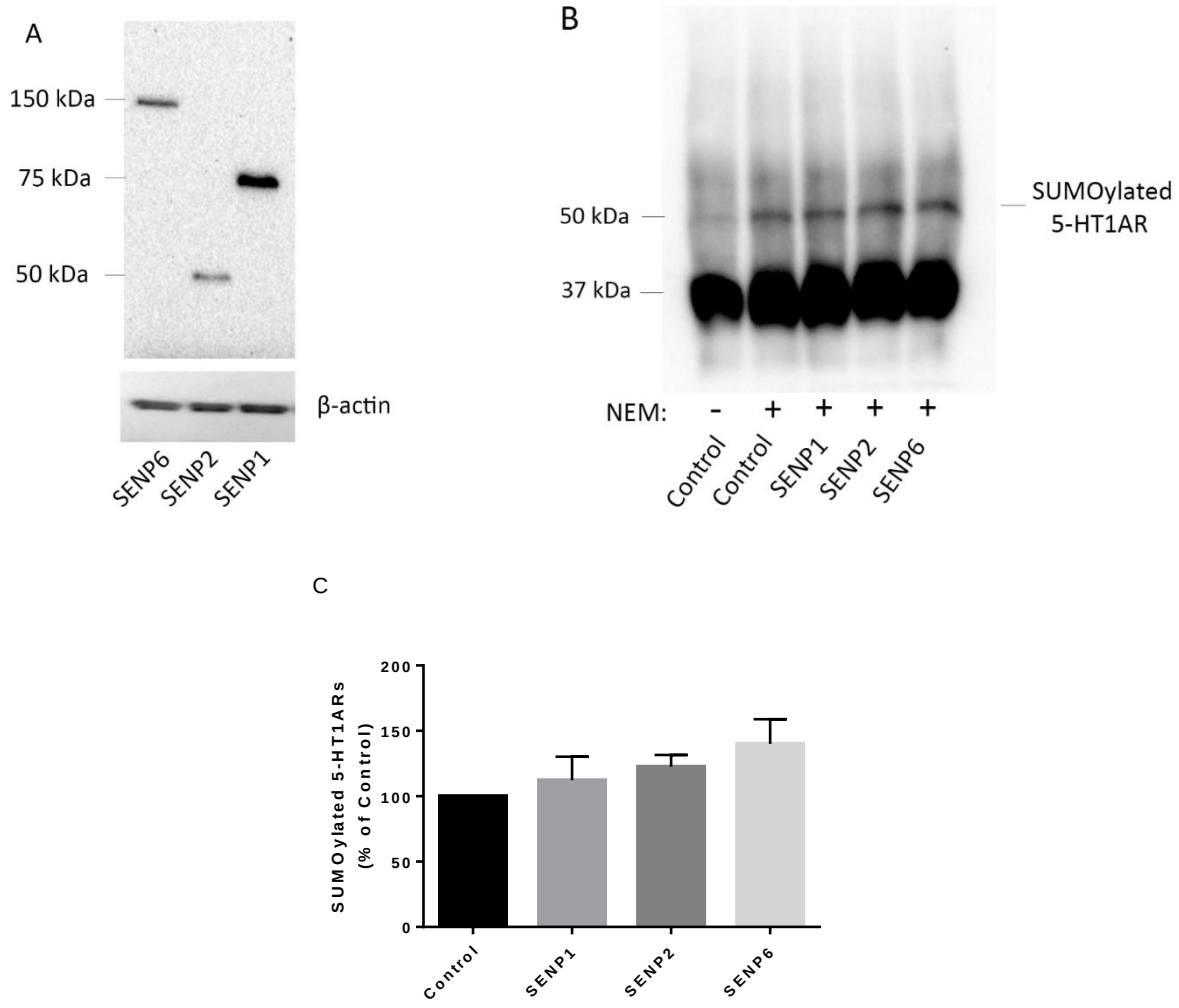

Figure 3. Effect of SENPs on SUMOylated 5-HT1ARs.

