Role of serotonin 4 receptor in the growth of axon and dendrites of hippocampal neurons during the embryonic development in mice

（マウス海馬ニューロンの軸索と樹状突起形成におけるセロトニン4型受容体の役割）

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Abstract

Brain development requires synergy in the various genetic, biochemical and physiological factors to maintain the energy homeostasis of this process, and imbalance in these factors adversely affects the brain development. Importantly, neurotransmitter system is one of the crucial biochemical machineries which fuel the growth of neurons, synaptogenesis and circuit formation in the brain. Among them, malfunctioning in the monoaminergic neurotransmitter systems such as serotonin (5-hydroxytryptamine, 5-HT) causes structural and functional impairment. The appearance of 5-HT during early embryonic development signifies its importance in the brain development. Altered 5-HT levels in the embryonic brain during critical periods of development has long-lasting effects on brain function, particularly on anxiety/depression-related behaviors in adulthood. In the emotional stress during postnatal life, 5-HT controls the remodeling of neural circuits via neurogenesis, which lends the idea about the developmental role of 5-HT in adult brain. Actions of 5-HT are mediated by its receptors, which are classified into 7 classes with 14 different subtypes. Each receptor exhibits a distinctive pharmacology, distribution and function in the brain. Among them, 5-HT4 receptor (5-HT4R) has become one of the foci of the recent research due to its pharmacological importance and distribution in the central nervous system. Additionally, emerging data suggest that 5-HT4R is abundant in certain brain areas and plays an important role in emotion and cognition. In continuation, it has been reported that activation of 5-HT4R increases the expression of neurotrophic factors such as brain derived neurotrophic factor (BDNF), which are known to promote the growth of neurons via TRK/Akt pathway. However, the specific role of 5-HT4R in the development of hippocampal neurons is still not well studied.

In the present *in vitro* study, I examined the roles of 5-HT4R in the growth of hippocampal neurons during the development of mouse brain. Hippocampal neurons of embryonic day 18 mouse (C57BL/6) were dissociated and cultured for 4 days. First, the role of 5-HT4R in the growth of hippocampal neurons were assessed using a 5-HT4R agonist RS67333. Further, immunohistochemistry using antibodies against microtubule associated protein 2 (MAP2) and SMI-31 as well as scanning electron microscopy were performed to investigate the effect of RS67333 on the formation of axons and dendrites in the culture of hippocampal neurons. I found that treatment with RS67333 increased axonal length, diameter and branching along with total dendritic length, number of primary dendrites and their branching. Additionally, I compared the effect of another 5-HT4R agonist BIMU8 and 5-HT to that of RS67333 on the growth promotion of neurites. Results confirmed that treatment with 5-HT4R agonists promoted the growth of axon and dendrites. Next, I examined the specificity of the effect of 5-HT4R agonist using 5-HT4R antagonist GR125487. The effects of RS67333 on growth of axons and dendrites were neutralized by the concomitant treatment of GR125487, which confirmed the specific role of the 5-HT4R. To investigate the molecular cascades underlying the 5-HT4R promoted growth of

neurites; link between 5-HT4R activation and mRNA expression of *BDNF, nerve growth factor (NGF), neurotrophin-3 (NT-3), tyrosine receptor kinase (TRK)* and *collapsin response mediator protein-2 (CRMP2)* were investigated. CRMP2 is expressed in early embryonic stage and facilitate the growth of axon and dendrite formation. Neurotrophic factors bind to specific TRK receptors (TRK-A, TRK-B, TRK-C) and induce their autophosphorylation, which initiates TRK/protein kinase B (Akt) pathway and dephosphorylates the CRMP2 which promotes the growth of neurites. RT-qPCR analysis showed that the treatment of RS67333 upregulated the mRNA expression of neurotrophic factors (*BDNF, NGF, NT-3*) and *TRK-A* together with *CRMP2*. Further, colocalization study using immunohistochemistry showed that 5-HT4R and CRMP2 both were colocalized in the dentate gyrus, CA1, CA2 and CA3 area of hippocampus at embryonic day 18. In addition, 5-HT4R and CRMP2 both were colocalized in cell bodies, dendrites from base to tip and axons from base to terminal in the primary culture of hippocampal neurons. It has been reported that dephosphorylation of CRMP2 promotes the growth of neurites. Based on this colocalization, I investigated the effect of 5-HT4R activation on the phosphorylation/dephosphorylation of CRMP2 using western blot technique. Phosphorylation/dephosphorylation of CRMP2 modulates the dynamics of cytoskeletal proteins such as tubulin and control the growth of neurons. Result from western blot suggested that the RS67333 treatment upregulated the expression of dephosphorylated CRMP2 in comparison to the control group. Furthermore, to investigate the effect of 5-HT4R mediated increase of *CRMP2* expression on the neurite growth, I performed knockdown study of the expression of *CRMP2* mRNA in the presence of 5-HT4R agonist. It was shown that transfection of hippocampal neurons with *CRMP2 siRNA* inhibited the RS67333-induced growth of axons and dendrites, which suggests that *CRMP2* is required for the 5-HT4R-mediated neurite growth. In conclusion, the current findings provide a new layer of understanding of the developmental role of 5-HT4R in the brain via modulating the expression of CRMP2 to promote the growth of hippocampal neurons in the developing brain.

Keywords

Brain development,

Collapsin response mediator protein-2,

Hippocampal development,

Neural developmental disorder,

Neurotrophic factors,

Formation of axons and dendrites,

Serotonin,

Serotonin 4 receptor,

Tyrosine receptor kinase

Abbreviations

5-HT: 5-hydroxytryptamine (Serotonin)

5-HT4R: 5-hydroxy trptamine-4 receptor (Serotonin-4 receptor)

5-HTR: 5-hydroxytryptamine receptor (Serotonin Receptor)

ADAM10: a disintegrin and metalloproteinase domain-containing protein 10

ADHD: attention deficit hyperactivity disorder

Akt/ PKB: protein kinase B

APP: amyloid precursor protein

Arc: activity-regulated cytoskeleton-associated protein

ASD: autism spectrum disorder

BACE1: beta-secretase 1

BDNF: brain derived neurotropic factor

CNS: central nervous system

CRMP2: collapsin response mediator protein-2

ECLs: extracellular loops

Erk: extracellular-signal-regulated kinase

GABA: gamma-aminobutyric acid

GPCR: G-protein-coupled receptor

GSK 3β: glycogen synthase kinase 3 beta

ICLs: intracellular loops

IHC: immunohistochemistry

IP3-kinase: phosphatidylinositol-4,5-bisphosphate 3-kinase

MAPK: mitogen-activated protein kinase

NGF: nerve growth factor

NT-3: neurotrophin-3

p75NTR: p75 neurotrophin receptor

pCRMP2: phosphorylated collapsin response mediator protein-2

PI-3: phosphoinositide-3

SEM: standard error of mean

SSRI: selective serotonin reuptake inhibitor

TMHs: trans-membrane helices

TRK: tyrosine receptor kinase

Introduction

1. Brain development and factors affecting the process

Developing brain is subject to endless modifications due to the continual changes in the genetic profile due to the stimulation provided by external environment. The elucidation of the different stages of embryonic brain development gives insights to understand the deficits that can arise due to lack of growth factors, genetic mutation and malfunctioning in neurotransmitter system such as serotonin (5-HT). Brain development is influenced by both endogenous genetic and exogenous environmental factors. Coordination of these factors is essential to lead the proper development of the brain. Endogenous factors involve genetic or molecular and neurotransmitter system in the brain. In continuation, the most important exogeneous environmental factors are temperature, gravity, stress, and mother-infant interaction (Mrozek et al., 2012; Rydze et al., 2017; Wagenführ et al., 2015; Wakayama et al., 2009). The importance of endogenous factors in the brain development is being explained in the following points.

1.1. Genetic factors which regulate brain development

Brain development is one of the most complex biological processes, orchestrated by the precisely timed and coordinated expression of thousands of genes (Douet et al., 2014; Lenroot and Giedd, 2008; Liscovitch and Chechik, 2013). Initial stages of brain development are strongly affected by genetic factors. As the brain develops, specialization of brain regions occurs, and their structure and function reflect unique sets of expressed genes (Douet et al., 2014). Genetic factors act in synergy with environmental factors to produce the distinct neuronal and molecular environment at different locations in the brain (Lenroot and Giedd, 2008). Additionally, some reports suggested the role of inherited genetics in the brain development, which changes over the course of development in a regionally specific fashion in the brain. For example, distinct biochemical nature and morphology of neurons are anatomically regionalized and specialized in function (Liscovitch and Chechik, 2013). Genetic factors also play important roles in brain development even after birth. In the initial postnatal 3 years significant development happens in the human brain. In the first year, brain becomes double in size due to extensive synapse formation (Douet et al., 2014; Leung and Jia, 2016). Till the end of third year, brain attains 80 percent volume of an adult brain. Thus, previous findings suggest that genetic factors play a vital role in shaping the brain architecture.

1.1.1. Role of neurotrophic factors and phosphoinositide-3 kinase pathway in the brain development

Neurotrophins such as NGF, BDNF, and NT-3/4 are essential for neural differentiation, growth and survival (Henderson, 1996; Tsai, 2017). Thus, neurotrophins play important roles to regulate the development and function of central and peripheral nervous systems. Neurotrophins promote the growth of neurons by activating two different classes of receptors, the receptor tyrosine kinases family (TRK-A, TRK-B and TRK-C) and p75 neurotrophin receptor (p75NTR, a member of the TNF receptor superfamily); through them neurotrophins regulate many signaling pathways (Stewart et al., 2008). These pathways can be activated, including those mediated by Ras and members of cdc-42/rac/rho like small signaling G-proteins, mitogen-activated protein kinase (MAPK), phosphoinositide-3 (PI-3) kinase, and jun kinase cascades (Huang and Reichardt, 2001). Early embryonic expression of TRK-C, a receptor for NT-3, in neural plate and neural tube suggests the significance of neurotrophins in the brain development (Averbuch et al., 1994). In contrast, TRK-B and TRK-A are expressed later than TRK-C (Bernd, 2008). However, both NT-3 and BDNF affect early brain development via promoting neuroblast survival, proliferation, and differentiation (Cohen et al., 2010; Zhou and Rush, 1996). This leads to the idea for the investigation of the role of neurotrophins in neuronal and glial development, neuroprotection, and modulation of synaptic interactions (Huang et al., 2007; Huang et al., 2012; Kowiański et al., 2018). In continuation, the secretion of neurotrophins in hippocampal neurons is evoked by long-term-potentiation-inducing electrical stimulation, which facilitates the increased plasticity via promotion of growth and arborization of neurites (Gartner and Staiger, 2002). Additionally, neurotrophins do not only control and ensure the proper development of the brain but also regulate the energy storage and expenditure in the brain, to maintain the biochemical synthesis and secretion of neurotransmitters (Fargali et al., 2012; Pedersen et al., 2009; Waterson and Horvath, 2015). Limited expression of neurotrophins is required locally in the brain for the coordinated growth and biochemical synthesis of neurotransmitters. BDNF and its receptor TRK-B are widely expressed in the brain (Waterson and Horvath, 2015) and control the energy balance via CNS innervation to autonomic nervous system and control the biosynthesis and release of neurotransmitters (Fargali et al., 2012). Additionally, NGF, NT-3, together with BDNF modulate the development of CNS via downstream signaling products such as VGF (non-acronymic), that plays a significant role in the controlled growth of neurons (Pedersen et al., 2009; Yang et al., 2018).

It has been well established that BDNF, NT-3 and NGF induce the increased plasticity, and facilitate the learning and memory by regulating the neurite outgrowth and branching by Akt pathway, as shown in Figure 1 (Cohen et al., 2010; Foltran and Diaz, 2016; Habtemariam, 2018; Henderson, 1996). Recently, our group reported that increased expression of BDNF and stimulation of TRK-B receptor promote the growth of dendrites in hippocampal neurons (Kozono et al., 2017). Further, growing body of evidences suggests that BDNF, NT-3 and NGF dephosphorylate CRMP2 via Akt/

extracellular-signal-regulated kinase (Erk) /Sema-3A cascades (Niisato et al., 2013; Yamashita et al., 2012; Yoshimura et al., 2005; Zhang et al., 2018) and promote the neurite outgrowth (Figure 1). Overexpression of these neurotrophins increases the phosphorylation of Akt at Ser-473 and GSK-3β at Ser-9, which results in the decrease of the phosphorylation level of CRMP2 at Thr-514. Resultant increase in dephosphorylated active CRMP2 promotes the formation of neurites (Yoshimura et al., 2005). Therefore, CRMP2 is an important target in studies which focus on the molecular mechanism of the brain development.

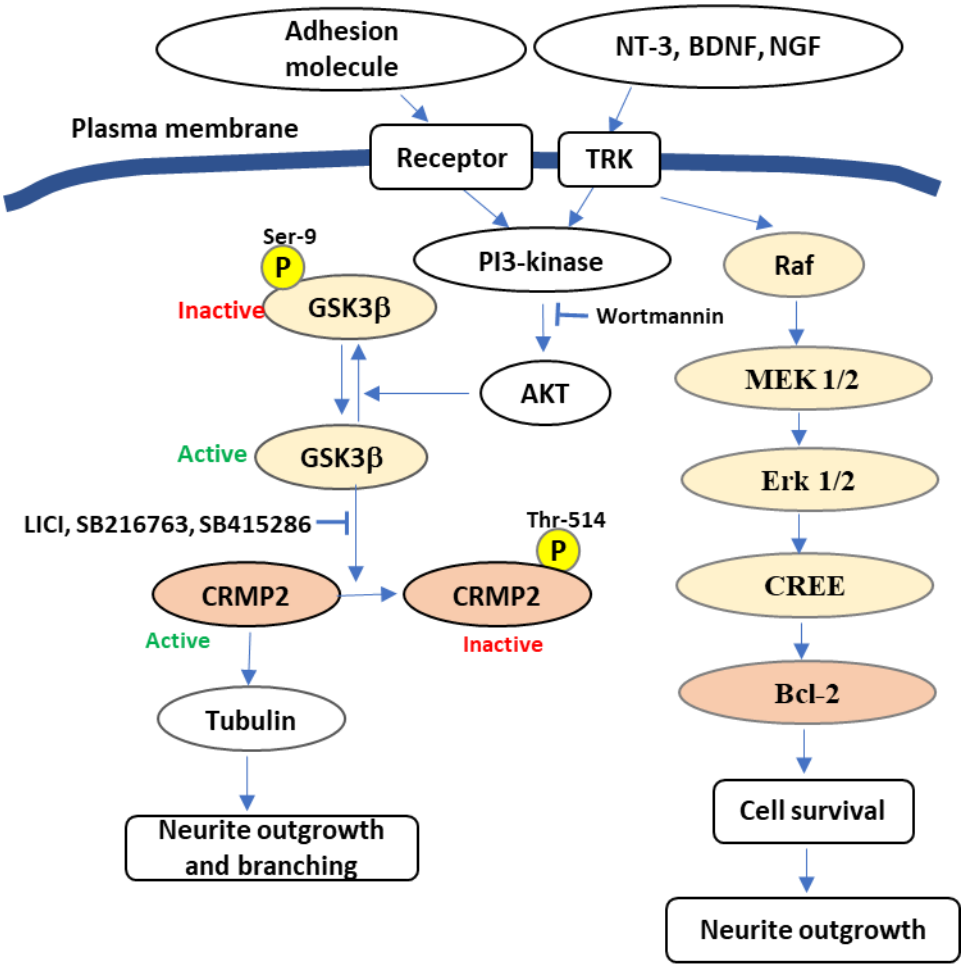

Figure 1. Schematic representation of PI-3/Akt pathway. Activation of TRK receptors (TRKA, B, and C) by neurotrophins, activate the PI-3 kinase which further inhibits the phosphorylation of CRMP2 and controls the availability of active CRMP2 (dephosphorylated), and promotes the growth of neurites.

1.1.2. Role of CRMP2 in the brain development

CRMP family of proteins were discovered to be one of the first proteins expressed in newly born neurons in the developing brain (Minturn et al., 1995), and CRMP2 expression has been shown to be induced by factors that promote neuronal differentiation such as noggin, chordin, glial cell line-

derived neurotrophic factor (GDNF), and fibroblast growth factor (FGF) (Schmidt and Strittmatter, 2007). CRMP family consists of five intracellular phosphoproteins (CRMP1, CRMP2, CRMP3, CRMP4, CRMP5) of similar molecular size (60–66 kDa) and high (50–70%) amino acid sequence identity. CRMPs are predominantly expressed in the nervous system during development and play important roles in neurites formation, guidance and collapse of growth cones through their interactions with microtubules (Arimura et al., 2004; Liu and Strittmatter, 2001). Cleaved forms of CRMPs have also been linked to neuron degeneration after trauma induced injury (Taghian et al., 2011). Among five members of the CRMP family, CRMP2 is highly expressed in the adult brain, especially in post-mitotic neurons of the olfactory system, cerebellum, and hippocampus (Kadoyama et al., 2015). CRMP2 is widely expressed in the embryonic brain and plays important role in neurite formation and synaptic plasticity through the interactions with cytoskeletal proteins (Yoneda et al., 2012). CRMP2 may form the hetero-oligomeric configuration due to interactions between its' dimeric and tetrameric form; Ca^{2+} and Mg^{2+} stabilizes its tetrameric form (Stenmark et al., 2007; Sumi et al., 2018). The C-terminus of CRMP2 consists of 80 amino acids and is the site of phosphorylation for various kinases (Schmidt and Strittmatter, 2007). CRMP2 expression is induced by neuronal growth promoting and differentiation factors such as noggin, chordin, GDNF, FGF, BDNF, NT-3 and NGF (Schmidt and Strittmatter, 2007). CRMP2 has been reported to control the growth of neurites via TRK/Akt pathway as shown in Figure 1 (Niisato et al., 2013; Yamashita et al., 2012; Yoshimura et al., 2005; Zhang et al., 2018). In continuation, CRMP2 regulates neuronal polarity by controlling microtubule dynamics and promotes the axon formation. In addition, CRMP2 plays important roles in growth cone guidance and collapse through interactions with microtubules (Inagaki et al., 2001; Yoshimura et al., 2005). CRMP2 expression is highest when neurons and synaptic connections mature actively during the first postnatal week in the mouse cerebellar granule neurons, suggesting the role of CRMP2 in neuronal network formation (Charrier et al., 2003).

In the adult brain, CRMP2 expression is significantly downregulated and limited in areas associated with brain plasticity, neurogenesis, or regeneration. Due to its high expression in adult hippocampus, CRMP2 is supposed to play an important role in the adult neurogenesis, learning and memory. In addition, CRMP2 has been proved an important therapeutic target in various psychiatric and neurodegenerative diseases such as Alzheimer disease (Charrier et al., 2003; Ryan and Pimplikar, 2005). Relation between BDNF, NT-3 and NGF with CRMP2 has been reported via Akt/Erk/Sema-3A cascades (Niisato et al., 2013; Yamashita et al., 2012; Yoshimura et al., 2005; Zhang et al., 2018). Overexpression of these neurotrophic factors increases the phosphorylation of Akt at Ser-473 and GSK-3β at Ser-9, which results in the decrease of the phosphorylation level of CRMP2 at Thr-514. Increase in non-phosphorylated active CRMP2 induces the formation of neurites (Yoshimura et al., 2005). Additionally, CRMP2 has been reported to promote axon formation (Inagaki et al., 2001) and arborization of dendrites in CA1 region of the hippocampus (Niisato et al., 2013).

Phosphorylation/dephosphorylation of CRMP2 undergoes small conformational changes at the C-terminal tail with shifting the surface charge, which not only alters the interactions within the CRMP2 tetramer but also alters the interactions with GTP-tubulin (Sumi et al., 2018). Therefore, based on above findings, in the present study, I examined the effect of 5-HT on the levels of *CRMP2* mRNA and phosphorylated and non-phosphorylated CRMP2 (Martinowich and Lu, 2007; Mattson et al., 2004).

1.2. Role of neurotransmitter systems in the brain development

Several neurotransmitters such as 5-HT, dopamine, glutamate and acetylcholine have been reported to promote or inhibit neurite outgrowth, depending on the neuronal group and the neurotransmitter involved (Torra and Britto, 2002). Pharmacological blocking and stimulation of neurotransmitter systems are one major tool to study the roles of neurotransmitters in the development. Some neurotransmitters change their effects during development, e.g. gamma-aminobutyric acid (GABA) is excitatory in the embryo, but inhibitory after birth due to a switch from a high to low chloride content in neurons (Herlenius and Lagercrantz, 2001; Levitt et al., 1997). It is possible that this is important for the wiring of neuronal network in early life. At birth, there is a surge of neurotransmitters such as monoamines, which may be of importance for the neonatal adaptation, differentiation and neuronal growth. Monoamines including 5-HT, dopamine, and norepinephrine are the principal neurotransmitters that mediate a variety of the CNS functions, such as motor control, cognition and emotion (Kobayashi, 2001). In this regard, dysfunction of monoamines and their metabolites as well as the maladaptive alternations of their receptors during the development may cause some neurologic and neuropsychiatric disorders (Huang et al., 2012; Zhang et al., 2012). Amongst them, 5-HT has been reported to play significant roles in the neuronal growth, synaptogenesis, brain's circuitry formation and plasticity. Therefore, in the present study, my focus was to explore the role of 5-HT system in the brain development.

2. Role of 5-HT and its receptors in the brain development

5-HT is a monoamine (Figure 2) which is known to play important roles during development (Daubert and Condron, 2010). The development of 5-HT starts early at embryonic days 11-12 in the rat (Lauder, 1990). The ontogeny of the axonal projections of 5-HT neurons may be divided into following overlapping four periods in rats: initial axon elongation (E12-E16), development of selective pathways (E15-E19), terminal field formation (E19-E21), and entrance into well-defined fiber tracts such as the fasciculus retroflexus, stria medullaris, external capsule, fornix, and supra-callosal stria. Axons from these tracts form terminal arborizations in the thalamus, hypothalamus, basal and limbic forebrain, and cerebral cortex (Lidov and Molliver, 1982).

