IDENTIFICATION OF SENTRIN SPECIFIC PROTEASES AND SUMOYLATION SITES OF 5-HT1A RECEPTORS

By

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Abstract

The serotonin-1A receptor (5-HT1A-R) is an abundant 5-HT receptor located in both pre-synaptic and post-synaptic membranes of neurons. Activation of this receptor has been involved in the mechanism of action of antidepressant, antipsychotic and anxiolytic drugs. Thus, studies of 5-HT1A-Rs might help to manage various psychiatric diseases.

SUMOylation of 5-HT1A-Rs has recently been reported and evidence suggests that SUMOylation might play an important role in the regulation of 5-HT1A-Rs. Previous studies in our lab found that 5-HT1A-Rs can be modified by SUMO-1 in rat brain and neuroblastoma2a cells. The majority of SUMO-1-5-HT1A-Rs are co-localized with endoplasmic-reticulum and trans-Golgi-network markers. Treatment with the 5-HT1A-R agonist, 8-OH-DPAT, increases SUMO-1-5-HT1A-Rs in the detergent-resistant membrane (DRM). SUMO-1-5-HT1A-Rs minimally bind to 5-HT1A-R agonist. Investigating the mechanism involved in the SUMOylation of 5-HT1A-R will help to understand the regulation of 5-HT1A-R and may lead to the development of novel therapeutic approaches for psychiatric diseases.

In this study, I focused on the SUMO machinery regulating deSUMOylation of 5-HT1A-Rs and the SUMOylation sites on 5-HT1A-Rs. I found that mouse Neuroblastoma 2a (N2a) cells can express exogenous 5-HT1A-Rs and verified expression of endogenous SUMOylation of 5-HT1A-Rs. The SUMOylation of 5-HT1A-Rs was detected around 55KD in N2a cells which is consistent to our previous finding in rat brain. In addition, N2a cells can express

transfected sentrin-specific proteases (SENPs) proteins in the membrane fraction. Transfection of SENP1, 2 and 6 causes a significant difference among groups but there is no significant difference between each transfected SENPs group compared to the non-transfected control group.

To identify the SUMOylation sites on 5-HT1A-Rs, six possible SUMOylation sites on 5-HT1A-R: K302, K332, K324, K232 and K235 were mutated into nonSUMOylatable arginine residues. I transfected each 5-HT1A-R mutant into N2a cells and found that transfection of each 5-HT1A-R mutant does not reduce SUMOylation of 5-HT1A-Rs. However, SUMOylation of transfected rat 5-HT1A-Rs has a different molecular mass compared to SUMOylation of endogenous 5-HT1A-Rs in N2a cells.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5-HT1A-Rs	serotonin 1A receptors
8-OH-DPAT	8-Hydroxy-2-dipropylaminotetralin
DRM	detergent-resistant microdomain
ANOVA	analysis of variance
DESI	DeSUMOylating isopeptidases
DEL	deSUMOylating isopeptidases
ER	endoplasmic reticulum
GPCR	G protein-coupled receptor
MT	mock transfection
NEM	N-Ethylmaleimide
PIAS	protein inhibitor activated STAT
PVN	hypothalamic paraventricular nucleus
SENP	sentrin-specific proteases
SERT	serotonin transporter
SIM	SUMO interacting motif

VMAT	vesicular monoamine transporter
SP-RING	Siz/PIAS RING
SSRI	selective serotonin reuptake inhibitor
UlPs	Ubiquitin-like proteases
SUMO	small ubiquitin-like modifier
TCA	tricyclic antidepressants
Ubc9	ubiquitin carrier protein 9
USPL1	ubiquitin-specific protease-like 1

Chapter I: Introduction

1.1. Serotonin and Serotonin neurons

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter derived from tryptophan. It is found primarily in the gastrointestinal tract, blood platelets, and central nervous system. The gastrointestinal tract produces around 95% of serotonin [1-3]. It is also involved in healing wounds, stimulating nausea, and maintaining bone health. In the central nervous system, serotonin is associated with temperature regulation, circadian rhythmicity, vomiting, aggression, and energy balance [4]. Serotonin plays a role in psychiatric diseases such as depression, anxiety, and schizophrenia. Serotonin can be taken up by cells such as platelets and neurons through the serotonin transporter (SERT). Serotonin can also be transported via a vesicular monoamine transporter (VMAT) into vesicles [5]. After it is transported into vesicles, serotonin is released via exocytosis into the synaptic cleft and thereafter binds to pre and post-synaptic serotonin receptors. Serotonergic neurons develop from mesopontine and medullary primordia, which group into rostral and caudal clusters that are maintained into adulthood [6]. Serotonergic neurons originate in the dorsal and median raphe nucleus of the caudal midbrain. Neurons within the rostral raphe complex project to the forebrain, neurons within the caudal raphe complex primarily project to the brainstem nuclei and to the spinal cord [6]. In fact, every cell in the brain is close to a serotonergic neuron [7].

All serotonin receptors belong to the G-protein coupled receptor family, except the 5-HT3 receptor which is a ligand-gated ionotropic receptor [8]. Serotonin receptors can be subdivided into 7 families based on the type of G proteins to which they are coupled. Serotonin 1 receptors (5-HT1-Rs) or serotonin 5 receptors (5-HT5-Rs) couple to Gαi/Gαo proteins; the serotonin 2 receptors (5-HT2-Rs) bind to Gαq/11 proteins; the serotonin 4 receptors (5-HT4-Rs), serotonin 6 receptors (5-HT6-Rs) and serotonin 7 receptors (5-HT7-Rs) couple to Gαs proteins [9]. Different subtypes of serotonin receptors activate different signaling pathways based on the Gα protein to which it couples. For example, activation of Gαs coupled receptors leads to elevation of cyclic AMP(cAMP), which is a second messenger associated with other proteins such as ion channels and protein kinase A (PKA). In contrast, activation of the Gαi receptor leads to downregulation of cAMP [10]. The activation of Gαq/11 coupled receptors activates protein kinase C (PKC) and results in the formation of diacylglycerol (DAG) and inositol-phosphate (IP3), which leads to the elevation of intracellular calcium [11]. Other factors also contribute to the diversity of serotonin receptors. There are thirteen genes coding for GPCR serotonin receptors that couple to almost every G-protein in the cell membrane and probably act without coupling to them [12]. Also, this diversity is made more complex by posttranslational modifications [12].

1.2. 5-HT1A receptor

5-HT1A-R is a subtype of serotonin receptor which binds to serotonin with a high

affinity. It has a molecular weight around 42kDa with 422 amino acids. 5-HT1A-Rs exist in many regions of the brain, but different regions express different densities of 5-HT1A-Rs. A recent study on the distribution of human 5-HT1A-R using autoradiography and positron emission tomography (PET) demonstrated higher levels of 5-HT1A-R in the infralimbic cortex, hippocampus (specifically within CA1), cingulate cortex, neocortex and raphe nuclei. Lower levels of 5-HT1A-R were reported in the basal ganglia and cerebellum [13]. From previous studies, the major effect of 5-HT1A-R stimulation is the activation of hyperpolarizing K+ channels, resulting in the inhibition of neuronal firing. 5-HT1A-Rs are also involved in different signaling pathways such as regulation of phospholipase C activity, inhibition of cAMP production and reduction of calcium currents [14]. 5-HT1A-Rs are located at pre- and post-synaptically. Pre-synaptic 5-HT1A-R are present in the serotonergic neurons with cell bodies located in raphe nuclei [15]. Serotonin release is thought to activate pre-synaptic auto-receptors, thus completing an autoinhibitory feedback loop [16]. Post-synaptic 5-HT1A-Rs are abundantly expressed in the hippocampus, septum, amygdala, and prefrontal cortex [17]. Activation of post-synaptic 5-HT1A-Rs mediates serotonin actions on fear, anxiety, stress and cognitive function [17].

5-HT1A-R is involved in many psychiatric disorders such as depression, anxiety, and schizophrenia. The role of 5-HT1A-Rs in anxiety disorders is well studied. A study in 5-HT1A-R knockout mice showed an increasing anxiety behaviors [1]. The role of 5-HT1A-Rs in schizophrenia remains unclear. However, a study in 5-HT1A-R knockout and knockdown mice showed that 5-HT1A-Rs are necessary for the atypical

antipsychotic drug induced elevation in cortical dopamine transmission [2]. Studies have shown an elevation in cortical 5-HT1A-R density in schizophrenia patients, which also suggests the role of 5-HT1A-Rs in schizophrenia [3]. The role of 5-HT1A-Rs in depression has been extensively studied. Antidepressants such as tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) both alter 5-HT1A-R signaling by direct or indirect mechanisms. Increased DNA methylation of the 5-HT1A-R promoters in brains of suicide victims has also been reported [18]. Further, a study in serotonin transporter knockout mice suggested that early life blockade of 5-HT1A-Rs normalizes depression-like behaviors [19]. Thus, 5-HT1A-Rs appear to play an important role in mental health and may be a useful target in the management of various psychiatric diseases.

5-HT1A-Rs can be modified by phosphorylation, glycosylation, palmitoylation and SUMOylation. Glycosylation is a process in which a carbohydrate is attached to a hydroxyl or other glycosyl acceptor molecule. Glycosylation of 5-HT1A-Rs takes place in the trans-Golgi network and is involved in transporting the receptor from the cytosol to the membrane [20].

Palmitoylation is a covalent attachment of the fatty acid and the palmitic acid to cysteine, serine and threonine residues of a protein. Palmitoylation of 5-HT1A-R occurs early after synthesis of the 5-HT1A-R [21]. Replacement of cysteine residues (417 or 420) prevented palmitoylation, reduced binding of 5-HT1A-R with Gi and

impaired inhibition of adenylyl cyclase activity [21]. In fact, when both palmitoylated cysteines were mutated, the association of 5-HT1A-R with Gi was completely abolished [21]. Moreover, non-palmitoylatable mutants were not able to inhibit forskolin-stimulated cAMP formation, suggesting that palmitoylation of the 5-HT1A-R is important for the Gi signaling [21].