N2a cells were transfected with flag-tagged SENP plasmid constructs and were harvested 48 hours after transfection. (A) Western blot showing the expression of SENPs in the membrane fractions of N2a cell lysates using an anti-flag antibody. (B) Membrane fractions were immunoprecipitated using an anti-SUMO1 antibody and immunoblotted with 5-HT1AR antibody to determine the effect of SENPs on the SUMOylated 5-HT1ARs (C) Graph showing the quantification of the SUMOylated 5-HT1AR. Data are shown as mean ± SEM (n=3). SENPs did not significantly alter the amount of SUMOylated 5-HT1ARs.

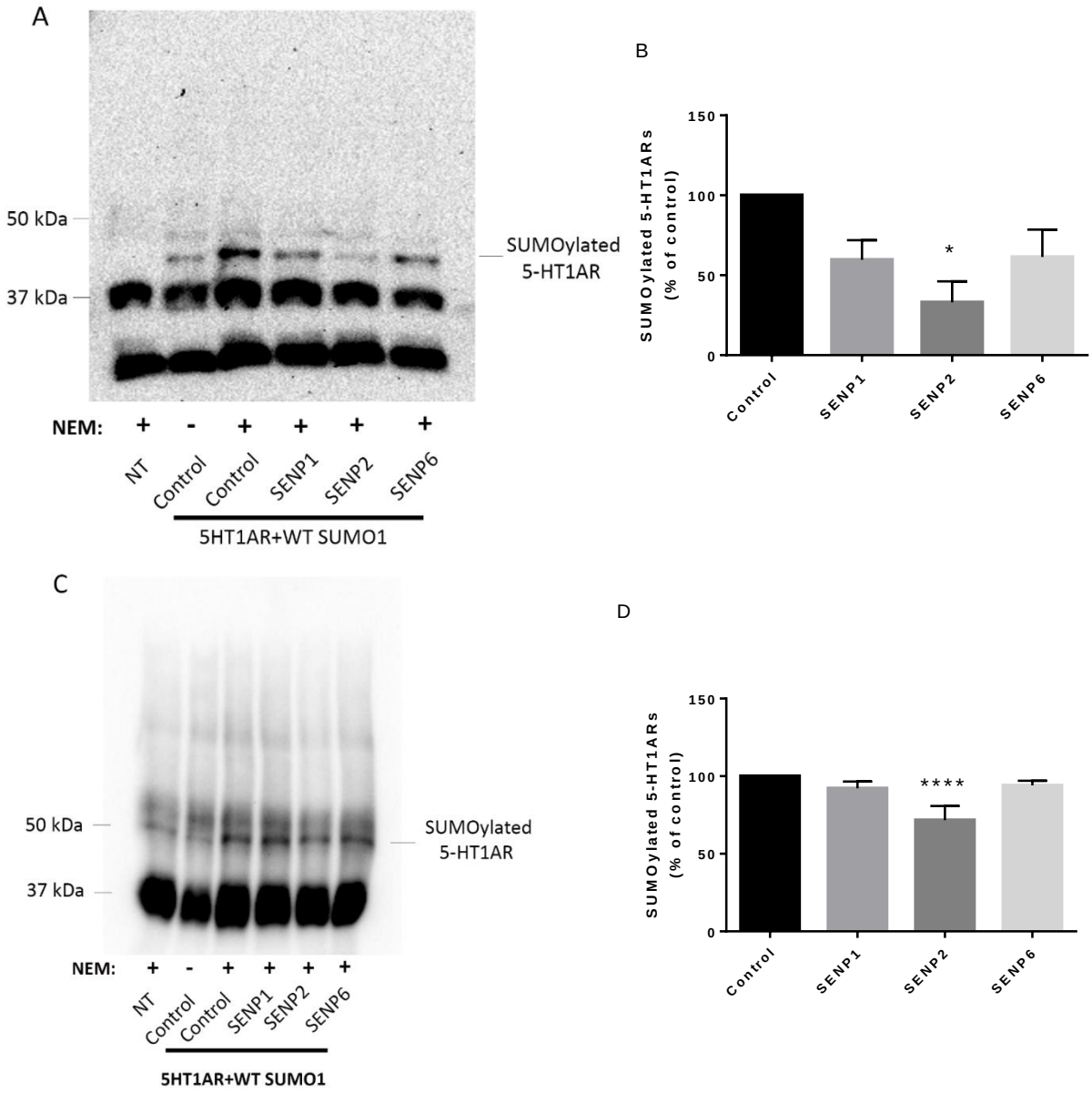

Figure 4. Effect of SENPs on SUMOylated 5-HT1ARs with overexpression of 5-HT1ARs and SUMO1.

