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Neuropharmacological study in the anti-depressive effects of rivastigmine

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Neuropharmacological study in the anti-depressive effects of rivastigmine

リバスチグミンの抗うつ様効果の作用機序に関する神経薬理学的研究

GRADUATE SCHOOL OF PHARMACEUTICAL SCIENCES

TOHOKU UNIVERSITY

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
ACh	acetylcholine
AD	Alzheimer`s disease
Akt	protein kinase
ANOVA	analysis of variance
BuChE	butylcholinesterase
BrdU	5-bromo-2`-deoxyuridine
ChAT	choline acetyltransferase
DCX	doublecortin
DG	dentate gyrus
DOI	2,5-dimethoxy-4-iodoamphetamine
5,7-DHT	5,7-dihydroxytryptamine
DRN	dorsal raphe nucleus
ERK	extracellular signal-regulated kinase
FST	forced swim test
GABA	gamma amino butyric acid
GCL	granule cell layer
5-HT	serotonin
5-HT$_{1A}$	serotonin 1A
5-HT$_{2A}$	serotonin 2A
I.P.	intraperitoneal
KO	knock out
mACh	muscarinic acetylcholine
NSFT	novelty suppressed feeding test
nACh	nicotinic acetylcholine
OBX	olfactory bulbectomy
8-OH-DPAT	7-8-hydroxy-2-(di-n-propylamino) tetralin
PBS	phosphate-buffered saline
PCPA	para-chlorophenylalanine
S.C.	subcutaneous
SGZ	sub granular zone
TCB-2	4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine
TST	tail suspension test
WAY-100635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide

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Chapter 1: General Introduction

1.1 Introduction

Dr Alois Alzheimer, in his classic early twentieth century case description of Alzheimer's disease noted behavioral symptoms as prominent manifestations in his brief case description (Barrios, 1990). The cognitive deficit symptoms are prevalent in up to 98% of patients (Steinberg et al, 2006). The non-cognitive symptoms of dementia are not well defined (Zaudig, 1996). In 2001, an estimated 24.3 million people had dementia and this is expected to double every two decades as life expectancy also increases; rising to 81.1 million in 2040 (Ferri et al, 2005). The cognitive impairments as fundamental to dementia had focused on the mechanism of cognitive deficits (cognitive paradigm), whereas less attention on the non-cognitive symptoms of dementia in particular behavioral disturbances had provided (Zaudig, 2000). The behavioral and psychological symptoms of dementia (BPSD) is a term used to describe a heterogeneous range of psychological reactions, psychiatric symptoms, and behaviors occurring in people with dementia of any aetiology (Finkel et al, 2000). Thus, BPSD is an umbrella term that catalogues and characterizes the non-cognitive or neuropsychiatric symptoms of dementias including AD (Cummings, 1987).

1.2 Depression of Alzheimer`s disease

The extent of symptoms of depression in AD patients has been reported as over 50%, of which 23% was major depression (Migliorelli et al, 1995). Burns et al (1990) and Lyketsos et al., (2000) reported over a 20% prevalence of depression in patients. Diagnosing depression in AD is difficult because there is difficulty in discriminating apathy symptoms from depressive symptoms. Over 50% of Alzheimer's patients with depression were co-morbid apathy symptoms (Lyketsos et al, 2001). The expression of depressive symptoms is thought to be changed during AD progression (Lee and Lyketsos, 2003). Depression is a complex and

multifactorial illness that involves multiple neural circuits and genetic and non-genetic risk factors. The core symptoms of depression are low mood, feeling of hopelessness, decreased ability to concentrate, anhedonia (diminished interest in pleasurable activities), daily insomnia or hypersomnia, weight gain or loss and thought of death or suicide (American Psychiatric Association, 2000).

A dysfunctional serotonergic system involved in the pathophysiology of depression is well supported by a wide range of experimental studies (Mahar et al, 2013). Currently, there are many treatments for depression, including psychotherapy, electroconvulsive therapy, and antidepressant medications. Despite the effectiveness of large number of antidepressants, those actions on serotonergic system and/or adult neurogenesis at the molecular and cellular levels still remains unclear.

1.3 Hippocampal neurogenesis

New neurons born in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus migrate into the granule cell layer (GCL) of the DG and eventually become mature granule cells. The newborn neurons are integrated into the existing circuit and receive functional input (Zhao et al, 2008). Therefore; adult neurogenesis in the hippocampus is defined as the progression from neural stem cell (NSC) to mature dentate granule neuron.

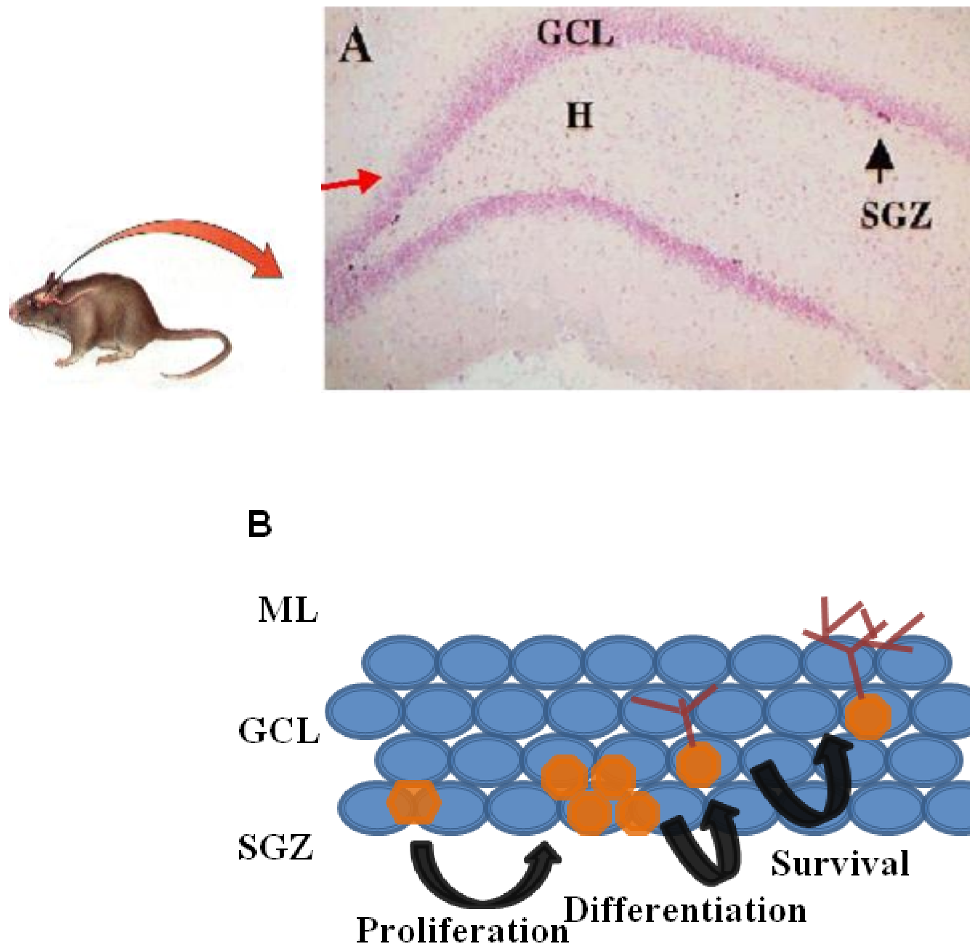

Fig 1.1 An illustration of the hippocampal neurogenesis (cited from Hippocampus.2006; 116, 239-249)

Based on our understanding of neurogenesis in the SGZ, all stages of adult neurogenesis are regulated by physiological activity, including proliferation, differentiation of neural NSCs and survival of newborn neurons (Zhao et al., 2008). Definition of proliferation and differentiation of newborn cells requires a specific protocol to define the DNA replication by administration of a synthetic thymidine analog, 5-bromo-2´-deoxyuridine (BrdU), which substitutes for thymidine incorporation into DNA synthesized during the S-phase of the cycle and the sacrifice of animals (Miller and Nowakowski,1988; Kempermann et al,1997).

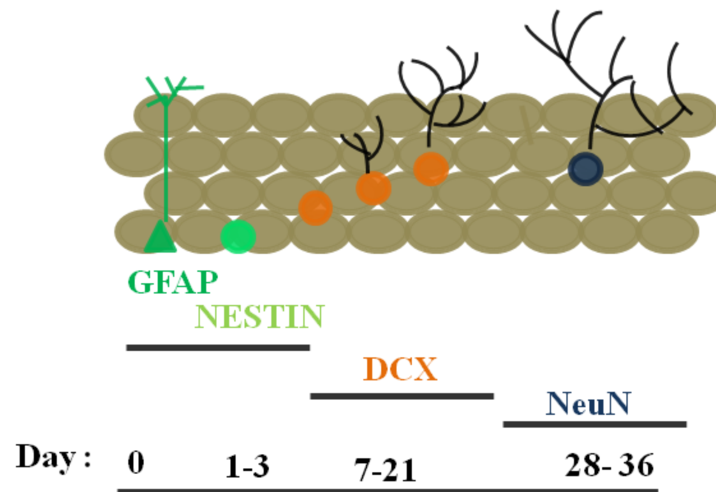

Fig 1.2 A schematic diagram for differentiation and maturation of neuronal stem cell. From left to right, positive radial glia-like progenitors (green) expressing glial fibrillary acidic protein (GFAP), rapidly amplifying neural progenitors (light green) expressing nestin, immature granule cells (orange) expressing immature neuronal marker doublecortin (DCX) and mature granule cells (blue) expressing mature neuronal marker NeuN (cited from The Neuroscientist, 2010; 16:578-591)

Soon after the discovery of adult hippocampal neurogenesis in humans (Eriksson et al, 1998), it was hypothesized that the deficits in hippocampal neurogenesis relates to the pathophysiology of depression (Kempermann, 2002). This is supported by studies showing that the increases in hippocampal neurogenesis are associated with antidepressants effects mirroring the time required for newly proliferated neurons to become functional (Mahar et al,2011), that antidepressant treatment increase hippocampal neurogenesis (Boldrini et al,2009), and that the behavioral effects of antidepressants may require intact neurogenesis (Perera et al,2011; Wang et al,2008). However, to understand the importance of adult hippocampal neurogenesis in the mechanism of antidepressants, it is important to discuss the regulation of neurogenesis by endogenous 5-HT systems in the hippocampus.

1.4 Serotonergic system

1.4.1 Serotonergic innervation in the dentate gyrus

The idea that serotonin (5-HT) influences neurogenesis was first proposed three decades ago (Lauder and Krebs, 1978). 5-HT is produced from the essential amino acid L-tryptophan in neurons of midbrain raphe nuclei. Serotonergic projections originating from the dorsal raphe nucleus (DRN) and median raphe nuclei (MRN) diffusely innervate multiple structures in the vertebrate forebrain (Jacobs and Azmitia, 1992). The serotonergic inputs to the hilus, molecular layers of the DG and the SGZ supports the possibility that 5-HT signaling may influence adult neurogenesis (Oleskvich et al, 1991). Interestingly, the effects of 5-HT mediated signaling on hippocampal neurogenesis appear to be receptor subtype-specific.

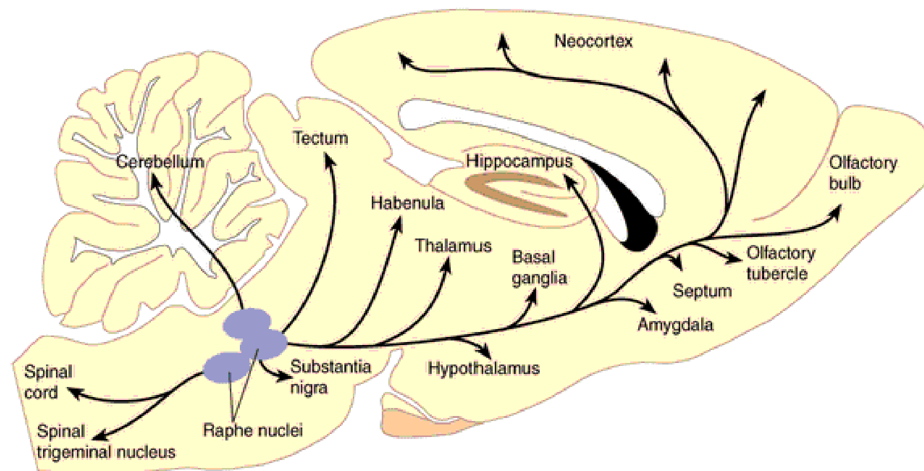

Fig 1.3 Serotonin pathways in the rat brain (cited from Biology of Serotonergic Transmission,1982; pp.29-61)

1.4.2 Serotonin receptors

5-HT activates fourteen different 5-HT receptor subtypes that are classified into seven families (5-HT_{1-7}) and almost all of these receptors are expressed in the DG (Djavadian,2004; Nichols and Nichols,2008). Most of the 5-HT receptor subtypes are G-protein coupled

metabotropic receptors, with the exception of $5\text{-}HT_3$ receptor which is a ligand-gated ion channel.

Out of the numerous serotonergic receptors, the $5\text{-}HT_{1A}$, a Gi/Go-protein-coupled receptor, is the one involved in regulation of neurogenesis in the DG (Tanti and Belzung, 2013). For example, acute administration of $5\text{-}HT_{1A}$ receptor antagonist, WAY-100635 decreases cell proliferation in the adult rat DG (Radley and Jacobs, 2002). Fluoxetine, selective serotonin reuptake inhibitor (SSRI), treatment fails to produce proliferation of neural progenitor cells in $5\text{-}HT_{1A}$ receptor knockout (KO) mice (Santarelli et al, 2003). Conversely, acute or chronic treatment with the $5\text{-}HT_{1A}$ receptor agonist, 8-OH-DPAT, promotes neurogenesis in the DG (Santarelli et al., 2003; Banasr et al., 2004). In addition, activation of $5\text{-}HT_{2A}$ receptors also increases neurogenesis in the DG, while agonists of the $5\text{-}HT_{1B}$ receptors increases the neurogenesis only after serotonergic depletion (Banasr et al, 2004).

Other types of serotonergic receptors that may be involved in mediating some effects of 5-HT on proliferation in the DG include $5\text{-}HT_{4,}$ $5\text{-}HT_6$ and $5\text{-}HT_7$ receptors. All of them, when activated, trigger cAMP-CREB (cAMP response element-binding protein) cascade, thereby increasing expression of BDNF (Brain derived neurotrophic factor). BDNF does not directly change the rate of neurogenesis in the DG, but it may increase the release of 5-HT (Siuciak et al, 1996) and in turn stimulates neurogenesis through increased activation of the $5\text{-}HT_{1A}$ receptors (Duman et al, 2001). Taken together, the differential effects of recruiting different post-synaptic 5-HT receptors on hippocampal neurogenesis.

1.4.3 Serotonin1A ($5\text{-}HT_{1A}$) receptor-mediated signaling pathway in hippocampal neurogenesis

Activation of $5\text{-}HT_{1A}$ receptors increases proliferation of neuronal progenitors and promotes development of neural precursors into adult neurons. Neurogenesis and neuroprotective

effects of 5-HT_{1A} receptors is mediated by the growth factor-associated signaling including extracellular signal- regulated kinase (ERK) and protein kinase B (Akt) pathway (Hsiung et al,2005). In hippocampus-derived HN2-5 cells, 5-HT_{1A} receptor agonists increase ERK phosphorylation, where growth factor-regulated ERK activation is mediated by the small GTPases Ras and Raf (Adayev et al, 1999). Activation of this pathway leads to changes in downstream pathway and activate transcription factor CREB (Xing et al, 1996).

