

Rivastigmine Restores 5-HT_{1A} Receptor Levels in the Hippocampus of Olfactory Bulbectomized Mice

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Abstract

Rivastigmine, a dual acetylcholinesterase and butyrylcholinesterase inhibitor, is used for symptomatic treatment of patients with mild to moderately severe dementia in Alzheimer's disease (AD) patients. In the present study, we found that 5-HT_{1A} receptor (5-HT_{1A}R) is downregulated, whereas 5-HT_{2A} receptor (5-HT_{2A}R) is upregulated in the hippocampal dentate gyrus (DG) and CA1 region by olfactory bulbectomy (OBX) in mice. Furthermore, chronic treatment with rivastigmine (1.0 mg/kg) for 2 weeks starting 2 weeks after OBX operation restored the decreased 5-HT_{1A}R and the increased 5-HT_{2A}R levels. To determine whether cholinergic receptor stimulation by rivastigmine is involved in the rivastigmine-induced regulation of 5-HT_{1A}R levels, we treated the mice with mecamylamine (2.5 mg/kg), or atropine (5.0 mg/kg) with rivastigmine (1.0 mg/kg) once a day for 2 weeks. Notably, the rivastigmine-induced 5-HT_{1A}R upregulation was eliminated by mecamylamine but not by atropine treatments. On the other hand, the restored 5-HT_{2A}R level by rivastigmine was not affected by either mecamylamine or atropine. Treatment with 8-OH-DPAT, a selective 5-HT_{1A}R agonist improved the decreased 5-HT_{1A}R and the increased 5-HT_{2A}R levels in OBX mice. On the other hand, treatment with TCB-2, a potent 5-HT_{2A}R agonist had no effects on the 5-HT_{1A}R and 5-HT_{2A}R dysregulation in OBX mice. Taken together, nicotinic acetylcholine receptor (nAChR) stimulation mediates rivastigmine-induced upregulation of 5-HT_{1A}R. Therefore, we speculate that the increased ACh levels by rivastigmine can stimulate nAChR located on serotonergic nerve terminals and stimulate 5-HT_{1A}R by the enhanced 5-HT release in the hippocampus. The 5-HT_{1A}R stimulation likely mediates the improvement of 5-HT_{1A}R levels as auto-receptor in OBX hippocampus.

Keywords

Rivastigmine, 5-HT_{1A} Receptor, 5-HT_{2A} Receptor, Nicotinic Acetylcholine Receptor, Olfactory Bulbectomized Mice

1. Introduction

The hippocampal functions are delicately modulated by cholinergic and serotonergic inputs from basal forebrain and the raphe nuclei, respectively. For example, nicotinic acetylcholine (nACh) receptor on presynaptic terminal regulates memory and attention function in the hippocampus [1]. Likewise, 5-HT_{1A}R regulates mood-related behaviors through somatodendritic inhibitory receptors pre-synaptically in the hippocampus [2]. 5-HT_{1A}R is located post-synaptically in pyramidal and granular neurons of the hippocampus [3]. 5-HT_{2A}R is also expressed in the pyramidal cell layers of CA1 and granule cells of dentate gyrus (DG) in the hippocampus of Sprague-Dawley rats [4], which has an important role in regulating network activity and neural oscillation in the hippocampal region [5]. In addition, 5-HT transporter (5-HTT) is localized to serotonergic neurons of the hippocampus, especially in the axons and nerve terminals [6] [7]. The presynaptically located 5-HTT is crucial for the regulation of 5-HT transmission because it controls the 5-HT availability at the site of postsynaptic receptors by high affinity reuptake of 5-HT in the synaptic cleft [8].

Olfactory bulbectomy (OBX) in rodents has been used as an Alzheimer disease (AD) model, because OBX induces various behavioral and biochemical phenotypes of AD such as increases in locomotor activity [9], aggressiveness [10] and cognitive defects [11]. Moreover, OBX in mice induces an elevation of amyloid beta ($A\beta$) in the brain [12]. We also reported neurodegeneration of cholinergic neurons in the medial septum [13] and impaired neurogenesis in the hippocampal DG [14]. Interestingly, OBX surgery degenerates 5-HT fibers innervating to the hippocampus, thereby reducing 5-HT synthesis and 5-HT receptor expression in rats. Therefore, this model is suitable for studying the cognitive deficits accompanied depressive symptoms [15] [16]. Likewise, post mortem examinations of brains reveal significant decline in both 5-HT and ACh levels as well as decreased hippocampal 5-HT_{1A}R expression [17]-[19].

