Activity-dependent bidirectional regulation of terminal neuronal maturation in the adult hippocampus

2015

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GENERAL INTRODUCTION

The hippocampus is one of the limbic structures and has a long-established role in learning and memory. In addition, it has become apparent that the hippocampus is an important regulator of emotion, and its dysfunction has been implicated in the pathophysiological basis of neuropsychiatric disorders, such as major depression and schizophrenia (Duman and Voleti, 2012; Decarolis and Eisch, 2010). The hippocampal structural connection is known as a trisynaptic circuit composed of the dentate gyrus (DG), and the CA1 and CA3 neurons. Granule cells (GCs) of the DG receive complex sensory information from the entorhinal cortex, and transfer the information to CA3 neurons via mossy fibers (MFs), which are axons of GCs of the DG. The information received by CA3 neurons is transmitted to CA1 neurons through the Schaffer collateral pathway. Finally, the processed information is returned to the entorhinal cortex.

The DG is located at the starting point of the hippocampal trisynaptic circuit. In the mammalian DG, generation of GC neurons, which is called neurogenesis, is sustained throughout life due to proliferation of neural stem cells in the subgranular zone (SGZ). Newborn GCs are integrated into granule cell layer (GCL), and finally become indistinguishable from pre-existing GCs within 2 months in rodents (Duan et al., 2008; Zhao et al., 2008). The continual supply of GCs is considered to be important for maintaining or reinforcing functions of the DG, although actual contribution of adult neurogenesis to neural functions has been still debated, because the number of additional newborn neurons would represent only a few percent of the total GC number (Ninkovic et al., 2007).

The principal cells of the DG are mature GCs. Given the high occupancy of mature GCs in the DG, functional modulation in mature GCs would have a great impact on hippocampal functions. Recent studies indicated that the maturation state of GCs may be modified even after they are completely maturated. Chronic treatment with selective serotonin reuptake inhibitor (SSRI) can reverse the established matured state of GCs to the late immature-like state in the adult mouse DG, which is called “dematuration” (Kobayashi et al., 2010). Dematured neurons show marked changes in physiological and functional properties, including expression of mature markers, neural excitability, and stimulus-induced responsibility (Kobayashi et al., 2010). However, the regulatory mechanism underlying the GC dematuration remains largely unknown. In addition, it has been reported that the dematured GC phenotype is observed concomitantly with enhanced adult neurogenesis in the DG (Kobayashi et al., 2010), although it is also unclear whether there is a relationship between the two phenomena.

In the present study, I set out to reveal the regulatory mechanism underlying GC dematuration (Chapter 1 and Chapter 2) and address whether there is a relationship between GC dematuration and adult neurogenesis (Chapter 3).

Chapter 1: Neural activation reverses mature phenotypes of granule cells in the dentate gyrus of the hippocampus

It has been generally believed that neural activity is known to regulate development of the nervous system. Although it has been reported that neural activity or excitation promotes structural and functional maturation of immature GCs (Overstreet-Wadiche et al., 2006; Ge et al., 2006; Piatti et al., 2011; Zhao et al., 2012), the facilitatory effects of the neural activity on GC maturation are typically observed at the early maturational stage. It remains largely unknown how neural activity modulates the late or terminal stage of GC maturation. In this chapter, I set out to reveal the role of neural activity in regulating the terminal neuronal maturation of GCs. To this end, I chose electroconvulsive stimulation (ECS) as excitatory stimulation. ECS is known to a model of electroconvulsive therapy, which is an effective and fast-acting treatment for depression, and robustly activate excitatory glutamatergic signaling in the DG of hippocampus (Ma et al., 2009; Stewart and Reid, 1994).