Another growth factor-regulated signaling pathway Akt can also be regulated by 5-HT_{1A} receptors. When tyrosine kinase receptors are activated by growth factors, they recruit phosphatidylinositol-3-kinase (PI3K) to activate Akt (Sale and Sale, 2008). Activation of Akt leads to inactivation of glycogen synthase kinase 3 (GSK3), downstream target of Akt and finally directly or indirectly activates CREB (Cross et al, 1995).

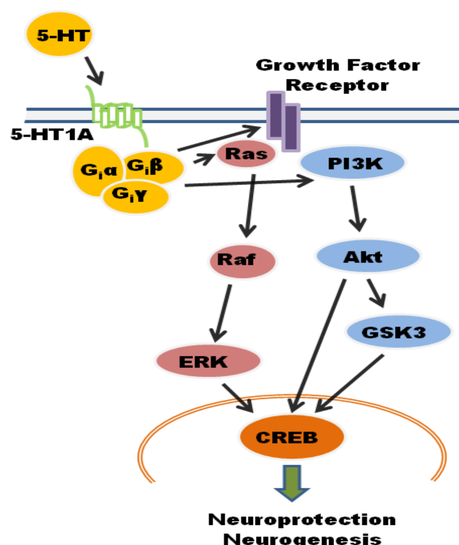

Fig 1.4 5-HT_{1A} receptor-regulated ERK and Akt signal transduction pathways (cited from Cellular Signaling 22:1406-1412)

1.5 Olfactory bulbectomy (OBX)

The olfactory bulbectomy (OBX) syndrome is a useful model for detecting anti-depressant activity since the behaviors and neurotransmitters related depression are observed in OBX

(Kelly et al, 1997). The olfactory bulbs are bilateral extensions of the rostral telencephalon and constitute about 4% of the total brain mass in the adult rat (Cain, 1974). Extensive efferent connections with limbic and higher brain center would implicate more far-reaching effects of OBX than anosmia alone.

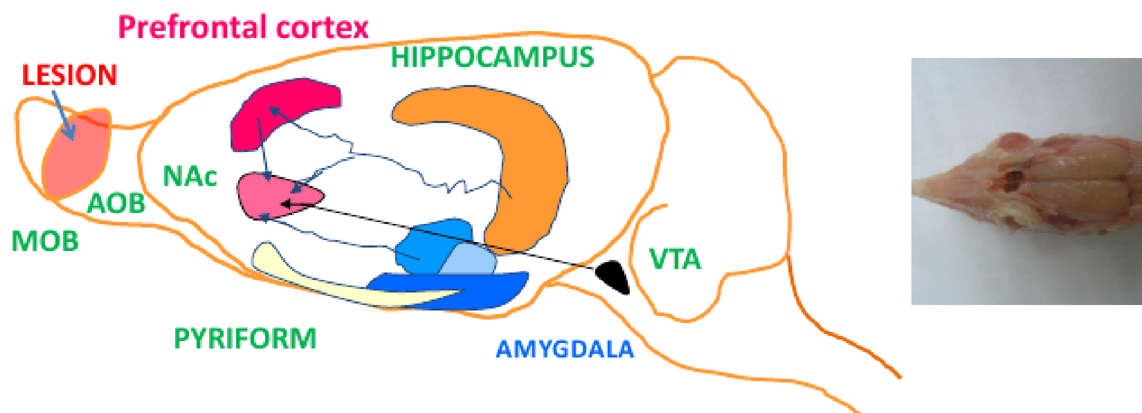

Fig 1.5 Schematic representation of the location of the burr holes relative to the main anatomical features of the rat skull and position of the olfactory bulb relative to important brain regions that are disputed following bulbectomy (cited from R Andrew Chambers presentation on Frontiers 2005 meeting)

As a result, it is not surprising to find that a cascade of changes emerges following the disruption of connections between the bulbs and other brain regions. Indeed, bilateral removal of the olfactory bulbs induces neurochemical, neuroanatomical, physiological, endocrine and behavioral changes in animals and a period of 2 weeks is usually allowed for recovery from the surgical procedure and is optimal for the development of the bulbectomy syndrome (van Riezen and Leonard, 1990). For example, due to the degeneration of serotonergic fibers innervating the main olfactory bulb (McLean and Shipley, 1987), OBX mice showed neurodegeneration (Nesterova et al, 1997) as well as reduced 5-HT synthesis (Neckers et al, 1975) in the DRN, can be reversed by chronic treatment with the SSRI fluvoxamine, citalopram (Watanabe et al, 2003; Hasegawa et al, 2005; Saitoh et al, 2007). Therefore, OBX

is unique in its sensitivity to chronic, but not acute administration of clinically efficacious antidepressants (Skelin et al, 2010).

Impairment of the central serotonergic system also mediate the behavioral changes observed after OBX. Lesions of the serotonergic projections to the olfactory bulb in rats following local injection of 5,7-DHT produced the same hyperactivity in the open field test as seen in OBX rats (Song and Leonard,2005). Furthermore, because of its cholinergic alterations, the OBX rats are also used as model of AD, as there is an elevation of beta-amyloid in the rat brain after bulbectomy (Aleksandrova et al, 2004).

1.6 Rivastigmine

The discovery and development of rivastigmine (ENA 713, or carbamoylatine; (+) (S)-N-ethyl-3-［(1-dimethyl-amino)ethyl］−N−methylphenylcarbamate hydrogen tartrate) arose from the search for a novel ChE inhibitor with enhanced clinical efficacy and improved pharmacokinetic and pharmacodynamic properties compared with earlier compounds (Ballard, 2002). In 2006, it was approved for treatment of mild to moderate dementia associated with Parkinson's disease and the rivastigmine transdermal patch was approved for the first patch treatment for dementia in 2007. Rivastigmine has no affinity for muscarinic, alpha- or beta-adrenergic, dopamine receptors or opioid binding sites (Enz et al., 1993).

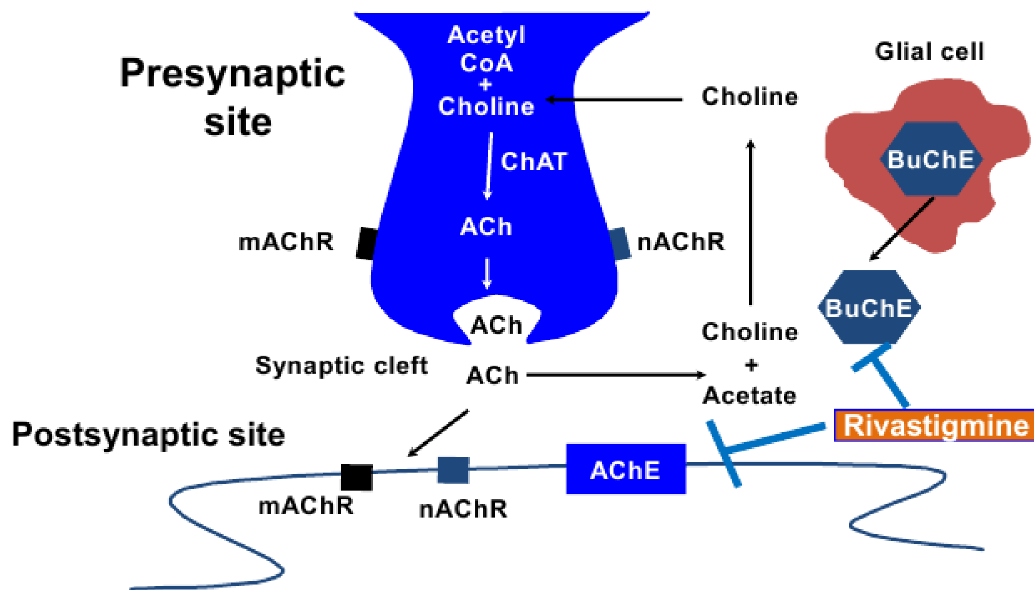

Fig 1.6 Mechnaism of action of dual ChE inhibitor, rivastigmine

The majority of cholinesterase in the brain, called acetylcholinesterase (AChE), is of neuronal origin and functions to metabolize ACh at synapses throughout the nervous system. The remainder, called butyrylcholineterase (BuChE), is of glial origin and has also been able to hydrolyze ACh in the brain (Guillozet et al, 1997). A decrease of ACh in the brain of AD patients appears to be a critical element in producing dementia. As AD progresses and cortical neurons are lost, levels of AChE in the brain progressively decline, while levels of BuChE increase (Perry et al, 1978). BuChE can and does take over the function to metabolize ACh at the synapse when AChE is lost, a phenomenon that has been demonstrated in an AChE KO mouse model and that probably occurs in AD (Mesulam et al, 2002). Sedimentation analysis of normal brain shows that the most abundant AChE forms are globular forms with tetramer (G_4) and monomer (G_1) structures. Interestingly, levels of G_4 form decline as AD progresses while G_1 form level relatively unchanged (Siek et al, 1990). Only one inhibitor among the

AChE inhibitors, rivastigmine, displayed preferential inhibition for the G_1 form (normal, IC_{50}:5100±100 nM; AD, IC_{50}:3500±100 nM) of AChE (Rakonczay,2003). In addition, for BuChE, the most specific was rivastigmine (IC_{50}:238±20 nM). So, drug inhibiting both AChE and BuChE is regarded better treatment strategy in AD than either selective AChE or BuChE. Rivastigmine is also effective in patients that no longer responded to donepezil (selective AChE ; IC_{50} :323±126 nM) treatment.

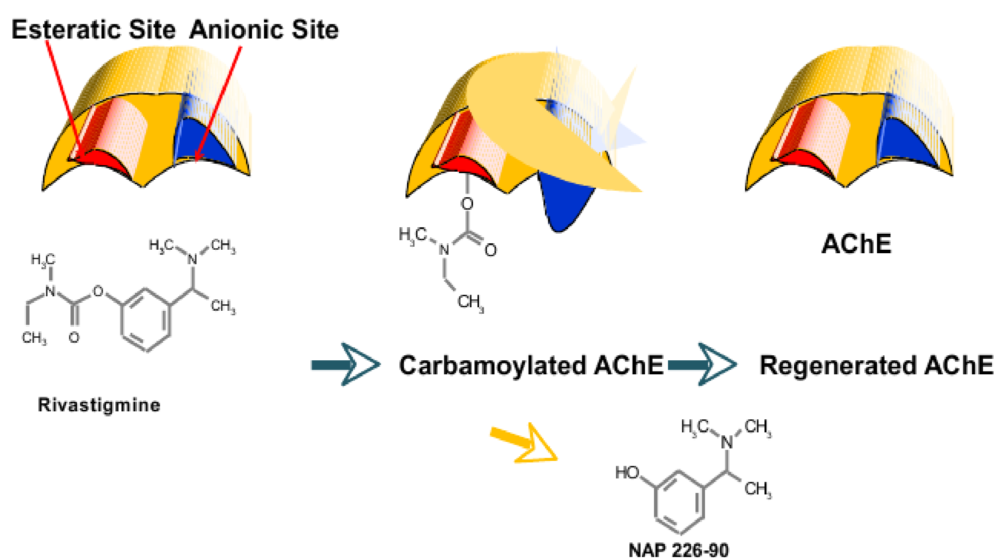

Fig 1.7 Model of rivastigmine (Alzheimer's disease: from molecular biology to therapy, 1996:211-215)

Rivastigmine fits into the enzyme's active site in a similar fashion to ACh, which results in a "flattening" of the carbamate moiety over the esteratic site, producing the prolonged inhibition of AChE. Like tacrine and donepezil, rivastigmine is hydrolyzed, but unlike these two agents, it leaves the esteratic site carbamylated and the phenolic derivative is excreted rapidly from the body (Polinsky, 1998). Sequestration of AChE in the carbamylated form precludes further enzyme hydrolysis of ACh. Based on administration of a single dose, the carbamate moiety can inhibit enzyme activity up to10 hours. Due to this longer duration

of action, rivastigmine is classified as an intermediate-acting (pseudo-irreversible) agent.

Rivastigmine is weakly bonded to plasma proteins (40%), explaining its short half-life ($t_{1/2}$)in plasma (~60 mins) as compared with a 8.5-10 hr half-life for ChE inhibition, allows twice daily dosing of rivastigmine. After oral administration, rivastigmine is rapidly absorbed and extensively metabolized, primarily by ChEs, to NAP 226-90, which then undergoes N-demethylation and sulphate conjugation. After metabolism, rivastigmine is rapidly eliminated by kidney, so the occurrence of clinically relevant drug-interactions is unlikely (Polinsky, 1998).

1.7 Objectives

1. I investigate whether rivastigmine treatment improves behavioral and psychological symptoms of dementia (BPSD) behaviors including hyperactivity and depression seen in Alzheimer's model mice.

2. I examine the mechanisms underlying improving depression by rivastigmine. I also define the stages of the hippocampal neurogenesis in response to rivastigmine and determine downstream signaling pathways responsible for rivastigmine-induced neurogenesis.

3. I elucidate involvement of serotonergic innervations to the hippocampus in the rivastigmine-mediated effects.

Chapter 2: 5-HT_{1A} receptor is involved in rivastigmine-induced improvement of depression-like behaviors in OBX mice

2.1 Introduction

Pharmacological studies in rodents indicate a central role for 5-HT receptors in modulating depressive behaviors. For example, among 5-HT receptor subtypes, serotonin receptor type 1A (5-HT_{1A}) and serotonin receptor type 2A (5-HT_{2A}) are of particular interest due to their association with drugs used to treat depression (Bhagwagar et al, 2006). 5-HT_{1A} receptor KO mice on various genetic backgrounds (specifically, 129/Sv, C57BL/6J, and CBA/J strains) also display increased anxiety-related behaviors (Klemenhagen et al, 2006), whereas 5-HT_{2A} receptor KO mice (from 129S6/SvEv strain) exhibit reduced anxiety in open field, elevated zero-maze, novel object, novelty-suppressed feeding and light-dark tests (Weisstaub et al, 2006). We recently reported that septal cholinergic neurons innervating the hippocampus degenerate in olfactory bulbectomized (OBX) mice, resulting in impaired learning behaviors (Han et al, 2008), and that reduced Akt and ERK activities in the hippocampal dentate gyrus (DG) correlate with both reduced neurogenesis and with depression-like behaviors (Shioda et al, 2011; Moriguchi et al, 2013). Notably, OBX also induces neurodegeneration in the raphe nuclei of C57B1/6j mice (Nesterova et al, 1997), possibly due to degeneration of serotonergic neurons innervating the olfactory bulb (Mclean and Shipley, 1987). Thus, the OBX mouse represents an attractive model of not only learning impairment but also depression-like behaviors (Harkin et al, 2003). Therefore, a study of a possible role of rivastigmine in depressive behaviors is an interesting theme in Alzheimer`s & dementia research.

2.2 Objectives

Aim of the present study were (1) to determine the mechanism underlying rivastigmine-induced improvement of depressive-like behaviors in OBX mice and (2) to

examine function for 5-HT_{1A} receptors in rivastigmine effects on OBX-induced hyperactivity and depressive-like behaviors.

2.3 Experimental design

After one week acclimatization period, OBX mice were prepared, as previously described by Hozumi et al. (2003). Sham operations were performed in the same way, but the olfactory bulbs were left intact. The 14-days post-surgery time interval was considered to be sufficient in order to guarantee an appropriate recovery of animals and development of persistent OBX-induced effects (Jarosik et al,2007; van Riezen and Leonard, 1990). As depicted in Fig 2.1.A, 14 days after surgery (1st-14th day, recovery period), I started drugs treatment (n=5~7 per group) and continued for a period of 14 days (15th-28th day, treatment period).