Rivastigmine is a second-generation, carbamate-based reversible non-competitive inhibitor of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) used to treat mild to moderate dementia in patients [20]. Recent studies report that AD patients administered rivastigmine show reversals in depression, as evaluated by the Hamilton Depression Scale [21]. Rivastigmine has been approved by the Food and Drug Administration (FDA) to enhance or maintain cognition in AD patients [22]. A current 6-month open-label observational study reported that treatment with a rivastigmine patch alone decreased the frequency and severity of major depression episodes (MDEs) in 50 patients with mild AD [23]. We recently documented that rivastigmine improves deficits in cognition and memory-related behaviors in OBX mice [24]. In addition, we documented that rivastigmine-induced hippocampal neurogenesis and improvement of depressive-like behaviors in OBX mice is mediated by 5-HT_{1A}R stimulation [25]. Since high dose (0.5 mg/kg, s.c.) of nicotine stimulates 5-HT release in the hippocampus and elicits anxiogenic effects in rats [26], rivastigmine-enhanced ACh levels likely stimulate 5-HT release in the hippocampus.

Although how rivastigmine stimulate 5-HT release in the hippocampus remains unclear, it is important to investigate the physiological relevance of increased serotonergic neural activity. Therefore, to extend our earlier observations, we examined whether 5-HT_{1A}R activation by rivastigmine regulates 5-HT_{1A}R and 5-HT_{2A}R levels in OBX hippocampus.

2. Materials and Methods

2.1. Animals

8 weeks-old male DDY mice weighing 23 to 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 ($23^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and humidity ($55\% \pm 5\%$) with a reversed 12:12 light/dark cycle (lights off at 21.00 p.m.) and had free access to food and water. Animal Experimentation Committee on Animal Experiments at Tohoku University approved all experimental animal procedures. All efforts were made to reduce animal suffering and minimize the total number of animals used.

2.2. Operation and Treatment Diagram

After one week of acclimatization period, OBX mice were prepared as described [11] [24]. Briefly, mice anesthetized with sodium pentobarbital (50 mg/kg i.p. Dainippon, Osaka, Japan) were placed in a stereotaxic instrument (KOPF, Tujunga, California, USA). After exposure of the skull, 1-mm diameter holes were drilled on ei-

ther 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°C) for 3 hr. After this period, mice were returned to their home cage.

After the 2 weeks of recovery and appearance of depression-like behaviors [27], rivastigmine (Exelon, Novartis Pharma, AG, Basel, Switzerland) was administered (1.0 mg/kg; s.c) 30 min after a nAChR antagonist, mecamylamine (2.5 mg/kg, s.c) or a mAChR antagonist, atropine (5.0 mg/kg, i.p.) administration once daily for 14 days. Similarly, a selective 5-HT_{1A}R agonist, (7)-8-hydroxy-2-(di-n-propylamino) tetralin(8-OH-DPAT) (1.0 mg/kg; s.c.; Tocris Bioscience, Bristol, United Kingdom); and a selective 5-HT_{2A}R agonist, (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine (TCB-2) (1.0 mg/kg; i.p.; Tocris Bioscience) was administered once daily for 14 days. The doses and administration protocols of drugs used in this study were selected based on findings from the literature employing these drugs in animal behavioral models [25] [28] [29]. 24 hrs after the last dose, all mice were sacrificed for western blot analysis and the lesions were verified histologically.

2.3. Experimental Animal Groups

Mice were first separated into the following six groups (n = 7): I: sham; II: 1.0 mg/kg rivastigmine-treated sham; III: OBX; IV: 1.0 mg/kg rivastigmine-treated OBX; V: 2.5 mg/kg mecamylamine co-administered with 1.0 mg/kg rivastigmine in OBX, and VI: 5.0 mg/kg atropine co-administered with 1.0 mg/kg rivastigmine in OBX. In experiments assessing receptor agonists, mice were again assigned to four groups (n = 7). Groups included: I: sham; II: OBX; III: 1.0 mg/kg 8-OH-DPAT treated OBX, and IV: 1.0 mg/kg TCB-2 treated OBX.