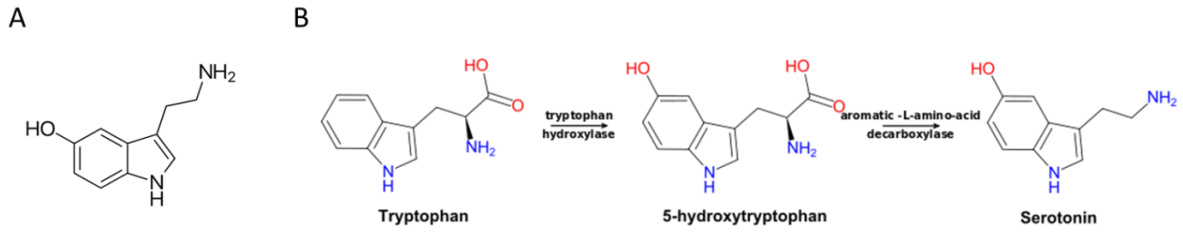

Figure 2. Molecular structure and chemical synthesis of 5-HT. Molecular structure of 5-HT (A), and endogenous synthesis route of 5-HT from tryptophan (B).

Early appearance of 5-HT neurons in the embryonic brain prior to synaptogenesis and the stabilization of neurotransmission systems suggests that 5-HT has crucial roles in neural development (Foote and Morrison, 1987; Gaspar et al., 2003; Hayashi et al., 2010; Lauder, 1990). New genetic models that target the 5-HT system show that alterations in 5-HT homeostasis modify the fine wiring of brain connections and cause permanent changes in adult behavior (Gaspar et al., 2003). In addition, evidence from molecular, pharmacological, and clinical studies also demonstrate the huge importance of 5-HT system for normal development and functioning of CNS (Gaspar et al., 2003; Lv and Liu, 2017; Sodhi and Sanders, 2004). 5-HT acts as a growth factor during development, and activity of 5-HT receptors (5-HTRs) forms a crucial part of the cascade of events leading to changes in brain structure. The 5-HT system interacts with BDNF, S100beta, and other neurotransmitter systems such as GABAergic, glutamatergic, and dopaminergic systems, which facilitate the initiation of various molecular cascades for the development of the brain circuitry (Barnes and Sharp, 1999; Sodhi and Sanders, 2004). Somehow, disruption in these cascades may contribute to the pathophysiology of CNS disorders that are associated with the impaired brain development. Clinically, many psychiatric drugs alter 5-HT activity and have been shown to lead changes in brain structure such as the hippocampus, which suggest the specific role of 5-HT in the development and functioning of the hippocampus (B. Mitchell et al., 1990; Berumen et al., 2012; Dale et al., 2015).

2.1. 5-HT receptor subtypes, expression and their functions

Activity of 5-HT is controlled by its receptors and transporters. 5-HTRs are classified into 14 subtypes with 7 families from 5-HT1R to 5-HT7R (Table 1) and are expressed in the brain and gut (Figure 3). Other than the 5-HT3R which is a ligand-gated ion channel, all the 5-HTRs are G-protein-coupled metabotropic receptors. The 5-HT4R is coupled with Gs protein and facilitates adenylate cyclase, leading to increase in intracellular cAMP concentration (Bockaert et al., 2006b; Pascual et al., 2012; Pilar et al., 2013).

Table 1. Classification of 5-HTRs. 7 classes of 5-HTRs, their coupled G protein working mechanism and further classification in sub types of each class have been shown.

Family	**Type**	**Mechanism**	**Postsynaptic Potential**	**Subtypes**
5-HT1	Gi/Go-protein coupled.	Decreasing cellular levels of cAMP.	Inhibitory	5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT1F,
5-HT2	Gq/G11-protein coupled.	Increasing cellular levels of IP3 and DAG.	Excitatory	5-HT2A, 5-HT2B, 5-HT2C,
5-HT3	Ligand-gated Na^+ and K^+ cation channel.	Depolarizing plasma membrane.	Excitatory	-
5-HT4	Gs-protein coupled.	Increasing cellular levels of cAMP.	Excitatory	-
5-HT5	Gi/Go-protein coupled.	Decreasing cellular levels of cAMP.	Inhibitory	5-HT5A, 5-HT5B
5-HT6	Gs-protein coupled.	Increasing cellular levels of cAMP.	Excitatory	-
5-HT7	Gs-protein coupled.	Increasing cellular levels of cAMP.	Excitatory	-

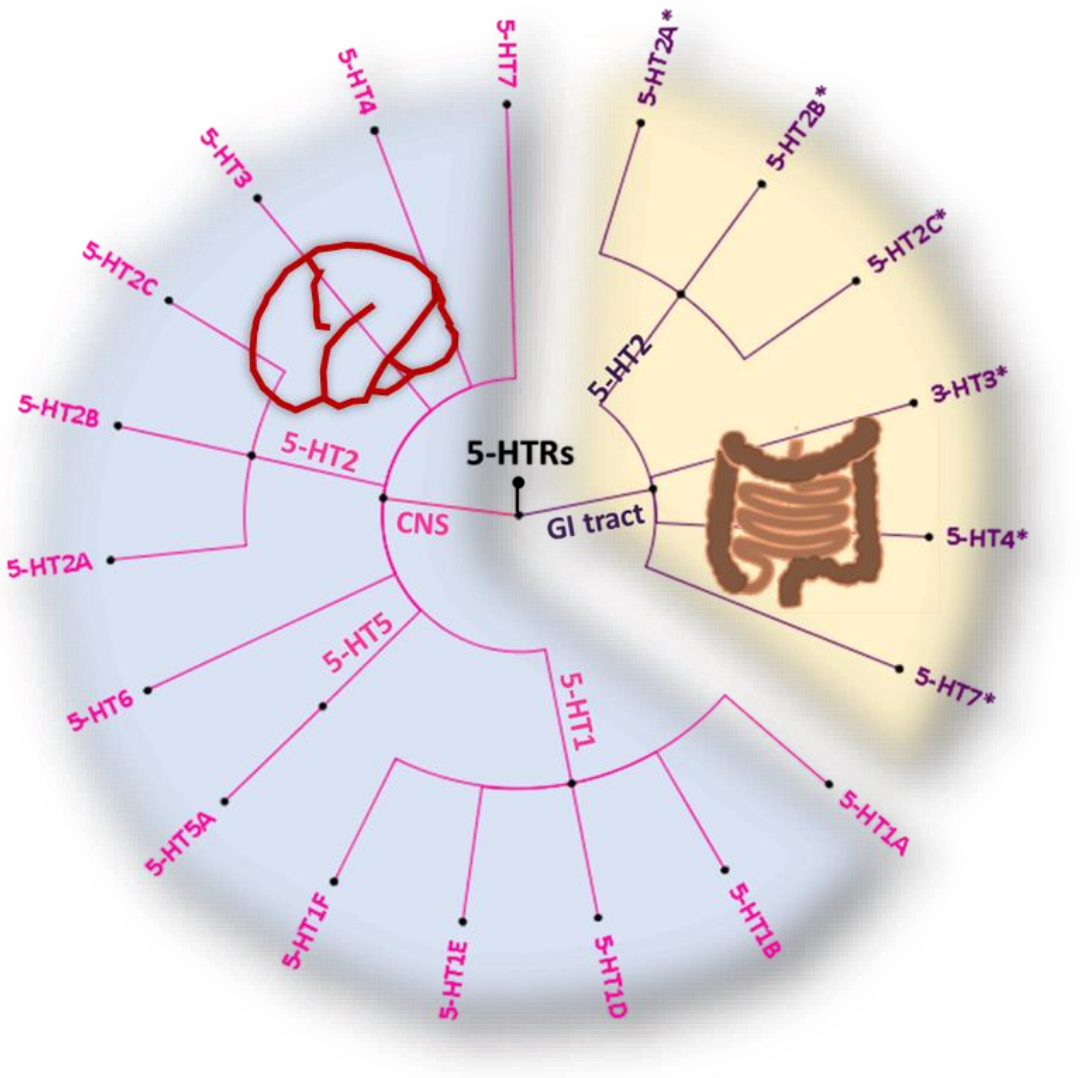

Figure 3. Circular tree representation of various 5-HTRs in CNS and gut

During the development inadequate functioning and distribution of 5-HTRs result in severe potential threats for the neural, behavioral and psychological health (Table 2) (Barnes and Sharp, 1999; Hurley and Tizabi, 2013; Marazziti, 2017; Sikander et al., 2009). Among 5-HTRs, 5-HT4R has become an important therapeutic target for the treatment of depression and Alzheimer's disease, due to their broad spectrum of expression and functioning (Bockaert et al., 2004; 2008; Bureau et al., 2010; Cho and Hu, 2007).

Table 2. **Classification, expression and function of 5-HTR subtypes**. Table shows the expression of all 14 subtypes of 5-HTR in the CNS, and their functions.

Type of 5-HTR	Expression in CNS	Function	References
5-HT1A	yes	Addiction, Autoreceptor, Aggression, Anxiety, Sleep, Appetite, Sexual behavior, Mood, Memory, Nausea, Nociception, Sociability, Thermoregulation,Vasocontraction	Garcia et al., 2014; Meneses, 2014a
5-HT1B	yes	Addiction, Autoreceptor, Aggression, Anxiety, Sexual Behavior, Mood, Memory, Learning, Locomotion, Penile erection	Clark and Neumaier, 2001; Meneses, 2014b
5-HT1D	yes	Anxiety, Autoreceptor, Locomotion	De Vries et al., 1999; Pullar et al., 2004; Skingle et al., 1996
5-HT1E	yes	Memory	Klein and Teitler, 2012; Meneses, 2014c
5-HT1F	yes	Migraine	Meneses, 2014c; Ramadan et al., 2003
5-HT2A	yes	Addiction, Anxiety, Appetite, Cognition, Imagination, Learning, Memory, Perception, Sleep, Thermoregulation, Sexual behavior	Guiard and Giovanni, 2015; Meneses, 2014d; Raote et al., 2007
5-HT2B	yes	Anxiety, Appetite, Cardiovascular function, Sleep, Vasodilation, GI motility	Diaz et al., 2012; Meneses, 2014d
5-HT2C	yes	Anxiety, Appetite, cardiovascular function, Sleep, Vasocontraction, GI motility, Thermoregulation, Sexual behavior, Mood, Locomotion, Penile erection, Locomotion	Canal and Murnane, 2017; Meneses, 2014d
5-HT3	yes	Addiction, Anxiety, Appetite, Learning, Memory, GI motility, Nausea	Lummis, 2012; Meneses, 2014e; Thompson and Lummis, 2007
5-HT4	yes	Anxiety, Appetite, Learning, Memory, GI motility, Mood, Respiration	Bockaert et al., 2004; Bureau et al., 2010; Hagena and Manahan, 2017; Kozono et al., 2017; Meneses, 2014f
5-HT5A	yes	Autoreceptor, Locomotion, Sleep	Gonzalez et al., 2013; Meneses, 2014g; Thomas, 2006
5-HT5B	No	Functions in rodents, Pseudogenes in humans	Grailhe et al., 2001; Meneses, 2014g
5-HT6	yes	Anxiety, Cognition, Learning, Memory, Mood	Geng et al., 2018; Meneses, 2014h; Ramírez, 2013; Woods et al., 2012
5-HT7	yes	Anxiety, Auto receptor, Respiration, Memory, Mood, Sleep, Thermoregulation	Ciranna and Catania, 2014; Hedlund, 2009; Meneses, 2014i; Nikiforuk, 2015

2.2. 5-HT4 receptor expression profile, structure and function

2.2.1. Early expression of 5-HT4R in the brain

It has been reported in the adult mouse that various regions of CNS such as olfactory system, basal ganglia, amygdala, septal regions, hippocampus, hypothalamus, thalamus, cerebral cortex, midbrain, pons, medulla, cerebellum, and spinal cord have abundant expression of 5-HT4R (Figure 4). However, early embryonic expression of 5-HT4R is limited in the limbic regions such as hippocampus and amygdala (Berthouze et al., 2005; Bockaert et al., 2004; 2008; Wang et al., 2017). Recently, it has been reported that 5-HT4R promotes the growth of dendrites in the hippocampus of rat embryos (Kozono et al., 2017; Trakhtenberg and Goldberg, 2012). Additionally, in early embryonic stage, 5-HT4R innervation is also important for the information processing in the hippocampus and cognitive functioning in brain (Hagena and Manahan, 2017). These findings suggest that the early expression of 5-HT4R is very essential for the proper development and functioning of the hippocampus.

2.2.2. Structure of 5-HT4R

5-HT4R is a Gs-protein-coupled receptor (GPCR). This membrane bound receptor has 3 extracellular loops (ECLs), 7 trans-membrane helices (TMHs), and 4 intracellular loops (ICLs), including C-terminal helix (Figure 5). The ECLs contain the N-terminal, ligand-binding domain and allosteric regulator sides. TMHs contain 3 important peptide domains at TM2, TM4 and TM7 helices, which control the signal transduction after binding of the ligand to the extracellular ligand-binding domain (Padayatti et al., 2013). Ligand binding to the binding pocket decreases oxidation rates at the peptide domains in TM helices. Resultant increase in the oxidation rates at C-terminal domain is observed, which facilitates the interaction between C-terminal domains to various proteins like CRMP2, SNX-27, MAGI-2, and NHERF-1 and controls trafficking, targeting and remodeling of neuronal cytoarchitecture (Bockaert et al., 2008; Castriconi et al., 2018; Padayatti et al., 2013). Hence, C-terminus of 5-HT4R may be important for the anatomical and functional characteristics of the brain. Therefore, these modifications in the oxidation of peptide domains in TM helices, ECLs, and ICLs are critical for ligand recognition and signal transduction. Human *htr4* gene encompassing 38 exons spanning over 700 kb, therefore, multiple C-terminal isoforms are expressed in specific tissues (Bockaert et al., 2004; Coupar et al., 2007; Rebholz et al., 2018).

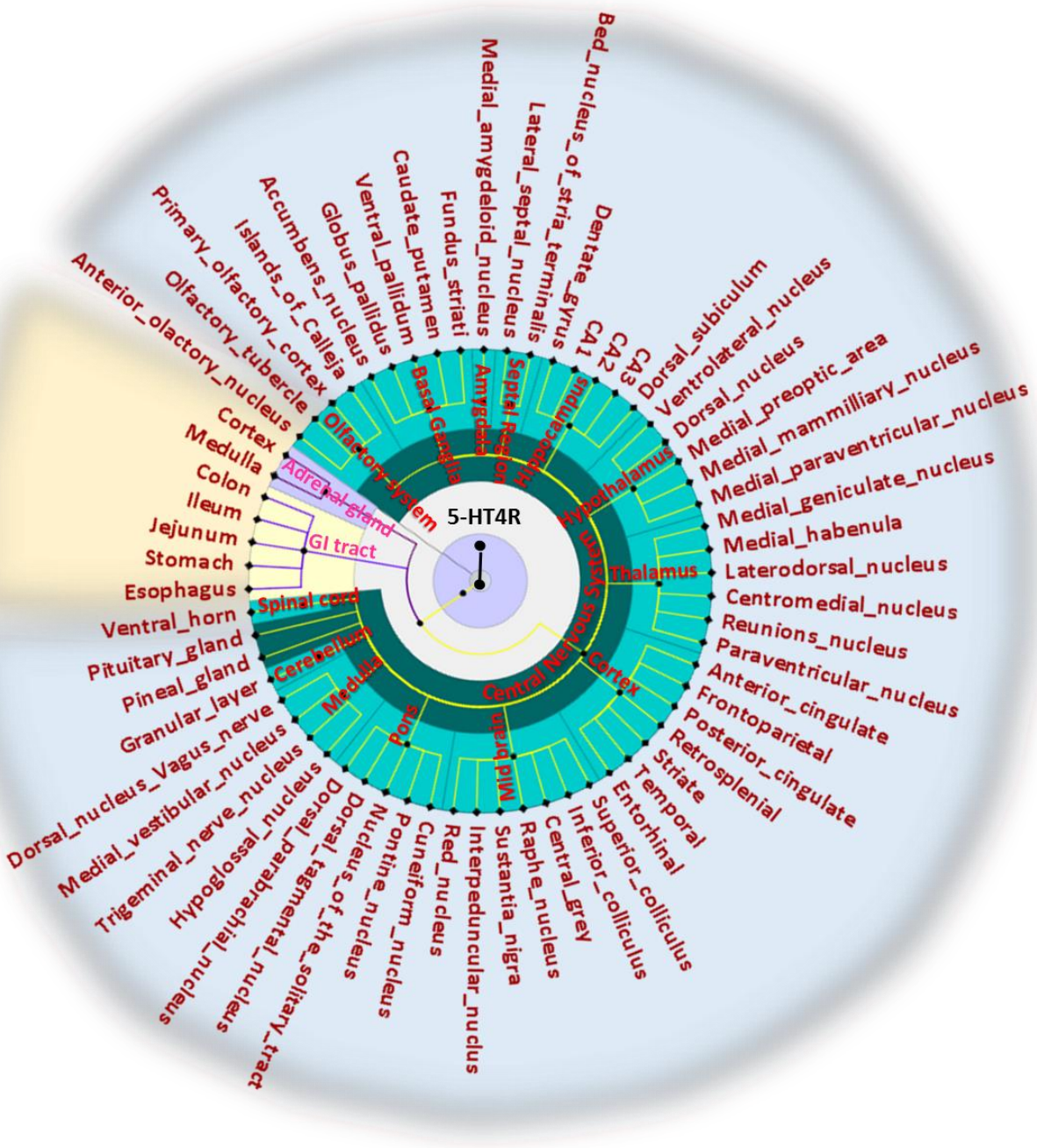

Figure 4. Expression of 5-HT4R. 5-HT4R is expressed in the various regions of CNS (olfactory system, basal ganglia, amygdala, septal regions, hippocampus, hypothalamus, thalamus, cortex, mid brain, pons, medulla, cerebellum, and spinal cord), and GI-tract.

There are 11 human 5-HT4R splice variant. Among them C-terminus of human 5-HT4R has 10 splice variants, a, b, c, long c, d, e, f, g, i and n (Table 3), which differ in their C-termini after a single position (L358) (Blondel et al., 1998; Bockaert et al., 2004; Brattelid et al., 2004; Coupar et al., 2007; Ray et al., 2009). 5-HT4R is widely expressed both in CNS and gut (Bockaert and Dumuis, 1998; Reynolds et al., 1995; S. Hegde, 1998). 5-HT4R isoforms (a–i and n) are highly expressed in the CNS except isoform (d) (Blondel et al., 1998; Bockaert et al., 2004; Vilaró et al., 2002). Among them,

isoform (a) and (b) is highly expressed in the amygdala, hippocampus, nucleus accumbens, caudate nucleus and pituitary gland. In continuation, isoform (c) is highly expressed in the pituitary gland and to a lesser degree in the caudate nucleus, hippocampus, and putamen. Further, isoform (g) seems to be highly expressed in the hypothalamus and cortex (Claeysen et al., 1998) and isoform (n), which lacks the alternatively spliced C-terminal exon is abundantly expressed in human brain regions involved in mood disorders such as frontal cortex and hippocampus (Vilaró et al., 2002). While isoform (d) is not present in the CNS, it is found in the small intestine together with isoform (a). Hence, selective therapeutic intervention of particular variant of 5-HT4R in the specific brain regions might be a very helpful to increase the specificity and effectiveness of the treatments, caused by the abnormality in the specific brain or gut regions.

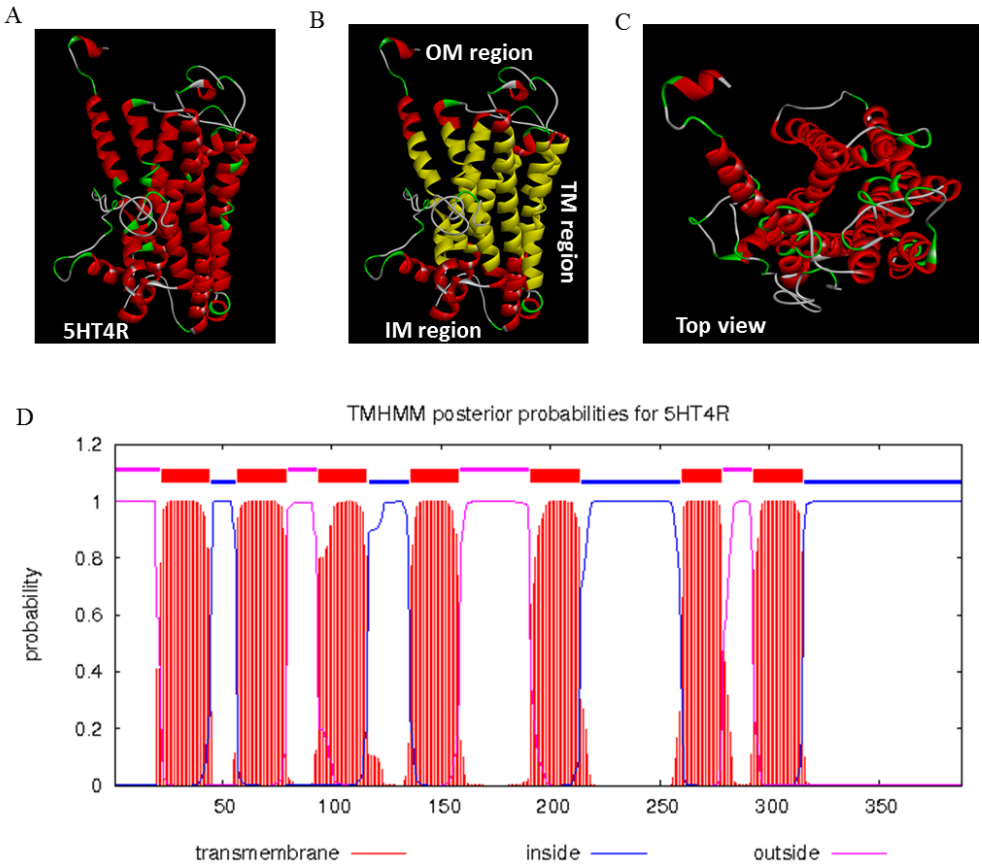

Figure 5. 3D structure of 5-HT4R. Homologous 3D model of 5-HT4R protein of *Mus musculus* generated using I-TASSER (A). Outer membrane (OM), trans membrane (TM) and inner membrane (IM) regions (B). Top-view of 5-HT4R showing the tunnel of the channel protein (C). Figure representing probability of the protein regions falling in OM, TM and IM regions, predicted using TMHMM server (D).