Rivastigmine from Novartis Pharma was administered subcutaneously (s.c.) in three doses (0.1, 0.3 and 1.0 mg/kg in a volume of 1 ml/100 g body weight) and dissolved in 0.9% saline immediately before injection. Sham and OBX mice received equal volumes of 0.9% saline (1.0 mg/kg, i.p.). To assess involvement of 5-HT_{1A} and 5-HT_{2A} receptor subtypes, mice were treated with 5-HT_{1A} receptor antagonist, 1.0 mg/kg WAY-100635 (s.c.) and the 5-HT_{2A} receptor antagonist 1.0 mg/kg ketanserin (i.p.). I administered antagonists 30 min before rivastigmine treatment, as described previously by Zeni et al.(2012). The administration schedule and the dose of various drugs used were chosen based on published studies of animal behavioral models (Abdel-Aal et al, 2011; Furukawa-Hibi et al, 2011; Moriguchi et al,2014).

For immunohistochemical studies (n=5 per group), I administered BrdU once daily for 5 consecutive days at 50 mg/kg (i.p.), as depicted in Fig 2.1.B. 35 days after OBX, coronal mouse brain sections were prepared according to a previously reported method by Shioda et al. (2010).

To define the time and order for behavioral tests, i performed the behavioral tasks from the least to the most stressful (Mcllwain et al, 2001; Teegarden S, 2012) after twenty-four hrs of last drug administration. Mice were first subjected to a Tail suspension test (TST), followed by a Forced swim test (FST) in the following day as previously described (Steru et al, 1985; Porsolt et al, 1977). Twenty four hours after the end of the last test (FST), mice were sacrificed for biochemical analyses and lesions were verified histologically. I also measured locomotor activities and the Novelty Suppressed Feeding Test (NSFT) in separate animal groups (n=5~7 per group).

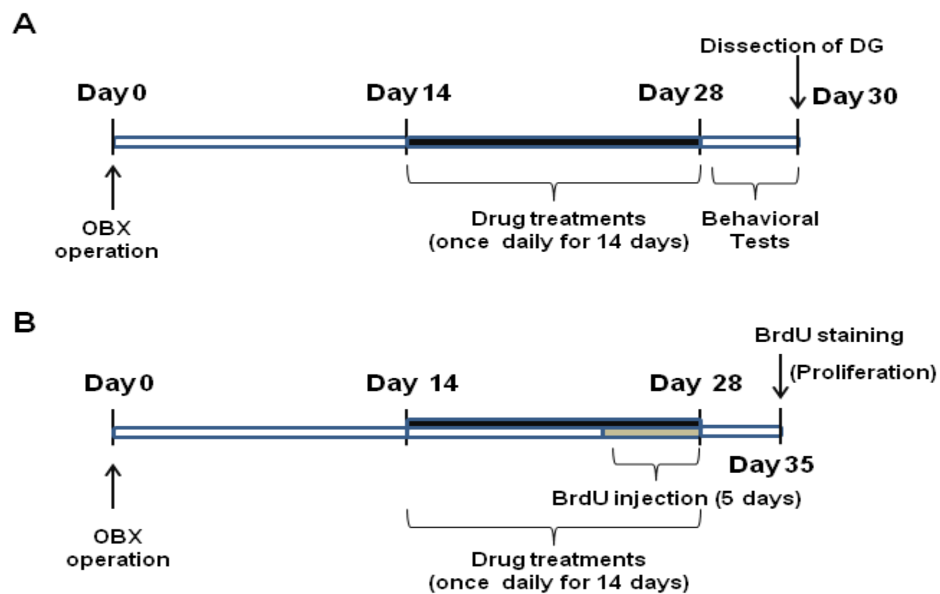

Fig 2.1 Schematic presentation of experimental design (cited from Neuroscience, 2014;272 :116-130)

2.4 Results

2.4.1 Effect of rivastigmine on depressive behaviors

I first investigated whether rivastigmine treatment improved depressive-like behaviors in OBX mice. Mice were first subjected to TST and then FST to determine rivastigmine effect. In the TST, I found a significant group effect on immobility time [$F(5, 31) = 11.609$, P <

0.01]. OBX mice experienced significantly greater immobility time than did sham-operated mice (sham: 80.28 ± 4.90 sec, n=7; OBX: 128.5 ± 6.21 sec, n=6; $P < 0.01$ vs. sham; Fig. 2.2.A), indicative of depression-like behavior. Interestingly, immobility time significantly decreased following administration of either dose (0.3 or 1.0 mg/kg) of rivastigmine relative to OBX mice (0.3 mg/kg: 94.83 ± 9.02 sec, n=6, $P < 0.05$ vs. OBX: 1.0 mg/kg: 84.67 ± 4.09 sec, n=6, $P < 0.05$ vs. OBX). There was no significant difference in terms of immobility time between Sham and 1.0 mg/kg rivastigmine-treated sham mice .

Next, when I analyzed immobility time using the FST, I also observed a significant group effect between treatment groups [$F(5,31) = 11.845$, $P < 0.01$]. OBX mice showed significantly increased immobility time compared to sham-operated mice (sham: 110.14 ± 4.58 sec, n=7; OBX: 157.33 ± 5.05 sec, n=6; $P < 0.01$ vs. sham; Fig. 2.2.B). No changes were observed in sham-operated mice injected with 1.0 mg/kg rivastigmine. Significant decreases in immobility time relative to untreated OBX mice were observed following either dose (0.3 or 1.0 mg/kg) of rivastigmine (0.3 mg/kg: 126.67 ± 6.10 sec, n=6, $P < 0.05$ vs. OBX; 1.0 mg/kg: 118.50 ± 5.77 sec, n=6; $P < 0.05$ vs. OBX).

The particular score on the NSFT reportedly differentiates chronic versus subchronic responses to antidepressant treatments and has been reported to neurogenesis-dependent (Wang et al, 2008; David et al, 2009). Therefore, I measured the latency to feed in the NSFT and found a significant group effect [$F(5, 31) = 10.297$, $P < 0.01$]. Compared to sham-operated mice, I observed a significant increase in latency to feed in OBX mice ($P < 0.01$ vs. sham; Fig. 2.2.C), indicating depression-like behavior. As expected, the change in latency to feed was reversed by rivastigmine administration (0.3 or 1.0 mg/kg) in OBX mice (0.3 mg/kg: $P < 0.05$ vs. OBX; 1.0 mg/kg: $P < 0.01$ vs. OBX; Fig. 2C). Home cage food consumption was not altered in any group (Fig. 2.2.D), and sham mice showed no significant

effect on latency to feed after 1.0 mg/kg rivastigmine. Overall, these data indicate that rivastigmine treatment improves depressive-like behaviors in OBX mice and suggest that improvements are due to enhanced hippocampal neurogenesis.

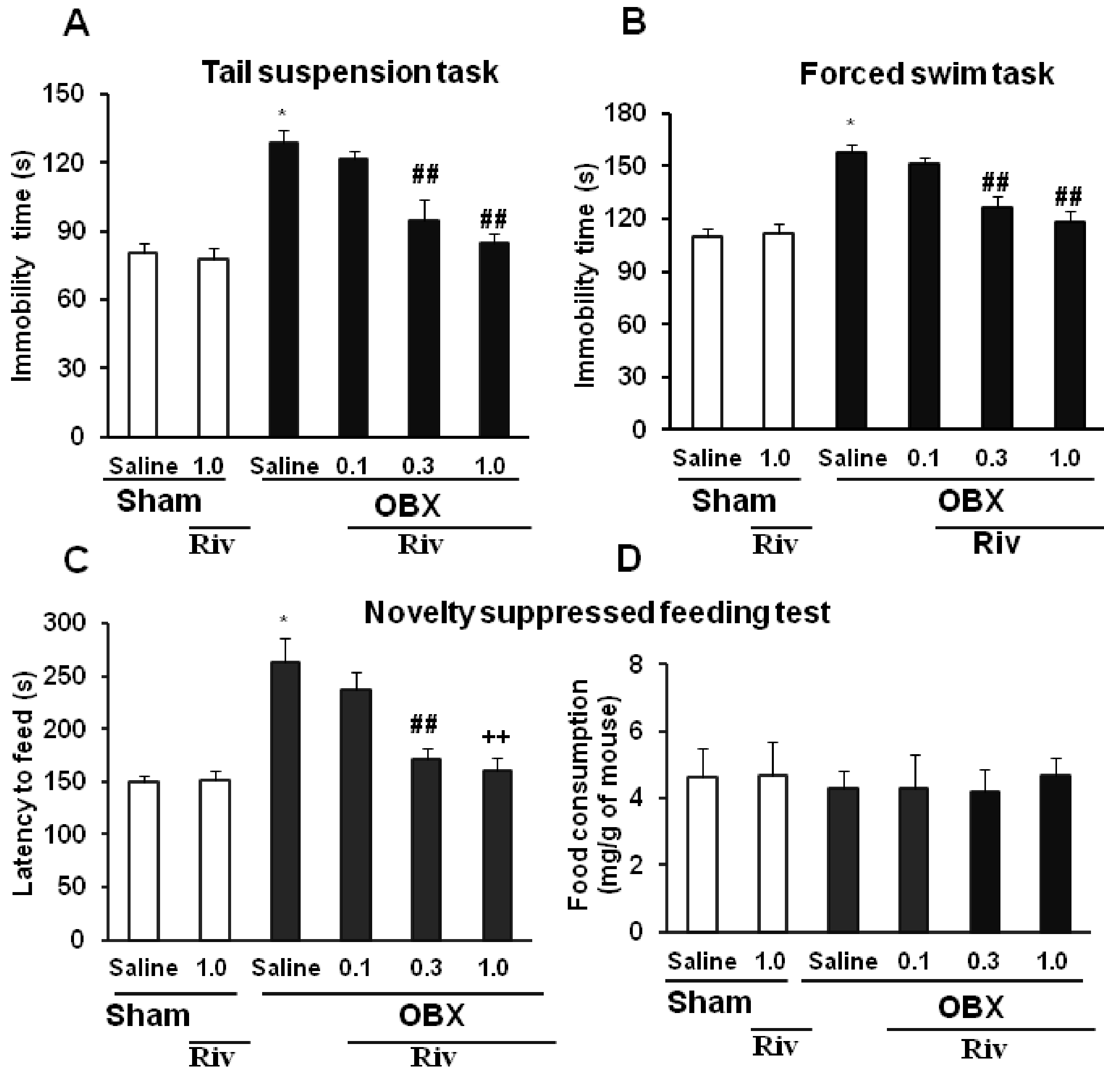

Figure 2.2. Rivastigmine improves depression-like behaviors in OBX mice A: Immobility time in a TST was measured after chronic rivastigmine (Riv) administration (at 0.1, 0.3 or 1.0 mg/kg, s.c.) for 14 days following OBX operation. B: Immobility time in a FST was measured in the same groups. C: Latency to feed in the NSFT was measured after chronic rivastigmine administration (at 0.1, 0.3 or 1.0 mg/kg, s.c.) for 14 days following OBX. D. Food consumption by the same groups was also measured in the NSFT Each bar represents the mean ± S.E.M. n= 5-7 in each group. (*p < 0.01 versus sham-operated mice, ##p < 0.05 versus OBX mice) (cited from Neuroscience, 2014; 272:116-130).

2.4.2 Effect of rivastigmine on increased locomotor activity

Next, I examined whether hyperactivity seen in OBX mice augments following rivastigmine and observed a significant group effect on locomotor activity [$F(5, 31) = 47.473$, $P < 0.01$]. Locomotor activities of OBX mice markedly increased compared to those seen in sham-operated mice ($P < 0.01$ vs. sham; Fig. 2.3), and those activities were significantly decreased relative to OBX only mice following administration of 0.3 or 1.0 mg/kg rivastigmine ($P < 0.05$ vs. OBX, Fig. 2.3). Sham mice showed no significant effect of 1.0 mg/kg rivastigmine. This observation indicated that rivastigmine attenuates OBX-related hyperactivity.

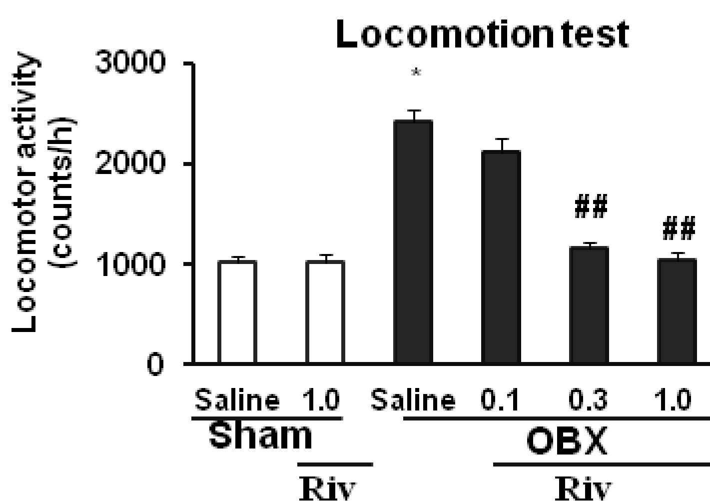

Figure 2.3. Rivastigmine antagonies OBX-related hyperactivity. Locomotor counts per hr were measured in the same groups after chronic rivastigmine administration (at 0.1, 0.3 or 1.0 mg/kg, s.c.) for 14 days following OBX. Each bar represents the mean ± S.E.M. n= 5-7 in each group. (*$p < 0.01$ versus sham-operated mice, ##$p < 0.05$ versus OBX mice) (cited from Neuroscience, 2014; 272:116-130).

2.4.3 Effects of WAY-100635 and ketanserin on anti-depressive effect of rivastigmine

I next elucidated whether 5-HT_{1A} and 5-HT_{2A} receptors function in rivastigmine-induced anti-depressive activity using WAY-100635, a 5-HT_{1A} antagonist, or ketanserin, a 5-HT_{2A}

antagonist. ANOVA analyses showed a significant group effect on immobility time in the TST [$F(6,36) = 11.88$, $P < 0.01$]. Increased immobility time observed in OBX mice (sham: 81.71 ± 4.97 sec, n=7; OBX: 133.67 ± 4.45 sec, n=6; $P < 0.01$ vs. sham; Fig. 2.4.A) was improved by 1.0 mg/kg rivastigmine treatment (1.0 mg/kg: 84.33 ± 5.96 sec, n=6; $P < 0.01$ vs. OBX). WAY-100635 pre-administration (0.3 or 1.0 mg/kg) partially eliminated rivastigmine-induced immobility time reduction (0.3 mg/kg: 119.50 ± 5.49 sec, n=6; 1.0 mg/kg : 116.33 ± 6.07 sec, n=6; $P < 0.05$ vs. rivastigmine-treated OBX mice), whereas administration of ketanserin (at 0.3 or 1.0 mg/kg) with rivastigmine did not result in a significant change in immobility time (0.3 mg/kg: 98.17 ± 6.74 sec, n=6; 1.0 mg/kg: 85.50 ± 4.89 sec, n=6; $P < 0.01$ vs. OBX).

Likewise, following FST analysis, I observed a significant group effect in all treatment groups [$F(6,36) = 10.582$, $P < 0.01$], and immobility time for OBX mice significantly increased (sham: 112.43 ± 4.74 sec, n=7; OBX: 168.5 ± 4.58 sec, n=6; $P < 0.01$ vs. sham; Fig. 2.4.B). However, this increase was reversed by treatment with 1.0 mg/kg rivastigmine (1.0 mg/kg: 122.33 ± 6.86 sec, n=6; $P < 0.01$ vs. OBX). Rivastigmine-dependent reduction was partially eliminated by WAY-100635 pre-administration (WAY-100635: 0.3 mg/kg: 151.50 ± 7.83 sec, n=6; 1.0 mg/kg: 148.33 ± 6.43 sec, n=6; $P < 0.05$ vs. rivastigmine-treated OBX mice), and no differences were detected following ketanserin treatment (ketanserin: 0.3 mg/kg: 125.33 ± 6.52 sec, n=6; 1.0 mg/kg: 116.83 ± 4.51 sec, n=6; $P < 0.01$ vs. OBX).