Phosphorylation of 5-HT1A-Rs is thought to be involved in desensitization of 5-HT1A-Rs [22]. Studies have shown that stimulation of protein-kinase C (PKC) by application of phorbol esters induces a rapid phosphorylation of the 5-HT1A-Rs [22]. PKC phosphorylation of the 5-HTlA-Rs leads to a decreased efficiency of coupling to G proteins [22]. Desensitization could be reversed by mutation of three putative PKC sites in the 5-HT1A-Rs, demonstrating a functional link between phosphorylation and desensitization of 5-HT1A-Rs [23].

Previous studies in our laboratory reported that 5-HT1A-Rs can be SUMOylated by SUMO1 protein [24]. The SUMO-1-5-HT1A-R is ~55 kDa, is located in the membrane fraction, but not the cytosol, and is distributed in all of the brain regions expressing 5-HT1A-Rs examined [24]. Acute stimulation of 5-HT1A-Rs significantly increased SUMO-1-5-HT1A-R in rat hypothalamus [24]. Pretreatment with estradiol for 2 days, which causes a partial desensitization of 5-HT1A-R signaling, potentiated agonist-induced increases in SUMO-1-5-HT1A-Rs in the hypothalamus of rats [24]. Using discontinuous gradient centrifugation followed by digitonin treatment, we found that the majority of SUMO-1-5-HT1A-Rs is co-localized with

endoplasmic-reticulum and trans-Golgi-network markers [24]. Although a small proportion of SUMO-1-5-HT1A-Rs are located in the detergent resistant microdomain (DRM) that contain active G-protein coupled receptors, their distribution was different from that of the Gαz protein that couples to the receptors [24]. These data suggest that the SUMO-1-5-HT1A-Rs are an inactive form of 5-HT1A-Rs, a finding further supported by results showing minimal 5-HT1A-R agonist binding to SUMO-1-5-HT1A-Rs [24]. Furthermore, SUMO1- 5-HT1A-Rs in the DRM were increased by treatment with a 5-HT1A-R agonist, 8-OH-DPAT [24]. Together, these data suggest that SUMOylation of 5-HT1A-Rs may be related to 5-HT1A-R trafficking and internalization, which may contribute to 5-HT1A-R desensitization [24]. Therefore, understanding the mechanism of SUMOylation of 5-HT1A-Rs will help us understand the regulatory mechanisms of these receptors in disease and provide potential targets for further diagnostic and therapeutic consideration.

1.3. SUMOylation

SUMOylation is the covalent binding of a SUMO group to a lysine residue of its substrates. It reversibly modulates the activity, stability, and the intracellular localization of proteins. Unlike chemical modifications such as phosphorylation, glycosylation, and methylation, SUMOylation is a small peptide called small ubiquitin-like modifier (SUMO). In fact, a large family of ubiquitin-like modifiers have been identified, SUMO, Nedd8, ISG15, FAT10, FUB1, UFM1, URM1, Atg12

and Atg8 [27].

The process of SUMOylation is carried out by an enzymatic cascade of 4 enzymes. The first step in SUMOylation is the maturation of pre-SUMO proteins by SUMO proteases. SUMO proteases can cleave the C-terminus of the SUMO precursor protein resulting in a C-terminal glycine residue to allow the formation of an isopeptide bond between this glycine residue of SUMO and the lysine residue of the substrate. Then SUMO protein needs to be activated by E1 enzymes, followed by conjugation to the E2 enzyme, Ubc9. E3 ligases may help to transfer SUMO protein to substrates. The SUMOylation process is reversed by SUMO proteases. SUMO proteases catalyze the removal of SUMO from SUMO-conjugated substrate proteins, and this process is called deSUMOylation. Although SUMOylation and ubiquitylation share many similarities, the number of related enzymes is different. There are several E2 and more than 600 E3 enzymes regulating ubiquitylation while only one E1 enzyme (SAE1/SAE2), one E2 enzyme (Ubc9) and only a few E3 enzymes that regulate SUMOylation have been discovered so far [25].

Previous studies revealed that most of the lysine residues that are SUMOylated lie within a specific consensus motif: ΨKXE (ψ, a hydrophobic amino acid; X, any amino acid residue) [28]. However, recent studies suggest that around 40% of SUMOylation site do not lie within this motif [29], and analysis of SUMOylation and SUMO interaction is still challenging.

Studies also suggest that SUMOylation contributes to disease onset and progression.

SUMO modification has been associated with cancer, pathogenic infections and neurodegeneration [26-28]. Notably, it has been reported that SUMOylation supports tumor growth [29]. Also, studies have suggested the that SUMOylation pathway plays an important role in regulating cardiovascular function [25]. Overexpression of Sentrin-specific protease 2 (SENP2) and expression of an SUMOylation-deficient mutant of the cardiac-specific homeobox protein (Nkx2.5) leads to congenital heart defects and cardiac dysfunctions in mice [30]. Many of the key proteins in various neurodegenerative diseases are SUMO targets such as huntingtin, tau, DJ1, α-synuclein and superoxide dismutase 1 [25]. There are some studies which indicate that SUMOylation helps to prevent neurons from damage triggered by transient ischemia [31]. Therefore, investigating the mechanism of SUMOylation will help us understand the mechanisms of disease and inform new therapeutic approaches for these diseases.

1.3.1. Small Ubiquitin-like Modifier (SUMO) proteins

In mammalian cells, there are 4 SUMO paralogues: SUMO-1, SUMO-2, SUMO-3, and SUMO-4. SUMO-1, SUMO-2, and SUMO-3 are detected in all tissues, whereas the existence of SUMO4 has only been demonstrated in the kidneys, dendritic cells, and macrophages [32]. SUMO-1 is 45% identical to SUMO-2 and SUMO-3. SUMO-2 and SUMO-3 share 95% identity and are often referred to as SUMO-2/3. Some SUMO substrates only associate with SUMO-1 protein, some only associate with SUMO-2/3, while others can associate with all SUMO proteins. For example,

RanGap1 is the major substrate for SUMO-1 but can still be modified by SUMO-2/3 [33]. Cells contain a larger pool of SUMO-2/3 compared to SUMO-1, which might be because SUMO-2/3 is responsible for polySUMO chain formation. A conserved lysine sequence **Ψ**KXE (ψ, a hydrophobic amino acid; X, any amino acid residue) in the N-terminal of SUMO-2/3 can be SUMOylated, and can lead to the formation of the polySUMO chains. SUMO-1 does not have this conserved lysine site and does not play a role in the formation of the polySUMO chain [34]. However, SUMO-1 might terminate polySUMO chains as a cap at the end of the polySUMO chain [34]. Furthermore, studies reveal that conjugation to SUMO-2/3 appears under stress conditions, but SUMO-1 conjugation does not [33]. Although the biological relevance of SUMO-4 remains unclear, SUMO-4 can be matured by the stress-induced hydrolase and is able to conjugate to its substrates when cells are under stress conditions [35].

1.3.2. SUMO-activating enzyme (E1)

There are two E1 enzymes in mammals, Sae1 and Sae2. The E1 enzyme catalyzes the reaction of activating SUMO protein and transferring it to SUMO conjugation enzyme (Ubc9). The reaction is a 3 step reaction: In the first step, the carboxyl group of SUMO C-terminal attacks ATP and forms an SUMO-adenylate intermediate with the release of pyrophosphate [36]. In the second step, the SUMO C-terminal is transferred to the catalytic cysteine of Sae1, forming a high-energy thioester bond between Sae1 and the C-terminal glycine of SUMO and releasing AMP [36]. Finally,

the SUMO is transferred to SUMO-conjugating enzyme Ubc9 and forms another thioester bond [36].

1.3.3. SUMO-conjugating enzyme (Ubc9)

Unlike the ubiquitin system, Ubc9 is the only SUMO conjugating enzyme in eukaryotic cells [37]. Human Ubc9 is a 17 kDa protein which is 100% identical to mouse [38]. In mammalian cells, Ubc9 is expressed in the nucleus, nuclear pore complex, nuclear envelope, cytoplasm and cell membrane [38]. It has a conserved cysteine residue in its active site and forms a thioester bond with SUMO [39]. It recognizes a specific motif of amino acid sequence in its substrates. The motif is described as ΨKxD/E where Ψ indicates a hydrophobic residue, followed by a lysine residue, a spacer, and an acidic residue. An isopeptide linkage between the C-terminus of SUMO and the target protein is formed, followed by the nucleophilic attack of the ε-amino group of a substrate lysine residue [38]. Studies have shown that Ubc9 can be SUMOylated at lysine14 and the SUMOylation of Ubc9 results in an enhanced interaction between SUMO interaction motif (SIM) of transcriptional factor Sp100 and SUMO protein [40].

1.3.4. SUMO ligases (E3)

SUMO ligases facilitate SUMO transfer from Ubc9 to its substrate. Some SUMO ligases have been described and can be divided into several classes such as: Siz/PIAS-family proteins, RanBP2, and other SUMO ligases. SUMO ligases might serve to (i) enhance the affinity of Ubc9 for a specific target, (ii) stabilize the Ubc9–

target intermediate, (iii) help to orientate the acceptor lysine, or (iv) contribute to conjugation.

1.3.4.1. Siz/PIAS-family proteins

The Siz/PIAS-family of E3 ligases contain a RING finger-like domain, which is also called SP-RING motif [41]. The Siz/PIAS family includes PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy in mammals. The RING finger-like domain is required for ligase activity but does not seem to contribute the catalytic residue to SUMO conjugation [39]. Siz/PIAS-family E3 ligases bind directly to Ubc9 and express substrate specificity. The five vertebrate PIAS proteins are involved in many processes, including gene expression, signal transduction and genome maintenance[42].

1.3.4.2. RanBP2

Studies have shown that RanBP2 expresses SUMO ligase activity in vitro [43]. RanBP2 is a nuclear pore complex protein that is located at the cytoplasmic pore. It contains an internal repeat domain (IR) which is two continuously repeated sequences of about 50 amino acids [44]. IR domain is required for SUMO ligase activity. RanBP2 facilitates SUMOylation in a substrate-independent manner. It promotes SUMOylation by folding around and aligning the Ubc9-SUMO intermediates in an optimal conformation that allows the interaction with lysine site of the substrate [45].

1.3.4.3. Other SUMO ligases

Other proteins that have been reported as SUMO ligases include histone deacetylase 4

(HDAC4), Pc2, Topors and KRAB-associated protein (KPA1) [43]. HDAC4 is involved in the SUMOylation of myocyte-specific enhancer factor 2 (MEF 2) [46]. It binds to Ubc9 and facilitates the SUMOylation of MEF 2 [46]. The krab-associated protein contains a PHD-finger domain that promotes SUMOylation of an adjacent KAP1 bromodomain [47]. The PHD-finger and bromodomain associate together and bind to Ubc9 thus facilitating SUMOylation [47].