Table 3. C-terminal splice variants of human 5-HT4R. Table represents all 10 splice variants of human 5-HT4R, a, b, c, long c, d, e, f, g, i and n, which differ in their C-termini after a single position (L358).

5–HT4R Splice Variant	Variance in the C-terminal sequence
a	STTTINGSTHVLRYTVLHRGHHQELEKLPIHNDPESLESCF
b	STTTINGSTHVLRDAVECGGQWESQCHPPATSPLVAAQPSDT
c	STTTINGSTHVLSSGTETDRRNFGIRKRRLTKPS
long c	STTTINGSTHVLSSGTETDRKKLWNKEEKIDQTIQMPKRKRKKKASLSYEDLI LLGRKSCFREGK
d	STTTINGSTHVLRF
e	STTTINGSTHVLSFPLLFCNRPVPV
f	STTTINGSTHVLSPVPV
g	STTTINGSTHVLSGCSPVSSFLLLFCNRPVPV
i	STTTINGSTHVLRTDFLFDRDILARYWTKPARAGPFSGTLSIRCLTARKPVLG DAVECGGQWESQCHPPATSPLVAAQPSDT
n	STTTINGSTHVLR

2.2.3. Functional roles of 5-HT4R in the brain

5-HT4R plays vital roles both in developing and adult brain (Figure 6). During development, 5-HT4R is highly expressed in the limbic region of the brain and plays an important role in the neuronal development. Recently, our group reported that 5-HT4R agonist BIMU8 promoted the growth of dendrite in the culture of hippocampal neurons from the rat embryo (Kozono et al., 2017), which suggests the importance of 5-HT4R in the hippocampal development.

In the adult brain 5-HT4R is involve in the pathophysiology of neurodegenerative diseases. Previous studies reported that 5-HT4R has complex variant C-terminus due to alternative splicing of the mRNA. It was reported that SNPs in C-terminus of 5-HT4R at IVS1+15T/C, IVS3+6G/A, IVS3–63C/T, IVS4−36T/C, g.83097C/T, g.83159G/A, g.83164(T)9–10, and g.83198A/G, were observed in schizophrenia and in depressive disorders in a Japanese population (Ohtsuki et al., 2002). In addition, several studies have found that depressed violent suicide victims have differential patterns of 5-HT4R binding and cAMP concentration levels in different brain regions (Rosel et al., 2004). Moreover, in major depressive disorder lower expression of 5-HT4R in the striatal region has been observed, which suggests the potential role of the 5-HT4R in mood disorders (Amigo et al., 2016; Madsen et al., 2014). Furthermore, roles of 5-HT4R in the inducing mechanism of Alzheimer's disease have been shown (Geldenhuys and Van der Schyf, 2011). A recent study reported that deposition of neurofibrillary tangles and amyloid precursor protein (APP) are cleared and patients showed significant improvements in motor activities when treated with the 5-HT4R based therapeutics (Coskuner and Uversky, 2018). 5-HT4R drives APP processing towards the beneficial non-amyloidogenic pathway via direct interaction with α-secretase, a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) or β-secretase (BACE1), which leads to the breakdown of APP into soluble APP without any toxic effect (Baranger et al., 2017; Cochet et al., 2013; Giannoni et al., 2013; Lalut et al., 2017). Through this

mechanism 5-HT4R also controls tau pathology by modulating the activity of GSK-3 via $G_{12/13}$ proteins. Additionally, recent studies have shown that 5-HT4R also controls the expression of myelin genes, which are involved in the synthesis of myelin sheet surrounding axons to facilitate the conduction of nerve impulses (Bockaert et al., 2008; Coskuner and Uversky, 2018; Geldenhuys and Van der Schyf, 2011). Thus, based on these studies it may be hypothesized that impaired expression and function of 5-HT4R induce serious threat for the neuronal, psychological and behavioral health, which further leads to severe form of neuronal disorder.

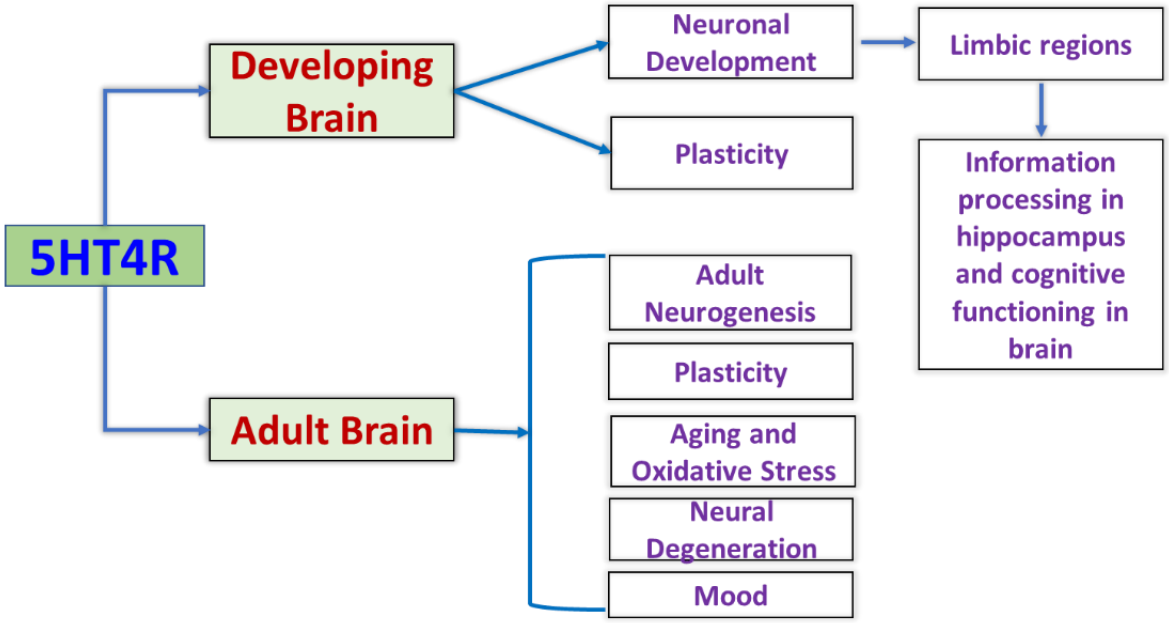

Figure 6. Schematic representation of roles of 5-HT4R in developing and adult brain.

3. Defining the problem and hypothesis of the current study

During development 5-HT4R is highly expressed in the emotional center of limbic forebrain but its' specific role in the hippocampus development is not well studied. Therefore, in the present study I hypothesized that "5-HT4R during development promotes the growth of axons and dendrites of hippocampal neurons via modulating the expression of neurotrophic factors and CRMP2". I defined the following objectives to validate my hypothesis:

- What is the role of 5-HT4R in the growth of axons and dendrites in hippocampal neurons?
- Does 5-HT4R function affect the expression of BDNF, NT-3 and NGF, which promote the growth of neurons?
- Does 5-HT4R function affect the expression of TRK receptors, which control the neurotrophins- mediated growth of neurons?
- Do 5-HT4R and CRMP2 both are co-expressed and colocalized in embryonic hippocampus?

- Does 5-HT4R affect the expression and phosphorylation of CRMP2 which promote the neurite growth and synapse formation in the embryonic brain?

4. Thesis outline

The present study aimed to explore and elucidate the developmental role of 5-HT4R in the mouse brain (Figure 7). I particularly focused on the hippocampus which is very important in the information processing, learning, memory and cognition. I measured the growth of hippocampal neurons in terms of axonal length, diameter and branching along with dendritic length and branching. Previous studies have reported that the neurotrophins (BDNF, NT-3, and NGF) phosphorylate the tyrosine receptor kinases (TRK-A, TRK-B, and TRK-C), which inhibit the phosphorylation of CRMP2 (Haddad et al., 2017; Jeanneteau et al., 2008; Rahajeng et al., 2010; Stewart et al., 2008; Usuki et al., 2018) and promote the growth of neurites. Based on these studies, to explore the downstream signaling, I investigated the association between the activation of 5-HT4R to the mRNA expression of neurotrophic factors (*BDNF*, *NT-3*, and *NGF*) and *CRMP2* altogether with the dephosphorylation of CRMP2, which are involved in the development and growth of neurites.

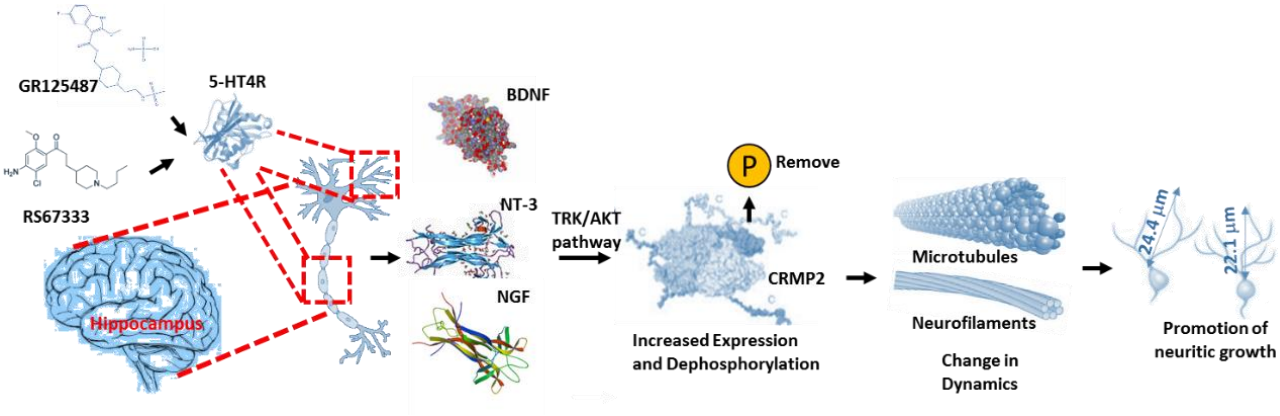

Figure 7. Pictorial representation of the study plan for role of 5-HT4R in the growth of neurites. 5-HT4R agonist RS67333 and antagonist GR125487 were used to activate and inhibit the function of the receptor in axon and dendritic formation.

Materials and methods

1. Materials

1.1. Animals

C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) were used in the present study. The use of animals followed the guide for the Care and Use of Laboratory Animals described by the National Institutes of Health (U.S.A.) and were approved by the Animal Experiment Committee of University of Tsukuba (Japan).

1.2. Chemicals and reagents

To culture embryonic hippocampal neurons, I used trypsin-EDTA (Life Technologies, Carlsbad, CA, U.S.A.), polyethyleneimine (Sigma Aldrich, St. Louis, MO, U.S.A.), minimal essential medium (Life Technologies), L-glutamine (Life Technologies), glutamate (Wako, Osaka, Japan), penicillin/streptomycin (Sigma Aldrich), heat-inactivated fetal bovine serum (FBS, Life Technologies), neurobasal medium (Life Technologies), and B-27 supplement (Life Technologies). As 5-HT related reagents, I used 5-HT4R agonists; RS67333 (Tocris Biosciences, Bristol, UK) and BIMU8 (Sigma Aldrich), 5-HT4R antagonist; GR125487 (Tocris Bioscience), and 5-HT (Sigma Aldrich). For histological examination, I used paraformaldehyde (Sigma Aldrich), glutaraldehyde (Sigma Aldrich), osmium tetroxide (OsO_4, Sigma Aldrich), OCT compound (Fisher Scientific, Pittsburgh, PA), normal goat serum (NGS, Invitrogen), Triton X-100 (Sigma Aldrich), rabbit anti-5HT4R polyclonal IgG (Bioss Inc, bs-12054R), chicken anti-microtubule-associated protein 2, polyclonal IgG (Chemicon, AB5543), mouse anti-SMI-31 IgG (Bio Legend, #801601), mouse anti-GAD65/67 IgG (Millipore, AB1511), rabbit anti-CRMP2 polyclonal IgG (Gene Tex, GTX113420), mouse anti-CRMP2 polyclonal IgG (Sant Cruz Biotech Inc, sc-376739), rabbit anti-pCRMP2 polyclonal IgG (Abcam, ab62478), anti-rabbit Alexa Fluor-488 (Life Technologies, Inc), anti-mouse Alexa flour 488 (Invitrogen), anti-mouse Alexa Fluor-594 (Life Technologies, Inc), anti-chick Alexa Fluor-594 (Life Technologies, Inc). For western blot analysis, 1x RIPA buffer (Santa Cruz Biotechnology, Inc.), Bradford reagent (Sigma Aldrich), ECL™ western blotting detection reagents (GE Healthcare), Amersham ECL anti-rabbit IgG, HRP linked secondary antibody (GE Healthcare), rabbit anti-β-actin polyclonal IgG (Cell Signaling Technologies, #4967S), Amersham ECL anti-mouse IgG, and HRP linked secondary antibody (GE Healthcare) were used. QuantiTect Reverse Transcription Kit (Qiagen) and SYBR Premix Ex Taq™ II (Takara Perfect Real Time) were used for quantitative RT-PCR. *CRMP2* siRNA transfection kit (Santa Cruz Biotechnology, Inc.) was used for knockdown study for *CRMP2*.

2. Methods

2.1. Dissociated culture of mouse hippocampal neurons

The day of the vaginal plug was considered the embryonic day (E) 0. Embryos at E18 were removed from pregnant mice under the deep anesthesia by isoflurane (Mylan, Tokyo, Japan). As shown in Figure 8, embryos were then quickly decapitated, and the brain was dissected. After the careful removal of meninges, the hippocampus was excised and incubated in 0.05% trypsin-EDTA for 5 min at 37˚C and cells were dissociated by trituration with a Pasteur pipette. After filtration with 70 µm nylon cell strainer (BD Falcon, San Jose, CA, U.S.A.), dissociated cells were plated at a density of 4 x 10^4 cells/well on 8-well chamber slides (NUNK, Rochester, NY, U.S.A.) which were coated with 0.2% polyethyleneimine. The cells were cultured in minimal essential medium, 0.5 mM L-glutamine, 25 µM glutamate and 25 µg/ml penicillin/streptomycin and 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 95% air and 5% CO2 at 37˚C. Later, 8 hours after plating, the serum medium was replaced by neurobasal medium with 2% B-27 supplement, 0.5mM L-glutamine and 25 µg/ml penicillin/streptomycin to remove proliferating glial and neuronal progenitors.

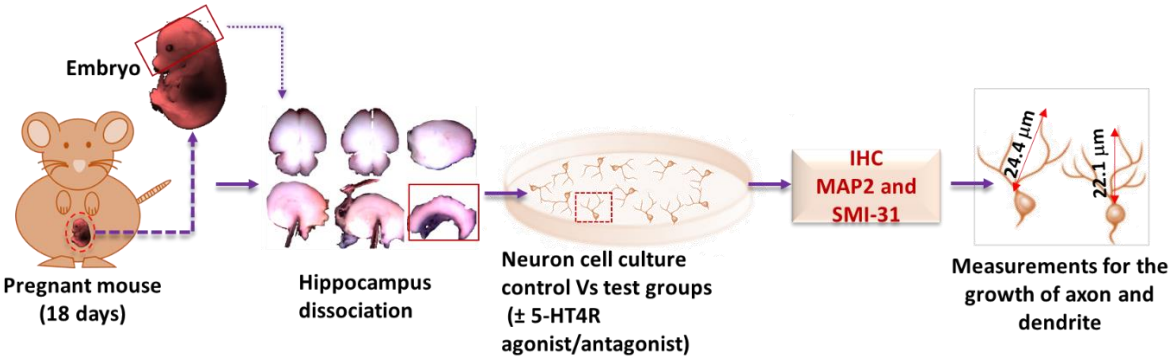

Figure 8. Flow chart representing the dissociation culture of mouse hippocampal neurons at E18. Hippocampi were dissected, and dissociation culture was performed. Further, drug treatment and immunohistochemistry were performed to analyze the role of 5-HT4R in the growth of axon and dendrites.

2.2. Immunohistochemistry of cultured neurons

Hippocampal neurons were cultured for 4 days as described above and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min at room temperature. Nonspecific antibody binding was blocked by incubation with 2% normal goat serum and 0.1% Triton X-100 in 0.1 M PB for 30 min. To examine the expression of 5-HT4R, CRMP2 and pCRMP2 in dendrites and axons, the cultured neurons were incubated overnight at 4˚C with the anti-5-HT4R, CRMP2, pCRMP2, MAP2, and SMI-31 primary antibodies (Table 4). I confirmed the specificity of the antibodies by immunostaining without the primary antibodies (negative staining), which yielded actually no staining. After the incubation with the primary antibodies, the cultured neurons were incubated with the

secondary antibodies for 1 h at room temperature. DAPI staining was performed to label the cell nuclei. X–Y plane or Z-stack images of the stained neurons were taken respectively at 40× or 63× with a confocal laser scanning microscope (LSM 510META ver.3.2, Carl Zeiss, Oberkochen, Germany).

Table 4. Primary antibodies and corresponding secondary antibodies used in the present study. IHC: immunohistochemistry.

Primary Antibody	Targeted Antigen	Secondary Antibody	Purpose
Rabbit anti-5HT4R poly clonal IgG (1:500, Bioss Inc, bs-12054R)	5HT4R	Anti-rabbit Alexa Fluor-594 and Alexa Fluor-488 (1:500, Life Technologies, Inc)	IHC
Chicken anti-MAP2 polyclonal IgG (1:400, Chemicon, AB5543)	MAP2 (Dendrite specific)	Anti-chick Alexa Fluor-594 (1:500, Life Technologies, Inc)	IHC
Mouse anti-SMI-31 IgG (1:500, Bio Legend, #801601)	Neurofilament-H (Axon specific)	Anti-mouse Alexa flour 488 (1:500, Invitrogen)	IHC
Rabbit anti-CRMP2 polyclonal IgG (IHC: 1:500; Western blot: 1:1000, Gene Tex, GTX113420) and Mouse anti-CRMP2 polyclonal IgG (IHC: 1:500; Western blot: 1:1000, Sant Cruz Biotech Inc, sc-376739)	CRMP2	Anti-mouse Alexa Fluor-488 and 594 (1:500, Life Technologies, Inc) and; Anti-rabbit Alexa Fluor-488 and 594 (1:500, Life Technologies, Inc) and, Amersham ECL anti-rabbit IgG, HRP linked secondary antibody (1:10,000, GE Healthcare) and; Amersham ECL anti-mouse IgG, HRP linked secondary antibody (1:10,000, GE Healthcare)	IHC, Western blot
Rabbit anti-pCRMP2 polyclonal IgG (IHC: 1:500; Western blot: 1:1000, Abcam, ab62478)	pCRMP2 (specific for phosphorylation at Thr-514)	Anti-mouse Alexa Fluor-488 and Alexa Fluor-405 (1:500, Life Technologies, Inc) & Amersham ECL anti-rabbit IgG, HRP linked secondary antibody (1:10,000, GE Healthcare)	IHC, Western blot
Rabbit anti-β -actin polyclonal IgG (IHC: 1:500; Western blot: 1:1000, Cell signaling Technologies, # 4967S)	β-actin	Amersham ECL anti-rabbit IgG, HRP linked secondary antibody (1:10,000, GE Healthcare)	IHC, Western blot
Mouse anti-GAD65/67 (1:400, Millipore, AB1511)	Glutamic acid decarboxylase (GAD)	Anti-mouse Alexa Fluor-594 (1:500, Life Technologies, Inc) s	IHC

2.3. Paradigm for the measurement of axon and dendrite growth

Previous studies reported dynamic changes of MAP2 immunoreactivity in developing neurites *in vitro* (De Lima et al., 1997; Hayashi et al., 2010). During the initial stages of neurite formation, all the neurites in cultured cortical neurons express MAP2 from base to the tip. Subsequently, the longest MAP2-positive neurite differentiates into an axon and loses MAP2-immunoreactivity gradually from the tip (De Lima et al., 1997; Hayashi et al., 2010). I confirmed that the longest MAP2-positive neurite

at 4 days in vitro (DIV) expressed SMI-31 (phosphorylated neurofilament-H), an axonal marker (Hayashi et al., 2010). Therefore, I identified the longest MAP2-positive neurite as a presumptive axon and remaining shorter neurites as dendrites in the present 4 DIV culture. I measured the total dendritic and total axonal length (total axon length = length of axon trunk + length of all axon collaterals) (Rockland, 2018), the number of primary dendrites which emerge directly from the cell body, the branching index of dendrites (number of branch points/number of primary dendrites), the branching index of axons (number of axon collateral branch points), the average dendritic length (total dendritic length/number of primary dendrites) using an image analyzing software (Neurocyte Image Analyzer ver. 14 1.5; Kurabo, Osaka, Japan) as shown in Figure 9. The branching index shows the ratio of the total number of branch points to the total number of primary dendrites or axons. Therefore, the branching index indicates the complexity of the arborization of dendrites and axons.