Overall, these effects suggest that rivastigmine antagonizes depressive behaviors by activating 5-HT_{1A} receptor

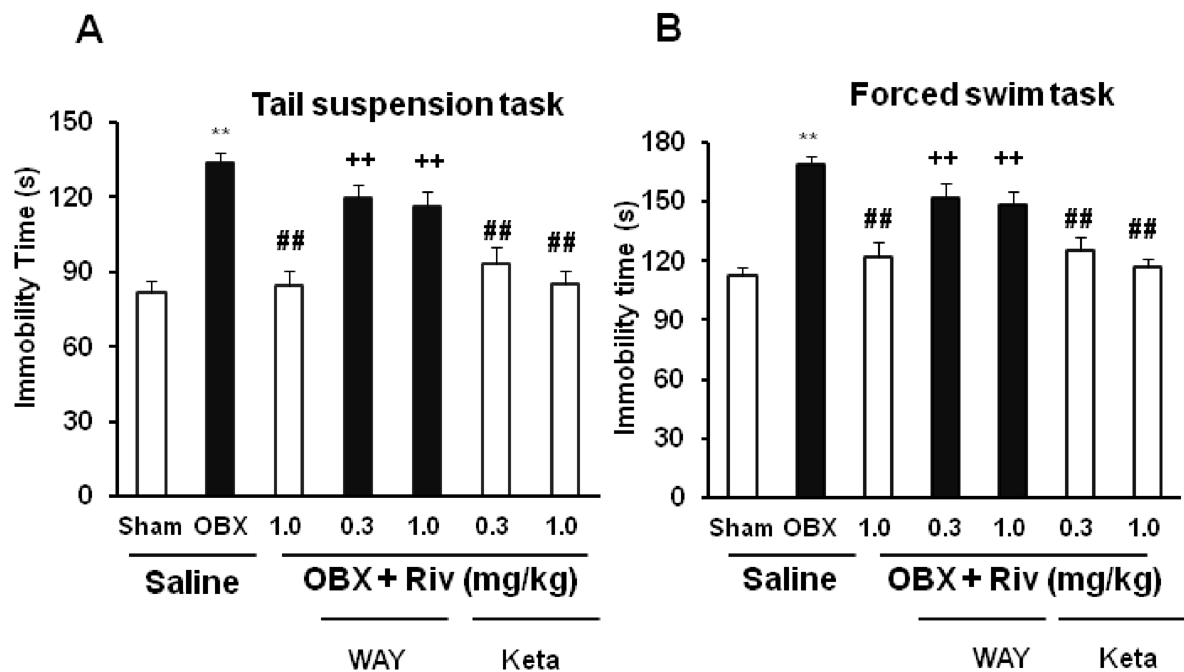

Figure 2.4. Rivastigmine improves depression-like behaviors in OBX mice through the 5-HT_{1A} receptor. A: Immobility time in a TST was measured after chronic co-administration of rivastigmine (0.3 or 1.0 mg/kg s.c) with WAY-100635 (1.0 mg/kg, s.c; WAY) or ketanserin (1.0 mg/kg, i.p.; Keta) for 14 days after OBX. B: Immobility time in a FST was measured in the same groups. Each bar represents the mean ± S.E.M. n= 5-7 in each group. (**$p < 0.01$ versus sham-operated mice, ##$p < 0.01$ versus OBX mice, ++$p < 0.05$ versus rivastigmine-treated OBX mice) (cited from Neuroscience, 2014;272:116-130).

2.4.4 Effect of rivastigmine, WAY-100635 and ketanserin on hippocampal DG neurogenesis

Based on the behavioral tests, I hypothesized that rivastigmine treatment ameliorates depression-like behaviors by stimulating neurogenesis in the hippocampal DG. To identify newborn DG neurons, I stained hippocampal slices with antibodies to BrdU and NeuN and counted double-positive cells. A significant group effect was observed in numbers of $BrdU^+/NeuN^+$ cells [$F(5,24) = 22.335$, $P < 0.01$]. That number significantly decreased in OBX mice (sham: 149.4 ± 4.09 cells, n=5; OBX: 93.4 ± 4.25 cells, n=5; $P < 0.01$ vs. sham;

Fig. 2.5.A, B). Rivastigmine administration to sham-operated mice did not alter the number of BrdU^{+}/NeuN^{+} cells (1.0 mg/kg, rivastigmine-treated sham: 149.0 ± 4.52 cells, n=5). Compared with OBX mice, rivastigmine treatment (1.0 mg/kg) significantly elevated the number of BrdU^{+}/NeuN^{+} cells (1.0 mg/kg: 135.60 ± 6.77 cells, n=5; $P < 0.01$ vs. OBX; Fig. 2.5.A,B). WAY-100635 treatment (1.0 mg/kg, s.c.) significantly reduced the number of BrdU^{+}/NeuN^{+} cells in rivastigmine-treated OBX mice (WAY-100635 1.0 mg/kg: 89.2 ± 3.44 cells, n=5; $P < 0.01$ vs. rivastigmine-treated OBX mice; Fig. 2.5.A,B), while ketanserin (1.0 mg/kg, i.p.) pre-administration did not (1.0 mg/kg: 141.60 ± 4.56 cells, n=5; $P < 0.01$ vs. OBX mice; Fig. 2.5.A,B).

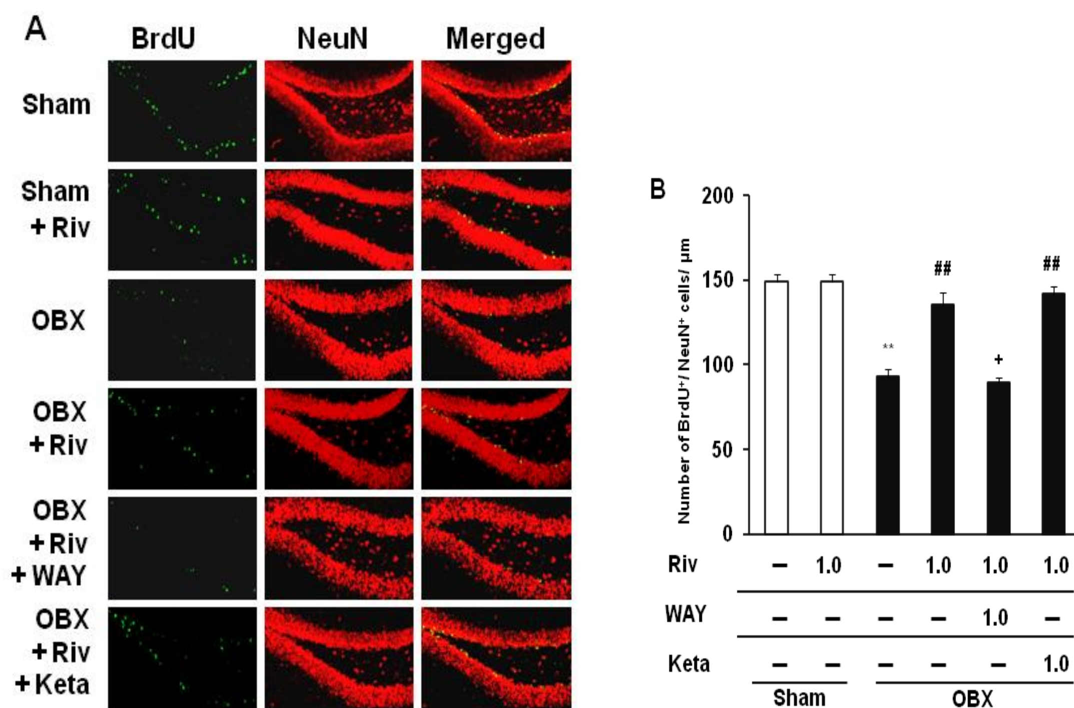

Figure 2.5. Rivastigmine treatment enhances hippocampal DG neurogenesis in OBX mice through the 5-HT$_{1A}$ receptor. A: Confocal microscopy images showing double staining for BrdU (green), NeuN (red) and merged images in hippocampal slices 35 days after OBX (n=5). B: Quantitative analyses of the number of BrdU/NeuN double-positive cells in the DG (n=5). Each bar represents the mean ± S.E.M. (**p<0.01 versus sham-operated mice, ##p< 0.01 versus OBX mice, +p < 0.01 versus rivastigmine-treated OBX mice) (cited from Neuroscience, 2014; 272:116-130).

Overall, these findings suggest that rivastigmine treatment increases hippocampal neurogenesis in OBX mice, an effect significantly eliminated by WAY-100635 pre-treatment but not by ketanserin, confirming that $5\text{-}HT_{1A}$ receptors likely mediate that effect.

2.4.5 Effect of only WAY-100635 and ketanserin on neurogenesis in sham and OBX mice

However, I did not observe a significant reduction in the number of $BrdU^{+}$ cells in the DG following treatment with either WAY-100635 or ketanserin (1.0 mg/kg) alone in sham (97.46 ± 7.12 % and 101.34 ± 6.59 % of control respectively). Similarly, WAY-100635 or ketanserin (1.0 mg/kg) alone OBX mice (96.57± 4.79 % and 98.29 ± 4.68 % of control respectively) did not show significant changes in the number of $BrdU^{+}/NeuN^{+}$ cells

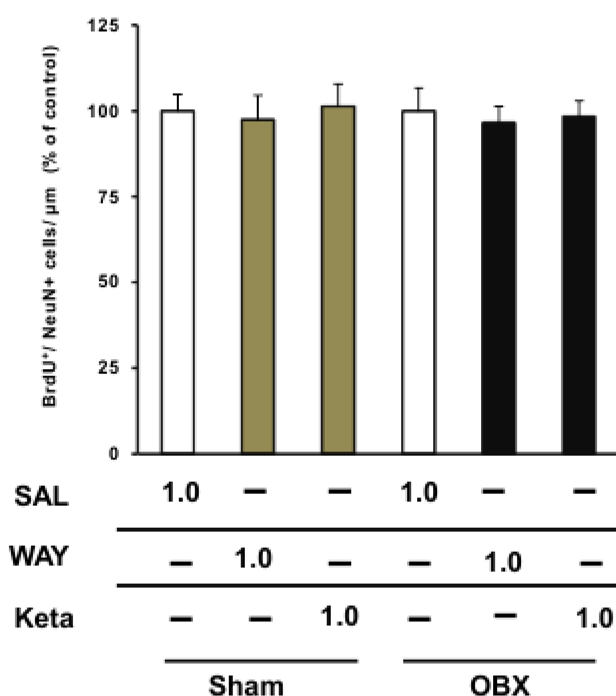

Figure 2.6. WAY-100635 and Ketanserin treatment only did not change neuronal proliferation in sham or OBX mice. Quantitative analyses of the % of BrdU/NeuN double-positive cells compared to control in the DG (n=5) of prepared hippocampal slices 35 days after OBX (n=5) (cited from Neuroscience, 2014; 272:116-130) .

2.4.6 Effects of rivastigmine, WAY-100635 and ketanserin on ERK and Akt signaling pathway in the DG of OBX mice

Since activation of extracellular signal-regulated kinase (ERK) and phosphatidylinostol 3-kinase (PI3K)/Akt pathways functions in progenitor cell proliferation during neurogenesis (Polter and Li, 2010), I asked whether ERK or Akt (Ser-473) activities were required for rivastigmine-induced neurogenesis. In sham-operated mice, rivastigmine administration (1.0 mg/kg) had no effect on phosphorylation of Akt (Ser-473) or ERK in the DG, as assessed by immunoblotting (Fig. 2.7.A). However, phosphorylation of Akt (Ser-473) and ERK in the DG markedly decreased in OBX compared to sham-operated mice (Akt (Ser-473): 59.92 ± 2.97 % of sham, n=4; $P < 0.05$ vs. sham; ERK: 39.75 ± 4.04 % of sham, n=4; $P < 0.05$ vs. sham). Rivastigmine (0.3 or 1.0 mg/kg) treatment significantly increased phosphorylation of Akt (Ser-473) and ERK in the DG of OBX mice (Akt (Ser-473) (0.3 mg/kg): 123.87 ± 5.07 % of sham, n=4; $P < 0.01$ vs. OBX; (1.0 mg/kg): 135.41 ± 4.04 % of sham, n=4; $P < 0.01$ vs. OBX; ERK (0.3 mg/kg): 95.90 ± 4.69 % of sham, n=4; $P < 0.05$ vs. OBX; (1.0 mg/kg): 101.85 ± 7.70 % of sham, n=4; $P < 0.05$ vs. OBX; Fig 2.7.A). The rivastigmine treatment increases phosphorylation of Akt (Ser-473) and ERK in the DG of in OBX mice.

Next i examined whether 5-HT receptor signaling mediated phosphorylation of these effectors in rivastigmine-treated OBX mice using the specific inhibitors WAY-100635 and ketanserin. Enhanced phosphorylation of ERK and Akt following rivastigmine treatment at either 0.3 or 1.0 mg/kg) was eliminated by WAY-100635 co-administration (1.0 mg/kg) (Fig. 5B), while ketanserin (1.0 mg/kg) co-administration had no effect on increased phosphorylation of Akt (Ser-473) and ERK stimulated by either dose of rivastigmine (Akt (Ser-473) (0.3 mg/kg): 123.94 ± 4.13% of sham, n=4; $P < 0.01$ vs. OBX; (1.0 mg/kg): 135.08 ± 5.06% of sham, n=4; $P < 0.01$ vs. OBX; ERK (0.3 mg/kg): 107.02 ± 4.47% of sham, n=4; P

< 0.05 vs. OBX; (1.0 mg/kg): 114.61 ± 4.03% of sham, n=4; $P < 0.05$ vs. OBX) (Fig 5B).

Taken together, the observations indicate that rivastigmine stimulation increases ERK and Akt phosphorylation in the DG via 5-HT_{1A} receptor activation.

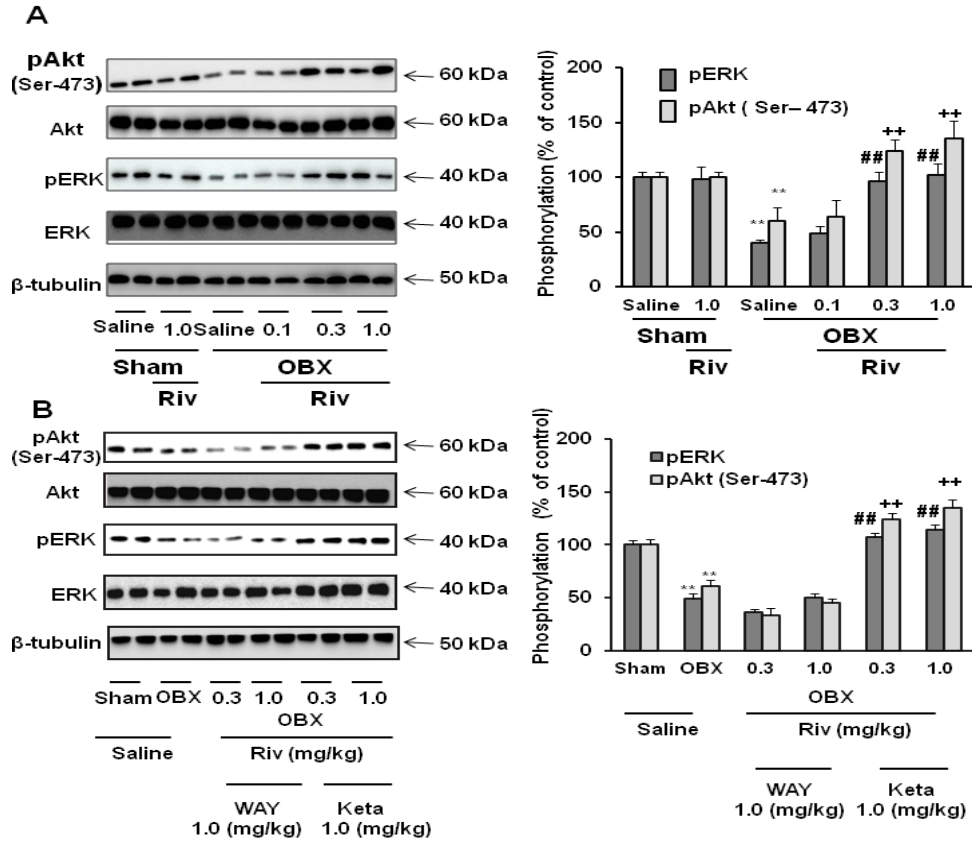

Figure 2.7. Rivastigmine treatment rescues reduced phosphorylation of Akt (Ser-473) and ERK in the DG of OBX mice. A: Representative images of immunoblots probed with antibodies against phosphorylated Akt (Ser-473), Akt, phosphorylated ERK, ERK and β-tubulin. Quantitative analyses of phosphorylated Akt (Ser-473) and phosphorylated ERK relative to respective total proteins are shown in the bar graph. B: WAY-100635 treatment significantly reduced phosphorylation of both Akt (Ser-473) and ERK in the DG of rivastigmine-treated OBX mice. Representative images of immunoblots using antibodies against phosphorylated Akt (Ser-473), Akt, phosphorylated ERK, ERK and β-tubulin. Quantitative analyses of phosphorylated Akt (Ser-473) and phosphorylated ERK relative to respective total proteins are shown in the bar graph. Each bar represents the mean ± S.E.M. (**p<0.05 versus sham-operated mice, ##p<0.05versus OBX mice, ++p < 0.01 OBX mice) (cited from Neuroscience, 2014; 272:116-130).