2.4. Immunoblot Analysis

Immunoblot analyses were performed as described previously [14]. Samples 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 Na₃VO₄, 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°C 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 70 V. 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°C with anti-5HT_{1A} receptor antibody (1:1000, ab85615, Abcam, Cambridge, MA, USA); anti-5HT_{2A} receptor antibody (1:1000, ab16028, Abcam, Cambridge, MA, USA), anti-Serotonin Transporter (5-HTT) antibody (1:2000, AB9726, Cell signaling, Woburn, MA, USA) and anti-beta-tubulin (1:5000, Sigma-Aldrich, St. Louis, MO, USA). Bound antibodies were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Chalfont St. Giles, UK) and analyzed semiquantitatively using the National Institute of Health Image program (Bethesda, MD, USA).

2.5. Statistical Evaluation

All data were expressed as means ±S.E.M. Comparison between two experimental groups was made using the unpaired student's *t* test. Statistical significance for multiple comparisons between control and other groups uses Scheffe's test by SPSS software (IBM). Statistical significance was considered $P < 0.05$.

3. Results

3.1. Involvement of Cholinergic Effect in Rivastigmine-Induced 5-HT_{1A}R and 5-HT_{2A}R

To explore the influence of rivastigmine on the expression of 5-HT_{1A}R and 5-HT_{2A}R, western blotting was first performed in DG samples. As shown in **Figure 1(a)**, in sham-operated mice, quantitative analyses revealed that rivastigmine treatment had no effect on expression of 5-HT_{1A}R or 5-HT_{2A}R in the DG.

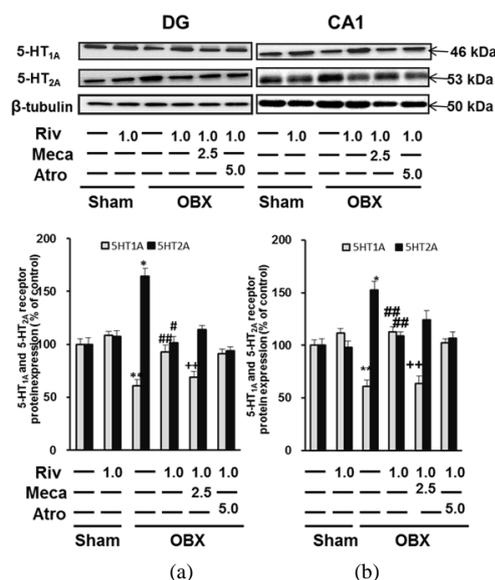


Figure 1. Rivastigmine treatment rescues reduced 5-HT_{1A}R expression levels in both DG and CA1 regions of OBX mice. (a) Representative images of immunoblots probed with antibodies against 5-HT_{1A}R and 5-HT_{2A}R in the DG and quantitative analyses are shown in the bar graph. (b) Representative images of immunoblots probed with antibodies against 5-HT_{1A}R and 5-HT_{2A}R in the CA1 and quantitative analyses are shown in the bar graph. Each bar represents the mean \pm S.E.M. ($p < 0.01$ versus sham-operated mice, $^{**}P < 0.05$ versus sham-operated mice, $^{\#}P < 0.01$ versus OBX mice, $^{##}P < 0.05$ versus OBX mice, $^{++}P < 0.05$ versus rivastigmine-treated OBX mice).

On the other hand, we first observed decreased 5-HT_{1A}R ($64.67\% \pm 2.05\%$ of sham, $P < 0.05$ vs. sham) and in contrast increased 5-HT_{2A}R ($164.12\% \pm 1.78\%$ of sham, $P < 0.01$ vs. sham) expression both in the DG region (**Figure 1(a)**). Interestingly, chronic rivastigmine treatment improves the dysregulation of 5-HT_{1A}R and 5-HT_{2A}R (5-HT_{1A}R: $96.16\% \pm 1.64\%$ of sham, $P < 0.05$ vs. OBX; 5-HT_{2A}R: $101.19\% \pm 2.26\%$ of sham, $P < 0.01$ vs. OBX). Notably, the improvement of 5-HT_{1A}R levels by rivastigmine was inhibited by mecamylamine co-administration ($65.72\% \pm 1.84\%$ of sham, $P < 0.05$ vs. rivastigmine-treated OBX mice), while atropine administration had no effect ($90.08\% \pm 3.51\%$ of sham). On the other hand, restoration of 5-HT_{2A}R levels by rivastigmine treatment was not blocked by mecamylamine and atropine. This suggests that nAChRs might be involved in rivastigmine-induced up-regulation of 5-HT_{1A}R expressions in the DG of OBX mice.