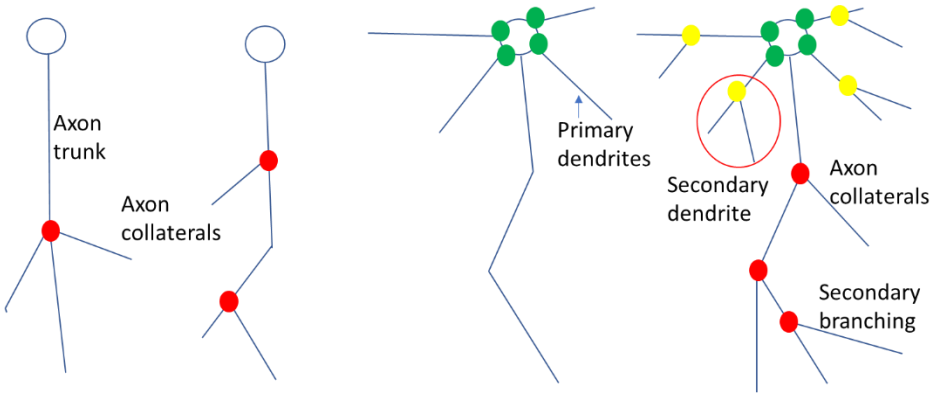

Figure 9. Diagrammatic representation of the terminologies and parameters used for the measurement of the growth of axon and dendrites. Total dendritic/axonal length = Sum (Length of all dendrites/axon trunk + collaterals); Average dendritic length = Total dendritic length / number of primary dendrites. Red circle shows the branching point of axon/or point of the emergence of axon collaterals. Green circle represents the point of emergence of primary dendrites from soma. Yellow circle denotes the point of emergence of secondary dendrites from primary dendrites.

2.4. Analysis of the axon and dendrite formation

As shown in Figure 10, hippocampal neurons were cultured for 4 days in the presence of 5-HT4R agonists RS67333 at concentrations of 1 nM, 10 nM, and 100 nM, and BIMU8 (100 nM) or 5-HT (100 nM). To confirm the specific effects of the 5-HT4R using a 5-HT4R antagonist GR125487 was added 30 minutes before the addition of 100 nM RS67333. RS67333, BIMU8, GR125487, and 5-HT were prepared in the neurobasal medium. I used the basal medium without the above compound as the control solutions in each experiment. The medium was changed at 2 DIV. At 4 DIV, neurons were fixed with 4% paraformaldehyde and incubated overnight at 4˚C with chick anti-MAP2 antibody and mouse anti-SMI-31 antibody as described in table 4. The neurons were then incubated with a mixture of Alexa

Fluor 594-conjugated goat anti-chick IgG antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG for 1 h at room temperature. X-Y plane images of the stained neurons were taken at 40x (1024*1024 pixel) with the confocal laser scanning microscope (LSM 510META ver.3.2, Carl Zeiss, Oberkochen, Germany).

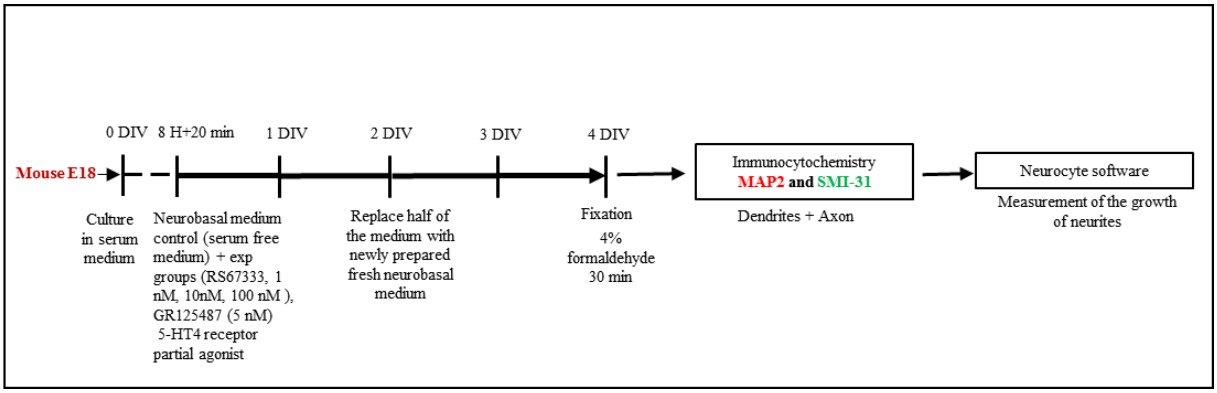

Figure 10. Flow chart represents the experimental paradigm for analysis of axon and dendrite growth. Dissociation culture was performed in the presence/absence of 5-HT4R agonist/antagonist, and neurons were fixed at 4 DIV. Immunocytochemistry was performed with SMI-31 and MAP2 and measurement was taken from neurocyte software for the growth of axon and dendrite.

2.5. Immunohistochemistry of brain sections

Brains were dissected from E18 mice and then fixed by rapidly replacing the phosphate buffered saline (PBS) with 4% paraformaldehyde for 24 hours at 4˚C. Further, brains were gradually immersed in 10%, 20% and 30% sucrose and then embedded in Tissue-Tek (OCT) compound. Embedded frozen brains were then coronally sectioned at 10 µm thickness by a cryostat (Leica CM3050 S, Leica Biosystems Inc., Buffalo Grove, IL). These sections were Nissl-stained to confirm the structure of hippocampus. Finally, brain sections containing hippocampus were immunostained with anti-5HT4R and anti-CRMP2 antibodies (Table 4).

2.6. Scanning electron microscope imaging of cultured neurons

To measure the axon diameter, hippocampal neurons were cultured on a glass coverslip using a 24 well plate (at a density of 4 x 10^4 cells/well) in the presence of 100 nM RS67333. At 4 DIV, the culture medium was replaced by pre-warmed (37˚C) fixative solution (3% glutaraldehyde). After the fixation for 20 mins at room temperature neurons were post-fixed in 2% OsO_4 for 30 minutes and then gradually dehydrated by 30%, 50%, 70%, 90% and 100% ethanol. Next, samples were dried in ‘critical point dryer’ to avoid shrinkage and collapse of surface artifacts. Later, sputter coating of platinum (1-2 nm thick layer) was done (B7340 Sputter Coater) for 1-2 sec on a completely dry sample Figure 11 (Heckman et al., 2007). Images were taken by scanning electron microscope (SNE 3200M Tabletop

Microscope, Nanoimages) at 3000x and 6000x magnification. Since axons and dendrites are difficult to distinguish in cell cultures, only isolated neurons in which the longest neurite could be distinguished were selected for the measurement of axon diameter. For the measurement of axon diameter, I selected the segment of the axon trunk just below the axon hillock to minimize the error in measurement of the neuronal process (Pesaresi et al., 2015).

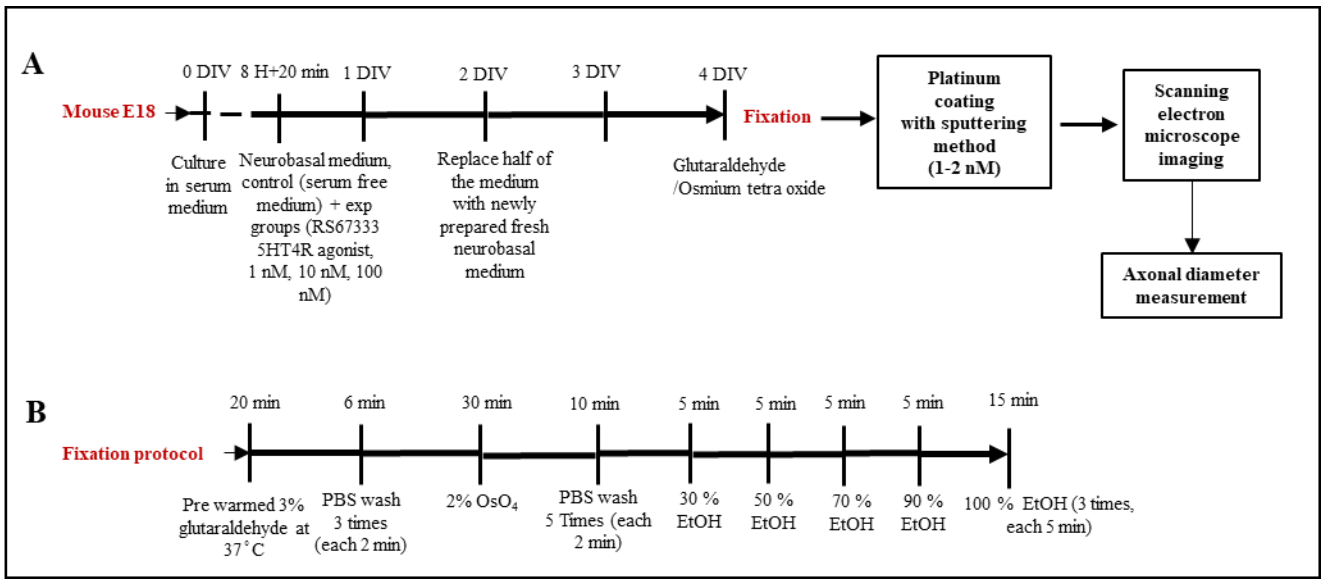

Figure 11. Flow charts explain the procedure of sample preparation for scanning electron microscope imaging for the measurement of axon diameter. (A) Flow chart represents the complete procedure of sample preparation for scanning electron microscope imaging and analysis. (B) Flow chart represents the fixation protocol for sample preparation.

2.7. Quantitative RT-PCR analysis

Hippocampal neurons were cultured for 4 days in the presence of 100 nM RS67333 on 24-well culture plates at a density of 2 x 10^5 cells/well (Figure 12). Control group was cultured without the treatment. The total RNA was then extracted from the cultured neurons using a NucleoSpin RNA XS kit (Takara, Shiga, Japan). The total RNA was diluted to 1:100 with RNase-free distilled water and the concentration of the total RNA was measured using a spectrophotometer (Pharmacia Biotech, Ultraspec 2000) to calculate 1 µg of cDNAs. The genomic DNAs were removed and the cDNAs were synthesized from 1 µg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). For PCR amplification, cDNA was added to the reaction mixture containing SYBR Premix Ex Taq™ II (Takara Perfect Real Time) and 0.2 M of the primers. Mouse *BDNF*, *NT-3*, *NGF*, *CRMP2*, *TRK-A*, *TRK-B*, *TRK-C*, and *GAPDH* primers (Table 5) were used for the quantification of their relative gene expression. GAPDH was used as an internal control. PCR was carried out on a Thermal Cycler Dice Real Time System (Takara TP800, software ver. 3.00) according to the following protocol: 5 s at 95°C and 30 s at 60°C - 50 cycles. The Ct values were calculated from the crossing point of the amplification curve and

threshold, and relative quantitative analysis of the targeted genes was carried out using a calibration curve. The expression of *GAPDH* as the internal control was used for compensation, and the relative expression of mRNA in the experimental groups was calculated when the expression of mRNA in the control group was set at 1.0.

Table 5. The sequence of mouse primers (5′ and 3′) used for RT-qPCR analysis.

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)
Akt	ATCCCCTCAACAACTTCTCAGT	CTTCCGTCCACTCTTCTCTTTC
BDNF	CAG CGCGAATGTGTTAGTGGTTA	CAGTGGACAGCCACTT TGTTTCA
CRMP2	TTTCCGCCACCTTGACTTG	ACAGGAACACGTGAACGGATT
GAPDH	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA
GSK-3β	TCCATTCCTTTGGAATCTGC	CAATTCAGCCAACACACACAGC
NGF	ATGGTGGAGTTTTGGCCTGT	GTACGCCGATCAAAAACGCA
NT-3	GCCCCCTCCCTTATACCTAATG	CATAGCGTTTCCTCCGTGGT
TRK-A	AGGGCCACATCATGGAGAAC	GTGCAGACTCCAAAGAAGC
TRK-B	GCGCGGCTCTGGGGCTTATG	CCTGAGTGTCGGGGCTGGATTTAG
TRK-C	TGCCCAGCCAAGTGTAGTTTCT	GCGCCTCCCCCTGTTCCT

2.8. Western blot analysis

Hippocampal neurons were cultured and treated with 100 nM RS67333 4 days on 24-well culture plates at a density of 2 x 10^5 cells/well (Figure 12). Control group was kept without the treatment. After washing with PBS, cells were lysed in 300 µl 1x RIPA buffer (Santa Cruz Biotechnology, Inc.) by gently rocking the 24 well plate. The lysate was centrifuged at 12,000 RPM (15,760 xg) for 10 mins at 4˚C, the supernatant was collected. Next, the protein concentration in the solution was measured using Bradford assay (Bradford, 1976). I used 20 µg/lane protein for the electrophoresis. Primary antibody staining was performed against pCRMP2, CRMP2 and β-actin proteins, which was followed by the HRP linked secondary antibody staining (Table 4). The relative amount of pCRMP2, CRMP2, β-actin in control and experimental group were quantified based on the ECL™ western blotting analysis system (GE Healthcare).

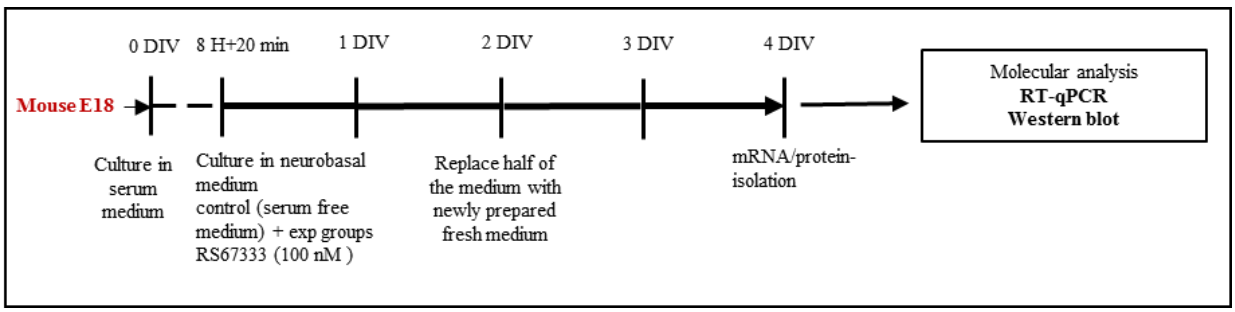

Figure 12. Flow chart represents the procedure for the molecular analysis.

2.9. Knockdown of the *CRMP2* expression in neuron culture

2.9.1. Chemical preparation for transfection reagent

I prepared transfection reagent mixture for the transfection of *CRMP2* siRNA according to the manufacturer's instruction (Santa Cruz Biotechnology, Inc.). Briefly, siRNA duplex (Solution A) was prepared for each transfection by diluting 2 µl of siRNA duplex (i.e. 0.25-1 µg or 20-80 pM siRNA) into 100 µl siRNA transfection medium (sc-36868). In continuation, transfection reagent (Solution B) was prepared by diluting 4 µl of siRNA transfection reagent (sc-29528) into 100 µl siRNA transfection medium for each transfection. Further, for the preparation of transfection reagent mixture (Solution A + Solution B), siRNA duplex solution (Solution A) was mixed into the transfection reagent (Solution B) and incubated for 15-45 min at room temperature prior to the transfection.

2.9.2. Transfection protocol

I followed the *CRMP2* siRNA transfection protocol for the knockdown of *CRMP2* expression according to the manufacturer's instruction (Santa Cruz Biotechnology, Inc.). In a 6-well tissue culture plate, neurons were cultured at a density of 2 x 10^5 cells/well in 2 ml minimal essential medium (Life Technologies), 0.5 mM L-glutamine (Life Technologies), 25 µM glutamate (Wako, Osaka, Japan) and 25 µg/ml penicillin/streptomycin (Sigma) and 10% heat-inactivated fetal bovine serum (FBS, Life Technologies). The serum medium was then replaced by neurobasal medium containing 100 nM RS67333 and cells were cultured for 16 hours (1 DIV). Further, I washed the neurons once with 2 ml of siRNA transfection medium. For each transfection, 0.8 ml siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture, the mixture was gently mixed and poured onto the neurons. Neurons were then incubated for 5 hours at 37° C in a CO_2 incubator. Further, I added 1 ml of 2x serum medium (contains the serum and antibiotics concentration double than the normal growth medium, and 100 nM RS67333) without removing the transfection mixture. After that, the neurons were incubated for an additional 24 hours (2 DIV). Later, the medium was aspirated and replaced with fresh 1x serum medium (with 100 nM RS67333) and cultured for an additional 43 hours (4 DIV). At 4 DIV, neurons were either fixed for immunohistochemistry or collected for western blot and RT-qPCR analysis.

2.9.3. Chemical treatments of other experimental groups

2.9.3.1. Control groups

Hippocampal neurons were treated with the complete neurobasal medium with or without 100 nM RS67333.

2.9.3.2. Negative control

Transfection reagent was prepared following the step 2.9.1 but instead of *CRMP2* siRNA, control siRNA *B* (sc-44230, Santa Cruz Biotechnology, Inc.) was used. Control siRNA *B* contains a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA.

2.10. Statistical analysis

Prism 6, GraphPad Software, (San Diego, CA, U.S.A.) was used for the statistical analysis. ANOVA with Tukey's post hoc test was performed for multiple comparisons. Each experiment was repeated 3 times. The paired t-test was performed in the experiments of mRNA expression and western blot analysis. Differences were considered significant if the probability of error was less than 5%. All the data were expressed as mean ± SEM.

2.11. Image processing

ImageJ Java 1.8.0 (NIH) and photoshop were used for image processing.

Results

1. The expression of 5-HT4R in axons and dendrites of the hippocampal neurons

The cellular and subcellular localization of the 5-HT4R in cultured hippocampal neurons were examined immunohistochemically, using antibodies against the 5-HT4R, MAP2 and phosphorylated neurofilament-H (SMI-31 antigen) (Figure 13A and 13B). I performed double staining of 5-HT4R in combination with MAP2 or SMI-31 to map the distribution of 5-HT4R in dendrites or axons, respectively, at 4 DIV. 5-HT4R was expressed in cell bodies, dendrites, and axons (Figure 13A and 13B). Furthermore, triple-staining with DAPI, anti-GAD65, and 5-HT4R antibodies demonstrated that both GAD65-negative and GAD65-positive neurons expressed 5-HT4R (Figure 13C). Next, I performed the immunostaining of 5-HT4R in the E18 brain section with DAPI and I found that *in vivo* expression of 5-HT4R was prominent in the CA1-CA3, and DG regions of the hippocampus (Figure 13D and 13E).

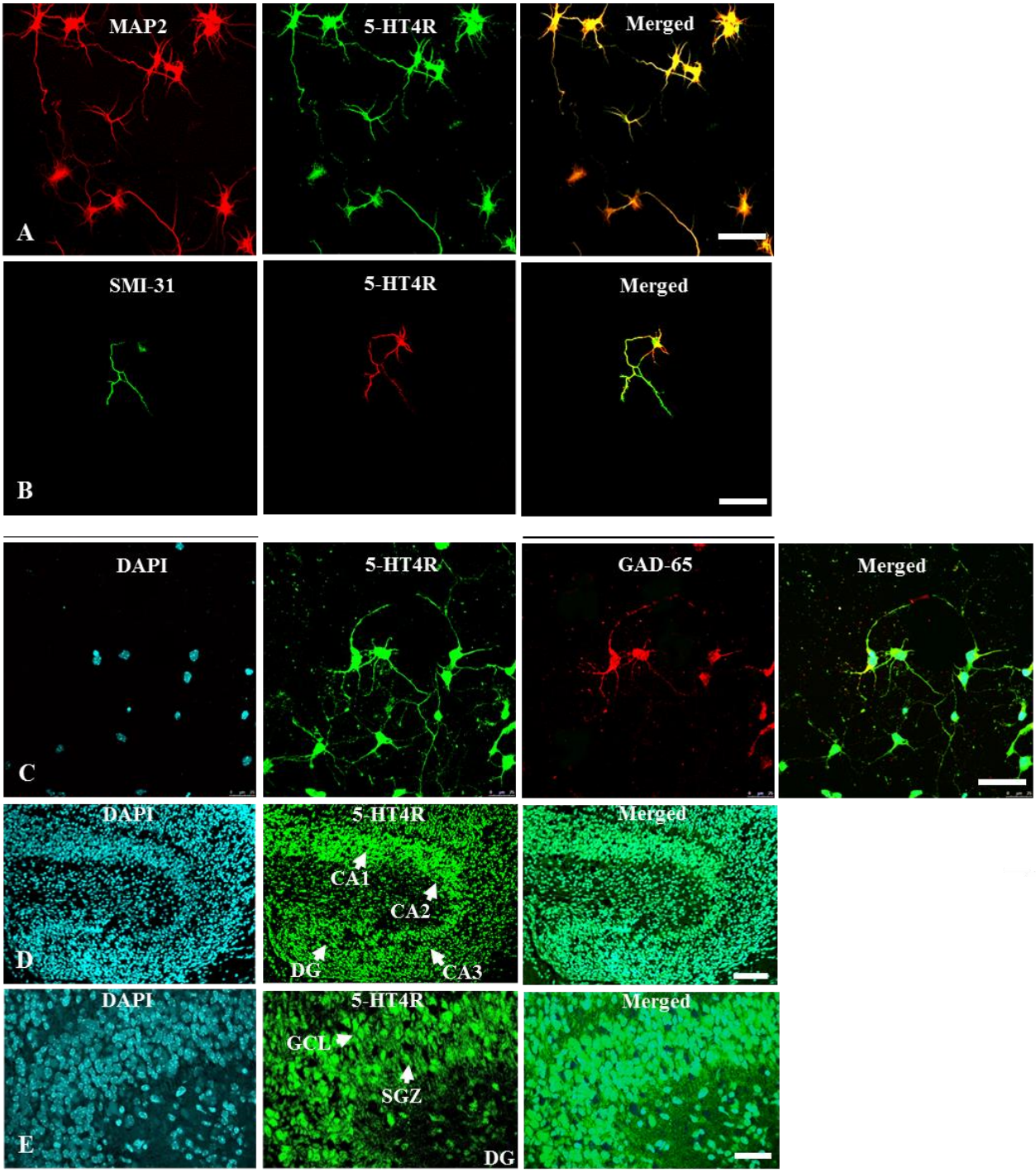

Figure 13. *In vitro and in vivo* expression of 5-HT4R in axons and dendrites of hippocampal neurons. (A-C) Hippocampal neurons at E18 were cultured for 4 days and *in vitro* expression of 5-HT4R was investigated. 5-HT4R in the dendrites was visualized by anti-MAP2 (red) and anti-5-HT4R (green) antibodies, MAP2 as dendrite marker (A). Staining with anti-SMI-31 (green) and anti-5-HT4R (red) antibodies shows the expression of 5-HT4R in the axon using SMI-31 as an axon marker (B). Fluorescent images are showing the expression of 5-HT4R in GABAergic (GAD65 positive neurons) and non-GABAergic neurons. Neurons were stained with DAPI (blue), 5-HT4R (green) and GAD65 (red) (C). (A-C) Scale bar: 75 μm. (D, E) Immunostaining of cryostat sections of hippocampus at E18 with anti-5-HT4R antibody. Nuclei were stained with DAPI. White arrows are showing CA1, CA2, CA3 and DG regions. DG: dentate gyrus. Magnified view of the DG is shown (E). GCL: granular cell layer, SGZ: sub-granular zone. Scale bar: 250 μm (D), 100 μm (E).