2.5 Discussion

The present study provides the first evidence that antidepressant activity of rivastigmine is closely associated with enhanced neurogenesis seen in the DG of OBX mice. Moreover, we found that 5-HT_{1A} receptor stimulation by rivastigmine treatment plays a critical role in these outcomes. Notably, others have reported loss of cholinergic neurons (Davies P and Maloney AFJ, 1976), reduced numbers of serotonergic neurons in the dorsal and median raphe nuclei (Alertino et al, 1992; Chen et al, 2000) and decreased serotonergic 5-HT_{1A} receptor sites in the hippocampus in postmortem brains of AD patients (Lai et al, 2011). Thus, our evidence supporting 5-HT_{1A} receptor stimulation by rivastigmine provides an attractive mechanism to explain improved BPSD seen following rivastigmine treatment of AD patients.

Interestingly, the hyperactivity, decreased neurogenesis and depressive-like behaviors seen in OBX mice were normalized by rivastigmine treatment in our study. Our observations are reinforced by rivastigmine-dependent improvement of memory deficits and long-term potentiation seen in OBX mice, an effect associated with cholinergic stimulation (Hozumi et al, 2003; Moriguchi et al, 2013b). Moreover, rivastigmine increases ACh levels in the hippocampus of AChE-/- mice of the 129S6/SvEvTac strain (Naik et al., 2009). Thus, we propose that elevated hippocampal ACh levels observed in OBX mice after rivastigmine treatment increase 5-HT levels and underlie neurogenesis via ERK and Akt activation in the DG. In this context, blockade of 5-HT_{1A} receptor would prevent neurogenesis in rivastigmine-treated OBX mice, an outcome we observe after WAY-100635 administration.

Although we report here that 5-HT_{1A} receptor stimulation increases hippocampal neurogenesis, as previously described by Klempin et al (2010), it remains unclear which signaling pathways function in neurogenesis in this context. Proliferation and survival of newborn cells in the DG are modulated by ERK and Akt pathways in the adult mouse

hippocampus (Polter and Li, 2010). We observed inhibition of rivastigmine-induced phosphorylation of Akt (Ser-473) and ERK following WAY-100635 administration.

To determine any confounding effect by the 5-HT receptor antagonists alone, I confirmed that 2-weeks treatment with WAY-100635 or ketanserin alone did not alter $BrdU^+$ cell numbers in control mice (both sham and OBX). In contrast, the same treatment of WAY-100635 antagonizes the increases number of new born cells following rivastigmine. The strong reduction in 5-HT innervations in the hippocampus of OBX mice allows me to conclude that the results obtained here reflect mostly the involvement of 5-HT_{1A} receptor stimulation in the regulation of cell survival, and suggest a role for 5-HT, via the 5-HT_{1A} receptor, in rivastigmine-induced neurogenesis.

In conclusion, my results demonstrate that OBX-induced depression-like behaviors in mice are rescued by chronic rivastigmine administration and stimulation of the 5-HT_{1A} receptor induced by rivastigmine accounts for hippocampal neurogenesis.

Chapter 3: 5-HT$_{1A}$ receptor stimulation improves depression-like behaviors and hippocampal neurogenesis in OBX mice

3.1 Introduction

The 5-HT$_{1A}$ receptor is a central component in the regulation of serotonin system activity, and is expressed in many neuronal structures including the hippocampus where it serves as a major postsynaptic receptor (Pompeiano et al., 1992) implicated in the pathophysiology of major depressive disorder in both animal models and human studies (Richardson-Jones et al, 2010). 5-HT$_{1A}$ receptors are divided into two functionally different receptor types, the presynaptic inhibitory autoreceptors and the postsynaptic 5-HT$_{1A}$ receptors (Riad et al, 2000). Previous research asserts that mice lacking this receptor primarily show increased anxiety behavior and excitation as well as reduced hippocampal-related cognitive functions (Sarnyai et al, 2000).

Increased neurogenesis can improve measures of cognition and behavior,even in aged and disease rodents, have led to interest in the molecular mediators regulating this beneficial effect (Grote and Hannan, 2007). Chronic alterations in 5-HT levels appear to have more robust and striking effects on hippocampal DG neurogenesis, perhaps mediated by specific serotonergic receptors present in the DG (Taupin and Gage, 2002). It was reported that 8-OH-DPAT increased cell proliferation in the hippocampal DG in rats (Huang and Herbert, 2005; Soumier et al., 2010), whereas blockade of 5-HT$_{1A}$ receptors resulted in reduced cell proliferation (Huang and Herbert, 2005; Radley and Jacobs, 2002).

Earlier reports have suggested that ERK and Akt phosphorylation in mouse hippocampus is significantly correlated with 5-HT$_{1A}$ receptor signaling pathway regulating neurogenesis (Li et al, 2009). Binding of 5-HT to the 5-HT$_{1A}$ receptor results in the release of βγ-subunits from pertussis toxin-sensitive G-protein and thereby activation of ERK and Akt

from sequence of several consecutive phosphorylation leading to cell proliferation (Albert and Tiberi, 2001). This study will provide the first evidence for a link between the above mentioned signaling proteins and 8-OH-DPAT in the DG of OBX mice.

3.2 Objectives

Aim of the present study were (1) to determine the ability of 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, to improve depressive behaviors in OBX mice; (2) to examine the effect of 8-OH-DPAT on three different stages of neurogenesis to elucidate the mode of action of 5-HT_{1A} receptor stimulation in hippocampal DG neurogenesis in OBX mice and (3) to examine the effect of 8-OH-DPAT to restore the decreased ERK and Akt phosphorylation responsible for reduced neurogenesis in OBX mice.

3.3Experimental design

I followed earlier described protocol for preparation of OBX mice and two weeks drug administration (n=5~7 per group). 5-HT_{1A} receptor agonist,8-OH-DPAT (s.c.) and the 5-HT_{2A} receptor agonist, TCB-2 (i.p.) were administered in two similar doses (0.3,1.0 mg/kg) like rivastigmine from earlier experiment.

Here also, following similar BrdU administration protocol of 5 days i prepared coronal mouse brain sections for immunohistochemical studies (n=5 per group) after 7 days and 1 month of last BrdU injection in animal groups,, The experimental design is schematically presented in fig 3.1.

To determine behavioral effects, I performed the TST after twenty-four hrs of last drug administration, followed by a Forced swim test (FST) in the following day Twenty four hours after the end of the last test (FST), mice were sacrificed for western blot analyses.

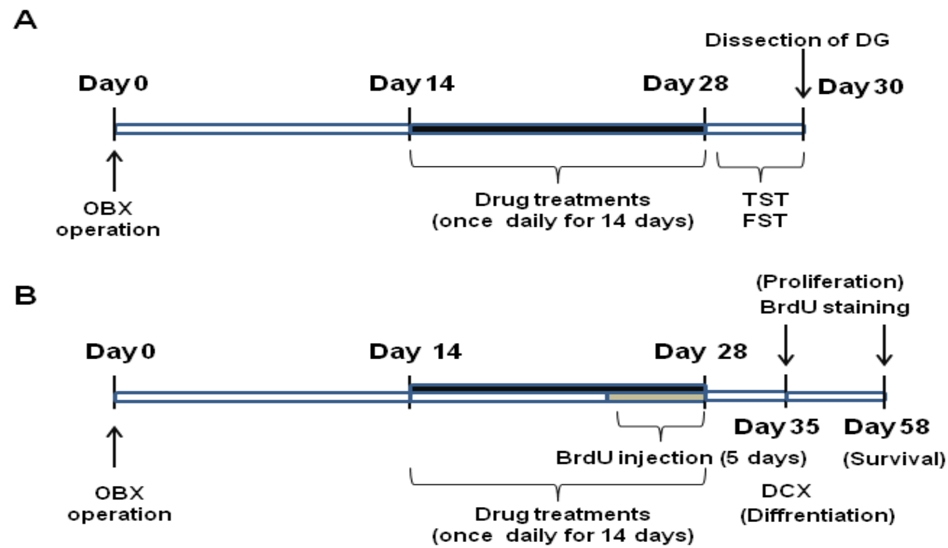

Fig 3.1 Schematic presentation of experimental design (cited from Neuroscience, 2014; 272:116-130)

3.4 Results

3.4.1 Changes in OBX-induced depressive behaviors following 8-OH-DPAT

To define whether treatment with 5-HT$_{1A}$ or 5-HT$_{2A}$ receptor agonists treatments might improve depressive behavior seen in OBX mice, I undertook TST and FST analysis after treating mice with 8-OH-DPAT (a 5-HT$_{1A}$ agonist) or TCB-2 (a 5-HT$_{2A}$ agonist), following the procedure shown in Fig 3.1. In the TST, I observed a significant group effect in all treatment groups [$F(6,33) = 9.067$, $P < 0.01$]. After verifying rivastigmine effects observed in Fig. 2.2. (sham: 78.28 ± 5.49 sec, n=7; OBX: 126.17 ± 5.74 sec, n=6; rivastigmine 1.0 mg/kg: 85.17 ± 5.56 sec, n=6; $P < 0.01$ vs. OBX; Fig. 3.2.A), we observed that 8-OH-DPAT treatment significantly decreased immobility time seen in OBX mice (0.3 mg/kg: 95.60 ± 4.75 sec, n=5; 1.0 mg/kg: 81.33 ± 5.58 sec, n=6; $P < 0.01$ vs. OBX). In contrast, TCB-2 administration (at 0.3 or 1.00 mg/kg, i.p.) did not improve immobility time in OBX mice (0.3 mg/kg: 115.40 ± 4.97 sec, n=5; 1.0 mg/kg: 110.20 ± 6.15 sec, n=5; $P < 0.05$ vs. rivastigmine-treated OBX mice).

Likewise, following the FST, ANOVA analysis showed a significant group effect in all treatment groups [$F(6,33) = 9.435$, $P < 0.01$]. After confirming a rivastigmine treatment effect (sham: 115.43 ± 5.79 sec, n=7; OBX: 166.17 ± 6.43 sec, n=6; rivastigmine 1.0 mg/kg: 123.5 ± 6.64 sec, n=6; $P < 0.01$ vs. OBX; Fig. 3.2.B), we found that 8-OH-DPAT treatment significantly improved immobility time (0.3 mg/kg: 133.60 ± 5.47 sec, n=6; 1.0 mg/kg: 119.33 ± 5.47 sec, n=6; $P < 0.01$ vs. OBX), while TCB-2 treatment did not (0.3 mg/kg: 158.80 ± 5.65 sec, n=5; 1.0 mg/kg: 155.80 ± 6.56 sec, n=5; $P < 0.05$ vs. rivastigmine-treated OBX mice).

Taken together, these studies confirm that stimulation of 5-HT_{1A} rather than 5-HT_{2A} antagonizes depression-like behaviors in OBX mice.

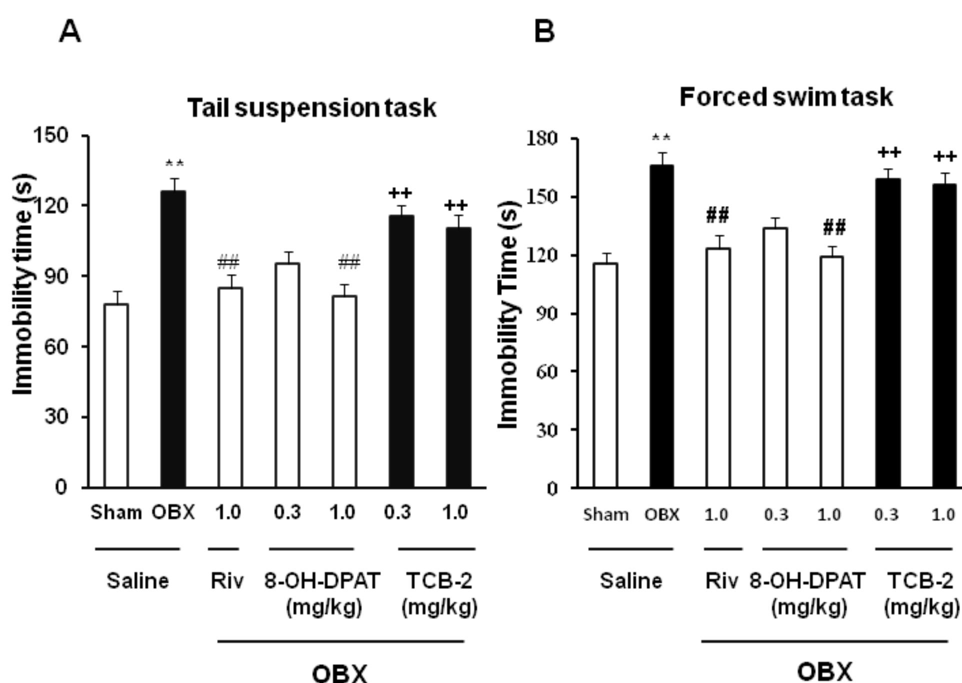

Figure 3.2. Chronic 8-OH-DPAT administration antagonizes OBX-induced depressive behaviors. A: Immobility time in a TST was measured after administration of 8-OH-DPAT (0.3 or 1.0 mg/kg, s.c.) or TCB-2 (0.3 or 1.0 mg/kg, i.p.) for 14 days after OBX. B: Immobility time in a FST was measured after identical treatments. Each bar represents the mean ± S.E.M. n= 5-7 in each group. (**p<0.01 versus sham-operated mice, ##p < 0.01

versus OBX mice, ++p < 0.05 rivastigmine-treated OBX mice) (cited from Neuroscience, 2014; 272:116-130).