Similarly, the dysregulation of 5-HT_{1A}R and 5-HT_{2A}R was observed in the CA1 region and rivastigmine treatment improved the decreased 5-HT_{1A}R ($112.58\% \pm 1.37\%$ of sham, $P < 0.05$ vs. OBX) and increased 5-HT_{2A}R expression ($108.70\% \pm 1.93\%$ of sham, $P < 0.05$ vs. OBX) in OBX mice (**Figure 1(b)**). Likewise, mecamylamine antagonized the rivastigmine effect on 5-HT_{1A}R ($63.81\% \pm 2.42\%$ of sham, $P < 0.05$ vs. rivastigmine-treated OBX mice), without alteration in 5-HT_{2A}R levels.

3.2. Effect of 8-OH-DPAT and TCB-2 on 5-HT_{1A}R and 5-HT_{2A}R Protein Expression

Since nAChR mediates regulation of 5-HT_{1A}R expressions by rivastigmine, we speculated nAChR stimulates 5-HT release, thereby up-regulating 5-HT_{1A}R in OBX mice. Therefore, we tested effects of 5-HTR agonists on the dysregulation of 5-HT_{1A}R and 5-HT_{2A}R expressions following OBX.

After administration of 8-OH-DPAT or TCB-2, we measured 5-HT_{1A}R and 5-HT_{2A}R expressions in both DG and CA1 regions. As expected, 5-HT_{1A}R agonist, 8-OH-DPAT treatments significantly improved the decreased 5-HT_{1A}R levels (DG: $134.67\% \pm 2.42\%$ of sham, $P < 0.01$ vs. OBX; CA1: $155.93\% \pm 3.62\%$ of sham, $P < 0.01$ vs. OBX) (**Figure 2(a)**, **Figure 2(b)**). In addition, the increased 5-HT_{2A}R levels in OBX mice (DG: $143.41\% \pm 2.48\%$ of sham, $P < 0.05$ vs. sham; CA1: $155.25\% \pm 2.99\%$ of sham, $P < 0.05$ vs. sham) were restored in 8-OH-DPAT treatment groups of OBX mice (DG: $96.05\% \pm 3.50\%$ of sham, $P < 0.01$ vs. OBX; CA1: $103.98\% \pm 1.70\%$ of sham, $P < 0.01$ vs. OBX) (**Figure 2(a)**, **Figure 2(b)**). Whereas, 5-HT_{2A}R, agonist, TCB-2 treatment

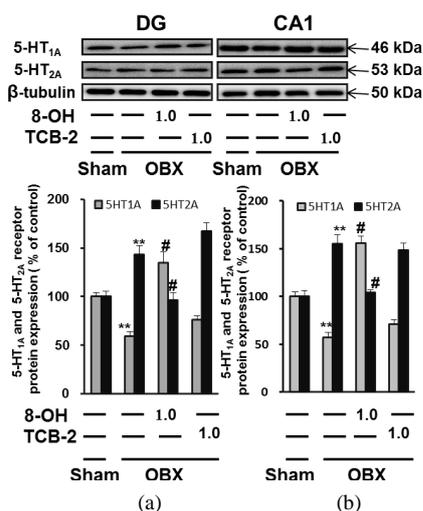


Figure 2. 8-OH-DPAT elevates 5-HT_{1A}R expression levels in both DG and CA1 of OBX mice. (a) Representative images of immunoblots probed with antibodies against 5-HT_{1A}R and 5-HT_{2A}R in the DG and quantitative analyses are shown in the bar graph. (b) Representative images of immunoblots probed with antibodies against 5-HT_{1A}R and 5-HT_{2A}R in the CA1 and quantitative analyses are shown in the bar graph. Each bar represents the mean \pm S.E.M. (** $P < 0.05$ versus sham-operated mice, # $P < 0.01$ versus OBX mice).

failed to improve 5-HT_{1A}R and 5-HT_{2A}R expression in the DG and CA1 of OBX (Figure 2(a), Figure 2(b)). These observations suggest that activation of 5-HT_{1A}R by 8-OH-DPAT restores both 5-HT_{1A}R and 5-HT_{2A}R expressions in the DG and CA1 of OBX mice.