2. Effects of 5-HT4R agonists and antagonist on axon growth

I analyzed the effects of 5-HT4R agonists (RS67333, BIMU8) and antagonist (GR125487) in the growth of axons *in vitro*. First, hippocampal neurons were treated with RS67333 and the effect was examined on axon formation (Figures 14, 15 and 16). I found that treatment with RS67333 (1 nM, 10 nM, and 100 nM) increased the total axon length by 32.8 ± 7.4% ($p < 0.01$), 40.7 ± 8.0% ($p < 0.001$), and 46.4 ± 11.3% ($p < 0.001$), respectively (Figure 14E), and axonal branching index by 95.6 ± 29.3% ($p < 0.01$), 112.5 ± 29.4% ($p < 0.001$), and 137.5 ± 37.8% ($p < 0.001$), respectively (Figure 14F), when compared with control. In addition, I confirmed the effects of analogous agonists using 100 nM BIMU8 and 100 nM 5-HT and found that both significantly increased the length and branching index of axon (Figure 15). Further, antagonist GR125487 was used to confirm the specific role of 5-HT4R on the axon growth (Figure 16). I found that the combination of 5 nM GR125487 and 100 nM RS67333 treatment significantly decreased total axon length by 21.29 ± 10.66% ($p < 0.05;$ Figure 16F) and branching index by 81.83 ± 27.71% ($p < 0.05;$ Figure 16G)**,** when compared with 100 nM RS67333 treated group (Figure 16C, D, F and G). Results suggest that GR125487 neutralized the RS67333-induced increased growth of axon. GR125487 alone had no significant effects on any of the parameters of axon growth as compared with the control (Figure 16F and G). Additionally, I analyzed the axon diameter in both control and 100 nM RS67333 treated groups using scanning electron micrographs (Figure 16H and I). I found that axon diameter was significantly increased by 68.6 ± 4.63% ($p < 0.0001$, Figure 16J) as compared to the control group.

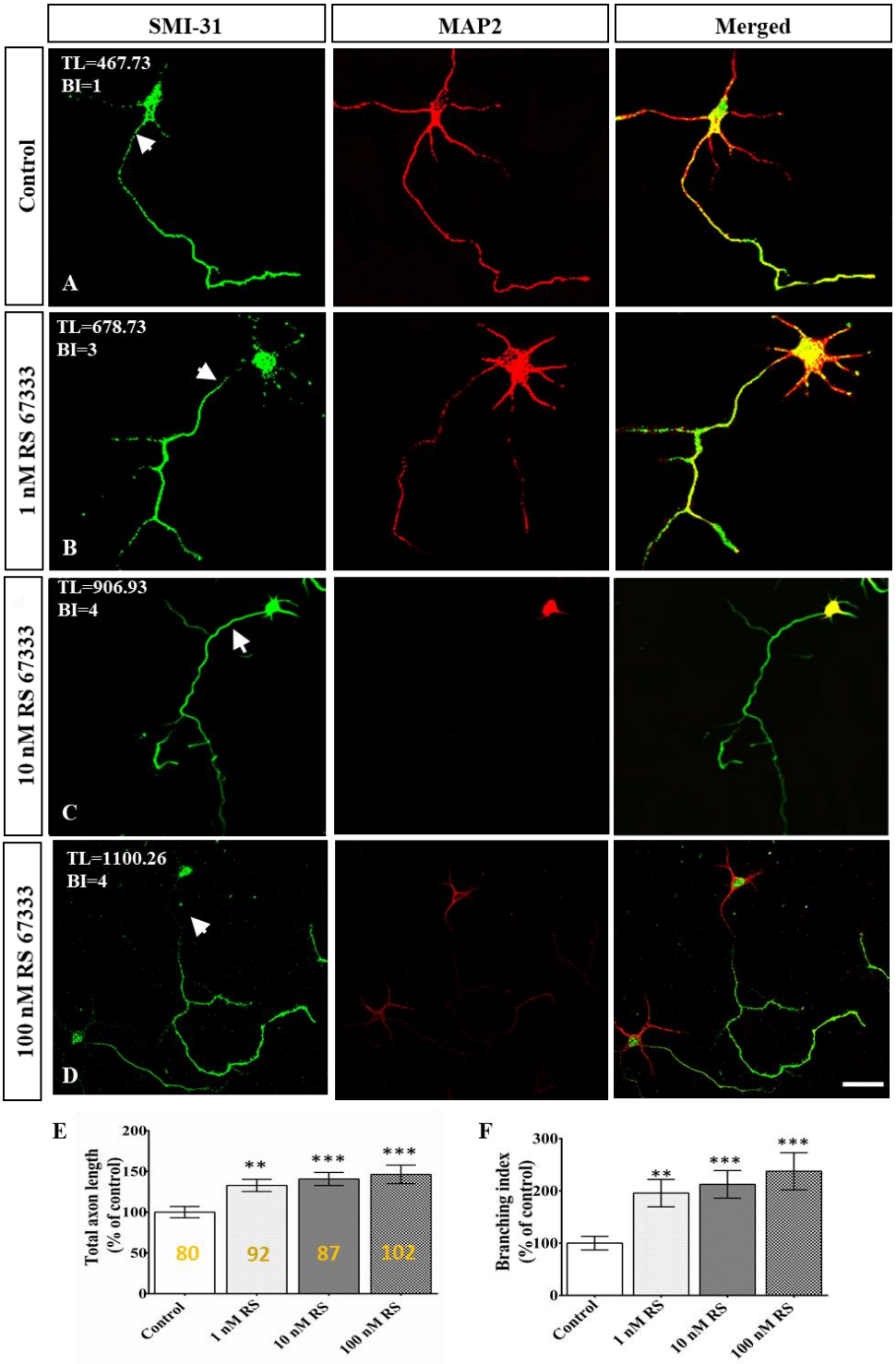

Figure 14. Effects of 5-HT4R agonist (RS67333) on the axon growth. (A-D) Hippocampal neurons were cultured for 4 days in the absence (A), and presence of 1 nM (B), 10 nM (C) and 100 nM (D) of RS67333. Morphological characterization of axons was performed using anti-SMI-31 (green) and anti-MAP2 antibodies (red), and effects of the treatment of RS67333 were analyzed on axon growth. BI=Branching index, TL=Total axon length. (A-C) Scale bar: 75 μm. Arrows indicate the longest neurite (axon). (E, F) Treatment with RS67333 significantly increased the total axon length (E) and branching index (F). RS= RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; $**p < 0.01$, $***p < 0.001$). The number of neurons analyzed is shown in each bar.

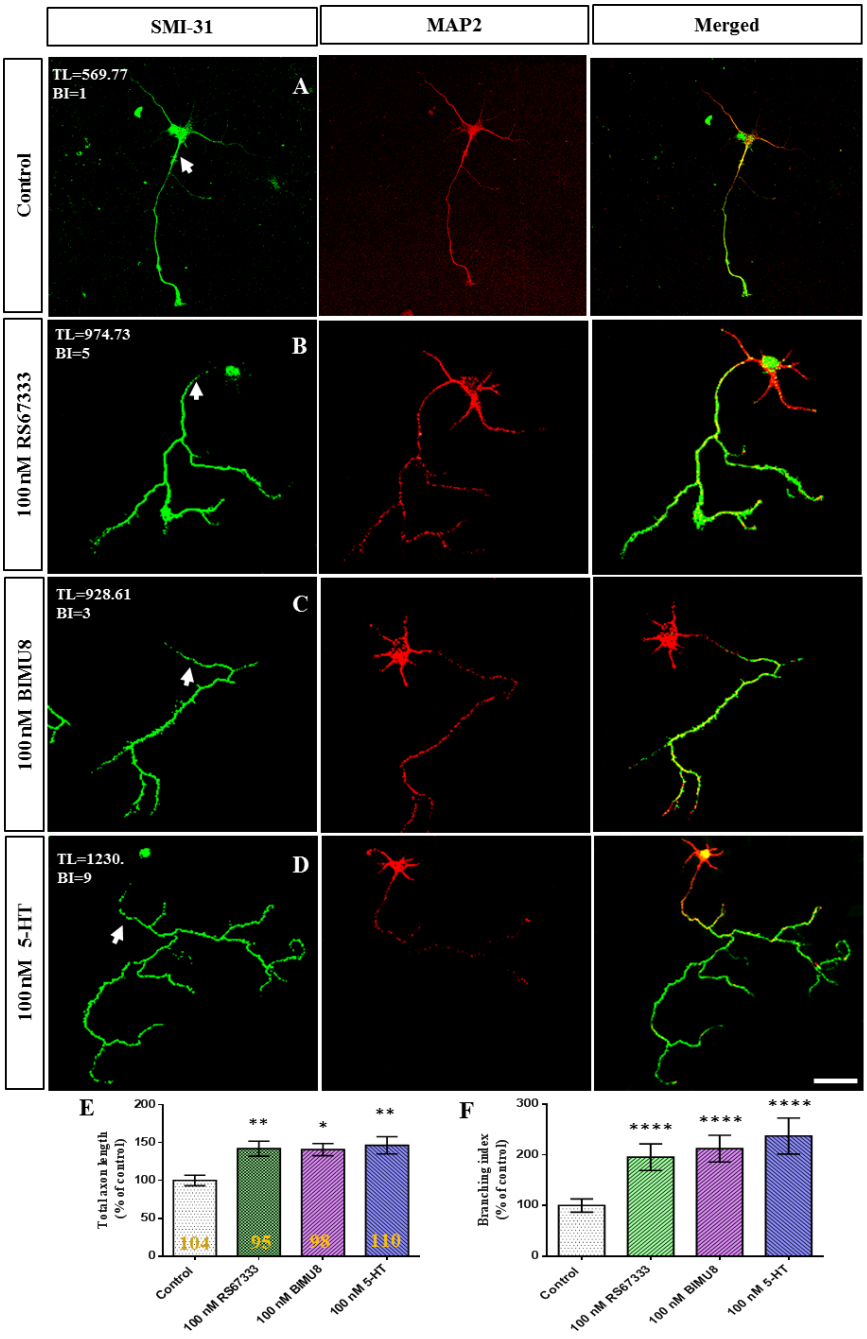

Figure 15. Effects of 5-HT4R agonists (RS67333, BIMU8) and 5-HT on the axon growth. (A-D) Hippocampal neurons were cultured for 4 days in the absence (A), and presence of 100 nM RS67333 (B), 100 nM BIMU8 (C) and 100 nM 5-HT (D). The neurons were immunostained with the anti-SMI-31 (green) and anti-MAP2 antibodies (red), and the effects on the axon growth were analyzed. BI=Branching index, TL=Total axon length. Scale bar: 75 µm. Arrows indicate the longest neurite (axon). (E-F) Treatment with RS67333, BIMU8 and 5-HT significantly increased the total axon length (E) and branching index (F). RS=RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

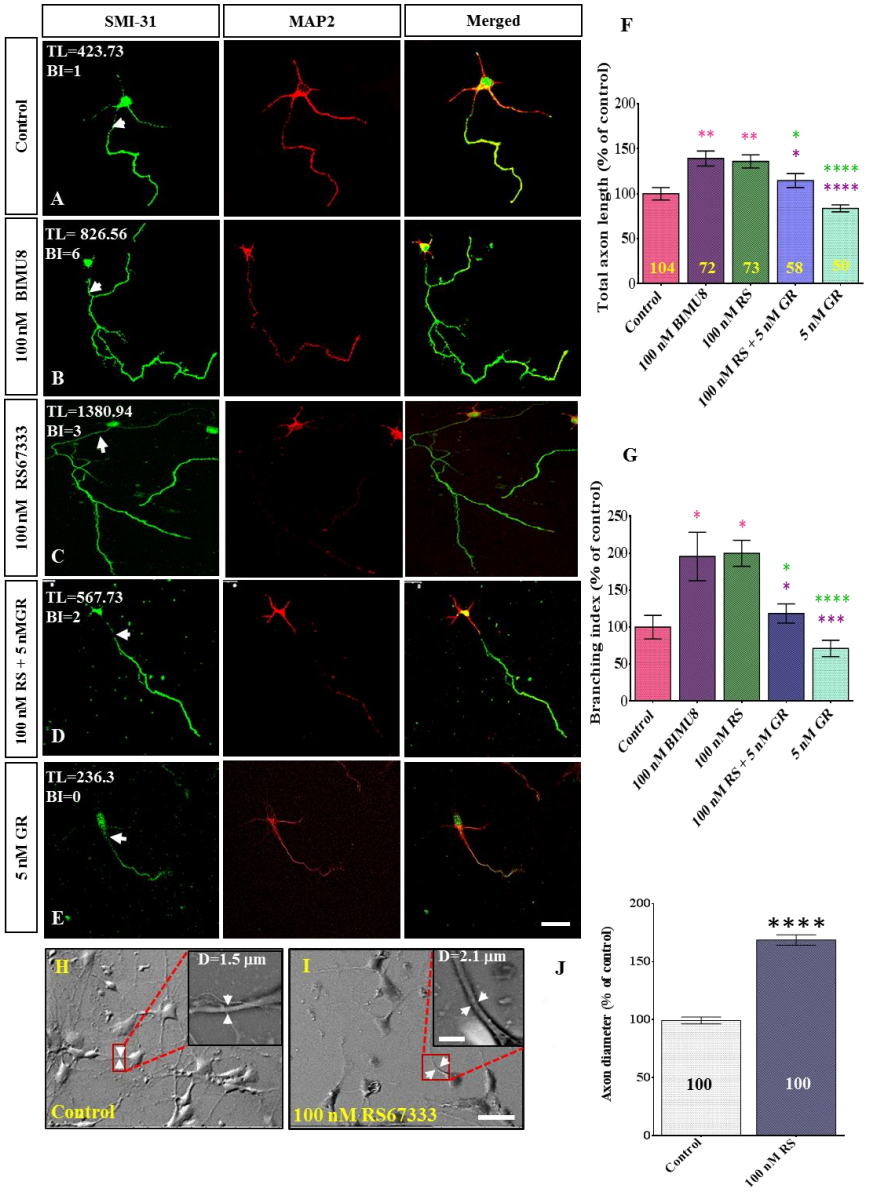

Figure 16. Effects of 5-HT4R agonists (BIMU8 and RS67333) and antagonist (GR125487) on the axon growth. (A-E) Hippocampal neurons were cultured for 4 days in the absence of agonist/antagonist (A), and the presence of 100 nM BIMU8 (B), 100 nM RS67333 (C), 100 nM RS67333 + 5 nM GR125487 (D), and 5 nM GR125487 (E). Morphological characterization of axons was performed using anti-SMI-31 (green) and anti-MAP2 antibodies (red), and effects of the treatment of RS67333 were analyzed on axon growth. Arrows indicate the longest neurite (axon). BI= Branching index, TL= Total axon length. Scale bar: 75 µm. (F, G) Treatment with GR125487 neutralized the effects of RS67333 on the total axon length (F) and axonal branching index (G). GR= GR125487, RS= RS67333. Data are shown as mean ± SEM. Purple, violet, green, blue and cyan asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$). (H, I) Hippocampal neurons were cultured for 4 days in the absence (H) and presence of 100 nM RS67333 (I). Scanning electron micrographs showing the ultrastructure of axons. Scale bar: 50 µm (1000x), 5 µm (6000x). (J) On the basis of scanning electron micrographs axon diameter was measured. Data are shown as mean ± SEM. Asterisks indicate statistical significance (Student's t-test, Paired, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

3. Effects of 5-HT4R agonists and antagonist on the growth of dendrites

In addition to axon formation, I also analyzed the effects of 5-HT4R agonists and antagonist on dendrite growth (Figure 17, 18 and 19). I found that treatment with 5-HT4R agonist RS67333 (1 nM, 10 nM, and 100 nM) increased the total dendritic length by 16.4 ± 3.27% ($p < 0.01$), 41.9 ± 4.1% ($p < 0.0001$), and 61.5 ± 3.70% ($p < 0.0001$), respectively (Figure 17E), and increased the number of primary dendrites by 15.4 ± 4.7% ($p < 0.05$), 26.5 ± 5.2% ($p < 0.05$), and 59.1 ± 3.4% ($p < 0.0001$), respectively (Figure 17G). In addition, 10 nM and 100 nM RS67333 increased the branching index by 21.8 ± 4.8% ($p < 0.05$) and 30.0 ± 7.6% ($p < 0.01$), respectively (Figure 17H). In contrast, RS67333 had no significant effects on the average dendritic length (Figure 17F). Simultaneously, I also examined the effects of analogous agonists BIMU8 and 5-HT. I found that 100 nM BIMU8 and 100 nM 5-HT significantly increased the total dendritic length, number of primary dendrites and branching index (Figure 18). Interestingly, 100 nM 5-HT significantly increased the average dendritic length (Figure 18F). Further, antagonist GR125487 was used to confirm the specific role of 5-HT4R on the dendritic growth (Figure 19). Treatment with 5 nM GR125487 in combination with 100 nM RS67333 neutralized the RS67333-induced growth of dendrites, thus, decreased the total dendritic length by 30.16 ± 9.7% ($p<0.01$; f Figure 19F), number of primary dendrites by 122.2 ± 34.44% ($p<0.01$; Figure 19H), and the branching index by 51.61 ± 31.42 ($p<0.05$; Figure 19I), when compared with 100 nM RS67333 treated group (Figure 19C, D, F, H and I). GR125487 alone had no significant effects on any of the parameters of dendrite development as compared with the control group (Figure 19F-I).

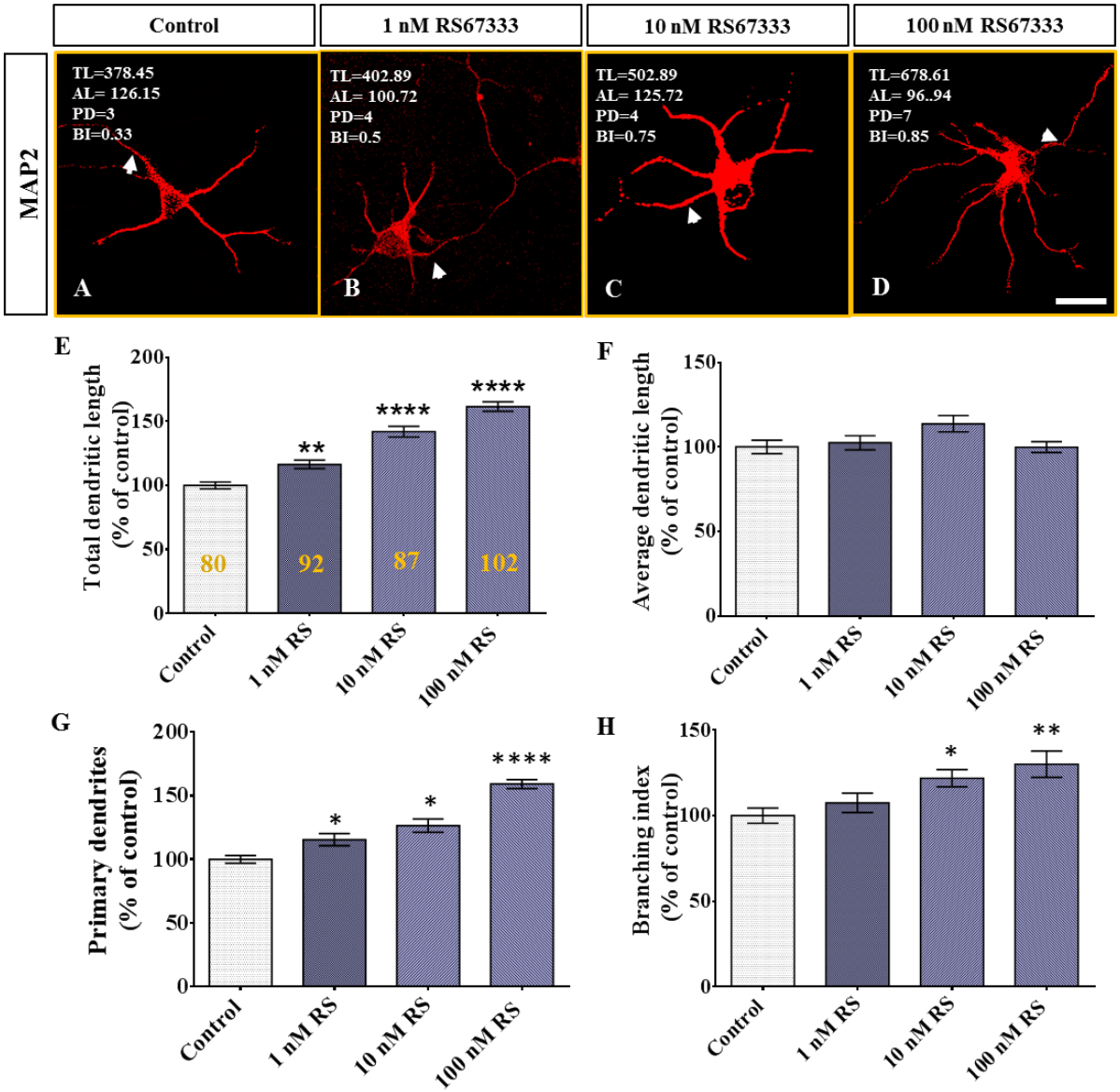

Figure 17. Effects of 5-HT4R agonist (RS67333) on the dendritic growth. (A-D) Hippocampal neurons were cultured for 4 days in the absence/control (A), and presence of 1 nM (B), 10 nM (C) and 100 nM (D) of RS67333. Morphological characterization of dendrites was performed using anti-MAP2 antibody (red) and effects of the treatment of RS67333 on total dendritic length (TL), average dendritic length (AL), number of primary dendrites (PD), and branching index (BI) were analyzed. Scale bar: 50 μm. Arrows indicate an axon (the longest neurite). (E-H) Bar graphs show that treatment with RS67333 significantly increased the total dendritic length (E), number of primary dendrites (G) and branching index (H), but no effect was observed on average dendritic length (F). RS= RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