The matured state of GCs is characterized by several distinct molecular and physiological features (Duan et al., 2008; Zhao et al., 2008). I first examined the gene expression of calbindin (*Calb1*), a marker for terminal maturation of GCs and found that single ECS strongly reduced *Calb1* expression in a DG-specific manner. Single ECS reduced the expression of *Calb1* and tryptophan 2,3-dioxygenase (*Tdo2*), another mature GC marker, to essentially the same extent as repeated ECS (11 times over 3 weeks), at 6 h after the stimulation. While *Calb1* and *Tdo2* expression levels returned toward the control level in 24 h after single ECS, they were continually downregulated at least for 14 days after repeated ECS. These results demonstrate that neural activation induces rapid downregulation of markers for GC terminal maturation and that repeated stimuli stabilize the downregulated state. I also confirmed the reduction in the calbindin expression after repeated ECS at the protein level (Tonder et al., 1994). In contrast, the same treatment did not affect expression of a neuronal marker, NeuN. In the SGZ of the DG, the expression of doublecortin and calretinin, markers for early immature GCs, was enhanced by repeated ECS probably due to increased adult neurogenesis. However, the majority of the NeuN-positive or calbindin-negative GCs in the DG did not express these markers after ECS, suggesting that ECS-treated GCs are in an intermediate or late immature state lacking calretinin and calbindin expression (Brandt et al., 2003). The rapid downregulation of the mature GC markers excludes a possibility that mature GCs are largely replaced by newly generated young neurons. Thus, these results suggest that

ECS changes the phenotype of mature GCs to that of immature GCs at the late immature stage.

Next, electrophysiological properties of ECS-treated GCs were examined. Immature GCs show higher somatic excitability, higher input resistance, and more depolarized resting membrane potentials than mature GCs (Ambrogini et al., 2004). Repeated ECS increased somatic excitability of GCs and reduced resting membrane potentials, but had no significant effect on input resistance. Presynaptic characteristics at the synapse between the GC axon MF and a CA3 pyramidal cell can be a good physiological index of the functional maturity of GCs (Kobayashi et al., 2010). The mature MF synapse exhibits strong frequency facilitation, a form of presynaptic short-term plasticity, and the magnitude of MF frequency facilitation well correlates with the expression level of the mature GC marker *Calb1* (Kobayashi et al., 2010). To further characterize the functional maturity of ECS-treated GCs, the MF-CA3 synaptic transmission was analyzed. Repeated ECS reduced the frequency facilitation of MF excitatory postsynaptic potentials (EPSPs) to the juvenile level, but had no significant effect on the basal synaptic efficacy. While 2 or 3 times of ECS had highly significant effects on frequency facilitation at 24 h after ECS, the magnitude of facilitation completely returned to the control level in 14 days. Repeated ECS caused a long-term reduction in frequency facilitation lasting more than 28 days. Taken together, these results strongly suggest that the neural activation by ECS rapidly initiates dematuration of GCs. The rapidly induced dematuration is not stable, and repeated ECS can convert the transient demature state into a long-lasting form and stabilize the state of GCs at the late immature stage, providing a novel regulatory mechanism of terminal maturation of GCs by neural activation.

Chapter 2: Alteration of excitation/inhibition balance bidirectionally regulates maturation of granule cells in the dentate gyrus of the hippocampus

In this chapter, I explored the regulatory mechanisms underlying GC dematuration by ECS. I firstly explored the signaling pathway involved in the induction of GC dematuration by ECS. Since ECS activates glutamate signaling in the hippocampus (Ma et al., 2009; Morinobu et al., 1997; Stewart and Reid, 1994), I examined whether N-methyl-D-aspartate receptor (NMDAR) is involved in the rapid induction of GC dematuration by using ketamine, an antagonist of NMDARs. High doses of ketamine attenuated the reduction in *Calb1* and *Tdo2* expression by single ECS. Ketamine also blocked the reduction in frequency facilitation at the MF synapse. In addition, i.c.v. administration of D-AP5, another NMDAR antagonist, attenuated the downregulation of *Calb1* and *Tdo2* expression by ECS. Thus, the rapid induction of GC dematuration by ECS requires NMDARs. Since NMDAR activation up-regulates expression of many genes in the DG, *de novo* protein synthesis might be necessary for ECS-induced neuronal dematuration. As expected, pretreatment of mice with cycloheximide, a protein synthesis inhibitor, attenuated the reduction in the expression of mature GC markers by single ECS. The reduction in the synaptic facilitation was also attenuated by pretreatments with cycloheximide. These results suggest that the protein synthesis following NMDAR activation is important for the rapid induction of neuronal dematuration by ECS.