1.3.5. SUMO de-conjugating enzymes

SUMO de-conjugating enzymes serve two major effects. First, the enzyme can cleave the C-terminus of the SUMO precursor protein resulting in a C-terminal glycine residue to allow the formation of an isopeptide bond between this glycine residue of SUMO and the lysine residue of the substrate. This reaction is referred to as the hydrolase activity of SUMO protease. Second, the enzyme can remove SUMO from its substrate by cleavage of an epsilon-linked peptide bond between the C-terminal glycine of the mature SUMO and the lysine epsilon-amino group of the target protein. This reaction is referred to as the isopeptidase activity of SUMO protease. SUMO de-conjugating enzymes were first described in yeast as UBL-specific proteases (UlPs) and include UlP1P, UlP2P. Later, six types of sentrin specific proteases (SENPs) were identified in humans followed by the discovery of 2 types of deSUMOylating isopeptidases (DESI1 and DESI2) and ubiquitin-specific proteases like-1 (USPL1) [48]. A list of various SUMO de-conjugating enzymes and their characteristics are summarized in Table 1 [49-52].

Table 1 The characteristics of SUMO proteases

SUMO protease	Sub-cellular localization	SUMO preference	Cleavage of pre-SUMO	Removal of SUMO from substrates	SUMO poly chain editing
Ulp1	Nuclear pole	Smt3	Yes	Yes	No
Ulp2	Nucleoplasm	Smt3	No	Yes	Yes
SENP1	Nuclear pole, Nuclear foci	SUMO-1, SUMO-2/3	Yes	Yes	Yes
SENP2	Nuclear pole, Nuclear foci, Cytoplasm	SUMO-1. SUMO-2/3	Yes	Yes	No
SENP3	Nucleolus, Nucleoplasm	SUMO-2/3	Unclear	Yes	No
SENP5	Nucleolus, Mitochondria	SUMO-2/3	Yes	Yes	No
SENP6	Nucleoplasm	SUMO-2/3	Yes	Yes	Yes
SENP7	Nucleoplasm	SUMO-2/3	No	Yes	Yes
DESI-1	Nucleus, Cytoplasm	SUMO-1, SUMO-2/3	Unclear	Unclear	Yes
DESI-2	Cytoplasm	Unclear	No	Yes	Unclear
USPL1	Cajal bodies	SUMO-2/3	Yes	Yes	Yes

1.3.5.1. Ubiquitin-like proteases (UlPs)

The family of UlPs contains two enzymes: UlP1P and UlP2P. UlPs/SENPs share a conserved catalytic domain at C-terminal which is around 200 amino acids in length [43]. UlP1P is localized in the nuclear pole and is involved in the maturation of Smt3p [48]. UlP2P is localized in the nucleoplasm and plays a role in regulating the formation of poly-Smt3p chains [53].

1.3.5.2. Sentrin specific proteases (SENPs)

In mammals, there are 7 SENPs (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7 and SENP8). Most of the SENPs are involved in the regulation of SUMOylation except SENP8. It is important to note that SENP8 is specific for Neddylation [54]. SENPs belong to the CE clan of cysteine proteases and share a common fold of their catalytic domain that encompasses the catalytic triad residues His478, Asp495, and Cys548 [49]. The domain architecture of each SENPs is shown in Figure 1. SENPs can be divided into 3 families. SENP1 and SENP2 belong to the first family because of the broad specificity for all SUMO isoforms [55]. The second family includes SENP3 and SENP5 for their preference on SUMO2/3 and localization in the nucleolus [55]. SENP6 and SENP7 also have a preference on SUMO2/3, but they have an additional loop inserted in the catalytic domain which makes them belong to the third family [55]. Different SENPs have distinct subcellular localizations. Studies have shown that their localizations are determined by their nonconserved N-terminal regions [56]. All SENPs can be activated by their substrate SUMO even though the mechanism remains unclear [49]. SENPs also exhibit selectivity toward SUMO substrates. Some studies indicate that substrate specificity of SENPs is an intrinsic characteristic while others suggest the importance of distinct localization of different SENPs [57]. Studies also suggest that the C-terminal end of each SUMO isoform might contribute to the preference of SENPs [58]. SENPs have been shown to play a pivotal role in cell cycle by regulating deSUMOylation.

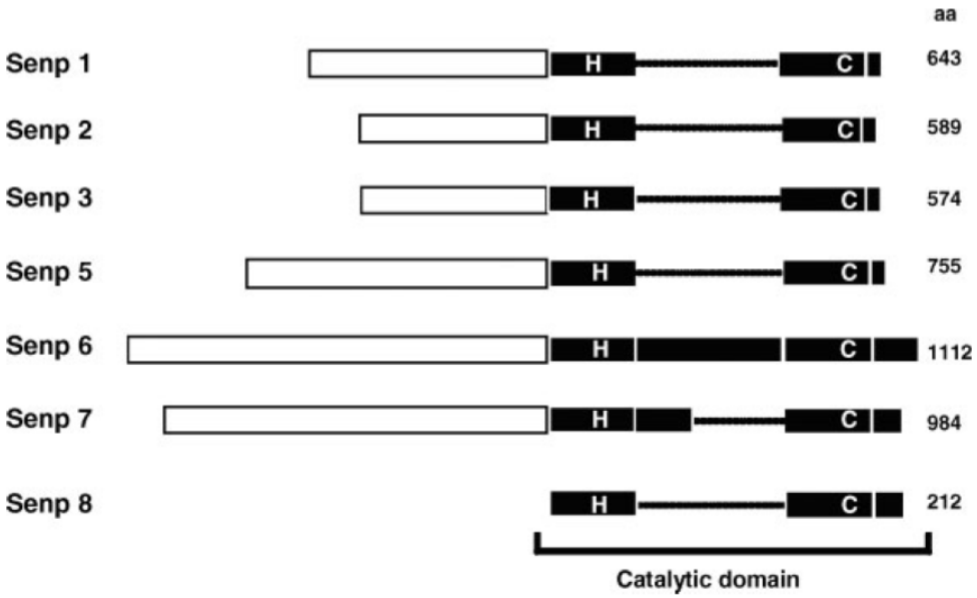


Figure 1. The conserved catalytic domain is represented in black. White bars represent the N-terminal region which might be involve in the localization of SENPs. SENP6 and SENP7 have insertions in the catalytic domain. SENP8 shares the same catalytic domain with other SENPs but is not involved in the SUMOylation process [49].

1.3.5.2.1. SENP1

Homo Sapien SENP1 is composed of 643 amino acids with a weight of 73 KD. The catalytic domain of SENP1 is formed by Cys602, His533 and Asp550 [59]. SENP1 exists at the nuclear envelope and can efficiently remove all three SUMO isoforms from their targets [60]. Studies have suggested that SENP1 is involved in the formation of polySUMO chains by removing the SUMO-1 protein from the end of the polySUMO chain [61]. Moreover, it processes the SUMO-1, -2 and -3 precursors to remove a C-terminal pro-peptide to expose the C-terminal diglycine. Some studies

show that SENP1 knockdown mice exhibit an increased level of SUMO-1 conjugation and accumulation of free SUMO-1, suggesting that SENP1 might have a preference to SUMO-1 [57]. Furthermore, studies have shown that SENP1 may itself be a target for SUMO-1 modification at a nonconsensus site [57]. Expression and localization of SUMO-1-conjugated target proteins play a pivotal role in the localization of SENP1 [57].

1.3.5.2.2. SENP2

Homo Sapien SENP2 is a 589 amino acids protein [58]. It is located at the nuclear pole, nuclear foci, and cytoplasm. It can be subdivided into 2 subdomains. An N-terminal domain with α helices that contain the protease nucleophile, and a C-terminal with a five-stranded β sheet and 2 α helices [58]. The SENP2 active site is formed by a catalytic triad of amino acid residue including Cys548, His478, and Asp495, just like other cysteine protease active sites [58]. SENP2 regulates the maturation of pre-SUMO and has a preference for pre-SUMO-2 compared to pre-SUMO-1 and pre-SUMO-3 [58]. It recognizes and cleaves all SUMO isoforms but is not involved in the editing of the poly-SUMO chain.

1.3.5.2.3. SENP3 and SENP5

Homo Sapien SENP3 is a 574 amino acids protein and Homo Sapien SENP5 has 755 amino acid residues. They are both found in the nucleolus while SENP3 can also be found in nucleoplasm and SENP5 also exists in the mitochondria. SENP3 has substrate specificity similar to that of SENP5; both of them exhibit a substrate

preference on SUMO2/3. The effect of SENP3 on SUMO maturation remains unclear, however, SENP5 exhibits hydrolase activity. The active site of SENP3 is formed by a catalytic triad of 3 amino acid residues including Cys532, His465, and Asp482. The SENP5 active site consists of Cys713, His646, and Asp663. Studies suggest that SENP3 plays an important role in regulating ribosome biogenesis by association with nucleophosmin1 [62, 63]. SENP5 might play a role in mitosis and cytokinesis [64]. Knockdown of SENP5 inhibits cell proliferation and alters the morphology of the nucleus [55]. SENP5 is also found in mitochondria and overexpression of SENP5 deconjugates SUMO-1 from a number of mitochondrial substrates, and rescues SUMO-1 induced mitochondrial fragmentation. Also, knock down of SENP5 results in abnormal mitochondrial development [55].

1.3.5.2.4. SENP6 and SENP7

Homo Sapien SENP6 consists of 1112 amino acids and Homo Sapien SENP7 has a 984 amino acid residues protein. Both SENP6 and SENP7 have a preference for SUMO2/3 but do not regulate the maturation of pre-SUMO. SENP6 was initially thought to localize to the cytoplasm. However, further studies suggest that SENP6 as well as SENP7 localize in the nucleoplasm [55]. Both are involved in the editing of polySUMO chains [65]. Active sites of SENP6 include amino acid residues 637–1112 [65]. The SENP7 catalytic domain includes amino acids 662–984[65]. Although the structure of SENP6 remains unclear, SENP6 and SENP7 share similar characteristics and have conserved sequence insertions in distinct positions within their catalytic

domains [65]. Structural studies of SENP7 revealed some unique features compared to other members from SENP/ULP family: 1. The lack of N-terminal α helix which exists in structures of SENP1, SENP2 and ULP1 [65]; 2. Four insertion loops; and 3. Alteration of some secondary structure elements in SENP7 compare to SENP1 or SENP2 [65]. Interestingly, recent studies note that the unexpected specificity of SENP6 and SENP7 distinguishes them from other members of SENPs family, suggesting that they might have substrates other than SUMO conjugates [66].