N2a cells were transfected with DYK-5-HT1AR and WT-SUMO1 plasmids along with either GFP (Control) or different flag-tagged SENPs. NT indicates non-transfected lysates. Cells were harvested 32 hours after transfection and membrane fractions of the cell lysates were

immunoprecipitated using an anti-SUMO1 antibody and immunoblotted using either (A) an anti-flag antibody or (C) an anti-5-HT1AR antibody. (B) Graph showing the quantification of SUMOylated 5-HT1AR on the blots labelled with an anti-flag antibody. Data are shown as mean ±SEM (n=3). One-way ANOVA shows a significant effect of SENP expression on SUMOylated 5-HT1AR expression levels, ($F_{(3,8)}$= 4.907, p = 0.0320). Dunnett's multiple comparisons post-hoc test shows that SUMOylated 5-HT1AR expression significantly decreased in the group transfected with SENP2 as compared to the control, * indicates $p<0.05$ (D) Graph showing the quantification of SUMOylated 5-HT1AR on the blots labelled with anti-5-HT1AR antibody. Data are shown as mean ±SEM (n=4). One-way ANOVA shows a significant effect of SENP transfection on SUMOylated 5-HT1AR levels ($F_{(3,12)}$ = 4.907, $p<0.0001$). Dunnett's multiple comparisons post-hoc test shows that SUMOylated 5-HT1AR significantly decreased in the group transfected with SENP2 as compared to the control.