3.4.2 Effect of 8-OH-DPAT and TCB-2 on hippocampal DG neurogenesis

I next assessed the effect of 5-HT_{1A} or 5-HT_{2A} stimulation on hippocampal neurogenesis in OBX mice, using BrdU labeling analysis similar to that shown in Fig. 2.5. We observed a significant group effect in terms of the number of $BrdU^+$/$NeuN^+$ cells [$F(5,24) = 21.43$, $P < 0.01$]. We then compared effects of 8-OH-DPAT or TCB-2 with that of rivastigmine on the number of $BrdU^+$/$NeuN^+$ cells (sham: 154.4 ± 4.35 cells, n=5; OBX: 89.6 ± 4.43 cells; rivastigmine 1.0 mg/kg: 142.40 ± 6.39 cells, n=5, Fig. 3.3.A,B). Like rivastigmine, 8-OH-DPAT (1.0 mg/kg, s.c.) administration to OBX mice significantly increased the number of $BrdU^+$/$NeuN^+$ cells (8-OH-DPAT 1.0 mg/kg: 155.8 ± 6.85 cells, n=5; $P < 0.01$ vs. OBX mice; Fig.3.3.A,B), whereas repeated TCB-2 administration ((1.0 mg/kg, i.p.) had no effect $BrdU^+$/$NeuN^+$ cell number (TCB-2 1.0 mg/kg: 98.80 ± 5.26 cells, n=5).

These findings indicate that amelioration of depression-like behaviors in OBX mice by specific stimulation of 5-HT_{1A} receptors is accompanied by enhanced hippocampal neurogenesis.

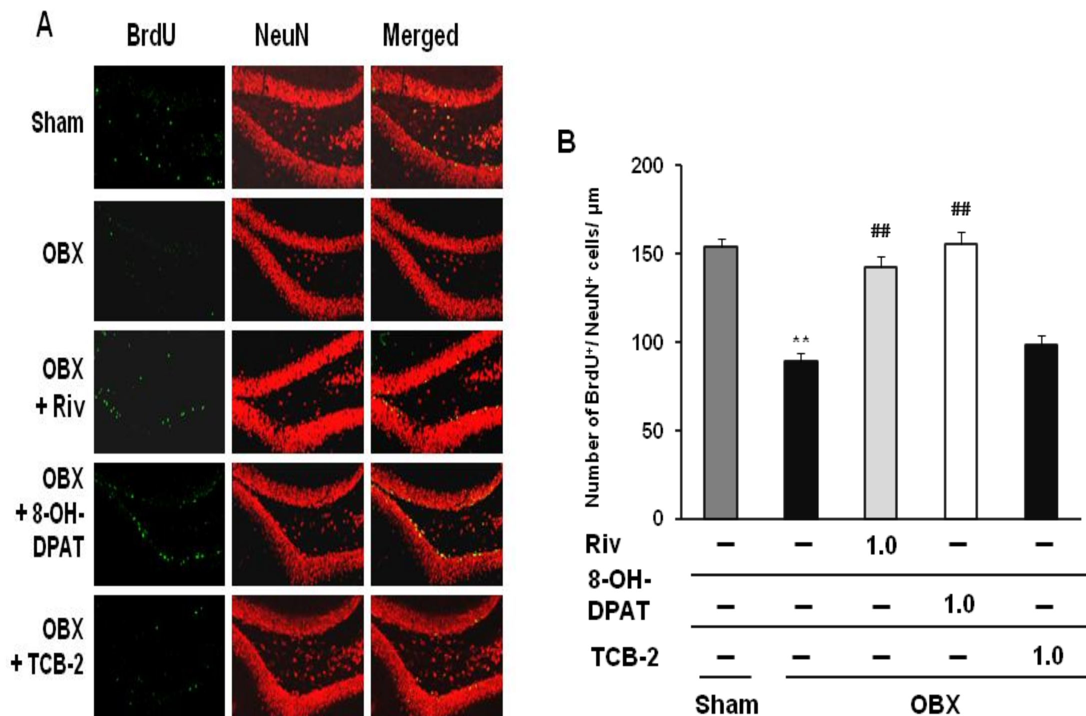

Figure 3.3. 8-OH-DPAT treatment restores neurogenesis in the DG of OBX mice. A: Confocal microscopy images showing double staining for BrdU (green), NeuN (red) and merged images in hippocampal slices, 35 days after OBX (n=5). B: Quantitative analyses of the number of BrdU/NeuN double-positive cells in the DG (n=5). Each bar represents the mean ± S.E.M. (***p*< 0.01 versus sham-operated mice, ##*p* < 0.01 versus OBX mice) (cited from Neuroscience, 2014; 272:116-130).

3.4.3 Effect of rivastigmine and 8-OH-DPAT on cell survival of neural precursor cells

In separate experiments designed to assess the effect of rivastigmine treatment on survival of newborn neurons, mice were sacrificed 1 month after the last BrdU injection and then hippocampal slices were prepared and double-stained with BrdU and NeuN antibodies. I found a significant group effect in terms of the number of $BrdU^+/NeuN^+$ cells [$F(5,24) = 12.19$, $P < 01$]. As expected, the number significantly decreased in OBX mice (sham: 81.40 ±6.13 cells, n=5; OBX: 38.8 ± 4.35 cells, n=5; $P < 0.01$ vs. sham; Fig. 3.4.A, B). Rivastigmine administration to sham-operated mice did not alter $BrdU^+/NeuN^+$ cell number

(1.0 mg/kg, rivastigmine-treated sham: 79.20 ± 5.75 cells, n=5). Compared with OBX only mice, OBX mice receiving rivastigmine treatment (1.0 mg/kg) showed significantly increased numbers of $BrdU^+/NeuN^+$ cells (1.0 mg/kg: 83.6 ± 6.75 cells, n=5; $P < 0.01$ vs. OBX; Fig. 3.4. A,B). Likewise, 8-OH-DPAT (1.0 mg/kg, s.c.) administration to OBX mice significantly increased the number of $BrdU^+/NeuN^+$ cells (8-OH-DPAT 1.0 mg/kg: 79.6 ± 5.62 cells, n=5; $P < 0.01$ vs. OBX mice; Fig. 3.4.A,B), whereas repeated TCB-2 administration ((1.0 mg/kg, i.p.) had no effect on $BrdU^+/NeuN^+$ cell number (TCB-2 1.0 mg/kg: 47.80 ± 4.95 cells, n=5). Taken together, these findings confirm that rivastigmine enhances survival of hippocampal DG cells in OBX mice.

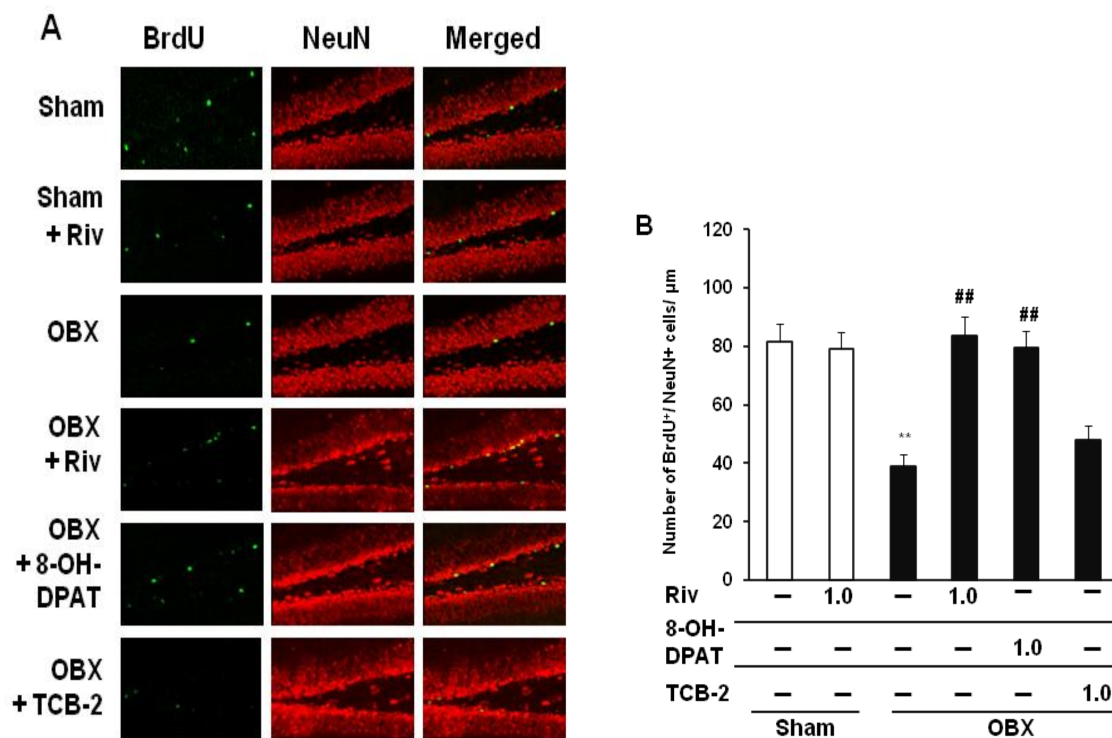

Figure 3.4. Rivastigmine treatment enhances newborn cell survival in the DG of OBX mice. A: Confocal microscopy images showing double staining for BrdU (green), NeuN (red) and merged images in hippocampal slices, 30 days after last BrdU injection (n=5). B: Quantitative analyses of the number of BrdU/NeuN double-positive cells in the DG (n=5). Each bar represents the mean ± S.E.M. (**p< 0.01 versus sham-operated mice, ##p < 0.01 versus OBX mice) (cited from Neuroscience, 2014; 272:116-130).

3.4.4 Effect of rivastigmine and 8-OH-DPAT on the number of immature granule cells

DCX is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in adult DG (Couillard-Despres et al., 2005). To assess whether newly generated granule cells born after the last BrdU injection differentiate into neuronal precursor cells (Kokaia et al, 2006; Wang et al, 2008; Chen et al, 2013), we performed BrdU and DCX dual immunostaining of DG regions where $BrdU^+/NeuN^+$ cells had been observed 1 wk after the last BrdU injection. We observed a significant group effect in terms of the number of $BrdU^+/DCX^+$ cells [$F(5,24) = 8.91$, $P < 0.01$]. Compared to sham-operated mice, the number of $BrdU^+/DCX^+$ cells significantly decreased in OBX mice (sham: 85.0 ± 5.04 cells, n=5; OBX: 45.6 ± 3.14 cells, n=5; $P < 0.01$ vs. sham; Fig. 9 A,B). Rivastigmine administration to OBX mice significantly elevated the number of $BrdU^+/DCX^+$ cells (1.0 mg/kg rivastigmine-treated OBX: 79.80 ± 8.12 cells, n=5; $P < 0.05$ vs. OBX; Fig. 9 A,B). This effect was not seen in sham controls (Fig. 9 A, B). Like rivastigmine, 8-OH-DPAT (1.0 mg/kg, s.c.) administration to OBX mice significantly increased the number of $BrdU^+/DCX^+$ cells (8-OH-DPAT 1.0 mg/kg: 76.6 ± 4.32 cells, n=5; $P < 0.05$ vs. OBX mice; Fig.9A,B), whereas TCB-2 administration ((1.0 mg/kg, i.p.) had no effect on the number of $BrdU^+/DCX^+$ cells (TCB-2 1.0 mg/kg: 52.00 ± 5.32 cells, n=5). Taken together, we conclude that rivastigmine-induced newborn cells differentiate to neuronal precursor cells, followed by maturating to adult granule cells in the DG of OBX mice.

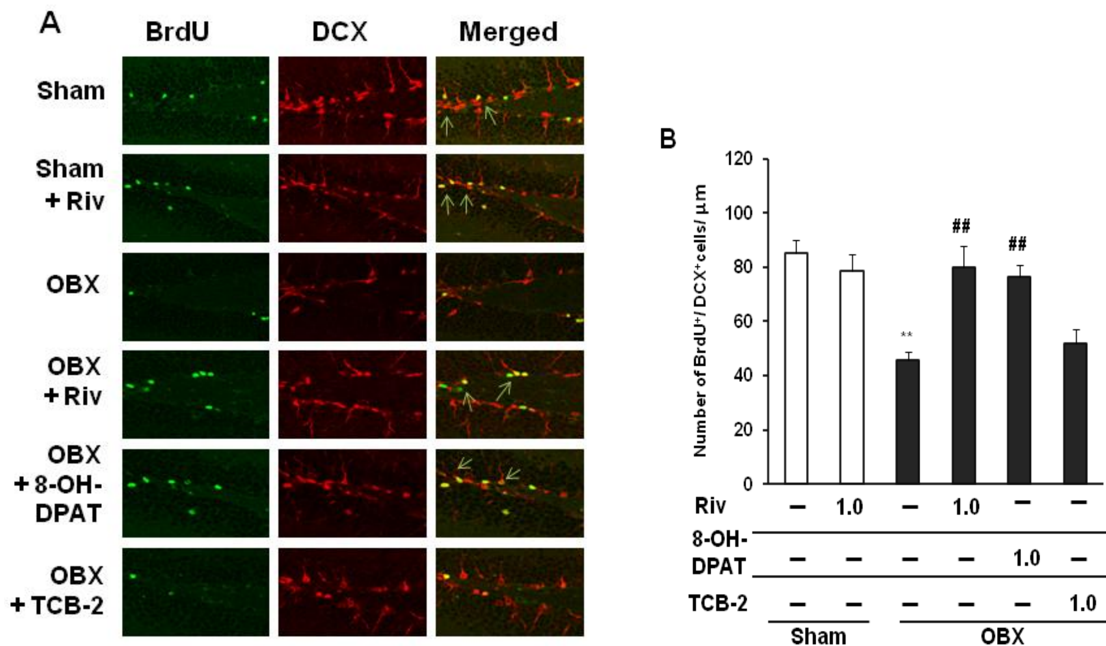

Figure 3.5. Both rivastigmine and 8-OH-DPAT treatment enhances newborn immature granule cells in the DG of OBX mice. A. Confocal microscopy images showing double staining for BrdU (green), DCX (red) and merged cells are shown as yellow (arrow) images in hippocampal slices, 35 days after OBX surgery (n=5). B. Quantitative analyses of the number of BrdU/DCX double-positive cells in the DG (n=5). Each bar represents the mean ± S.E.M. (**p<0.01 versus sham-operated mice, ##p < 0.05 versus OBX mice) (cited from Neuroscience, 2014; 272:116-130) .

3.4.5 Effect of 8-OH-DPAT and TCB-2 on ERK and Akt signaling in OBX mice DG

Finally, i examined phosphorylation status of Akt (Ser-473) and ERK following 8-OH-DPAT or TCB-2 treatment of OBX mice using immunoblotting analyses of lysates made from DG tissues. The significantly reduced phosphorylation of Akt (Ser-473) and ERK seen in the DG of OBX mice (Akt (Ser-473): 63.53 ± 4.68% of sham, n=4; P < 0.05 vs. sham; ERK: 53.97 ± 5.71% of sham, n=4; P < 0.05 vs. sham; Fig 3.6.A,B) was improved by 8-OH-DPAT treatment (8-OH-DPAT; Akt (Ser-473): 141.79 ± 3.39% of sham, n=4; P < 0.05 vs. OBX; ERK: 109.86 ± 3.19% of sham, n=4; P < 0.05 vs. OBX; Fig 3.6.A,B). However, TCB-2

administration (at 0.3 or 1.0 mg/kg) did not rescue phosphorylation of either Akt (Ser-473) or ERK in the DG.