1.3.5.3. DeSUMOylating isopeptidases (DESI)

DeSUMOylating isopeptidases are a set of newly discovered SUMO proteases belonging to a putative isopeptidase superfamily. Two types of deSUMOylating isopeptidases have been reported so far, including DESI-1 and DESI-2. DESI-1 and DESI-2 belong to a group of proteins called PPPDE (Permuted Papain fold Peptidases of Ds-RNA viruses and Eukaryotes) and contain around 140 amino acids [52]. The active sites of DESI-1 and DESI-2 are formed by the catalytic domain containing two conserved cysteine and histidine residues [67]. DESI-1 deconjugates both SUMO-1 and SUMO2/3 while DESI-2 remains unclear [67]. In contrast to Ulp/SENP family members, the DESI-1 and DESI-2 enzymes have isopeptidase activity on a selected substrate but do not seem to process the maturation of pre-SUMO [52]. Although there are very limited studies about the substrate of DESIs, it is suggested that DESIs have different substrate specificity compared to SENPs [67].

1.3.5.4. Ubiquitin-specific proteases like-1 (USPL1)

Ubiquitin-specific protease like-1 is a recently discovered SUMO protease. It belongs to cysteine protease USP family and has a molecular weight at 120 KD [68]. A structural study shows that a catalytic triad residues of Cys236, His 456, Asp472 forms the active site of USPL1. Studies suggest that USPL1 can process pre-SUMO-1 and pre-SUMO2 and has a preference for SUMO2/3 [68]. It also influences the editing of poly-SUMO chains, especially SUMO-3 chains [68]. USPL1 is found in the Cajal bodies colocalized with coilin [68]. Interestingly, global SUMOylation is not affected by the depletion of USPL 1, however, the distribution of coilin and cell proliferation was affected [68]. The mechanism remains unclear, but the catalytic domain of USPL 1 is not involved [68].

1.3.6. SUMO-interacting motif

Substrates containing a specific motif can bind with SUMO non-covalently [69]. This specific motif is called SUMO-interacting motif (SIM). It was first observed in the SUMOylation of P73 [69]. Scientists found that certain proteins containing a Ser-X-Ser sequence flanked by a hydrophobic core on one side and acidic amino acids on the other can bind with SUMOylated P73 [69]. Further studies suggest that the hydrophobic core, with the consensus Val/ Ile-X-Val/Ile-Val/Ile (V/I-X-V/I-V/I) facilitates the interaction between SUMO and SIM-containing peptides [70]. Recent studies suggest that the hydrophobic core is an essential component of the SIM and it is usually juxtaposed to a negatively charged cluster of amino acids [71]. In some cases, SIM-containing proteins do not have this negatively charged amino acid

juxtaposed to the hydrophobic core. Phosphorylated serine and threonine residues all found adjacent to the hydrophobic SIM domains, which is a good way to introduce a negatively charged cluster into the SIM domains [72]. Interestingly, some studies even indicate that SUMO-2 prefers to conjugate with SIMs that lacks negatively charged amino acids, suggesting that the negatively charged cluster might affect the affinity, orientation and paralogue specificity of SUMO conjugation [72].

The identification of SUMOylation sites in proteins and enzymes involved in SUMOylation is fundamental for improving the understanding of SUMOylation recognition, understanding the biological functions and regulatory mechanisms of SUMOs, and provides potential targets for further diagnostic and therapeutic considerations [73]. To study the mechanism of SUMOylation of 5-HT1A-Rs, we need to know what enzymes regulate the SUMOylation of 5-HT1A-Rs and the SUMO consensus sites on 5-HT1A-Rs. A previous study on the PIAS proteins in neuroblastoma 2a (N2a) cells demonstrated that PiasXα facilitates the SUMOylation of 5-HT1A-Rs in N2a cells. SUMO proteases involved in the SUMOylation of 5-HT1A-Rs remains unclear. In this study, I focused on the deSUMOylation of 5-HT1A-Rs and examined possible SUMOylation sites on 5-HT1A-Rs. The specific aims of my thesis are as follows:

Aim1: To determine which SENPs regulate the deSUMOylation of 5-HT1A-Rs.

Aim2: To determine the SUMOylation sites on 5-HT1A-Rs.

Chapter II: Materials and Methods

2.1. Plasmid constructs

The plasmid constructs used in this project are listed in Table 1. QIAGEN®Plasmid Midi Kit (25) (Cat#12143, QIAGEN, USA) was used to isolate and purify plasmids. RGS-Senp3 and RGS-Senp5 were cut at BamH1/ Xho1 sites. Human Senp3 and Senp5 coding sequences were isolated and inserted in-frame into the pCMV-3Tag 2B vector at BamH1/ Xho1 sites.

Table 2. Summary of the plasmid constructs used

Plasmid constructs	**Vector**	**Tag**	**Source**
pcDNA4 HisMax C	pcDNA4 HisMax C	His, Xpress	ThermoFisher# V86420
pcDNA4 HisMax C- 5-HT1A-R	pcDNA4 HisMax C	His, Xpress	
pcDNA4 HisMax C- 5-HT1A-R -K332R	pcDNA4 HisMax C	His, Xpress	
pcDNA4 HisMax C- 5-HT1A-R-K302R	pcDNA4 HisMax C	His, Xpress	
pcDNA4 HisMax C- 5-HT1A-R -K332.302R	pcDNA4 HisMax C	His, Xpress	

pcDNA4 HisMax C- 5-HT1A-R -K324R	pcDNA4 HisMax C	His, Xpress	
pcDNA4 HisMax C- 5-HT1A-R -K232.235R	pcDNA4 HisMax C	His, Xpress	
pcDNA4 HisMax C- 5-HT1A-R -K232.235.324R	pcDNA4 HisMax C	His, Xpress	
Flag-SENP1	pFLAG-CMV(4700bp)	Flag	Plasmid# 17357, Addgene
Flag-SENP2	pFLAG-CMV(4700bp)	Flag	Plasmid# 18047, Addgene
Myc-SENP3	pCMV3Tag-2B(4200bp)	3×Myc	
Myc-SENP5	pCMV3Tag-2B(4200bp)	3×Myc	
Flag-SENP6	pFLAG-CMV(4700bp)	Flag	Plasmid# 18065, Addgene
RGS-SENP3	pcDNA3-RGS(5400bp)	RGS	Plasmid# 18048, Addgene
RGS-SENP5	pcDNA3-RGS(5400bp)	RGS	Plasmid# 18053, Addgene

2.2. Cell culture and harvesting

Mouse Neuroblastoma 2a cells (N2a cells) were cultured 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). N2a cells were transfected with mammalian expression plasmids using Lipofectamine 3000 (Cat# L3000015, Thermo Fisher, USA). The 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). The cells were harvested in hypotonic buffer (20mM NEM, 1/100 dilution of phosphatase inhibitors and protease inhibitors were added before use), sonicated three times for 10s in ice with ~30s interval to cool down the solution. After sonication, cells were centrifuged at 25000 x g 4ºC for 1 hour. The supernatant was collected as the cytosol fraction. The pellet was suspended in solubilization buffer (20 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl. 1% sodium cholate hydrate, 20mM NEM, 1/100 dilution of phosphatase inhibitors and protease inhibitors) and sonicated 3 times for 10s each in ice with ~30s interval. After sonication, the vials were shaken horizontally at high speed, at 4ºC for 1 hour, and samples were then centrifuged at 25000 x g, at 4 ºC for 1 hour. The supernatant was collected as the solubilized membrane fraction. Protein samples were stored at -80 ºC. Protein concentration was measured using the BCA protein assay (Cat# 23228, Cat# 1859078, BCA Protein Assay, Thermo Fisher, USA).

2.3. Immunoprecipitation assay

Protein from the N2a cell membrane fraction was used for the immunoprecipitation assays and incubated with mouse anti-SUMO-1 antibody, 1:40 (Cat# sc-5308, Santa Cruz, USA), rabbit anti-SUMO-1 antibody, 1:125 (donated by Dr. Yoshi Azuma), rabbit IgG control, 1:40 (Cat# 170-6515, Bio-Rad, USA) or mouse IgG control, 1:80 (Cat# sc-2025, Santa Cruz, USA) overnight at 4 ºC. After incubation, 50ul of recombinant Protein G agarose (Cat# 15920-010, Invitrogen, USA) or Protein A affinity resins (Cat# 10-2003-01, Repligen, USA) were used for precipitation. The beads were washed 3 times with IP buffer (50 mM Tris, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100, containing 20 mM NEM, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail I and II,) and incubated with protein samples and antibody at 4ºC overnight. The beads were centrifuged at 1000 x g at 4 ºC for 3min. The pellets were collected, and the bound proteins were eluted with 50ul 2×SDS sample buffer (10g SDS in 100ml ddH_2O with β-mercaptoethanol) and used for immunoblot assays.

2.4. Immunoblot assay

Protein samples were separated using SDS-PAGE as previously described [74] and then transferred to 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 as described and listed in Table 2. The proteins were detected with

an ECL detection kit (Cat# WBLUR0500, Millipore, USA, Cat# 10026384, Cat# 10026385, BioRad, USA).