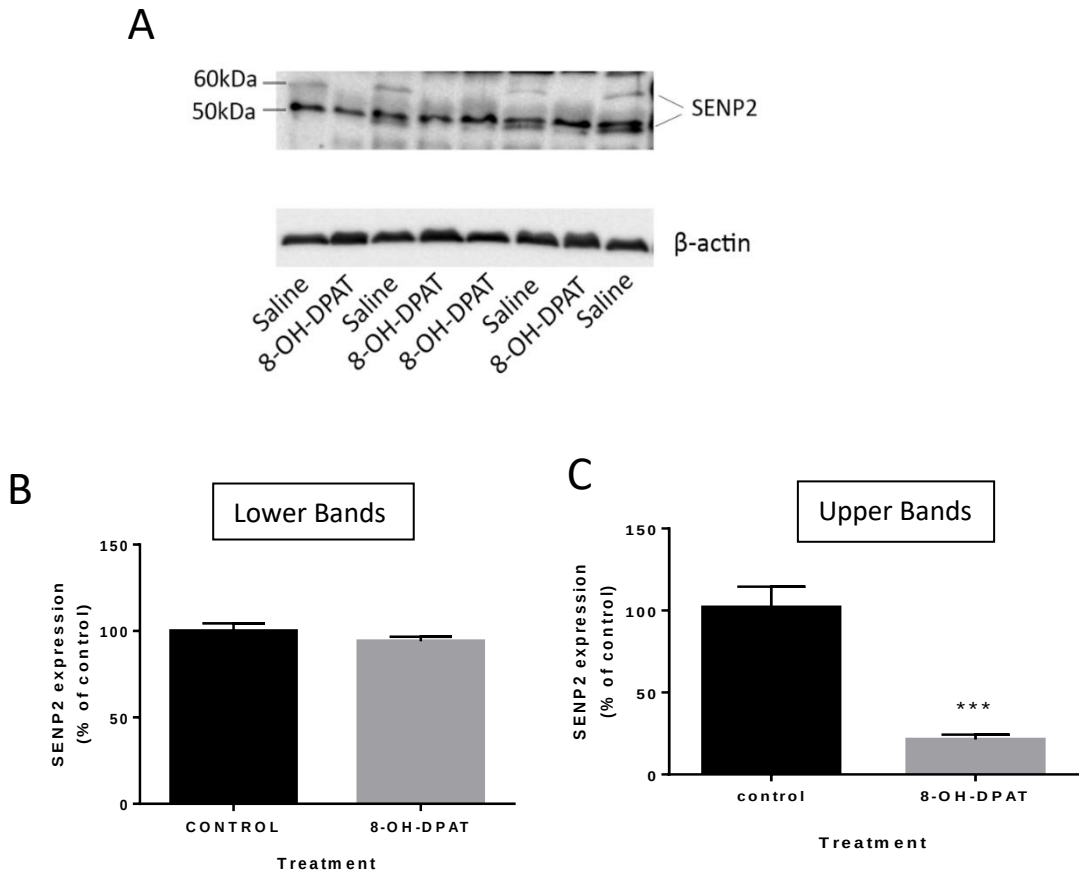

Figure 5. Effect of 8-OH-DPAT treatment on SENP2 expression in the rat frontal cortex.

Ovariectomized female rats were injected with either saline or 8-OH-DPAT (200 µg/kg s.c.) and then decapitated after 5 minutes. (A) Western blot showing the effect of 8-OH-DPAT treatment on SENP2 expression. (B) Graph showing the quantification of the lower SENP2 bands. (C) Graph showing the quantification of the upper SENP2 bands. Unpaired t-test shows significant effect of 8-OH-DPAT treatment on SENP2 expression. ***p=0.0008 Data are shown as mean ±SEM (n=4).