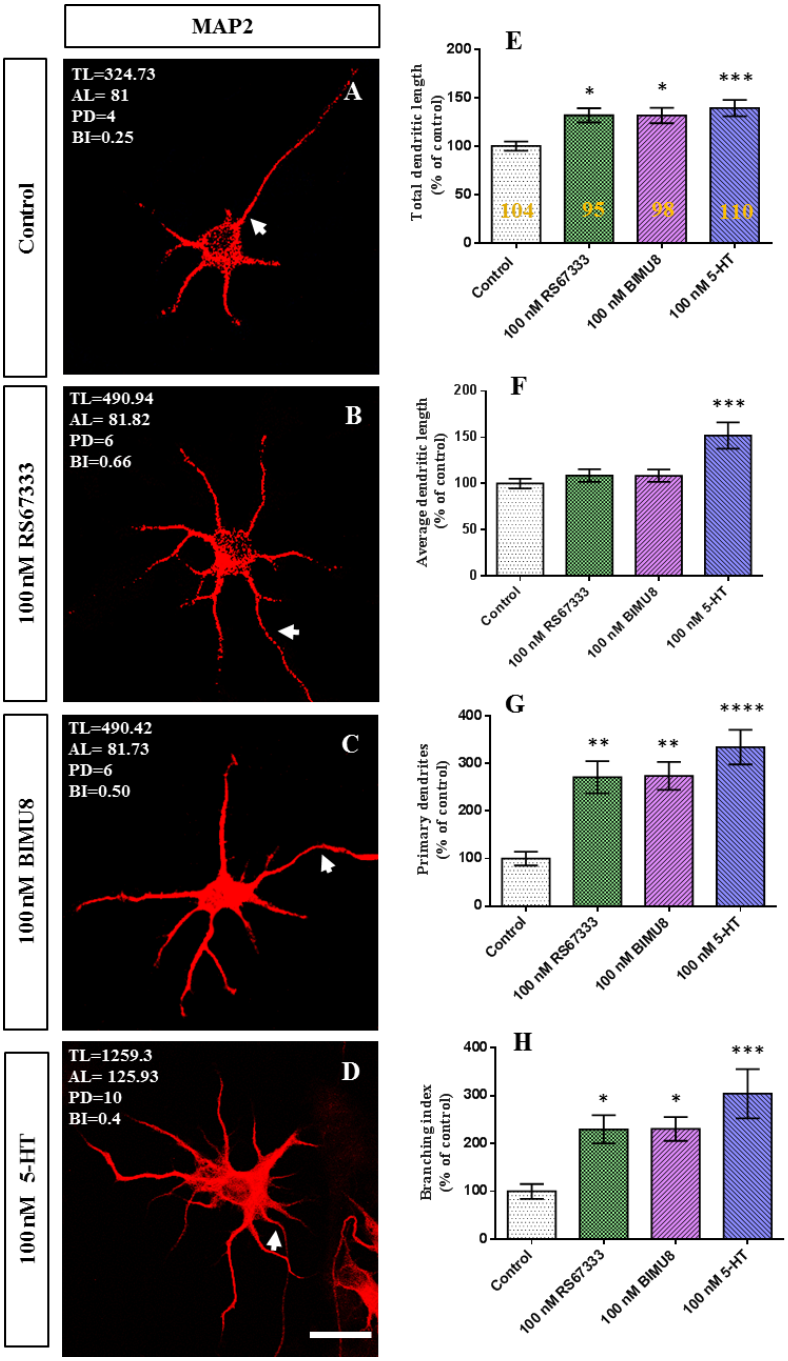

Figure 18. Effects of 5-HT4R agonists (RS67333, BIMU8) and 5-HT on the dendritic growth. (A-D) Hippocampal neurons were cultured for 4 days in the absence (A), and presence of 100 nM RS67333 (B), 100 nM BIMU8 (C) and 100 nM 5-HT (D). The neurons were immunostained with the anti-MAP2 antibody, and the effects on the dendritic growth were analyzed. AL=Average dendritic length, BI=Branching index, PD=number of primary dendrites, TL=Total axon length. Scale bar: 50 μm. Arrows indicate an axon (the longest neurite). (E-H) Treatment with RS67333, BIMU8 and 5-HT significantly increased the total axon length (E), number of primary dendrites (G) and branching index (H). RS=RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The number of neurons analyzed is shown in each bar.

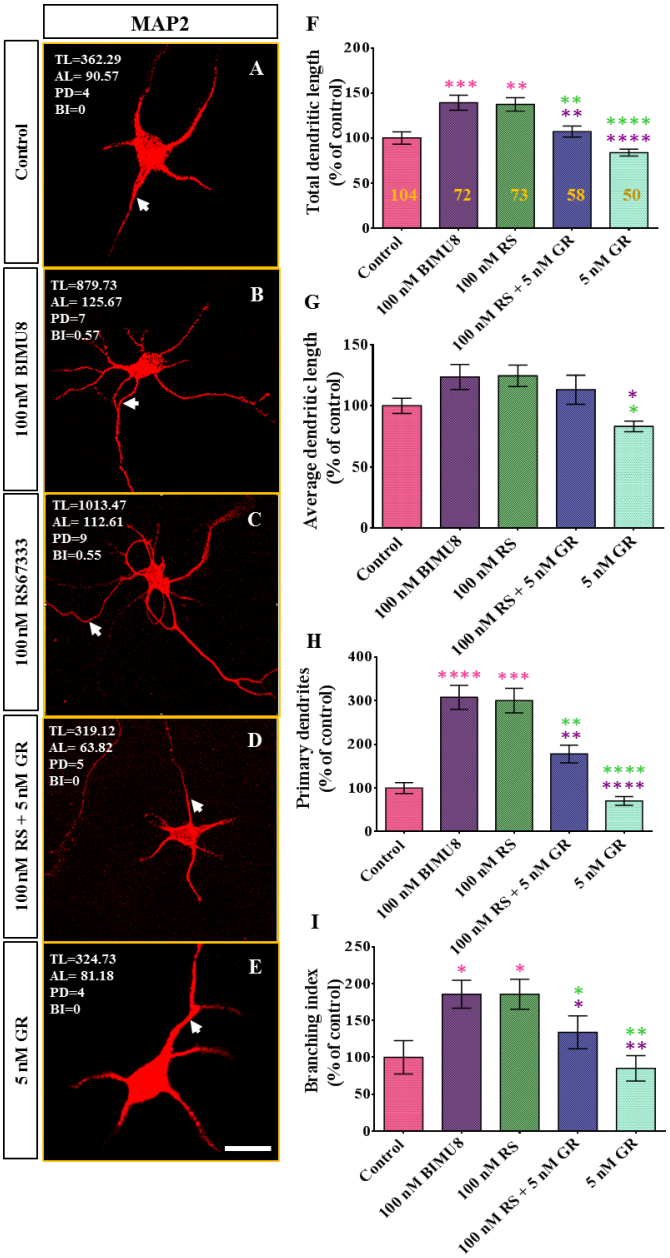

Figure 19. Effects of 5-HT4R agonists (BIMU8 and RS67333) and antagonist (GR125487) on the dendritic growth. (A-E) Hippocampal neurons were cultured for 4 days in the absence of agonist/antagonist (A), and the presence of 100 nM BIMU8 (B), 100 nM RS67333 (C), 100 nM RS67333 + 5 nM GR125487 (D), and 5 nM GR125487 (E). Morphological characterization of dendrites was performed using anti-MAP2 antibody (red) and effects of the treatment of RS67333 on total dendritic length (TL), average dendritic length (AL), number of primary dendrites (PD), and branching index (BI) were analyzed. Scale bar: 50 µm. (F-H) Treatment with GR125487 neutralized the effects by RS67333 in the total dendritic length (F), number of primary dendrites (H) and branching index (I). No significant effect of any agonists and antagonist was observed on average dendritic length as compared with the control (G). GR= GR125487, RS= RS67333. Data are shown as mean ± SEM. Purple, violet, green, blue and cyan asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The number of neurons analyzed is shown in each bar.

4. Colocalization of 5-HT4R and CRMP2 in hippocampal neurons *in vitro* and *in vivo*

I analyzed the cellular and subcellular localization of the 5-HT4R and CRMP2/pCRMP2 in hippocampal neurons immunohistochemically, using antibodies against the 5-HT4R, CRMP2, and pCRMP2 (Figure 20). At 4 DIV, all the 5-HT4R-positive neurons showed immunoreactivity to CRMP2 in cell bodies, dendrites from base to tip and axons from base to terminal**.** In contrast, pCRMP2 was expressed both in cell bodies, axon trunk except axon collaterals and dendrites (Figure 20A-C). *In vivo* expression in E18 embryos were analyzed by triple staining with anti-5-HT4R, anti-CRMP2 antibodies and DAPI. Results showed that 5-HT4R and CRMP2 were prominently colocalized in the hippocampus (Figure 20D and E).

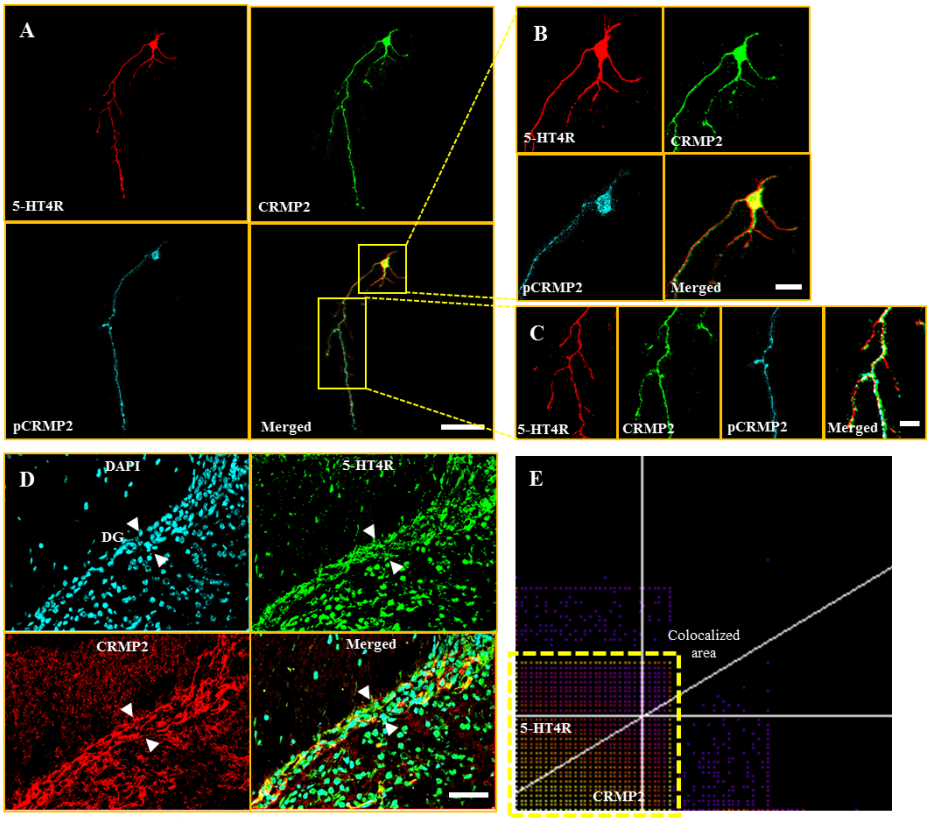

Figure 20. Fluorescent images showing the 5-HT4R and CRMP2 colocalization in hippocampal neurons. (A-C) Neurons were immunostained with antibodies against 5-HT4R (red), CRMP2 (green), and pCRMP2 (blue). B and C are enlarged images shown in A. pCRMP2, CRMP2 and 5-HT4R were expressed in the soma and dendrites (B) and the axon and its collateral branches (C). Scale bar: 75 μm (A), 25 μm (B), 20 μm (C). (D, E) Colocalization of 5-HT4R (green) and CRMP2 (red) in the hippocampus at E18. DAPI staining was performed to label the cell nuclei (D). Arrowheads represent the hippocampal area (DG: dentate gyrus). Scale bar: 100 μm (D). 2D scattered plot (pixel map) for the green and red channel. The colocalized area of 5-HT4R and CRMP2 was shown in yellow line (dotted) square box. Y-axis shows the green channel intensity, X-axis shows the red channel intensity and diagonal line shows the ratio of green to red channel, which represents the linear regression from both channels. Manders' Coefficients for the overlapping of green and red channels are M1 (0.693) and M2 (0.447), which show fraction of green channel overlapping red channel and fraction of red channel overlapping green channel respectively (E).

5. Role of *CRMP2* in 5-HT4R-mediated growth of axon and dendrites

I knockdown the expression of *CRMP2* to investigate the role of CRMP2 in the 5-HT4R-mediated axon growth (Figure 21). In this set of experiment, cultured neurons were treated with 100 nM RS67333 in the presence of *CRMP2* siRNA (Figure 21C), which significantly decreased the growth of axon. This decrease in axon growth was observed in terms of total axon length by 151.4 ± 11.15% ($p<0.0001$; Figure 21E) and branching index by 175.7 ± 27.40% ($p<0.0001$; Figure 21F) as compared to RS67333 treated group (Figure 21B). Further, treatment with control siRNA *B* (Figure 21D) did not induce any significant difference in axon growth in comparison with the RS67333 treated group (Figure 21B, E and F). The effects of *CRMP2* siRNA and siRNA *B* were confirmed by examining the relative expression of *CRMP2* mRNA in all the experimental groups (Figure 21G).

Similarly, the role of CRMP2 in 5-HT4R-mediated growth of dendrites were assessed (Figure 22). As shown above, neurons which were treated with 100 nM RS67333 showed significant enhancement in the growth of dendrites in comparison with control (Figure 22A, B, E-H). However, this enhancement was significantly decreased when the neurons were cultured in combination with 100 nM RS67333 and *CRMP2* siRNA (Figure 22C, E-H). Results showed that total dendritic length was decreased by 151.9 ± 15% ($p<0.0001$; Figure 22E), average dendritic length by 76.10 ± 7.5% ($p<0.0001$; Figure 22F), number of primary dendrites by 198.1 ± 31.03% ($p<0.0001$; Figure 22G) and branching index by 160.2 ± 40.05% ($p<0.0001$; Figure 22H), in comparison with RS67333 treated group. Further, treatment with control siRNA *B* did not induce any significant difference in dendritic growth in comparison with the RS67333 treated group (Figure 22B, D, E-H).

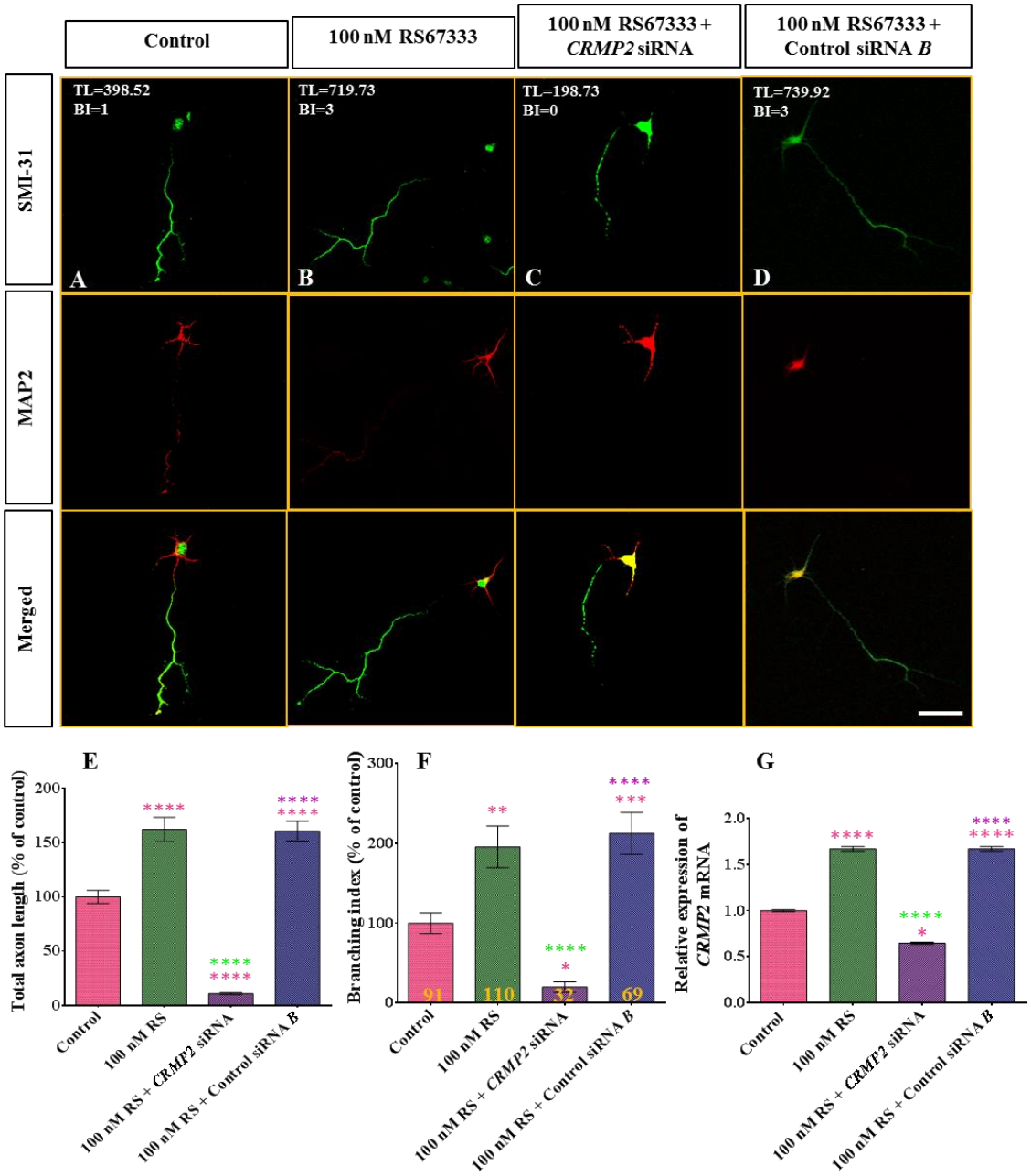

Figure 21. Effects of CRMP2 expression on 5-HT4R mediated axon formation analyzed by *CRMP2* knockdown study. (A-D) Hippocampal neurons were cultured for 4 days in the absence of RS67333 (A), the presence of 100 nM of RS67333 (B), 100 nM RS67333 + *CRMP2* siRNA (C), and 100 nM RS67333 + control siRNA *B* (D). Axon growth was evaluated using the immunostaining with anti-SMI-31 (green) and anti-MAP2 antibodies (red) and total axon length (TL) and branching index (BI) were analyzed. Scale bar: 75 µm. (E-G) Bar graphs represent the effects of the *CRMP2* knockdown on total axon length (E) and branching index (F). Expression of *CRMP2* mRNA was analyzed by qRT-PCR (G). RS= RS67333. Data are shown as mean ± SEM, purple, green, violet and blue asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). 8 culture wells were analyzed in each experimental condition. The number of neurons analyzed is shown in each bar.