Table 2. Antibodies used for immunoprecipitation and immunoblots

Antibodies	**Dilution**	**Source**
Rabbit anti-5-HT1A-Rs	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
Mouse-anti-Xpress	1:5000	Catalog#: R910-25, Invitrogen, USA
Mouse-anti-β-actin	1:20000	Cat# 691001, MP Biomedicals, LLC
Goat-Anti-Mouse IgG	1:10000 for β-actin antibody 1:5000 for Xpress antibody	Cat# 119380, Jackson ImmunoResearch, USA
Goat Anti-Rabbit IgG	1:10000	Cat# 120745, Jackson ImmunoResearch, USA
Goat Anti-Rat IgG	1:10000	Cat# 112-005-003
Rabbit-anti-SUOM-1	1:125 (for immunoprecipitation)	Gift from Dr. Yoshiaki Azuma

2.5. Data analysis and statistics

Densitometric analysis of immunoblots was implemented with ImageLab 3.0 software (BioRad, Hercules, CA). The SUMOylated 5-HT1A-R proteins levels were normalized to β-actin which is measured on a SDS-PAGE gel performed with membrane fraction used as the input for the immunoprecipitation. All data were analyzed by one-way analysis of variance (ANOVA) using the GraphPad Prism (version 6.02) followed Dunnett's multiple comparisons test and the criterion of significance was set at $p<0.05$. Quantitative data are presented as the mean±SEM.

2.6. Prediction of SUMOylation sites

Previous studies indicated that most of SUMOylation sites follow a consensus motif of ΨKXE (ψ, a hydrophobic amino acid; X, any amino acid residue). It is possible to predict the SUMOylation sites in silico in order to narrow down the number of candidate sites and generate helpful information for further exploration. Here we used SUMOplot and PCI-SUMO programs to predict possible SUMOylation sites on the rat 5-HT1A-R. The sequence of rat 5-HT1A-R was obtained from The National Center for Biotechnology Information (NCBI). Data are shown in Table 3. Lysine 302 has the highest score which suggests that it has the highest chance to be the SUMOylation site.

Lysine Location	Window of Residues	Score
232	FRIRKTVRKVEKKGAGT	0.1458
235	TVKKVEKTGADTRH	0.33
302	EVHRVGNSKEHLPLPSE	0.5633
324	YAPACLERKNERNAEAK	0.2620
332	KNERNAEAKRKMALARE	0.2643

Table 3. Possible SUMOylated lysine residues and the sequence at which they are located. The higher the score the higher chance to be a SUMOylation site.

Chapter Ⅲ. Results

3.1. Identification of Sentrin specific proteases of 5-HT1A-Rs.

To investigate which SENP proteins are involved in the de-SUMOylation of 5-HT1A-Rs, SENP proteins were transfected into N2a cells. I used several SENPs plasmids, FLAG-SENP1, FLAG-SENP2, MYC-SENP3, MYC-SENP5, RGS-SENP3, RGS-SENP5, and FLAG-SENP6. Cell membrane fractions were collected and the expression levels of each transfected SENPs were examined by immunoblotting with anti-FLAG, anti-MYC and anti-His antibodies. In the cell membrane fraction, transfected SENP1 was detected at 75kDa, transfected SENP2 was detected at 55kDa and transfected SENP6 was detected at 150kDa, all of which are the appropriate size (Figure 1A). Among the three SENPs, the SENP1 construct expressed most abundantly. The expression levels of SENP2 and SENP6 are very similar, and express at a lower level (Figure 1A). In the cell cytosol fraction, transfected SENP1, 2 and 6 were detected at appropriate size as well however, the expression of SENP1 and SENP6 were quite abundant, SENP2 remained at a low expression level (Figure 1B). Several anti-MYC antibodies and anti-His antibodies were used to try to detect the transfected proteins, however none of them detected transfected proteins or positive controls. These results verify transfection and the expression of only FLAG-SENP1, FLAG-SENP2 and FLAG-SENP6 in N2a cells.

To determine the effects of SENPs on SUMOylation of 5-HT1A-Rs, the cell membrane fraction was collected and immunoprecipitated with a SUMO-1 antibody

followed by immunoblotting with a 5-HT1A-R antibody. A 55kDa band in the membrane fraction was detected (Figure 1C) which is consistent with our previous findings that 5-HT1A-R can be SUMOylated by SUMO-1 protein in rat brain [24]. Based on our ability to confirm the over-expression of FLAG-SENP1, FLAG-SENP2 and FLAG-SENP6, I used one-way ANOVA to compare the effects of these SENPs to the non-transfected control group. Statistical analysis shows that there is a significant difference in SUMO-1-5-HT1A-R levels among non-transfected, SENP1, 2 and 6 transfected groups (n=5, One-way ANOVA, $F(3, 15) = 4.15$, $p=0.025$) (Figure 1D), but the Dunnett's multiple comparisons test shows no significant difference between the non-transfected group compared to each SENP transfected group.

Na+, K+ ATPase is a plasma membrane marker and was selected to verify separation of membrane and cytosolic fractions (Figure 1E). The results show that there was abundant Na+, K+ ATPase in the membrane fraction while there was no Na+, K+ ATPase detected in the cytosolic fraction, indicating successful separation of membrane and cytosol fraction.

3.2. Overexpression of rat 5-HT1A-Rs in N2a cells.

To identify the SUMOylation sites on 5-HT1A-Rs, we first determined whether the N2a cells can express transfected 5-HT1A-Rs and determined the optimal amount of 5-HT1A-Rs constructs for overexpression. A rat 5-HT1A-R sequence was inserted into the pcDNA4HisMaxC plasmid. N2a cells were transfected with different

amounts of pcDNA4HisMaxC-5-HT1A-Rs plasmids. Immunoblotting with an Xpress antibody was used to identify the overexpressed 5-HT1A-Rs in both cytosol and membrane fraction. Our results show that the expression levels of transfected 5-HT1A-Rs increased with the increasing amounts of 5-HT1A-Rs plasmids and 14ug of pcDNA4HisMaxC-5-HT1A-Rs plasmids resulted in an optimal expression level (Figure 2A).

Next, to examine the SUMOylated 5-HT1A-Rs, different amounts of membrane proteins were collected and immunoprecipitated with a mouse SUMO-1 antibody followed by immunoblotting with mouse Xpress antibody. N2a cell membrane preparations containing 250, 350, 450, 550, 650 and 750ug were used for immunoprecipitation assays followed by immunoblotting with Xpress antibody. As Fig. 2B shows, we observed bands around 48kDa, 55kDa and above 70kDa (Figure 2B). Unfortunately, mouse IgG heavy chain was detected at 55kDa which is the expected position of SUMO-1-5-HT1A-Rs, because the same species of antibody was used for immunoprecipitation and detection on the immunoblot. The SUMOylated 5-HT1A-R levels detected were increased and correlated to the protein concentration in the range between 250 and 750 mg of protein. Based on this study, we used 450 mg of protein in subsequent experiments (Figure 2B).

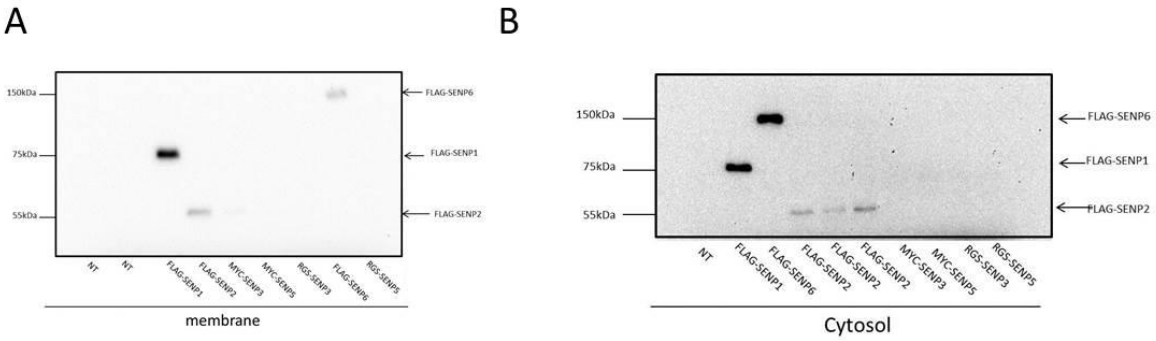
To prevent detection of IgG heavy chain, we chose a polyclonal rabbit SUMO-1 antibody (donated by Dr. Yoshiaki Azuma) [75] to pull down the SUMOylated

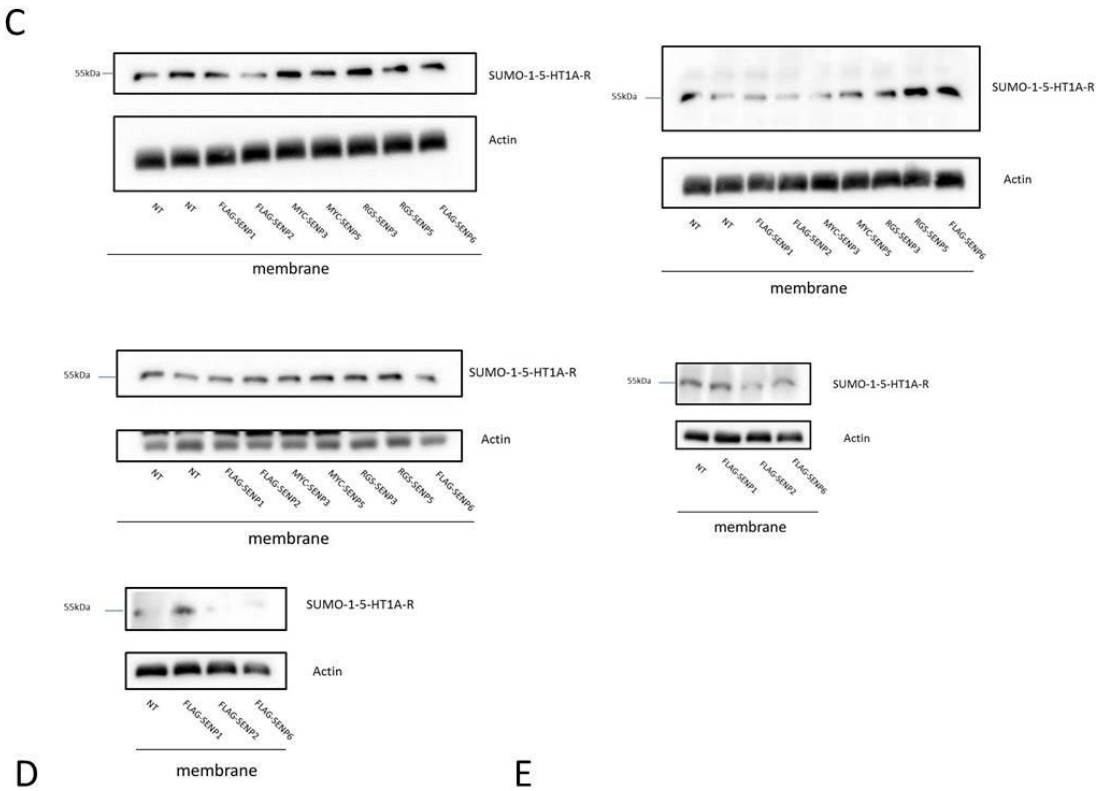
proteins. The mouse Xpress antibody detected protein bands at ~48kDa and above ~70kDa (Figure 2C). Bands at 48kDa and above 70kDa are not the predicted molecular size for SUMO 1- 5-HT1A-R complex as previously detected in different regions of rat brain tissue. In order to determine if the protein bands detected by Xpress antibody are SUMOylated 5-HT1A-Rs, we removed NEM (which inhibits deSUMOylation) from the buffers. When NEM was not used to harvest N2a cells and conduct immunoprecipitation experiments, the intensity of SUMOylated 5-HT1A-Rs was dramatically decreased, thus confirming that the band around 48 kDa and bands above 70kDa represents the SUMOylated 5-HT1A-Rs (Figure 2C).