3.3. Effect of Rivastigmine, 8-OH-DPAT and TCB-2 on 5-HTT Protein Expression

Since 5-HT transporter (5-HTT) has crucial role in the regulation of 5-HT levels in synaptic regions, we next assessed the effect of rivastigmine on hippocampal 5-HTT protein levels in OBX mice. The significantly elevated 5-HTT levels seen in the hippocampus of OBX mice ($159.04\% \pm 2.98\%$ of sham, $P < 0.05$ vs. sham) was restored by rivastigmine treatment ($108.01\% \pm 4.27\%$ of sham, $P < 0.05$ vs. OBX) (Figure 3(a)). Mecamylamine co-administration, but not atropine prevented the increased hippocampal 5-HTT protein levels ($158.92\% \pm 5.25\%$ of sham, $P < 0.05$ vs. rivastigmine-treated OBX) (Figure 3(a)). Similarly, treatment with 8-OH-DPAT but not TCB-2 restored increased 5-HTT levels ($147.09\% \pm 1.41\%$ of sham, $P < 0.05$ vs. sham; $103.46\% \pm 3.03\%$ of sham, $P < 0.05$ vs. OBX) (Figure 3(b)). Taken together, these findings confirm that rivastigmine improves the enhanced 5-HTT protein levels seen in the hippocampus of OBX mice and 5-HT_{1A}R stimulation mediates the 5-HTT regulations by rivastigmine.

4. Discussion

The novel findings in the present study are that the depression-like behaviors are closely associated with down-regulation of 5-HT_{1A}R and upregulation of 5-HT_{2A}R in the hippocampal DG and CA1 after OBX and that rivastigmine restores the reduced 5-HT_{1A}R in nAChR-dependent manner. Because mecamylamine co-administration, but not in atropine prevented the rivastigmine-induced 5-HT_{1A}R expression in the hippocampus, the elevated 5-HT_{1A}R expression was not due to a direct effect of rivastigmine on 5-HT_{1A}R. We conclude that rivastigmine-induced ACh increase stimulates nAChR, which mediates the 5-HT_{1A}R regulation by rivastigmine treatment. Furthermore, 5-HT_{1A}R stimulation by release 5-HT accounts for regulation of both 5-HT_{1A}R and 5-HT_{2A}R in the hippocampus.

Our results are supported by evidences that nAChRs are located on hippocampal serotonergic neurons in rats and are capable of modulating 5-HT release [30] [31] and that rivastigmine treatment increases the availability and binding of endogenous ACh at nicotinic receptors in methamphetamine-dependent individuals [32]. Furthermore, nicotine-induced increase in 5-HT release in rat hippocampal slices is antagonized by mecamyla-

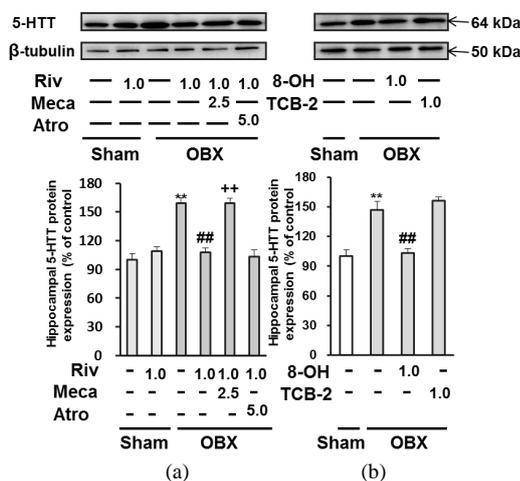


Figure 3. Rivastigmine and 8-OH-DPAT restored elevated 5-HTT expression level in the hippocampus of OBX mice. (a) Representative images of immunoblots probed with antibodies against 5-HTT in the hippocampus and quantitative analyses are shown in the bar graph. (b) Representative images of immunoblots probed with antibodies against 5-HTT in the hippocampus and quantitative analyses are shown in the bar graph. Each bar represents the mean \pm S.E.M. (** $P < 0.05$ versus sham-operated mice, ## $P < 0.05$ versus OBX mice, ++ $P < 0.05$ versus rivastigmine-treated OBX mice).

mine [33].