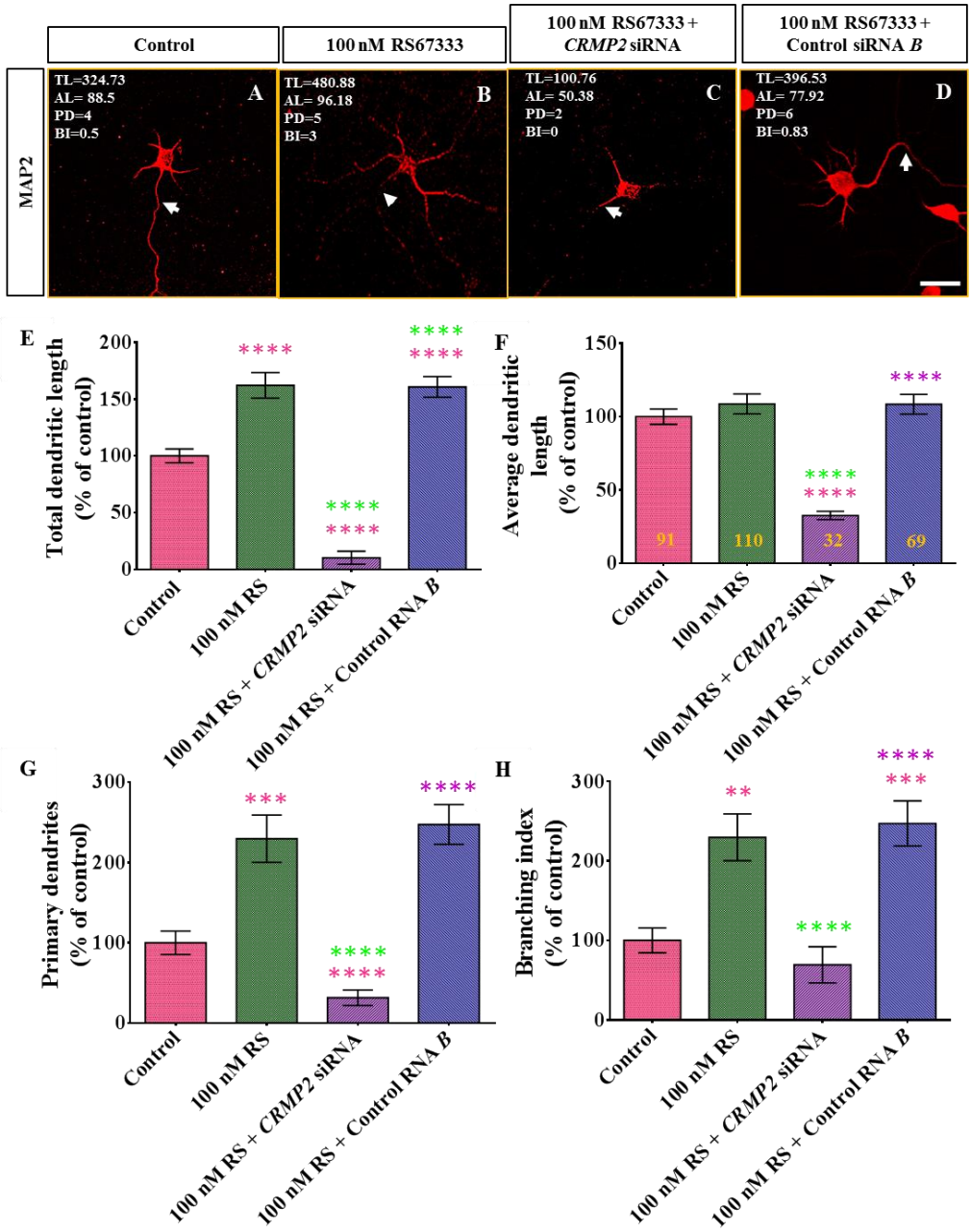

Figure 22. Effects of CRMP2 expression on 5-HT4R mediated dendrite growth analyzed by *CRMP2* knockdown study. (A-D) Hippocampal neurons were cultured for 4 days in the absence of RS67333 (A), the presence of 100 nM of RS67333 (B), 100 nM RS67333 + *CRMP2* siRNA (C), and 100 nM RS67333 + control siRNA *B* (D). Dendritic growth was evaluated using the immunostaining with anti-MAP2 antibody (red), and total dendritic length (TL), average dendritic length (AL), number of primary dendrites (PD), branching index (BI) were measured. Scale bar: 50 μm. Arrows indicate an axon (the longest neurite). (E-H) Bar graphs represent the effects of the *CRMP2* knockdown on the total dendritic length (E), average dendritic length (F), number of primary dendrites (G), branching index (H). RS= RS67333. Data are shown as mean ± SEM. Purple, green, violet and blue asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; **p < 0.01, ***p < 0.001, ****p<0.0001*). The number of neurons analyzed is shown in each bar.

6. RS67333 increased mRNA expression and dephosphorylation of CRMP2

To examine the signaling mechanism mediating the effects of the 5-HT4R on dendrite and axon formation, I focused on the expression of *CRMP2*. Quantitative RT-PCR analysis was performed to examine the role of CRMP2 in axon and dendrite formation. The treatment with 100 nM RS67333 increased the expression of *CRMP2* mRNA by 4.3 ± 1.5 times ($p<0.05$) (Figure 23A). Next, western blot study was performed to analyzed 5-HT4R induced posttranslational modification in CRMP2 (Figure 23B). The treatment with 100 nM RS67333 decreased the average band area of pCRMP2 (0.41 ± 0.14 times; $p<0.05$; Figure 23C), while increased average band area of CRMP2 (7.3 ± 1.8 times; $p<0.001$; Figure 23C) as compared with the control group.

7. RS67333 increased the mRNA expression of *BDNF, NT-3, NGF* and *TRK-A*

I examined the effects of RS67333 on the expression of neurotrophins (*NGF, BDNF, and NT-3*) and their receptors (*TRK-A, TRK-B, and TRK-C*) by quantitative RT-PCR (Figure 23D, E). Treatment of 100 nM RS6733 significantly increased the mRNA expression of *BDNF*, *NT-3* and *NGF* by 5.9 ± 2.3 times ($p<0.05$), 2.0 ± 0.8 times ($p<0.05$), and 1.25 ± 0.18 times ($p<0.05$), respectively, when compared with the control group (Figure 23D). Additionally, 100 nM RS67333 increased the mRNA expression of *TRK-A* receptor by 1.08 ± 0.23 times ($p<0.05$), although no significant difference was observed in the expression of *TRK-B*, *TRK-C* (Figure 23E), Akt and GSK-3β (Figure 23F).

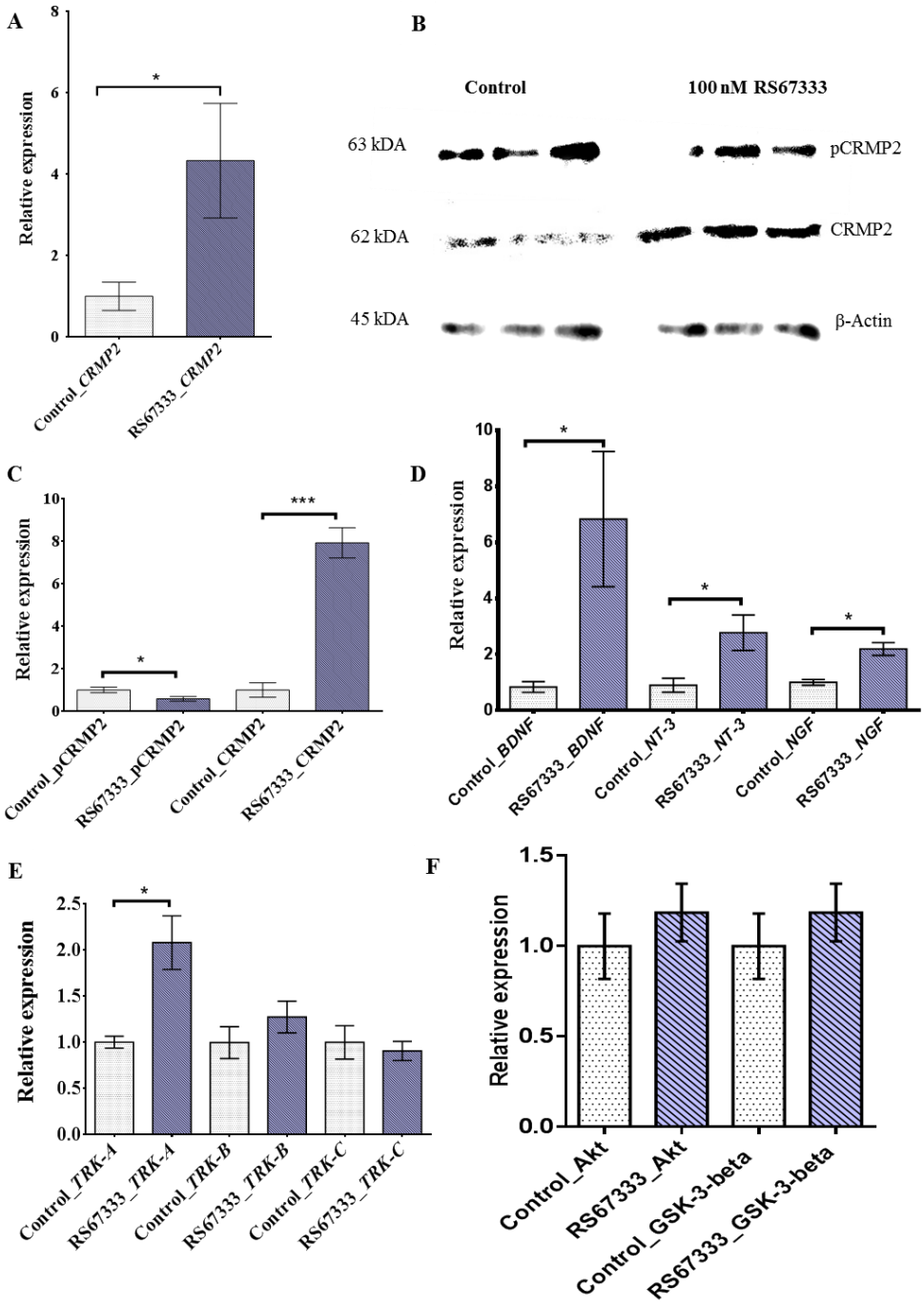

Figure 23. Analysis of molecular pathways associated with 5-HT4R. (A-E) Hippocampal neurons were cultured for 4 days in the absence (control) and presence of a 5-HT4R agonist (RS67333). qRT-PCR showed that treatment of RS67333 increased the mRNA expression of *CRMP2* (A). Lysates of cultured hippocampal neurons were separated by SDS-PAGE and immunoblotted with anti-pCRMP2 and anti-CRMP2 antibodies. β-actin was used as an internal control (B). Saturated immunoblot analysis showed that treatment of RS67333 decreased pCRMP2, while increased CRMP2 (C). qRT-PCR showed that treatment of RS67333 increased the mRNA expression of neurotrophic factors (*BDNF*, *NT-3* and *NGF*) (D), *TRK* receptors (*TRK-A*, *TRK-B*, and *TRK-C)* (E), and Akt and GSK-3β (F). (A-F) Data are shown as mean ± SEM. Asterisks indicate statistical significance (Student's t-test; paired, *$p < 0.05$, ***$p < 0.001$). At least 4 wells were analyzed in each experimental condition.

Discussion

1. Role of 5-HT4R in axon and dendrite growth

In the present *in vitro* study, I investigated the role of 5-HT4R using agonist RS67333. I found that RS67333 increased length, branching, and diameter of the axon in hippocampal neurons during the embryonic development of mice. This is the intriguing finding of the present study, which previous studies have not properly addressed. The results revealed that even minimum 1 nM concentration of RS67333 showed significant effects on the axon growth by increasing their total length and initiating the growth of axon collaterals. Similarly, I investigated the effect of agonist RS67333 on dendritic growth and found the significant increase in total dendritic length, number of primary dendrites, and dendritic branching, but there was no significant effect observed on average dendritic length. However, treatment with 5-HT increased the average length of dendrites (Fig.18F). This might be due to the interaction between 5-HT and other 5-HTRs apart from 5-HT4R expressed in the hippocampus. A literature suggests the relative expression of 5-HT4R (75.60-fold), 5-HT3R (4.2-fold), 5-HT2R (54.04-fold), 5HT6R (4.74-fold) and 5-HT7R (4.79-fold) in the hippocampus (BIoGPS database). Thus, 5-HT2R may be the most influential candidate after 5-HT4R, because it has almost 10 times larger fold expression than other 5-HTRs except 5-HT4R. However, 5-HT2R, 5-HT3R, 5-HT6R and 5-HT7R have been reported to promote the growth and survival of neurons (Dayer et al., 2015; Ohtani et al., 2014; Rojas et al., 2017; Yoshida et al., 2011; Yoshimura et al., 2016). Therefore, further study is needed to investigate absolute expression profile of these 5-HTRs during specific developmental stages to get more reliable answer for the same. Additionally, Mnie et al. in their study compared the putative effect of 5-HT and RS67333 on the isoforms 5-HT4(a) and 5-HT4(b), which are highly expressed in limbic region of the brain, in their ability to undergo endocytic regulation. Both ligands differed in their ability to induce internalization of either isoform, 5-HT being more effective than RS67333 in HEK293 cells and in neurons, in contrast, trafficking induced by 5-HT was isoform-specific (Mnie et al., 2010). Thus, results from current study combined with the previous studies lend the idea that 5-HT treatment may be more effective than RS67333 due to the activation of not only 5-HT4R but also other 5-HTRs. In addition to that it increases the internalization of 5-HT4R isoforms (a) and (b), which might cause significant increase the overall growth of dendrites. I also confirmed the specificity of 5-HT4R actions through the antagonist GR125487 which neutralized the effects of RS67333 on axon and dendrite growth.

Previously, our group reported that 5-HT3R inhibit the growth of dendrites and axon in cortical neurons (Hayashi et al., 2010). However, our group also reported that treatment of 5-HT4R agonist BIMU8 increased total dendritic length, number of primary dendrites and dendritic branching without affecting average dendritic length of hippocampal neurons (Kozono et al., 2017), which is consistent

with the results of the current study. In continuation, Yoshimura et al., explored the role of 5-HT1AR and 5-HT2AR, which elevated the expression of *BDNF*, regulated assembly of microtubule and promoted dendritic growth, arborization and synaptogenesis in the cortex (Ohtani et al., 2014; Yoshida et al., 2011; Yoshimura et al., 2005; Yoshimura et al., 2016). In addition, the current findings indicate that 5-HT4R increased the length, diameter and branching of axon. Axon length and diameter along with intramembrane resistance and membrane capacitance determine the nerve conduction velocity of electrical signal through the axon fiber (Barazany et al., 2009), which are quantified for the investigation of neuropathy (Finsterer and Grisold, 2015; Perge et al., 2012; Rao et al., 2012). Additionally, increased dendritic length, branching, and arborization are known to form the long-term synapses, which facilitate the increased plasticity (Donnell et al., 2011; Paulin et al., 2016; Weber et al., 2016). Thus, aforementioned studies suggest the differential role of various 5-HTRs in the brain development. In this regard, the current study emphasizes the importance of 5-HT4R in the synaptogenesis and plasticity in the brain by facilitating the growth of hippocampal neurons.

2. Downstream signaling via 5-HT4R to promote the growth of axons and dendrites

2.1. Roles of 5-HT4R increased CRMP2 expression and dephosphorylation in the growth of neurites

Aim of this study was to elucidate and explore the possible downstream molecular mechanism underlying the effects of 5-HT4R on the promotion of dendrite and axon growth. Previously, it has been reported that cytosolic protein CRMP2 and 5-HT4R both are highly expressed in the brain during early embryonic development (Berthouze et al., 2005; Bockaert et al., 2006b; Bockaert et al., 2008; Inagaki et al., 2001; Kozono et al., 2017). Furthermore, during the embryonic stage, the increased expression of npCRMP2 in the neurons has been reported, which is localized on microtubules, clathrin-coated pits, and actin filaments in growth cones, and controls the growth of axon and dendrites (Rahajeng et al., 2010; Ryan and Pimplikar, 2005; Yamashita et al., 2012). In contrast, phosphorylated CRMP2 is localized only on actin filaments and control the axon growth (Arimura et al., 2005; Gu and Ihara, 2000). Recently, it has been reported that the overexpression and/or dephosphorylation of CRMP2 induce the formation and maturation of dendritic spines (Niisato et al., 2013; Zhang et al., 2018).

Moreover, it has also been reported that 5-HT4R could modulate the phosphorylation-dependent changes in protein activity (Barthet et al., 2007). Based on the earlier findings (Bockaert et al., 2006a; Bockaert et al., 2006b; Inagaki et al., 2001; Kozono et al., 2017; Ryan and Pimplikar, 2005; Yamashita et al., 2012), it may be hypothesized that the 5-HT4R could modulate the *CRMP2* mRNA expression and/or dephosphorylation of CRMP2 which facilitate the promotion in axon and dendrite growth. Therefore, I performed colocalization study of 5-HT4R with CRMP2 and found that these proteins were colocalized in the axons, dendrites and cell bodies of embryonic hippocampal neurons. Furthermore,

pharmacological activation of 5-HT4R by RS67333 increased the mRNA expression of *CRMP2* and decreased expression of pCRMP2. Moreover, the growth of axon and dendrites was neutralized when I knockdown *CRMP2* in the presence of RS67333. These results suggest, the activity of 5-HT4R upregulates the expression and dephosphorylation of CRMP2, which promotes the growth of axon and dendrites. These observations suggest the functional relationships between 5-HT4R and CRMP2.

2.2. Roles of 5-HT4R upregulated neurotrophic factors and TRK receptors in the phosphorylation and expression of CRMP2

Recently, our group reported that 5-HT4R upregulates expression of *BDNF* and blocking of TRK-B inhibit the growth of dendrites in rat hippocampal neurons (Kozono et al., 2017). Therefore, in the current follow up study I confirmed the effect of 5-HT4R on the expression of *BDNF*, and further showed that activation of 5-HT4R increased the mRNA expression of *NT-3*, *NGF* and *TRK-A* in mouse hippocampal neurons. It has been well established that NGF, BDNF and NT-3 dephosphorylate the CRMP2 and upregulate the expression of npCRMP2 through the activation of TRK-A, TRK-B and TRK-C respectively (Martin-Iverson et al., 1994; Niisato et al., 2013; Shimazu et al., 2006; Stewart et al., 2008; Usuki et al., 2018; Yamashita et al., 2012; Yoshimura et al., 2005; Zhang et al., 2018). npCRMP2 promotes the axon and dendrite formation, while pCRMP2 inhibits the neurite growth (Yoshimura et al., 2005). Collectively, there is a conceivable pathway which may interlink the activity of 5-HT4R with CRMP2 expression and dephosphorylation through elevated mRNA expression of *BDNF*, *NT-3*, *NGF*, and *TRK-A* via 5-HT4R. Upregulation of the neurotrophic factors inhibits the phosphorylation of CRMP2 protein, and increases npCRMP2, which promotes the growth of axon and dendrites.

3. Significance of 5-HT4R promoted growth of hippocampal neurons in learning, memory and emotions

Hippocampus works as an indexer by relaying the information to the cingulate and subiculum cortex, which facilitate the long-term storage of the information. Additionally, hippocampus in concert with amygdala, works as an emotional motor center and controls the emotional responses and memory formation during the adverse life situations. It is particularly important in forming new memories and connecting emotions and senses such as smell and sound to memories (Opitz, 2014). In this regard, the present study may provide some clue for the possible mechanism behind the roles of 5-HT4R in the learning, memory formation and emotion. I found that pharmacological activation of 5-HT4R promoted the growth of neurites in the hippocampus via modulating the dephosphorylation and expression of *CRMP2*, which could facilitate the circuit formation in the hippocampus via promoting the growth of axons and dendrites, resultant, increases its plasticity. In addition, increased circuit formation in the hippocampus facilitates the processing of the information and its' long-term storage; thus, facilitates

the higher cognitive processes in the brain. Therefore, 5-HT4R expression and function in the hippocampus in the embryonic brain has great importance in the mechanism of learning, memory and emotions in the brain.

Various groups reported that the stimulatory effect of 5-HT4R in other limbic regions and frontal cortex in the memory and cognition (Consolo et al., 1994, Teixeira et al., 2018, Siniscalchi et al., 1999). Recent studies reported that various 5-HT4R agonists improve cognition and memory function in rodents by stimulating the release of acetylcholine in the frontal cortex (Consolo et al., 1994). Siniscalchi and colleagues reported that BIMU8 increased the outflow of acetylcholine in the hippocampus and enhanced memory and cognition, which were blocked by concomitant treatment with a 5-HT4R antagonist GR125487 (Siniscalchi et al., 1999). There are some reports showing that the stimulatory role of 5-HT4R agonists RS67333 in memory enhancement and facilitation of learning, which could be reversed with treatment of antagonists SDZ 205-557 and GR125487 (Fontana et al., 1997; Meneses and Hong, 1997; Orsetti et al., 2003).

4. Association between 5-HT4R and neurological disorders

5-HT4R has an excitatory role on neurons and is widely expressed in limbic regions such as the amygdala, septum, hippocampus and mesolimbic region (Figure 4), which are the emotional motor centres, and functioning of these areas are highly affected during the stressful situations (Hannon and Hoyer, 2008; Tanaka et al., 2012). Emotional, physical, and other stressors in the external environment are the main cause of depression and anxiety. The results of the current study can give some insights of the mechanism by which RS67333 shows its anxiolytic and antidepressant effects. Additionally, most of the anxiolytic and antidepressant drugs increase the level of 5-HT in the brain, which increases the expression of neurotrophic factors such as BDNF. This increased expression of BDNF has been reported to promote the neurogenesis and growth of hippocampal neurons and increase synaptogenesis and plasticity. Present study also suggests such role of 5-HT4R agonists in the hippocampus and can give important insights for the development of 5-HT4R based medications for the treatment of anxiety and depressive disorders (Amigo et al., 2016; Yohn et al., 2017). A recent study reported that treated with 5-HT4R-based therapeutics cleared the deposition of neurofibrillary tangles and APP in Alzheimer disease patients. In addition, these patients showed significant improvements in motor activities after the treatment (Coskuner and Uversky, 2018). 5-HT4R can also regulate tau pathology by modulating the activity of GSK-3β via G12/13 proteins (Butzlaff and Ponimaskin, 2016).

5. Importance of 5-HT4R promoted growth of hippocampal neurons in neuronal developmental disorders

It has been explained that hippocampus is associated with attention deficit hyperactivity disorder (ADHD) and autism spectral disorder (ASD) through the role in regulating emotion and memory (Basu et al., 2016; Dager et al., 2007; Plessen et al., 2006). Numerous studies over the past decade on animal models have reported detailed changes in epigenetic modifications in gene expressions in the hippocampus under various learning paradigms to support the role of hippocampus in mental retardation or intellectual disability disorders (Lagali et al., 2010). Recently, some groups reported the specific role of 5-HT4R in the information processing and neuronal plasticity of hippocampus (Amigo et al., 2016; Hagena and Manahan, 2017). In this regard, present study can provide insights to develop 5-HT4R drugs-based models for the treatment of neuronal developmental disorders. Pharmacological activation of 5-HT4R increased the neuronal growth in the hippocampus. Additionally, CRMP2 has been reported to facilitate neuronal regeneration, survival and differentiation (Ip et al., 2014; Kondo et al., 2019; Na et al., 2017; Zhang and Koch, 2017). Therefore, 5-HT4R increased expression of CRMP2 can facilitate the growth and survival of neurons in the hippocampus. This will promote synaptogenesis and will strengthen the plasticity, which might improve the learning and memory function in such patients.