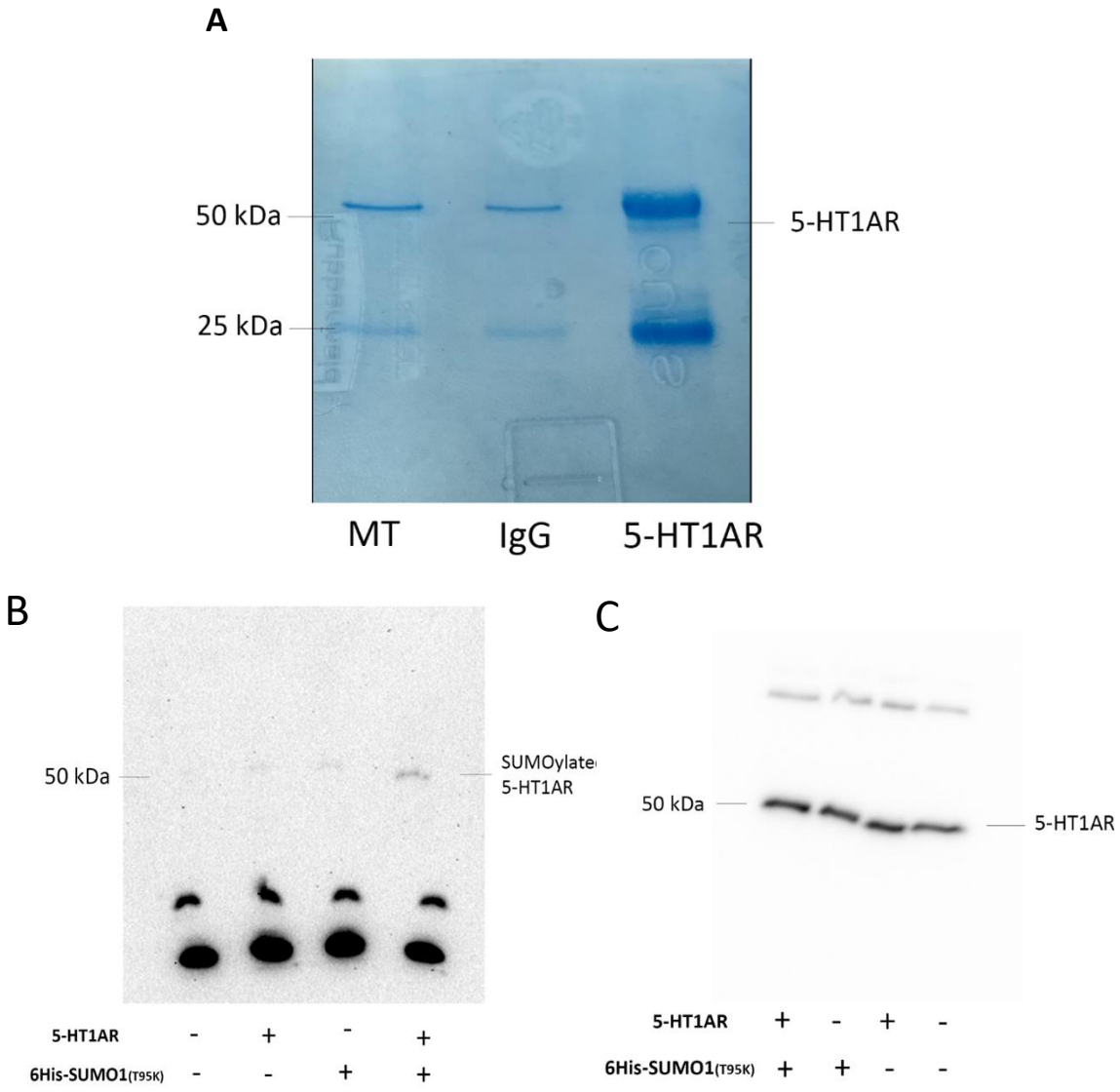

Figure 6. Purification of 5-HT1AR and SUMOylated 5-HT1AR.

N2a cells were either mock transfected (MT) or transfected with 5-HT1AR and/or 6His-SUMO1$^{(T95K)}$. (A) Coomassie gel showing the purified 5-HT1AR when immunoprecipitated with anti-flag antibody. (B) Western blot showing the expression of purified SUMOylated 5-HT1AR using his-tagged pull down upon immunoblotted with anti-5-HT1AR antibody. (C) Western blot showing the 5-HT1AR expression in the membrane fraction of N2a cells (input).

CHAPTER 4: DISCUSSION

In this study, we identified that SENP2 is involved in the deSUMOylation of 5-HT1ARs. For these experiments, we used mouse neuroblastoma (N2a) cells which express endogenous 5-HT1AR and SUMO1 machinery [76]. We determined which SENP enzymes are involved in the deSUMOylation of 5-HT1ARs using two different approaches. First, we overexpressed different flag-tagged SENPs in N2a cells and observed no difference in the amount of SUMOylated 5-HT1AR in the presence of any SENP. Second, we overexpressed flag-tagged 5-HT1AR and SUMO1 along with the SENPs and observed that the amount of SUMOylated 5-HT1AR decreases in the presence of SENP2. This difference in the results could be because overexpressing the SENPs in N2a cells leads to an increased amount of SUMO1 moieties available for SUMOylation since SENPs are also involved in the maturation of the SUMO precursor proteins. The availability of SUMO1 moieties might be a rate limiting step in the SUMO pathway and hence we were not able to see the deSUMOylating effects of SENPs. Overexpressing SUMO1 in the N2a cells may have resulted in observing the deSUMOylating effects of SENP2 on the SUMOylation of 5-HT1ARs separate from the effects of increasing mature SUMO1. We speculate that the effects we observe on the SUMOylated 5-HT1AR due to SENPs is because of overexpression of SUMO1 alone. The levels of SUMO1 might be too low in N2a cells to see the effects of deSUMOylation independent of the effects of SUMO1 maturation. Further studies are needed to confirm this interpretation.

The effects of SENPs on the SUMOylated 5-HT1AR was studied using two different antibodies. We observed that the SUMOylated 5-HT1AR band when immunoblotted with the anti-flag antibody was of a different molecular size from the band labeled with the anti-5-H1AR antibody. The anti-flag antibody labeled the transfected flag-tagged 5-HT1AR at 42kD and the 5-

HT1AR antibody labeled the endogenous 5-HT1AR which was observed at 52kD. This could be because the N2a cells might be processing the transfected 5-HT1AR differently as compared to the endogenous 5-HT1AR.