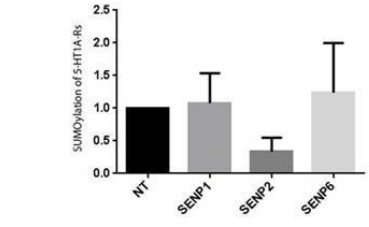
3.3. Identification of SUMOylation sites on 5-HT1A-Rs.

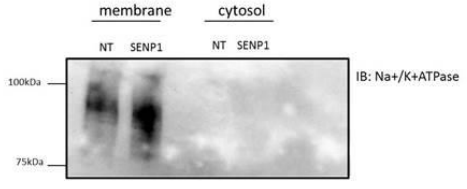
SUMO proteins associate with target proteins at lysine residues. Thus, to identify the SUMOylation sites on 5-HT1A-Rs, possible SUMOylation sites: K302, K332, K324, K232 and K235 were mutated into nonSUMOylatable arginine residues. Each 5-HT1A-R mutant was transfected into N2a cells. Membrane fractions were collected after 48 hours and immunoprecipitated with a rabbit SUMO-1 antibody followed by immunoblotting with a mouse Xpress antibody. Bands at 48kDa and above 70kDa were detected. As shown in Figure 3A, we quantified the 48kDa band and the 75kDa bands. Statistical analysis shows that there is no significant difference between mutants and wild-type 5-HT1A-R for either the 48kDa band (n=3, One-way ANOVA, $F(6, 17) = 0.9182$, $p=0.5059$) (Figure 3A) or the 75kDa band (n=3, One-way ANOVA, $F(6, 14) = 0.1483$, $p=0.9864$) (Figure 3A).

Next, we compared SUMOylation of endogenous 5-HT1A-Rs and SUMOylation of transfected 5-HT1A-Rs. The same PVDF membrane was incubated with a rabbit 5-HT1A-R antibody to look at the SUMOylation of endogenous 5-HT1A-Rs. The endogenous SUMO-1-5HT1A-Rs were detected at 55kDa (Figure 3B). Next, we merged Figure 3A and Figure 3B together to compare the SUMOylation of endogenous 5-HT1A-Rs and transfected 5-HT1A-Rs. As shown in Figure 3C, the band of SUMOylated endogenous 5-HT1A-R is at 55kDa while the bands of SUMOylated transfected 5-HT1A-R is at 48KD, and above ~70kDa, suggesting that the transfected receptors that are SUMOylated are a different size than the endogenous receptor (Figure 3C).





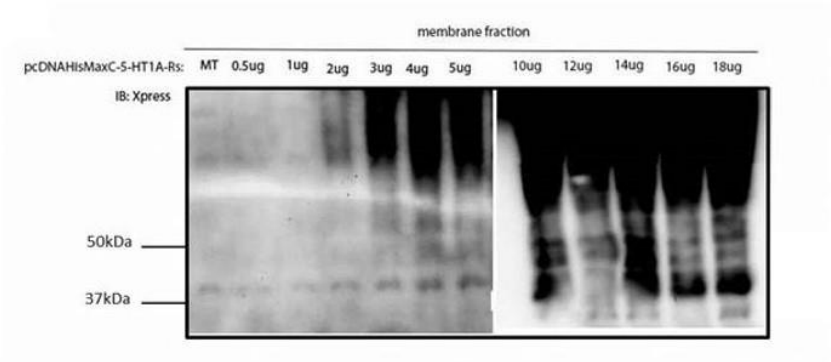




A
150kDa
75kDa
55kDa
FLAG-SENP6
FLAG-SENP1
FLAG-SENP2
NT
NT
FLAG-SENP1
FLAG-SENP2
MYC-SENP3
MYC-SENP5
RGS-SENP3
FLAG-SENP6
RGS-SENP5
membrane
B
150kDa
75kDa
55kDa
FLAG-SENP6
FLAG-SENP1
FLAG-SENP2
NT
FLAG-SENP1
FLAG-SENP6
FLAG-SENP2
FLAG-SENP2
FLAG-SENP2
MYC-SENP3
MYC-SENP5
RGS-SENP3
RGS-SENP5
Cytosol
C
55kDa
SUMO-1-5-HT1A-R
Actin
NT
NT
FLAG-SENP1
FLAG-SENP2
MYC-SENP3
MYC-SENP5
RGS-SENP3
RGS-SENP5
FLAG-SENP6
membrane
55kDa
SUMO-1-5-HT1A-R
Actin
NT
NT
FLAG-SENP1
FLAG-SENP2
MYC-SENP3
MYC-SENP5
RGS-SENP3
RGS-SENP5
FLAG-SENP6
membrane
55kDa
SUMO-1-5-HT1A-R
Actin
NT
NT
FLAG-SENP1
FLAG-SENP2
MYC-SENP3
MYC-SENP5
RGS-SENP3
RGS-SENP5
FLAG-SENP6
membrane
55kDa
SUMO-1-5-HT1A-R
Actin
NT
FLAG-SENP1
FLAG-SENP2
FLAG-SENP6
membrane
55kDa
SUMO-1-5-HT1A-R
Actin
NT
FLAG-SENP1
FLAG-SENP2
FLAG-SENP6
membrane
D
SUMOylation of 5-HT1A-Rs
2.5
2.0
1.5
1.0
0.5
0.0
NT
SENP1
SENP2
SENP6
E
membrane
cytosol
NT
SENP1
NT
SENP1
100kDa
75kDa
IB: Na+/K+ATPase

Figure 1. Identification of SENPs proteins in N2a cells. N2a cells were transfected with FLAG-SENP1, FLAG-SENP2, MYC-SENP3, MYC-SENP5, RGS-SENP3, RGS-SENP5, and FLAG-SENP6 plasmid constructs. The SUMO-1-5-HT1A-Rs were immunoprecipitated with a mouse SUMO-1 antibody followed by immunoblotting with a rabbit 5-HT1A-Rs antibody. **(A).** The expression of transfected SENPs in membrane fraction. **(B).** The expression of transfected SENPs in cytosol fraction. **(C).** The effect of SENPs proteins on SUMOylation of 5-HT1A-Rs (blots from all five independently repeated experiments are shown). **(D).** Quantification of SUMO-1-5-HT1A-Rs. Data are presented as mean±SEM (n=5). One-way ANOVA shows there is a significant difference among groups ($F(3, 15) = 4.15$, $p=0.0251$) but Dunnett's multiple comparison test post-hoc test shows there is no significant difference comparing NT group to each SENPs group. **(E).** The separation of membrane fractions was verified by immunoblotting using Na+/K+ ATPase as plasma membrane marker. MT: Mock transfection. IgG: immunoprecipitation with the same amount of mouse IgG instead of a mouse anti-SUMO-1 antibody. NT: None transfection.

A


membrane fraction
pcDNAHisMaxC-5-HT1A-Rs:
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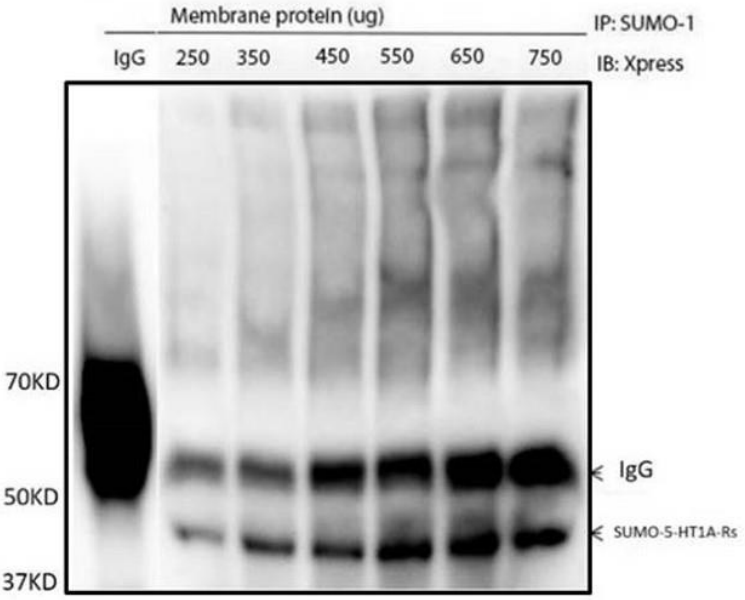
IB: Xpress
50kDa
37kDa

B


Membrane protein (ug)
IP: SUMO-1
IgG 250 350 450 550 650 750
IB: Xpress
70KD
50KD
37KD
IgG
SUMO-5-HT1A-Rs