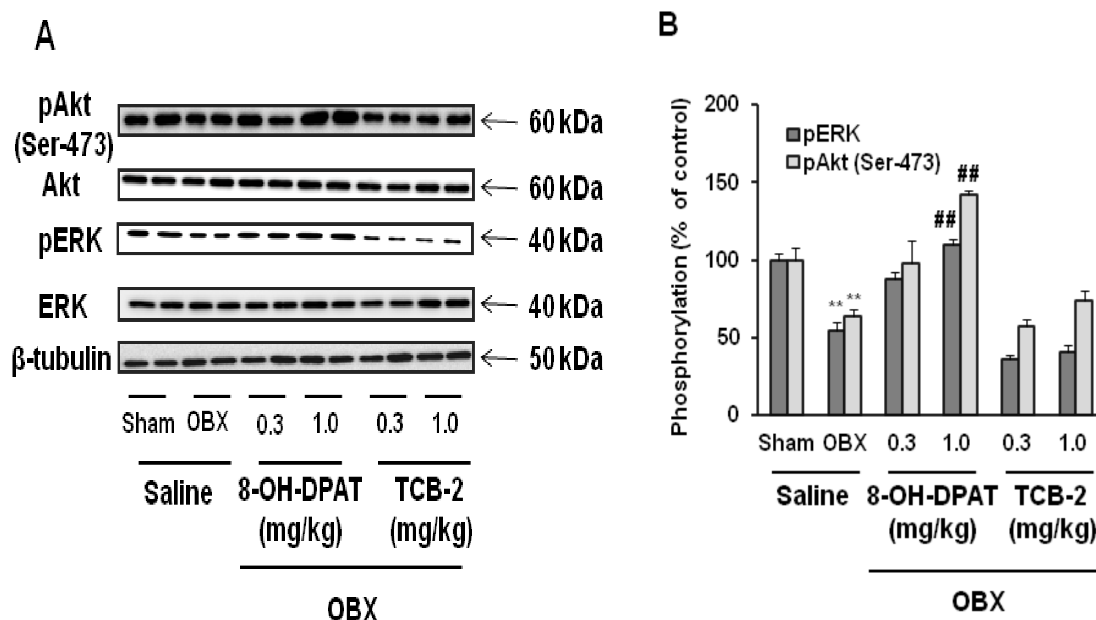

Figure 3.6. 8-OH-DPAT stimulates Akt (Ser-473) and ERK phosphorylation in the DG of OBX mice. A: Representative images of immunoblots probed with antibodies against phosphorylated Akt (Ser-473), phosphorylated ERK, and total proteins of each. B: Quantitative analyses of phosphorylated Akt (Ser-473) and phosphorylated ERK relative to respective total proteins are shown in the bar graph. Each bar represents the mean ± S.E.M. (***p*<0.05 versus sham-operated mice, ##*p*<0.05 versus OBX mice) (cited from Neuroscience, 2014; 272:116-130).

3.5 Discussion

The main discovery of the present study is that 8-OH-DPAT (5-HT_{1A} agonist) but not TCB-2 (5-HT_{2A} agonist) improves the depressive behaviors and decreased DG neurogenesis in OBX mice. The increase in neurogenesis was supported by three different independent measures of neurogenesis including counts of total number of BrdU/NeuN double-positive cells in the granular layer, the survival of new born granule cells after 1 month as indicated by the number of BrdU/NeuN double-positive cells and counts of immature granule cells by co-labeling with DCX (a marker of immature neurons). The increased BrdU/ DCX double

positive cell numbers together with increased BrdU/ NeuN double positive cell counts in my study suggest that 8-OH-DPAT as well as rivastigmine increased numbers of new neurons by increasing the proliferation, differentiation and survival of new cells into neurons. These findings are in agreement with a recent report on 129SvEv mice strain, in which proliferation, differentiation and survival steps of DG neurogenesis are enhanced following chronic fluoxetine treatment (David et al, 2010).

Furthermore, Huang and Herbert reported the influence of p-chlorophenylalanine, which inhibits 5-HT synthesis, on the effects of 8-OH-DPAT (Huang and Herbert, 2005). p-Chlorophenylalanine followed by 8-OH-DPAT resulted in increased cell proliferation in the hippocampal DG compared with p-chlorophenylalanine alone. Further, 8-OH-DPAT stimulated cell proliferation in the SGZ of the hippocampal DG following the microinjection of the 5-HT neurotoxin 5,7-dihydroxytryptamine, which induces hippocampal 5-HT depletion (Huang and Herbert, 2005). These results suggest that 5-HT_{1A} receptors modulate cell proliferation in the hippocampus via direct post-synaptic 5-HT_{1A} receptor action.

8-OH-DPAT-induced neurogenesis is reportedly closely associated with elevated levels of phospho-ERK (pERK) in the brain of Sprague–Dawley rats (Buritova et al, 2009) and with activation of phospho-Akt (pAkt) in hippocampal neurons of Sprague-Dawley rats (Cowen et al, 2005). Although there is increasing evidence that the 5-HT_{1A} receptor directly modulates hippocampal ERK and Akt activity (Polter and Li, 2010), some discrepancies remain. For example, rats treated with 8-OH-DPAT fail to activate ERK in hippocampus (Chen et al, 2002) or in cultured hippocampal neurons (Cowen et al, 2005). Here, we found

It has been known that 5-HT_{1A} receptor activation madiates 5-HT-induced neurogenesis in depression therapy. In fact, the antidepressant effects of chronic fluoxetine treatment are associated with increased neurogenesis in 129 Sv/Ev mice; however, in the

same study, similar treatment failed to increase neurogenesis in 5-HT_{1A} receptor KO mice generated on a 129/Sv background (Santarelli et al, 2003). These findings suggest that fluoxetine-mediated serotonin elevation may stimulate hippocampal neurogenesis through 5-HT_{1A} receptor signaling. In agreement, we confirmed that prolonged 5-HT_{1A} receptor stimulation promotes increased cell proliferation, and that 5-HT_{1A} receptor inhibition decreases formation of new neurons in OBX mice.

The majority of cells generated in the DG of WT mice die during the cell differentiation process (Synder et al,2009). Administration of the 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) has no effect on the number of $BrdU^{+}$ cells in the DG of adult male Wistar rats (Jha et al, 2008). Also, stimulating the 5-HT_{1A} receptor with 8-OH-DPAT (1 mg/kg) for 14 days has no effect on the number of $BrdU^{+}$ cells in the DG of Lister hooded male rats (Huang and Herbert,2005). By contrast, OBX model showed a robust increase in new born neuronal survival, an effect that is observed as early as 1 week after their birth and that seems to last at least for 4 weeks. Interestingly, TCB-2 treatment did not increase neurogenesis in our study, suggesting that in OBX mice, 5-HT_{1A} receptor activity has a direct effect on neurogenesis.

Chapter 4: General Discussion

4.1 Summary discussion

In this study, I examined whether chronic treatment with rivastigmine, a non-competitive AChE/BuChE inhibitor, reverses depressive-like behaviors in OBX mice using several behavioral paradigms such as TST, FST, locomotion and NSFT. Again, I evaluated the rivastigmine-induced antidepressive effects using agonists and antagonists of 5-HT_{1A} and 5-HT_{2A} receptors. Furthermore, I examined the effects of rivastigmine on different stages of

hippocampal neurogenesis accompanied by ERK and Akt signaling in OBX mice.

Serotonergic innervation of the DG, a region where adult neurogenesis occurs, has been characterized (Moore and Halaris, 1975). The 5-HT$_{1A}$ receptor is predominantly expressed on the soma of DG neurons and dendrites of raphe neurons, and also on dendrites of excitatory neurons of the prefrontal cortex (PFC) and amygdala (Xia et al, 2012). By contrast, the 5-HT$_{2A}$ receptor is expressed on GABAergic interneurons in the hippocampal DG and CA1 and CA3 regions, and on glutamatergic neurons of the DG (Lüttgen et al., 2004). Impaired DG neurogenesis seen following OBX in Wistar rats is reportedly restored by chronic citalopram treatment (Jaako-Movits et al, 2006). Others report that intraperitoneal administration of WAY-100635 (at 5 mg/kg) reduces the number of BrdU-immunoreactive cells in the DG of adult male Sprague-Dawley rats by 30% (Radley and Jacobs, 2002).

It remains unclear whether cholinergic innervation regulates serotonergic neuronal activity in the hippocampal DG. In rats, DG regions enriched in 5-HT$_{1A}$ receptors receive serotonergic afferents from the raphe nuclei (Vanderwolf, 1988), and serotonergic neurons in dorsal raphe nucleus (DRN) express functional nACh receptors in that species (Galindo-Charles et al, 2008). In addition, ACh enhances 5-HT neuronal firing via presynaptic modulation of glutamate release in Wistar rats (Garduno et al, 2012) or via activation of nACh receptors expressed in serotonergic neurons in the DRN of adult male Sprague-Dawley rats (Chang et al, 2011). WAY-100635 antagonism of nicotine-induced amelioration of cognitive dysfunction suggests that cholinergic regulation via the α7 nACh receptor is 5-HT$_{1A}$ receptor-dependent (Uchiumi et al, 2013). Abrogation of cholinergic input produced by 192IgG-saporin infusion in male Fischer-344 albino rats significantly decreases the number of BrdU^{+} cells in the DG (Cooper-Kuhn et al, 2004). Enhanced cholinergic signaling following donepezil treatment of C57BL6 mice (Kaneko et al, 2006) and physostigmine treatment of

Sprague-Dawley rats (Mohapel et al, 2005) promotes survival of newborn DG neurons. However, direct innervation of newborn neurons by cholinergic fibers has not been observed. One group reported that chronic lithium treatment enhances hippocampal cholinergic neurotransmission through 5-HT_{1A} receptor-mediated pathways in rats (Fujii et al, 2000). Taken together, these findings suggest that cholinergic inputs to the hippocampal DG indirectly stimulate neurogenesis through the 5-HT_{1A} receptor.

5-HT reportedly regulates DG neurogenesis. For example, when acute 5-HT depletion or destruction of brain serotonergic neurons is induced by injections of the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) into the raphe nuclei of Wistar rats, DG cell proliferation is largely impaired (Brezun and Daszuta, 1999; Brezun and Daszuta, 2000). Conversely, stimulation of serotonergic neurotransmission by treatment of C57BL/6J mice with serotonin transporter-small interfering RNA (SERT-siRNA) (Ferres-Coy et al, 2013) or by treatment of male Sprague-Dawley rats with citalopram (Wang et al, 2010) enhances hippocampal neurogenesis. Using adult male Sprague Dawley rats, Malberg and colleagues (2000) also found that increased 5-HT levels resulted in increased rates of proliferation of DG cells. Serotonin depletion by para-chlorophenylalanine (PCPA) treatment also reduces survival and proliferation of cultured neurospheres derived from adult wild-type C57B6 mouse hippocampus tissue (Benninghoffe et al, 2010). Expression of the immediate-early gene c-FOS in the DG in response to experimental conditions that favor neurogenesis, such as environmental enrichment or physical activity (Kempermann et al, 1997; Pragg et al, 1999), serves as a marker of newborn DG granule cells that might be important for hippocampus-dependent behaviors such as spatial pattern separation or Morris water maze performance (Durpet et al, 2007; Synder et al, 2009). 5-HT depletion using 5,7-DHT reportedly reduces c-FOS expression in the dorsal and ventral hippocampus of male rats after

social stress (Chung et al, 2000). Rivastigmine treatment rescues c-FOS expression in the DG following PCPA-induced serotonergic depletion in male Sprague Dawley rats (Kornum et al, 2006). These observations suggest that increased numbers of newborn granule cells stimulated by rivastigmine treatment are functional in the hippocampus. Indeed, we recently confirmed that chronic rivastigmine treatment of OBX mice enhances memory-related behaviors as assessed by a Y-maze task, a novel object recognition task, passive avoidance tasks, the Barnes maze task and analysis of long term potentiation activity (Moriguchi et al, 2014). In the present study, we document improvement of depression-like behaviors, in addition to memory behaviors.

Cholinergic and serotonergic dysfunction seen in OBX rodents is consistent with the pathophysiology of depression (Hellweg et al, 2007; Dagyte et al, 2011). OBX mice exhibit decreased hippocampal acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) levels (Nakajima et al, 2007; Moriguchi et al., 2006). However, an increase in hippocampal AChE activity was also reported in OBX mice (Machado et al, 2012). Also, nicotine-induced acetylcholine release in the hippocampus is largely impaired in OBX mice (Yamamoto et al, 2013a). Donezepil, a classical AChE inhibitor, can rescue cholinergic neurons in the medial septum from neurodegeneration in OBX mice (Yamamoto and Fukunaga, 2013b). Serotonergic nerve endings in rat brain are inhibited and/or activated by stimulation of muscarinic or nicotinic cholinergic receptors (Haubrich and Reid,1972; Héry et al,1977). Like OBX, cholinergic neuron-specific lesions in the nuclei basali magnocellularis (NBM) caused by treatment with neurotoxin AF64A reduce 5-HT levels in the rat hippocampus (Nakamura et al, 1992). Therefore, reduced cholinergic activity in the hippocampus may account for serotonergic neuronal degeneration in OBX mice. Accordingly, OBX rats exhibit decreased 5-HT in the hippocampus (Xu et al, 2005), and OBX mice exhibit reduced hippocampal

5-HIAA/5-HT (Hellweg et al, 2007). Moreover, rats with lesions of serotonergic projections to the olfactory bulb following local injection of 5,7-DHT display the same hyperactivity in an open field test as seen in OBX rats, further implicating impaired serotonergic activity in behavioral changes seen in these rats (Song and Leonard, 2005; Kelly et al,1997). Decreased 5-HT activity of OBX rats result from either pre- or post-synaptic 5-HT_{1A} receptor dysfunction (Watanabe et al, 2006; Skelin et al, 2010).

Evidence suggests that nAChRs are located on hippocampal serotonergic neurons in male hooded lister rats and are capable of modulating 5-HT release (Rao et al,1996; Kenny et al,2000a). It is noteworthy that rivastigmine treatment also increased the availability and binding of endogenous ACh at nicotinic receptors in methamphetamine-dependent individuals. Nicotine-stimulated concentration-dependent increase of 5-HT release in rat hippocampal slices was antagonized by mecamylamine (Kenny et al, 2000b). Rivastigmine significantly increased [^{3}H]-EPI binding (to measure non-α7 nAChRs) only, not [^{3}H]-MLA binding (to measure α7 nAChRs), in the hippocampus of Sprague-Dawley rats (Reid and Sabbagh,2008). Previous behavioral findings also demonstrated that rivastigmine-improved neurological deficits induced by closed head injury in male Sabra rats was prevented by the simultaneous injection of mecamylamine (2.5 mg/kg.), but not scopolamine (1.0 mg/kg), a mAChR antagonist (Chen et al, 1998). Rivastigmine ameliorated neurological dysfunction and memory deficits after a chronic autoimmune encephalomyelitis model in female C57BL/6 mice induced by myelin oligondendrocyte glycoprotein was reversed by co-administration of mecamylamine, where mecamylamine alone did not exacerbate disease severity (Nizri et al,2008). Therefore, my findings suggests a hypothesis that sensitizing nAChRs of 5-HT neurons through increased ACh levels might influence 5-HT release, which activates 5-HT_{1A} receptor in the hippocampus of rivastigmine-treated OBX mice.