To further confirm the results of our immunoprecipitation and western blot analysis, we performed a control experiment to determine the size of SUMOylated 5-HT1AR in different species. Membrane fractions of rat and mouse frontal cortex tissue were used in addition to non-transfected and transfected N2a cell lysates with rat and human 5-HT1AR constructs. First, it was confirmed that the anti-5-HT1AR antibody is identifying the SUMOylated 5-HT1AR across different species tested. The size of SUMOylated 5-HT1AR was found to be similar in rat, mouse and human species i.e. around 52-53 kDa.

In N2a cells, it was found that SENP2 is involved in the deSUMOylation of 5-HT1ARs. Therefore, we examined if the expression of SENP2 decreased in rats treated with 8-OH-DPAT as compared to the control, a treatment that leads to the increase in SUMOylation of 5-HT1ARs. We observed a significant decrease in a 60kDa isoforms of SENP2 with 8-OH-DPAT treatment but not in 50kDa isoform of SENP2.

There are multiple isoforms of SENP2. Previous studies observed four different isoforms at 60 kDa, 50 kDa, 44 kDa and 40 kDa in HeLa cells [71]. These different isoforms of SENP2 may arise due to alternative splicing of the *senp2* mRNA. Previous studies have suggested that SENP2 encodes for three different isoforms generated by alternative splicing, SENP2, SENP2-M and SENP-S in human HCT116 cells [72]. Another isopeptidase found in mice, SUMO1 Protease 1 (SuPr-1) had 87% sequence identical to human SENP2. Based on genomic sequence analysis, SuPr-1 appears to be an alternatively spliced product of the *senp2* mRNA because it was observed that SuPr-1 excised an entire exon when compared to the *senp2* genomic sequence [73, 74]. We

quantified two proteins bands one at 50 kDa and one at approximately 60 kDa. We chose the 50 kDa band because flag-tagged SENP2 is expressed at 50 kDa. We also identified a SENP2 isoform at 60 kDa but could not distinguish a 44 or 40 kDa band. With 8-OH-DPAT treatment, no difference in SENP2 expression was seen for the 50 kDa, whereas a significant decrease in the SENP2 expression was seen in the 60 kDa protein band. Further experiments are needed to confirm these findings. Mass spectrometry analysis could also be done to distinguish between the two isoforms of SENP2. These mass spectrometry analyses could be used to confirm the amino acid sequence of these protein bands. siRNAs could be designed to knockdown specific SENP2 isoforms, which could be used to determine which SENP2 isoform is directly involved in the SUMOylation of 5-HT1ARs. These studies would be important to confirm which SENP2 isoform regulates deSUMOylation of 5-HT1ARs.

We observed that the bands at 60 kDa molecular weight diminished in the membrane fraction of 8-OH-DPAT treated in frontal cortex tissue. This could be due to several reasons. First, the treatment with 8-OH-DPAT could cause SENP2 to translocate to the cytoplasm from the membrane. Second, 5-HT1AR activation further leads to the activation of several downstream signaling pathways such phosphoinositide-3-kinase (PI3K)-Akt pathway, extracellular signal-regulated kinases1 and 2 (ERK1/2) pathway[2]. Activation of these signaling pathways results in phosphorylation of different proteins. Activation of these kinases might have resulted in phosphorylation of SENP2, which would have resulted in a conformational change in the SENP2 structure that could not be recognized by the antibody. To identify the 60 kDa isoform of SENP2, which may have undergone a conformational change, other anti-SENP2 antibodies could be used. To confirm the translocation of SENP2 in the cytoplasm, immunoblotting analysis on the cytoplasmic fractions could be done using an anti-SENP2 antibody. Immunocytochemistry studies

could also be performed in order to determine the translocation of SENP2 in the cytoplasm. Phosphorylation of SENP2 could be tested using anti-phospho-SENP2 antibodies. Radiolabeling assays using radiolabeled 32P-orthophosphate could be performed to determine if SENP2 is indeed phosphorylated.