C

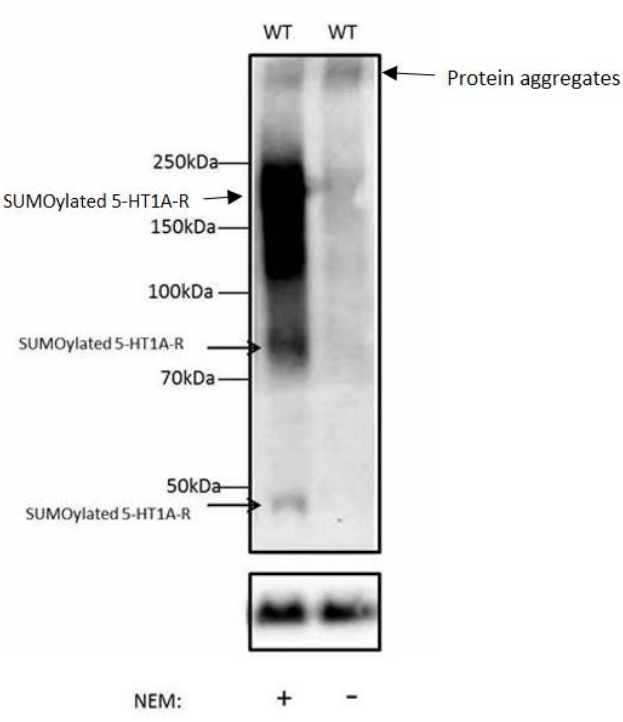

WT WT
Protein aggregates
250kDa
SUMOylated 5-HT1A-R
150kDa
100kDa
SUMOylated 5-HT1A-R
70kDa
50kDa
SUMOylated 5-HT1A-R
NEM: + -

Figure 2. Overexpression of rat 5-HT1A-Rs in N2a cells. (A). The N2a cells were transfected with different amount of pcDNA4HisMaxC-5-HT1A-R plasmids. Membrane fractions were isolated 48 hours after transfection and immunoblotted with a mouse Xpress antibody. Several bands were detected. A band near 42kDa might indicate the unmodified transfected 5-HT1A-Rs; a band around 50kDa might represent SUMO-1-5-HT1A-Rs; several bands above 50kDa might indicate other post translational modifications of transfected 5-HT1A-Rs. N2a cells can express pcDNA4HisMaxC-5-HT1A-R construct and 14ug of plasmids results in optimal expression levels. **(B).** 450ug of protein is an optimal amount for immunoprecipitation. 250-750ug protein of cell membrane preparation was immunoprecipitated with a mouse SUMO-1 antibody and immunoblotted with a mouse Xpress antibody. **(C).** Protein bands at 48kDa, 70kDa~250kDa indicate the SUMOylation of transfected 5-HT1A-Rs. Membrane fractions were isolated in the absence or presence of NEM. The bands at 48kDa and 70kDa~250kDa significantly decreased in the absence of NEM. A band at the top of the blot does not decreased in the absence of NEM treatment, indicating nonSUMOylated protein aggregates.

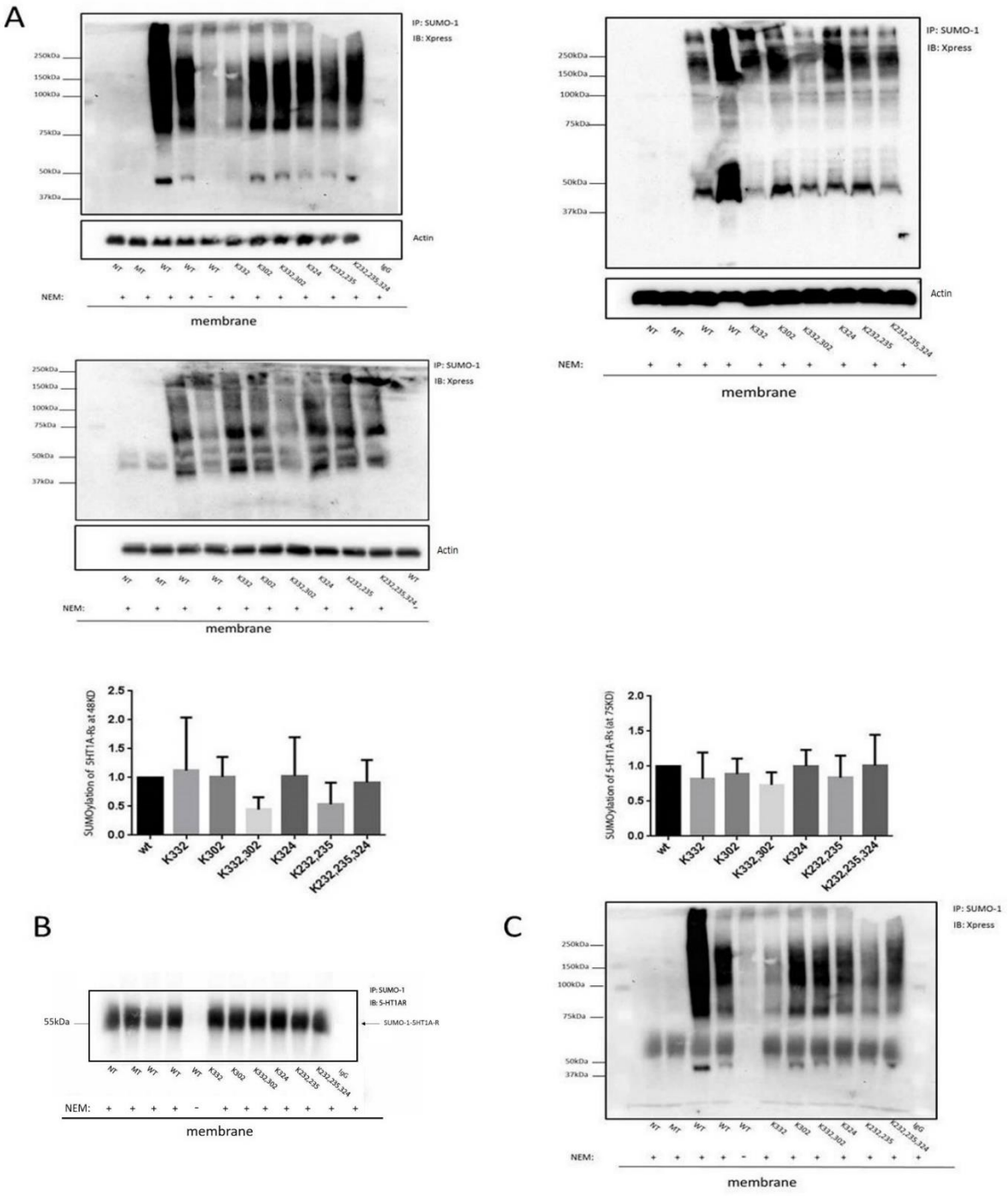

A
IP: SUMO-1
IB: Xpress
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
Actin
NT MT WT WT WT K332 K302 K332,302 K324 K232,235 K232,235,324 IgG
NEM: + + + + - + + + + + + +
membrane
IP: SUMO-1
IB: Xpress
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
Actin
NT MT WT WT K332 K302 K332,302 K324 K232,235 K232,235,324
NEM: + + + + + + + + + +
membrane
IP: SUMO-1
IB: Xpress
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
Actin
NT MT WT WT K332 K302 K332,302 K324 K232,235 K232,235,324 WT
NEM: + + + + + + + + + +
membrane
SUMOylation of 5HT1A-Rs at 48KD
2.5
2.0
1.5
1.0
0.5
0.0
wt
K332
K302
K332,302
K324
K232,235
K232,235,324
SUMOylation of 5-HT1A-Rs (at 75KD)
2.0
1.5
1.0
0.5
0.0
wt
K332
K302
K332,302
K324
K232,235
K232,235,324
B
IP: SUMO-1
IB: 5-HT1AR
55kDa
SUMO-1-5HT1A-R
NT MT WT WT WT K332 K302 K332,302 K324 K232,235 K232,235,324 IgG
NEM: + + + + - + + + + + + +
membrane
C
IP: SUMO-1
IB: Xpress
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
NT MT WT WT WT K332 K302 K332,302 K324 K232,235 K232,235,324 IgG
NEM: + + + + - + + + + + + +
membrane

Figure 3. Identification of SUMOylation sites on 5-HT1A-Rs. Six 5-HT1A-R mutants were constructed and transfected into N2a cells, including K332R; K302R; K332,302R; K324R; K232,235R; K232,235,324R. **(A).** Membrane fraction was collected, immunoprecipitated with a rabbit SUMO-1 antibody and examined on immunoblots with a mouse Xpress antibody. The experiment was repeat five times (all blots from five repeats are shown). Quantification of 48kDa band and 75kDa band from 3 separate experiments shows that there is no significant difference between transfection groups for the 48kDa band (n=3, one-way ANOVA, $F(6, 17) = 0.9182$, $p=0.5059$). Quantification of 75kDa bands shows no significant difference between groups due to transfection of the mutant or wild-type 5-HT1A-R (n=3, one-way ANOVA, $F(6, 14) = 0.1483$, $p=0.9864$). **(B).** The immunoblot shown in the upper right corner was next incubated with a 5-HT1A-R antibody to look at the endogenous SUMOylation of 5-HT1A-Rs. A band at 55kDa was detected, which indicates the expression of SUMO-1-5-HT1A-Rs in the presence of NEM. SUMO-1-5HT1A-Rs were not detected in the absence of NEM which further verifies the band at 55kDa represents the SUMOylation of 5-HT1A-Rs. **(C).** Figure 3A and Figure 3B were merged. The 48kDa band does not overlap with 55kDa band, demonstrating that these are different molecular mass protein bands.

Chapter Ⅳ. Discussion

In this study, we focused on the deSUMOylation of 5-HT1A-Rs and the SUMOylation sites on 5-HT1A-Rs. Our studies have demonstrated that 1). Transfected SENP1, 2 and 6 can be expressed in the membrane fraction of N2a cells; 2). Transfection of SENP1, 2 and 6 had a significant effect on de-SUMOylation of 5-HT1A-Rs. 3); N2a cells express 5-HT1A-Rs and transfected rat 5-HT1A-Rs can be SUMOylated. 4); Transfection of 5-HT1A-R mutant has no significant effect on SUMOylation of 5-HT1A-Rs after statistical analysis of protein bands at 48kDa and 75kDa; 5). SUMOylation of transfected rat 5-HT1A-Rs has different molecular mass bands compared to SUMOylation of endogenous 5-HT1A-Rs in N2a cells.