6. Clinical significance of the present study

Pharmacological stimulation of 5-HT4R in rodents has been reported to improve learning and memory function (Haahr et al., 2013; Orsetti et al., 2003; Stenbæk et al., 2017). Additionally, pharmacological intervention of 5-HT4R showed its critical role in anxiety and depression via mediating the expression and function of BDNF, TRK-B, Activity-regulated cytoskeleton-associated (Arc) protein and 5-HT1AR (Amigo et al., 2016; Yohn et al., 2017). Preclinical studies on 5-HT4R knockout mice showed crucial roles of 5-HT4R in anxiety and depression via mediating the expression and function of BDNF, TRK-B, Arc and 5-HT1AR (Amigo et al., 2016; Yohn et al., 2017). Administration of 5-HT4R agonist (RS67333) has shown rapid anxiolytic effect in rodents via desensitizing 5-HT1A auto-receptors and increasing hippocampal neurogenesis (Lucas et al., 2007; Yohn et al., 2017). These results are consistent with the findings of the present study, where treatment with 5-HT4R agonist increased the expression of neurotrophic factors (*BDNF*, *NT-3*, *NGF*), *TRK-A* and *CRMP2*, which has been reported to control neural development, plasticity and information processing in the hippocampus (Cho and Hu, 2007; Hagena and Manahan, 2017; Inagaki et al., 2001; Rebholz et al., 2018) (Figure 24). Various 5-HT4R agonists and antagonists have been investigated for their therapeutic potential are shown in Table 6, however, the selection of 5-HT4R agonist is very crucial to investigate specific effect of 5-HT4R. Most of the 5-HT4R agonists work as antagonists for 5-HT3R and similarly 5-HT4R antagonists as 5-HT3R agonists, but RS67333, GR125487 and BIMU8 are selective high affinity ligands specifically interact with 5-HT4R only.

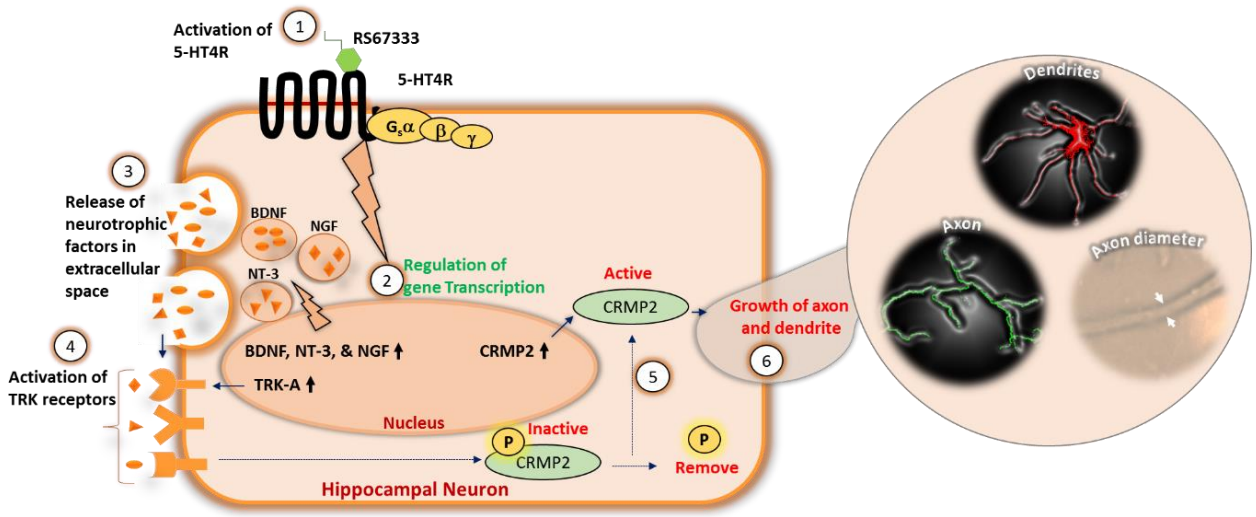

Figure 24. Effect of 5-HT4R agonist RS67333 on neurite formation. Role of 5-HT4R on neurite formation. (1) Pharmacological activation of 5-HT4R through agonist RS67333, (2) Overexpression of CRMP2, BDNF, NT-3, NGF and TRK-A. (3) Increased secretion of BDNF, NT-3 and NGF. (4) Autocrine and paracrine activation of cell membrane receptors via BDNF, NT-3 and NGF. (5) Dephosphorylation of CRMP2. Increase in non-phosphorylated active CRMP2. (6) Growth of axon (length, diameter and branching) and dendrites (length and branching).

Table 6: List of potential agonists and antagonists of 5-HT4R

5-HT4R agonists	References	5-HT4R antagonists	References
5-HT BIMU8 RS67333 RS17017 Cisapride Dazopride Matoclopramide Mosapride Prucalopride Cinitapride Renzapride Tegaserod Zacopride Mosapride citrate (MOS) CJ-033466	(Bockaert et al., 2008; Maeyer et al., 2008; Manabe et al., 2010)	L-lysine Piboserod GR125487 GR113808 NS-3389 SB204070 SB203186 SB205800 SB207266 SDZ205557 SC-53116 SC-53606 SC-56184 RS39604 RS100235	(Torres et al., 1994; Meneses and Hong, 1997; Smriga and Torii, 2003)

Conclusion

Function of 5-HT neurotransmitter system is critical for the development of brain. The distinctive distribution pattern of 5-HTRs in different brain areas suggests important roles in their development. 5-HT4R is present in abundance in limbic forebrain. Early embryonic expression of this receptor may regulate the development of emotional motor centers such as hippocampus and amygdala. In the present study, I explored and extensively investigated the roles of 5-HT4R in mouse embryonic hippocampal neurons *in vitro*. I found that activation of 5-HT4R promoted the axonal growth by increasing the length, diameter and number of axon collaterals, together with the dendritic development by increasing the number of primary dendrites, total dendritic length, and branching. Furthermore, the inside mechanism revealed that the enhanced mRNA expression of *BDNF*, *NT-3*, *NGF*, *TRK-A* and the npCRMP2 may possibly contribute to the axon and dendritic development, which is triggered by 5-HT4R activation. Overall, based on the current investigation, I conclude that 5-HT4R plays a crucial role in the brain development through the promotion of both axon and dendritic growth. The current study focused on cultured neurons; thus, *in vivo* analysis will be required for the futuristic clinical implication. Importantly, the present key findings add a new layer of understanding to provide a platform to establish models for preclinical studies, where 5-HT4R can be targeted for the therapeutic intervention for the treatment of various psychiatric and neurodevelopmental diseases.

Highlights of the study

- 5-HT4R agonist increased the length and branching of axon and dendrites of hippocampal neurons.
- Pharmacological activation of 5-HT4R significantly increased the axonal diameter.
- Treatment with 5-HT4R agonist induced a remarkable increment in the mRNA expression of neurotrophic factors *BDNF*, *NT-3*, and *NGF*.
- 5-HT4R agonist treatment induced elevation in the mRNA expression of *TRK-A* receptor and *CRMP2*.
- 5-HT4R agonist RS67333 increased the expression of non-phosphorylated CRMP2.

Acknowledgments

It gives me immense pleasure to express my deepest gratitude and indebtedness to my supervisor Prof. Takashi Shiga. I've relied on both your amazing capacity to generate many exciting new directions, and your amazing capacity to prune down and recognize what to focus on. You are a joy to work with. I'll forever be inspired by how you practice science, together with being as a good supervisor you are a very good human being also. Your simple, humble, polite, dedicated, punctual and honest personality has inspired me a lot to improve myself at personal and professional level. Next, I would like to thank Dr. Sunil Kumar Vimal (Southwest university China), Dr. Minhua chen (Chung Yuan Christian University, Taiwan), Dr. Ranjith Kumar bakku (University of Tsukuba) and Dr. Masuda (University of Tsukuba) for co-supervising me in my research. Thank you for your inspiration, constructive criticism, support and for sharing your valuable time and attention during the research. Additionally, I would like to thank our collaborators Dr. Sanjib Bhattacharya (Southwest university China) and Dr. Anirban Bandyopadhyay (National Institute for Material Sciences, Japan), who brought an entirely new perspective at the beginning of my research career.

I want to convey my deepest regard and special thanks to all the members of my thesis review committee Takashima sensei, Takei sensei, Koganezawa sensei, and Kaneko sensei; for their valuable suggestions, helpful perspectives, support, and guidance, which significantly improved my thesis. I would also like to show my sincere gratitude towards Yamanaka sensei (Director of Kansei Behavioral and Brain sciences department) and the administration of Kansei department, Graduate school of comprehensive human sciences, and International student support office for their support and guidance. Specially, I would like to convey my deep regards to Yamada san, Akiko san (International student support office) and Sakai San for their unconditional support. I am grateful to all current and former members of the Shiga laboratory for their help, discussion and co-operation special thanks to Kasegai Kun, Li san, Ichikawa san and Nana san for their friendly and supportive nature. Additionally, I wish to express my sincere gratitude to Hattori San and Nagano San from National Institute for Material Sciences, Tsukuba, Japan for their incredible support, company, enough time, cooperation and for extending all possible help during my experiments. Moreover, I would like to acknowledge the open facilities of medical science department at University of Tsukuba.

I would like to show my sincere gratitude to my parents and siblings for supporting me and inspiring me during my hardships. I am forever indebted to you, without whom this work could have never been accomplished. My special thanks go to my friends Ohmori Yoko, Manoj Kumar Yadav, Lalhaba Oinamcha, Arun Kumar, Mustafa Korkutata, Namiko Onodera, Manpreet Kaur Randhawa, Thanasis Poullikkas, Meetu Bharti, Ritesh Kumar Patel and Asami Suga for their unconditional love and unquestioning support. You always pushed me in right direction during my hard times.

I would like to thank to the following funding sources for their support: Toshimi Otsuka Scholarship Foundation, for the financial support during the research (2018-2020), Grant-in-Aid for Scientific Research (26640024) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Finally, I express all my gratitude to almighty God, who helped me directly or indirectly to get this opportunity. I could not have been able to keep my self-confidence and patience without grace of God.

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Figure 11. Flow charts explain the procedure of sample preparation for scanning electron microscope imaging for the measurement of axon diameter. (A) Flow chart represents the complete procedure of sample preparation for scanning electron microscope imaging and analysis. (B) Flow chart represents the fixation protocol for sample preparation.

Figure 12. **Flow chart represents the procedure for the molecular analysis**.

Figure 13. ***In vitro and in vivo*** **expression of 5-HT4R in axons and dendrites of hippocampal neurons**. (A-C) Hippocampal neurons at E18 were cultured for 4 days and *in vitro* expression of 5-HT4R was investigated. 5-HT4R in the dendrites were visualized by anti-MAP2 (red) and anti-5-HT4R (green) antibodies, MAP2 as dendrite marker (A). Staining with anti-SMI-31 (green) and anti-5-HT4R (red) antibodies shows the expression of 5-HT4R in the axon using SMI-31 as an axon marker (B). Fluorescent images are showing the expression of 5-HT4R in GABAergic (GAD65 positive neurons) and non-GABAergic neurons. Neurons were stained with DAPI (blue), 5-HT4R (green) and GAD65 (red) (C). (A-C) Scale bar: 75 μm. (D, E) Immunostaining of cryostat sections of hippocampus at E18 with anti-5-HT4R antibody. Nuclei were stained with DAPI. White arrows are showing CA1, CA2, CA3 and DG regions. DG: dentate gyrus. Magnified view of the DG is shown (E). GCL: granular cell layer, SGZ: subgranular zone. Scale bar: 250 μm (D), 100 μm (E).

Figure 14. **Effects of 5-HT4R agonist (RS67333) on the axon growth**. (A-D) Hippocampal neurons were cultured for 4 days in the absence (A), and presence of 1 nM (B), 10 nM (D) and 100 nM (D) of RS67333. Morphological characterization of axons was performed using anti-SMI-31 (green) and anti-MAP2 antibodies (red), and effects of the treatment of RS67333 were analyzed on axon growth. BI=Branching index, TL=Total axon length. (A-C order) Scale bar: 75 μm. Arrows indicate the longest neurite (axon). (E-F) Treatment with RS67333 significantly increased the total axon length (E) and branching index (F). RS= RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$**p < 0.01$*, *$***p < 0.001$*). The number of neurons analyzed is shown in each bar.

Figure 15. **Effects of 5-HT4R agonists (RS67333, BIMU8) and 5-HT on the axon growth**. (A-D) Hippocampal neurons were cultured for 4 days in the absence (A), and presence of 100 nM RS67333 (B), 100 nM BIMU8 (C) and 100 nM 5-HT (D). The neurons were immunostained with the anti-SMI-31 (green) and anti-MAP2 antibodies (red), and the effects on the axon growth were analyzed. BI=Branching index, TL=Total axon length. Scale bar: 75 μm. Arrows indicate the longest neurite (axon). (E-F) Treatment with RS67333, BIMU8 and 5-HT significantly increased the total axon length (E) and branching index (F). RS=RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$*p <0.05$*, *$**p < 0.01$*, *$****p<0.0001$*). The number of neurons analyzed is shown in each bar.

Figure 16. **Effects of 5-HT4R agonists (BIMU8 and RS67333) and antagonist (GR125487) on the axon growth**. **(**A-E) Hippocampal neurons were cultured for 4 days in the absence of agonist/antagonist (A), and the presence of 100 nM BIMU8 (B), 100 nM RS67333 (C), 100 nM RS67333 + 5 nM GR125487 (D), and 5 nM GR125487 (E). Morphological characterization of axons was performed using anti-SMI-31 (green) and anti-MAP2 antibodies (red), and effects of the treatment of RS67333 were analyzed on axon growth. Arrows indicate the longest neurite (axon). BI= Branching index, TL= Total axon length. Scale bar: 75 μm. (F, G) Treatment with GR125487 neutralized the effects of RS67333 on the total axon length (F) and axonal branching index (G). GR= GR125487, RS= RS67333. Data are shown as mean ± SEM. Purple, violet, green, blue and cyan asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$). (H, I) Hippocampal neurons were cultured for 4 days in the absence (H) and presence of 100 nM RS67333 (I). Scanning electron micrographs showing the ultrastructure of axons. Scale bar: 50 μm (1000 X), 5 μm (6000 X). (J) On the basis of scanning electron micrographs axon diameter was measured. Data are shown as mean ± SEM. Asterisks indicate statistical significance (Student's t-test, Paired, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

Figure 17. **Effects of 5-HT4R agonist (RS67333) on the dendritic growth**. (A-D) Hippocampal neurons were cultured for 4 days in the absence/control (A), and presence of 1 nM (B), 10 nM (C) and 100 nM (D) of RS67333. Morphological characterization of dendrites was performed using anti-MAP2 antibody (red) and effects of the treatment of RS67333 on total dendritic length (TL), average dendritic length (AL), number of primary dendrites (PD), and branching index (BI) were analyzed. Scale bar: 50 μm. Arrows indicate an axon (the longest neurite). (E-H) Bar graphs show that treatment with RS67333 significantly increased the total dendritic length (E), number of primary dendrites (G) and branching index (H), but no effect was observed on average dendritic length (F). RS= RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

Figure 18. **Effects of 5-HT4R agonists (RS67333, BIMU8) and 5-HT on the dendritic growth**. (A-D) Hippocampal neurons were cultured for 4 days in the absence (A), and presence of 100 nM RS67333 (B), 100 nM BIMU8 (C) and 100 nM 5-HT (D). The neurons were immunostained with the anti-MAP2 antibody, and the effects on the dendritic growth were analyzed. AL=Average dendritic length, BI=Branching index, PD=number of primary dendrites, TL=Total axon length. Scale bar: 50 μm. Arrows indicate an axon (the longest neurite). (E-H) Treatment with RS67333, BIMU8 and 5-HT significantly increased the total axon length (E), number of primary dendrites (G) and branching index (H). RS=RS67333. Data are shown as mean ± SEM. Asterisks indicate statistical significance (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

Figure 19. **Effects of 5-HT4R agonists (BIMU8 and RS67333) and antagonist (GR125487) on the dendritic growth**. (A-E) Hippocampal neurons were cultured for 4 days in the absence of agonist/antagonist (A), and the presence of 100 nM BIMU8 (B), 100 nM RS67333 (C), 100 nM RS67333 + 5 nM GR125487 (D), and 5 nM GR125487 (E). Morphological characterization of dendrites was performed using anti-MAP2 antibody (red) and effects of the treatment of RS67333 on total dendritic length (TL), average dendritic length (AL), number of primary dendrites (PD), and branching index (BI) were analyzed. Scale bar: 50 μm. (F-H) Treatment with GR125487 neutralized the effects by RS67333 in the total dendritic length (F), number of primary dendrites (H) and branching index (I). No significant effect of any agonists and antagonist was observed on average dendritic length as compared with the control (G). GR= GR125487, RS= RS67333. Data are shown as mean ± SEM. Purple, violet, green, blue and cyan asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

Figure 20. **Fluorescent images showing the 5-HT4R and CRMP2 colocalization in hippocampal neurons**. (A-C) Neurons were immunostained with antibodies against 5-HT4R (red), CRMP2 (green), and pCRMP2 (blue). B and C are enlarged images shown in A. pCRMP2, CRMP2 and 5-HT4R were expressed in the soma and dendrites (B) and the axon and its collateral branches (C). Scale bar: 75 μm (A), 25 μm (B), 20 μm (C). (D, E) Colocalization of 5-HT4R (green) and CRMP2 (red) in the hippocampus at E18. DAPI staining was performed to label the cell nuclei (D). Arrowheads represent the hippocampal area (DG: dentate gyrus). Scale bar: 100 μm. (D) 2D scattered plot (pixel map) for the green and red channel. The colocalized area of 5-HT4R and CRMP2 was shown in yellow line (dotted) square box. Y-axis shows the green channel intensity, X-axis shows the red channel intensity and diagonal line shows the ratio of green to red channel, which represents the linear regression from both channels (E).

Figure 21. **Effects of CRMP2 expression on 5-HT4R mediated axon formation analyzed by *CRMP2* knockdown study**. (A-D) Hippocampal neurons were cultured for 4 days in the absence of RS67333 (A), the presence of 100 nM of RS67333 (B), 100 nM RS67333 + *CRMP2* siRNA (C), and 100 nM RS67333 + control siRNA *B* (D). Axon growth was evaluated using the immunostaining with anti-SMI-31 (green) and anti-MAP2 antibodies (red) and total axon length (TL) and branching index (BI) were analyzed. Scale bar: 75 μm. (E-G) Bar graphs represent the effects of the *CRMP2* knockdown on total axon length (E), and branching index (F). Expression of *CRMP2* mRNA was analyzed by qRT-PCR (G). RS= RS67333. Data are shown as mean ± SEM, purple, green, violet and blue asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$). 8 culture wells were analyzed in each experimental condition. The number of neurons analyzed is shown in each bar.

Figure 22. **Effects of CRMP2 expression on 5-HT4R mediated dendrite growth analyzed by *CRMP2* knockdown study**. (A-D) Hippocampal neurons were cultured for 4 days in the absence of RS67333 (A), the presence of 100 nM of RS67333 (B), 100 nM RS67333 + *CRMP2* siRNA (C), and 100 nM RS67333 + control siRNA *B* (D). Dendritic growth was evaluated using the immunostaining with anti-MAP2 antibody (red), and total dendritic length (TL), average dendritic length (AL), number of primary dendrites (PD), branching index (BI) were measured. Scale bar: 50 μm. Arrows indicate an axon (the longest neurite). (E-H) Bar graphs represent the effects of the *CRMP2* knockdown on the total dendritic length (E), average dendritic length (F), number of primary dendrites (G), branching index (H). RS= RS67333. Data are shown as mean ± SEM. Purple, green, violet and blue asterisks indicate statistical significance with the bar of corresponding color (One-way ANOVA with Tukey's post hoc test; *$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$). The number of neurons analyzed is shown in each bar.

Figure 23. **Analysis of molecular pathways associated with 5-HT4R**. (A-E) Hippocampal neurons were cultured for 4 days in the absence (control) and presence of a 5-HT4R agonist (RS67333). qRT-PCR showed that treatment of RS67333 increased the mRNA expression of *CRMP2* (A). Lysates of cultured hippocampal neurons were separated by SDS-PAGE and immunoblotted with anti-pCRMP2 and anti-CRMP2 antibodies. β-actin was used as an internal control (B). Immunoblot analysis showed that treatment of RS67333 decreased pCRMP2, while increased CRMP2 (C). qRT-PCR showed that treatment of RS67333 increased the mRNA expression of neurotrophic factors (*BDNF*, *NT-3* and *NGF*) (D), and *TRK* receptors (*TRK-A, TRK-B,* and *TRK-C)* (E). (A-E) Data are shown as mean ± SEM. Asterisks indicate statistical significance (Student's t-test; paired, *$p <0.05$, ***$p < 0.001$). At least 4 wells were analyzed in each experimental condition.

Figure 24. Effect of 5-HT4R agonist RS67333 on neurite formation. Role of 5-HT4R on neurite formation. (1) Pharmacological activation of 5-HT4R through agonist RS67333, (2) Overexpression of *CRMP2*, *BDNF*, *NT-3*, *NGF* and *TRK-A*. (3) Increased secretion of BDNF, NT-3 and NGF. (4) Autocrine and paracrine activation of cell membrane receptors via BDNF, NT-3 and NGF. (5) Dephosphorylation of CRMP2. Increase in nonophosphorylated active CRMP2. (6) Growth of axon (length, diameter and branching) and dendrites (length and branching).

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Appendix

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Agrawal L, Vimal SK, & Shiga T (2019) Role of serotonin 4 receptor in the growth of hippocampal neurons during the embryonic development in mice. *Neuropharmacology*, 158, 107712. (ISSN: 0028-3908, doi.org/10.1016/j.neuropharm.2019.107712).

Agrawal L, Korkutata M, Vimal SK, Yadav MK, Bhattacharyya S, & Shiga T (2020) Therapeutic potential of serotonin 4 receptor for chronic depression and its associated comorbidity in the gut. *Neuropharmacology*, 166, 107969. (ISSN: 0028-3908, doi.org/10.1016/j.neuropharm.2020.107969).

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