Rivastigmine significantly increased [3 H]-EPI binding (to measure non- $\alpha 7$ nAChRs) only, not [3 H]-MLA binding (to measure $\alpha 7$ nAChRs), in the hippocampus of Sprague-Dawley rats [34]. Previous behavioral findings also demonstrate that rivastigmine-improved neurological deficits induced by closed head injury in male Sabra rats were prevented by the simultaneous injection of mecamylamine (2.5 mg/kg.), but not scopolamine (1.0 mg/kg), a mAChR antagonist [29]. Rivastigmine ameliorates neurological dysfunction and memory deficits after a chronic autoimmune encephalomyelitis model in female C57BL/6 mice induced by myelin oligodendrocyte glycoprotein. The effects are reversed by co-administration of mecamylamine [35]. Together with the previous findings, our results suggest that stimulation of nAChRs on 5-HT neurons through increased ACh causes 5-HT release, which activates 5-HT_{1A}R in the hippocampus of rivastigmine-treated OBX mice.

The finding on up-regulation of 5-HT_{1A}R expressions in OBX mice following 8-OH-DPAT presented here fit well with 5-HT-mediated mechanism of up-regulation of 5-HT_{1A}R. Furthermore, we confirmed that treatment with mecamylamine, atropine, 8-OH-DPAT or TCB-2 alone in sham or OBX mice had almost no marked effects on 5-HT_{1A}R, 5-HT_{2A}R and 5-HTT expressions (data not shown). Our data provide evidence that variation in hippocampal 5-HT_{1A}R expressions can also affect the level of 5-HT_{2A}R expressions. However, the precise causal pathway involved in the 5-HT_{1A}R-mediated 5-HT_{2A}R expressions did not define in the present study. To the best of our knowledge, this is the first study to demonstrate that rivastigmine restores 5-HT_{1A}R and 5-HT_{2A}R expression in the hippocampus of OBX mice.

It is well documented that both 5-HT_{1A}R and 5-HTT play an important role to maintain 5-HT homeostasis in the hippocampus. For example, the increased 5-HTT densities were observed in the hippocampus of OBX rats by autoradiography [36]. The elevation of the 5-HTT in the OBX rats is likely mediated by proliferation of the glia after the neuronal cells die [37]. Elevated 5-HTT protein levels in our study were decreased following rivastigmine or 8-OH-DPAT treatments. The improvement of 5-HTT levels by rivastigmine was prevented by mecamylamine co-administration. Taken together, nAChRs present in serotonergic neurons likely modulate the serotonergic transmission in the hippocampus as shown in Figure 4.

In conclusion, nAChR is involved in rivastigmine-induced 5-HT_{1A}R upregulation in the hippocampus of OBX mice. Stimulation of 5-HT_{1A}R mediates improvement of impaired 5-HT_{1A}R and 5-HT_{2A}R in the hippocampus. To define the site of action of nAChR on the serotonergic neurons, further extensive studies are required. The novel site of action on 5-HT_{1A}R induced by rivastigmine is attractive to explain the effects on the behavioral and psychological symptoms in Alzheimer disease patients.

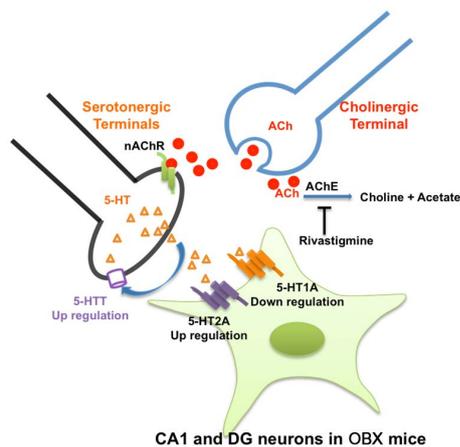


Figure 4. Working model of rivastigmine in the modulation of hippocampal 5-HT neural activity. The elevation of ACh concentration by blocking of ChE activity by rivastigmine results in stimulation of nicotinic acetylcholine receptors located on serotonergic nerve terminals in the hippocampus, thereby increasing extracellular 5-HT levels. The stimulation of 5-HT_{1A} receptors likely mediates upregulation of 5-HT_{1A}R. 5-HT, serotonin; ACh, acetylcholine; ChE, cholinesterase; nAChR, nicotinic acetylcholine receptor; 5-HTT, serotonin transporter protein; 5-HT_{1A}R and 5-HT_{2A}R, serotonin receptor subtypes.

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Conflicts of Interest

The authors declare that we have no conflicts of interest to disclose.

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