There are 7 SENPs (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7, and SENP8) in mammals. All these SENPs are involved in the regulation of SUMOylation except SENP8. Previous studies on SENPs demonstrated the distribution of SENPs in nucleus and cytosol, however, the SUMO machinery can also translocate to different regions of neurons during development [76, 77]. SUMO-1-5-HT1A-Rs are located at ER and Golgi, and in the detergent-resistant membrane (DRM) where the active 5-HT1A-Rs are located. A previous study in which PIAS proteins were transfected into N2a cells confirmed the distribution of PIAS protein in the membrane. Here, I verified the expression of SENP1, 2 and 6 in membrane fraction of N2a cells and found that SENP1 was expressed more abundantly (Figure 1A) in the membrane fraction. 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. To determine if SENP1 expressed more abundantly than other SENPs in the cell membrane, more evidence is required. For example, we can further isolate cell membrane by ultracentrifugation.

In the experiment to identify the effect of SENPs on deSUMOylation of 5-HT1A-Rs, I also transfected MYC-SENP3, MYC-SENP5, RGS-SENP3, and RGS-SENP5 constructs into N2a cells, but I was not able to detect the expression of these proteins. The MYC-SENP3, MYC-SENP5, RGS-SENP3 and RGS-SENP5 constructs were sequenced by ACGT, Inc. in order to rule out the possibility about incorrect sequence. Different MYC antibodies and RGS antibodies were used in the immunoblot but none of them can detected the expression of transfected proteins including the positive control. Since I could not verify the expression of these constructs, the effects of these SENPs on SUMOylation of 5-HT1A-Rs are unreliable. To solve this problem, we could try different antibodies for the MYC tagged and RGS tagged proteins or to choose SENPs specific antibodies to verify the expression of SENP proteins.

Transfection of SENP2 seemed to decrease the SUMOylation of 5-HT1A-Rs (Figure 1B), and one-way ANOVA showed there is a significant difference of SUMOylation of 5-HT1A-Rs after transfection with SENP1, 2 and 6. However the post-hoc test showed there was no significant difference when each transfected SENPs group was compared to the non-transfected control group. There are two possible explanations.

First, the variability of the data might contribute to the lack of statistical significance. Since SENP1, SENP2 and DESI-1 are the only three SUMO proteases that have a preference for SUMO-1 and 5-HT1A-Rs can be SUMOylated by SUMO-1, SENP1, SENP2, and DESI-1 are possibly involved in the deSUMOylation of 5-HT1A-Rs [50]. Moreover, there are no reports about the isopeptidase effects of DESI-1, suggesting that DESI-1 might not be involved in the cleavage of SUMO protein from its substrate [50]. Thus, SENP1 and SENP2 are more likely to play a role in the deSUMOylation of 5-HT1A-Rs, especially SENP2 according to the present study (Figure 1B). Another possible explanation is that deSUMOylation of 5-HT1A-Rs is processed by an undiscovered SUMO protease belonging to deSUMOylating isopeptidases. To further identify the SUMO proteases involved in the deSUMOylation of 5-HT1A-Rs, a fluorescence resonance energy transfer (FRET) assay can be performed [39]. FRET is a process by which the excited state energy of a fluorescent donor molecule is transferred to an acceptor molecule [39]. Efficient energy transfer requires very close proximity, and can therefore be used as a read-out for covalent protein interactions such as SUMOylation [39]. Moreover, a SUMO-CHOP-Reporter assay platform would be able to test the SUMO proteases activity to further study the mechanism of SUMOylation of 5-HT1A-Rs [39]. The SUMO-CHOP-Reporter assay platform was developed by Progenra, Inc. A reporter construct was created by fusing His6-tagged small ubiquitin-like modifier (SUMO) to the amino terminus of the reporter enzyme phospholipase A2 (PLA2) [39]. Following cleavage by a SENP, free PLA2 is able to hydrolyze its substrate, resulting in the release of a fluorescent product which is

readily quantifiable using a fluorimeter or a fluorescence plate reader [39].

Here, I expressed rat 5-HT1A-Rs in N2a cells and verified that rat 5-HT1A-Rs can be SUMOylated in N2a cells. In the initial experiment, I immunoblotted the membrane fraction with the same rabbit 5-HT1A-R antibody as I used in the SENPs experiment. However, there was no change in the level of 5-HT1A-Rs after transfection of rat 5-HT1A-Rs (data not shown). The lack of expression of 5-HT1A-Rs might be due to the rabbit 5-HT1A-R antibody only recognizing endogenous 5-HT1A-Rs. So, we immunoblotted membrane fraction with a mouse Xpress antibody to detect Xpress tagged 5-HT1A-Rs in N2a cells (Figure 2A). A concentration-response assay showed that 14ug of plasmids transfection can express transfected 5-HT1A-Rs. To only look at SUMOylation of transfected 5-HT1A-Rs, I next immunoprecipitated membrane fractions with a mouse SUMO-1 antibody and immunoblotted with a mouse Xpress antibody. Unfortunately, mouse IgG heavy chain was detected at 55kDa which is the expect position of SUMO-1-5-HT1A-Rs. To prevent detection of IgG heavy and light chain, we chose another rabbit SUMO-1 antibody from Dr. Yoshiaki Azuma. The mouse Xpress antibody detected protein bands at ~48kDa and above ~70kDa. The bands were further verified in the absence of SUMO protease inhibitor NEM (Figure 2C), suggesting SUMOylation of transfected 5-HT1A-Rs. To further confirm the SUMOylation of transfected 5-HT1A-Rs, N2a cells can be treated with SUMOylation inhibitors such as ginkgolic acid and measure the level of SUMOylation.

Our lab previously reported several SUMOylated protein bands which were detected by a 5-HT1A-R antibody at ~75kDa, ~55kDa. The 55kDa band was consistently present but the 75kDa was not. The protein band at 55kDa was shown to be a SUMO-1 modified 5-HT1A-Rs, but the identity of the protein band at 75kDa remains unclear [24]. However, here we observed protein bands at ~48kDa and 70kDa~250kDa but not 55kDa. Possible explanations include that 5-HT1A-Rs might be SUMOylated at multiple lysine sites, a cross-talk between SUMOylation and other post-translational modifications or the formation of polySUMO chains. The formation of polySUMO chains would significantly increase the molecular weight of SUMO-conjugation, which is consistent to the high molecular weight of bands. The editing of polySUMO chains by SENPs generate variable length SUMO chains, reflected on the SDS-PAGE as multimeric cleavage intermediates. The protein band at ~48kDa might indicate one SUMO protein added to 5-HT1A-Rs as an intermediate since the predicted molecular weight of 5-HT1A-R is ~42kDa and a single SUMO-1 protein is around 8~10kDa. Thus, the previously reported 55kDa protein band might result from two SUMO proteins bound to 5-HT1A-Rs. To further verify the formation of polySUMO chains, a SUMO-2/3 antibody could be used for immunoblotting, since polySUMO chains are formed by SUMO-2/3. Another alternative explanation is the crosstalk of other post-translational modifications on SUMO-1-5-HT1A-Rs. Studies have shown post-translational modifications involved in 5-HT1A-Rs include palmitoylation, glycosylation and phosphorylation. Glycosylation usually results in an additional high molecular weight polysaccharide to the protein and thus causes a high

molecular mass complex. Thus, the crosstalk between glycosylation and SUMOylation might contribute to the 70kDa~250kDa bands. Palmitoylation can add a single palmitoyl modification or be dually modified with one or more palmitoyl groups to cysteine and less frequently to serine and threonine residues of proteins. It is reported that palmitoylation of 5-HT1A-Rs occurs at Cys 417 and Cys 420 and the molecular weight of palmitoylated 5-HT1A-Rs is around 46kDa [21], it is possible that two palmitoyl groups are added to SUMO-1-5-HT1A-Rs and results in a molecular weight at ~55kDa. However, phosphorylation only provides an additional 80Da phosphate group to the protein which does not seems to change the molecular weight of the protein by much. So, it is likely that the protein band at 55kDa is caused by the cross-talk between SUMOylation and palmitoylation, and two palmitoyl groups might added to the SUMO-1-5-HT1A-Rs.

I speculate that the 55kDa band might be caused by two SUMO protein SUMOylation or two palmitoyl groups added to SUMO-1-5-HT1A-Rs and the 48kDa band from transfected 5-HT1A-Rs might be only one SUMO-1 protein SUMOylation. One possible reason that may have happened is that transfected 5-HT1A-R does not process through ER and Golgi and might not be palmitoylated, thus exists as a 48kda band instead of the 55kDa band. In my experiment, I used a lipofectamine 3000 transfection method. This method can deliver foreign DNA plasmids into nucleus, but foreign DNA will not integrate with the host genome. In this way, it is possible that the expressed proteins do not process through ER and Golgi. In addition, the

70kDa~250kDa bands are more likely caused by SUMOylation at multiple sites than the glycosylation, because most of the glycosylation also occurs in ER and Golgi [78]. There is another possibility that those modified proteins aggregated together and formed high molecular complex. Thus, without processing through ER and Golgi network, the transfected 5-HT1A-Rs are unable to process post translational modifications like endogenous 5-HT1A-Rs and thus, causes different molecular weight bands. A transfection approach such as lentivirus might help solve this problem. Lentivirus are RNA viruses that carry a gene for a reverse transcriptase that transcribes the viral genetic material into a double stranded DNA intermediate [79]. This DNA intermediate is then incorporated into the host DNA allowing the host cell machinery to produce the desired protein [79]. Additionally, because the viral genome is stably integrated into the host DNA, any modification that has been made will be passed to all daughter cells that are derived from the transfected cell [79]. In this way, the transfected 5-HT1A-Rs can process post translational modifications and enter proper signaling pathways similar to the endogenous 5-HT1A-Rs.

After transfection of rat 5-HT1A-R mutants into N2a cells, there is no significant difference in SUMOylation of 5-HT1A-Rs, which means the mutations failed to prevent 5-HT1A-Rs from SUMOylation. As I discussed above, the rat 5-HT1A-R might not be expressed properly. Another possible scenario is that other lysine residues got SUMOylated instead of 6 candidate lysine residues. A proteomic mass spectrometry approach would be able to tell us all the SUMOylation sites on

5-HT1A-Rs, however this approach requires a large amount of protein.

In conclusion, my experiments found that N2a cells can express transfected 5-HT1A-Rs, and that transfected SENP1, 2 and 6 are expressed in the membrane fraction of N2a cells. I also found that transfection of SENP1, 2, and 6 causes a significant difference among groups but additional works is needed to improve the observed variability and to allow for a more definitive conclusion with respect to which SENP may regulate the SUMOylation of 5-HT1A-Rs.

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