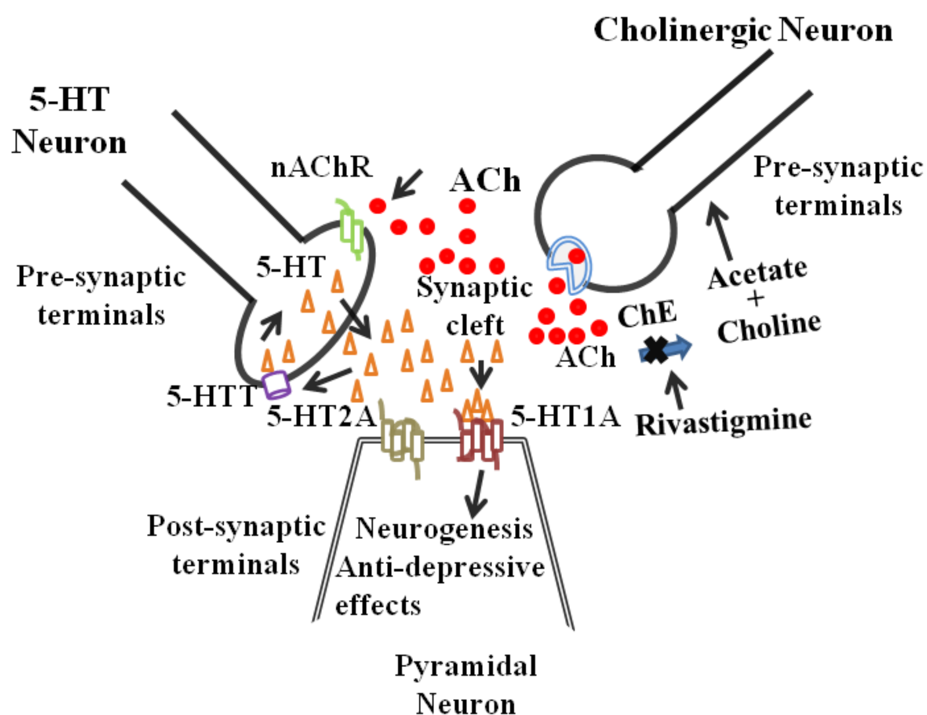

Figure 4.1 Proposed mechanism of 5-HT_{1A} receptor stimulation by rivastigmine. 5-HT, serotonin; The rise in the acetylcholine concentration at the synaptic cleft, caused by the blocking of acetylcholinesterase activity following rivastigmine, may have resulted in an over-stimulation of nicotinic acetylcholine receptors located on serotonergic neurons in the hippocampus which in turn increases extracellular 5-HT levels near the pyramidal neurons and up-regulates 5-HT_{1A} receptors. This regulation appears to underlie increased hippocampal neurogenesis and antidepressant-like behavioral responses observed after rivastigmine treatment for 14 days (cited from Neuroscience, 2014; 272:116-130).

4.2 Future expansion

The meta-analyses of current AD treatments, including the ChEIs and memantine, have examined numerous trials that included thousands of AD patients. While the results of these analyses consistently show efficacy with statistical significance, conclusions about their clinical significance differs between clinicians. Preclinical studies in mouse models have identified dozens more potential therapeutic targets, which await validation in humans but

will hopefully keep the drug development pipeline stocked for years to come. AD is a devastating neurodegenerative disease which affects millions of people, and threatens to become a public health crisis in coming years. Great strides have been made in our understanding of the underlying disease mechanisms and in early diagnosis, and the first experimental efforts to treat AD presymptomatically are beginning, potentially heralding a new era of AD management. In conclusion, although several facets of 5-HT_{1A} receptor-mediated signaling in the rivastigmine-induced neurogenesis have been determined, it seems that even more questions have been generated about the signaling and regulation of this receptor in the brain, and much more research is needed to fully elucidate the role of this receptor in various AD mouse model following rivastigmine.

In AD, hippocampal neurogenesis appears to be decreased in most mouse model. A recent study of PDAPP (Transgenic mice overexpressing mutant human amyloid precursor protein) mouse model of AD showed that old, but not young, PDAPP mice have 50% reduction in neurogenesis and new SGZ neurons exhibit abnormal maturation (Grote and Hannan,2007). It would be interesting to see whether rivastigmine treatment in other mouse model of AD exhibit similar improvement of neurogenesis or non-dementia symptoms.

There are several courses of action that could be pursued to further clarify the mechanisms of 5-HT receptor-mediated signaling and expression in the hippocampus of AD model mouse. First of all, a few of the pharmacological tools employed in the study did not reflect the whole scenario of serotonergic systems on AD model mouse. To completely clarify the role of 5-HT_{1A} receptor in DRN or hippocampus or other 5-HT receptors present, a more specific and wide investigation could be useful for opening a new therapeutic dimension. Future research is also necessary to determine the involvement of GABA-ergic interneuron or 5-HT transporter protein in the regulation of serotonergic neurotransmission following ChEIs.

Chapter 5: Materials and Methods

5.1 Materials

5.1.1 Chemicals

The following reagents were obtained from respective sources: Rivastigmine (Exelon, Novartis Pharma, AG, Basel, Switzerland); BrdU (Sigma-Aldrich, St. Louis, MO, USA); (7)-8-hydroxy-2-(di-n-propylamino)tetralin(8-OH-DPAT)and(4-bromo-3,6-dimethoxybenzoc yclobuten-1-yl) methylamine (TCB-2) (Tocris Bioscience, Bristol, United Kingdom); N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide (WAY-100635) (Abcam, Cambridge, MA, USA); ketanserin (Sigma-Aldrich, St. Louis, MO, USA). Other reagents were of the highest quality available (Wako Pure chemicals, Osaka, Japan).

5.1.2 Antibodies

The following antibodies were obtained from respective sources : anti-phospho ERK 1/2 (Thr-202/Tyr-204) (Cell Signaling, Woburn, MA, USA); anti-ERK 1/2 (Thr-202/Tyr-204) (Sigma-Aldrich, St. Louis, MO, USA), anti-Akt antibody (Cell Signaling, Woburn, MA, USA), anti-phosphorylated Akt (ser 473) antibody (Cell Signaling, Woburn, MA, USA), anti-beta-tubulin (1:5000, Sigma-Aldrich, St. Louis, MO, USA); anti-NeuN monoclonal antibody (Millipore,Temecula, CA92590,USA); a rat anti-BrdU monoclonal antibody (Accurate Chemical and Scientific, Oxford Biotechnology, Oxfordshire, UK); rabbit anti-DCX polyclonal antibody (ab18723, Abcam, Cambridge, MA, USA); Alexa 488-labeled anti-rat IgG (anti-BrdU), Alexa 594-labeled anti-mouse IgG (anti-NeuN) and Alexa 594-labeled anti-rabbit IgG (anti-DCX) (Life technologies, Eugene,OR,USA).

5.1.3 Animals

Eight-weeks-old male DDY mice weighing 23-26 g were obtained from Nippon SLC (Hamamatsu, Japan). All animals were group-housed in our animal facility in propylene cages at a constant temperature (23 ± 1℃) and humidity (55 ± 5%) with a reversed 12:12 light/dark cycle (lights off at 21.00 p.m.) and had free access to food and water. An acclimation period of at least 1 week was provided before initiating the experimental protocol. All experimental animal procedures were approved by the Animal Experimentation Committee on Animal Experiments at Tohoku University. All efforts were made to reduce animal suffering and minimize the total number of animals used.

5.2 Methods

5.2.1 Experimental animal groups

Initially, I separated mice into the following six groups (n=12, except for groups IV and V, n=7): I: sham; II: 1.0 mg/kg rivastigmine-treated sham; III: OBX; And IV-VI: 0.1, 0.3 and 1.0 mg/kg rivastigmine-treated OBX, respectively. For BrdU incorporation and immunohistochemical studies, 5 animals from groups I, II, III and VI were used.

In other experiments assessing 5-HT antagonist effects, mice were assigned to seven groups (n=7, except for groups V and VII, n=12). Groups included: I: sham; II: OBX; III. 1.0 mg/kg rivastigmine-treated OBX; IV-V: 1.0 mg/kg WAY-100635 co-administered with rivastigmine (0.3 and 1.0 mg/kg) in OBX; and VI-VII: 1.0 mg/kg ketanserin co-administered with rivastigmine (0.3 and 1.0 mg/kg) in OBX. Five animals from groups V and VII were isolated for immunohistochemical tests.

In experiments assessing receptor agonists, I again divided the animals into another seven groups (n=7, except for groups IV and VI, n=7), which included: I: sham; II: OBX; III.

1.0 mg/kg rivastigmine-treated OBX; IV-V: 0.3 and 1.0 mg/kg 8-OH-DPAT-treated OBX, respectively; and VI-VII: 0.3 and 1.0 mg/kg TCB-2-treated OBX, respectively. Five animals from groups I, II, III, V and VII were isolated for immunohistochemical procedures.

To determine antagonists effect alone, mice were divided into following four groups for immunohistochemical analyses (n=5 per group). Groups included: I: 1.0 mg/kg WAY-100635 treated sham; II: 1.0 mg/kg WAY-100635 treated OBX; III. 1.0 mg/kg ketanserin-treated sham; IV: 1.0 mg/kg ketanserin-treated OBX.

5.2.2 Bilateral olfactory bulbectomy surgical procedure in mice

After a one-week acclimatization period, olfactory bulbectomy was performed according to the procedure described by Hozumi et al. (2003). Briefly, mice anesthetized with sodium pentoparbital (50 mg/kg i.p.; Dainippon, Osaka, Japan) were placed in a stereotoxic instrument (KOPF, Tujunga, California, USA). After exposure of the skull, 1-mm diameter holes were drilled on either side of the olfactory bulbs, which were then bilaterally aspirated using a suction pump. Care was taken not to damage the frontal cortex. Holes were filled with a homeostatic sponge (Spongel, Astellas Pharma Inc., Tokyo, Japan) to avoid further bleeding, and the skin was closed with sutures. Sham-operated animals underwent the same procedure without excision and aspiration of olfactory bulbs. After surgery, all animals were allowed to recover in a post-operative cage (maintained at 23℃) for 3 hr. After this period, mice were returned to their home cage.

5.2.3 Behavioral tests

5.2.3.1 Tail Suspension Test

Immobility was observed by the tail suspension test according to the procedure of Steru et al.

(1985). Briefly, mice are suspended by the tail via attachment to a tail hanger with adhesive tape.and recorded over a 10-min period, similar to earlier protocol performed on same mice strain (Han et al, 2009; Shioda et al, 2010; Moriguchi et al, 2013). Mice were considered immobile when they hung passively and remained completely motionless. The duration of immobility was calculated during a 10-min test session.

5.2.3.2 Forced Swim Test

The FST was conducted as described by Porsolt et al. 1977. Briefly, mice were dropped individually in an open cylindrical container (diameter, 10 cm; height, 30 cm) filled with water to a depth of 18 cm and maintained at 23-25℃. The total duration of immobility within the course of a 5-min test was scored. Each mouse was judged immobile when it ceased struggling and remained floating motionless in the water.

5.2.3.3 Locomotion Test

Locomotor hyperactivity is the key behavioral feature of bulbectomized rodents. To investigate locomotor activity, mice housed individually in standard plastic cages were positioned in an automated open-field activity monitor using digital counters with an infrared sensor (Digital Acquisition System; Neuroscience, Inc., Tokyo, Japan). During the experimental period, mice had free access to food and water. Locomotor activity was measured during a 1 h period under lighted conditions.

5.2.3.4 The Novelty Suppressed Feeding Test

The Novelty Suppressed Feeding Test (NSFT) was administered to a separate group of mice during a 5-min period as described (Santarelli et al., 2003; David et al, 2010). Briefly, the test

apparatus consisted of a white plastic box (50×50×20 cm, containing bedding). Food was removed from the home cage for 24 h prior to testing. At the time of testing, a single food pellet (regular chow) was placed on a white paper platform positioned at the center of the brightly lit box. A mouse was placed in a corner of the box and a stopwatch was immediately started. The latency to eat (defined as the mouse sitting on its haunches and biting the pellet with use of forepaws) was scored. Immediately afterwards, mice were transferred to their home cage, and the amount of food consumed by the mouse in a 5 min period was measured. This measurement served as a control for a change in appetite as a possible confounding factor.

5.2.4 Western Blotting Analysis

Immunoblot analyses were performed at 31 days after OBX according to a previously reported method by Moriguchi et al. (2013). Hippocampal DG regions were dissected out from experimental mice brain and were homogenized in 70 μl buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na_3VO_4, 40 mM sodium pyrophosphate, 50 mM NaF, 100 nM calyculin A, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor and 1 mM dithiothreitol. Insoluble material was removed by a 10-min centrifugation (15,000 rpm). After determining supernatant protein concentration using Bradford`s solution, samples were heated at 100 ℃ for 3-min in Laemmli`s sample buffer.

Now samples containing equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to an Immobilon polyvinylidene difluoride membrane for 2-h at 70V. After blocking with TTBS solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) containing 5% nonfat dry milk

for 1-h at room temperature, membranes were incubated overnight at 4℃ with anti-$5HT_{1A}$ receptor antibody (1:1000, Abcam, ab85615); anti-$5HT_{2A}$ receptor antibody (1:1000, Abcam, ab16028), anti-Serotonin Transporter antibody (1:2000, Cell signaling, AB9726), and anti-beta-tubulin (1:5000,Sigma-Aldrich). Bound antibodies were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Chalfont St. Gilws, UK) and analyzed semiquantitatively using the National Institute of Health Image program (Bethesda, MD, USA).

5.2.5 Immunohistochemistry

At 35 days after OBX, immunohistochemistry was performed according to a previously reported method by Shioda et al. (2010). Briefly, mice were anesthetized with sodium pentoparbital (50 mg/kg i.p.; Dainippon, Osaka, Japan) and perfused via the ascending aorta with phosphate-buffered saline (PBS) until the outflow became clear, followed by perfusion with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 15 min. The brain was removed and post-fixed in the same fixative for 24 h at 4 °C. The brain was sliced at 50 μm thickness using a vibratome (Dosaka EM Co. Ltd., Kyoto, Japan).

Coronal mouse brain sections prepared using a vibratome were incubated as follows: 30 min in PBS, 30 min 2N HCl, and 1 h in PBS with 3% bovine serum albumin (blocking solution). Sections were then incubated overnight at 4℃ with mouse anti-NeuN monoclonal antibody (1:1000; Millipore) and a rat anti-BrdU monoclonal antibody (1:500; Accurate Chemical and Scientific, Oxford Biotechnology, Oxfordshire, UK) in blocking solution. After thorough washing in PBS, sections were incubated 3 h in Alexa 488-labeled anti-rat IgG (anti-BrdU) and Alexa 594-labeled anti-mouse IgG (anti-NeuN).

For BrdU and Doublecortin (DCX) double immunostaining, immunohistochemistry

was performed in the following steps: 30 min in PBS; 30 min 2N HCl, 1 h in PBS with 3% bovine serum albumin (blocking solution) and overnight at 4℃ with rabbit anti-DCX polyclonal antibody (ab18723, 1:500; Abcam) and rat anti-BrdU monoclonal antibody (1:500; Accurate Chemical and Scientific, Oxford Biotechnology, Oxfordshire, UK) in blocking solution. After thorough washing in PBS, sections were incubated 3 h in Alexa 488-labeled anti-rat IgG (anti-BrdU) and Alexa 594-labeled anti-rabbit IgG (anti-DCX). Finally, after several PBS washes, sections were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent images were analyzed using a confocal laser-scanning microscope (Nikon EZ-C1, Nikon, Tokyo, Japan).

To count $BrdU^+/NeuN^+$ and $BrdU^+/DCX^+$ cells, six hippocampal sections were cut every 50 μm beginning at 1.7 to 2.2 mm caudal to the bregma. The number of BrdU+/NeuN+ and BrdU+/DCX+ cells was determined in a 300×300 μm area per section in the DG region. In the DG, the number of cells in the GCL (approximately 50 μm wide) and the SGZ defined as a zone two cell bodies (5μm) wide along the border of the GCL and hilus) was determined. Numbers of $BrdU^+/NeuN^+$ and BrdU+/DCX+ cells in each mouse were expressed as the number of double-positive cells per 300 × 300 μm area. Six

5.2.6 Statistical Evaluation

All data were expressed as means ± S.E.M. Comparisons between two experimental groups were made using the unpaired Student`s *t*-test. Statistical significance for differences among groups was determined by one-way analysis of variance (ANOVA), followed by multiple comparisons between control and other groups using Scheffe’s test and SPSS software (IBM). Statistical significance was considered $P < 0.05$.

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Published publication related to this thesis

1. **Islam MR**, Moriguchi S, Tagashira H, and Fukunaga K (2014) Rivastigmine improves hippocampal neurogenesis and depression-like behaviors via 5-HT_{1A} receptor stimulation in olfactory bulbectomized mice. *Neuroscience*, **272**,116-130.