In the study to determine the effect of 8-OH-DPAT treatment on SENP2 expression, animals were treated with 8-OH-DPAT with a single dose of 200 µg/kg and then decapitated after 5 minutes. It was observed that treatment with 8-OH-DPAT increased SUMOylation of 5-HT1ARs [32]. However, in order to determine the optimal dose of 8-OH-DPAT required to increase SUMOylation of 5-HT1ARs, dose response studies are needed to be performed. Time course studies are also important to study the onset and peak of SUMOylation of 5-HT1AR due to agonist treatment.

Previous studies in our lab have also shown that acute treatment with 8-OH-DPAT increase in SUMOylation of 5-HT1ARs which was further increased by a two-day treatment with EB, treatments that lead to desensitization of 5-HT1ARs. Previous data also suggests that SUMOylation may be playing a role in the desensitization of the 5-HT1ARs [32]. 5-HT1AR desensitization is required for anti-depressant action [21]. Hence, understanding the regulation of 5-HT1ARs by SUMOylation would be useful in the development of better therapeutic targets for depression.

To determine the primary site of SUMOylation, previous studies in our lab have used acceptor photobleach FRET to demonstrate that lysine residues 232, 235 and 324 of the amino acid sequence of the 5-HT1AR could be possibly involved in SUMOylation. However, further studies are needed to confirm these results. We chose to use mass spectrometry to identify the primary site of SUMOylation on 5-HT1AR. Mass spectrometry is a direct, sensitive and an

unbiased technique to identify post translational modification sites. In order to perform mass spectrometry, we needed to purify the SUMOylated 5-HT1AR. For this study cell lysates were harvested 32 hours after transfection because the receptor expression was maximal at that timepoint. A time-course experiment was performed to determine the optimum time for 5-HT1AR expression. We use a hexahistidine (6His)-tagged mutant form of SUMO1 with a single amino acid substitution at the C-terminal end of the molecule, where the amino acid preceding the diglycine motif has been mutated to lysine (6His-SUMO1$^{(T95K)}$), which can be recognized by mass spectrometry [75]. Proteins modified by 6His-SUMO1$^{(T95K)}$ are purified using nickel affinity purification. Further, these samples will be used for tandem MS/MS analysis of the sites that are SUMOylated on the 5-HT1AR.

Recently, many proteins associated with G-proteins are found to be SUMOylated. Metabotropic glutamate receptors are the first G-protein coupled receptors that were shown to be SUMOylated. However, function of SUMOylation on mGLuRs is not clear, it is believed that SUMO might provide an interface for the binding of many interacting proteins [67]. Cannabinoid receptor 1 (CB1) is another GPCR that is known to be SUMOylated [68]. It was found that agonist treatment decreased SUMOylation of CB1. Another GPCR, M1 muscarinic acetylcholine receptors are SUMOylated [69]. Results suggest that SUMOylation plays an important role in regulating ligand-binding affinity and signal transduction of M1 muscarinic acetylcholine receptors. Besides GPCRs, many regulators of G-protein signaling such as RGSZ1 and RGSZ2 are also SUMOylated upon the activation of μ-opioid receptors [70]. Studies suggest that SUMOylation of RGSZ1 and RGSZ2 mediate desensitization of GPCRs. Since, these studies demonstrate that SUMOylation alters GPCR function, it is important to study the role of SUMOylation on other GPCRs that are SUMOylated such as 5-HT1ARs.

In conclusion, this study identified that SENP2 is involved in the deSUMOylation of 5-HT1ARs. We also found decreased levels of one of the SENP2 isoforms in the frontal cortex of rats with the 8-OH-DPAT treatment. However, further studies are needed to confirm this result and the mechanism responsible for decrease in the expression of SENP2 with 8-OH-DPAT treatment. Hence, SENP2 can used as a target for the regulation of SUMOylation of 5-HT1ARs and could be used to modulate 5-HT1AR function. Mass spectrometry studies will be conducted to find the primary site of SUMOylation on the 5-HT1ARs. Determining the site at which SUMOylation occurs will help us further understand the 5-HT1AR structure and function. Together these studies, further our understanding of the mechanism regulating SUMOylation of 5-HT1ARs and will provide tools to investigate the role of SUMOylation in 5-HT1AR functioning.

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