The above results showed that activation of excitatory synaptic transmission triggers the mature-to-demature state transition of GCs. I next explored the mechanism underlying the long-lasting dematuration. Dematured GCs show higher somatic excitability (Kobayashi et al., 2010 and Chapter 1). This raises a possibility that enhanced excitability is involved in the long-lasting dematuration. I therefore examined the role of GABAergic inhibition in regulating the maturation state of GCs. Diazepam, a positive allosteric modulator of the $GABA_A$ receptor, was administered during and after the period of ECS treatments. Diazepam had no significant effect on the reduction in frequency facilitation at 1 day after 3 times of ECS, suggesting that the enhanced GABAergic inhibition does not affect the induction of dematuration. However, in diazepam-treated mice, 11 times of ECS failed to induce a long-lasting reduction in frequency facilitation at 14 days after ECS, and the reduction in the expression of

mature GC markers, *Calb1* and *Tdo2*, was significantly reversed in 14 days after 11 times of ECS. Thus, augmented GABAergic inhibition prevents stabilization of the dematured state of GCs. Taken together, these results suggest that the enhanced GABAergic inhibition prevents the stabilization, but not the induction phase, of GC dematuration, and that the terminal maturation of GCs is reversed and advanced by neuronal excitation and inhibition, respectively.

Chapter 3: Involvement of mature granule cell dematuration in the adult neurogenesis in the dentate gyrus of the hippocampus

Chronic administration of antidepressants, such as SSRIs, increases adult neurogenesis in the DG (Malberg et al., 2000; Eisch et al, 2012), and a recent pharmacological study suggested that the 5-HT_4 receptor partly mediates SSRI-induced neurogenic action in the DG (Mendez-David et al., 2014). Furthermore, it has been reported that the 5-HT_4 receptor is essential for dematuration of GCs by chronic SSRI treatment (Kobayashi et al., 2010). However, it remains largely unknown how the 5-HT_4 receptor contributes to both neurogenic and dematuration effects of chronic SSRI administration within the DG. In this chapter, using 5-HT_4 receptor knockout (5-HT4R KO) mice with the C57BL/6J background, I explored regulatory roles of the 5-HT_4 receptor in both neurogenesis and GC dematuration.

Mice were intraperitoneally administered with 22 mg/kg/day fluoxetine, an SSRI, for 21 days, and 5-Bromodeoxyuridine (BrdU) was given to label proliferating cells 2 h before sacrifice on the next day of the last fluoxetine administration. The chronic fluoxetine treatment significantly increased the number of BrdU-positive cells in the SGZ of the DG compared with saline treatment in WT mice, whereas no significant difference was observed between saline and fluoxetine treatments in 5-HT4R KO mice. I next assessed the number of immature neurons by immunostaining for doublecortin (DCX), a marker of neurogenesis. The number of DCX-positive cells in the DG was significantly increased in fluoxetine-treated WT mice, but not significantly changed in 5-HT4R KO mice. These results demonstrate that the neurogenic effect of chronic fluoxetine treatment in the DG is mediated by the 5-HT_4 receptor.

It has been reported that the 5-HT_4 receptor is abundantly expressed in the DG (Tanaka et al., 2012; Warner-Schmidt et al., 2009). To determine the cell types expressing the 5-HT_4 receptor in the DG, I examined β-galactosidase immunoreactivity (LacZ-IR) in the hippocampus of the 5-HT4R KO mice, in which the β-galactosidase gene is ‘knocked-in’ at the *Htr4* gene locus. I found that the LacZ-IR was co-localized with the immunoreactivity of a neural marker, NeuN, and that of a marker for mature GCs, calbindin. However, LacZ-IR was not co-localized with the immunoreactivity of DCX, a marker for neural progenitors and immature neurons, or that of calretinin, a

marker for immature GCs, in the SGZ, indicating that the 5-HT_4 receptor is mainly expressed in mature GCs.

The 5-HT_4 receptor is necessary for fluoxetine-induced GC dematuration (Kobayashi et al., 2010). Dematured GCs exhibit various phenotypic changes, including reduced expression of mature GC markers, increased somatic excitability, and reduced activity-dependent synaptic facilitation (Kobayashi et al., 2010). Because expression of the 5-HT_4 receptor in the DG is mainly restricted to mature GCs, I hypothesized that progression of GC dematuration via the 5-HT_4 receptor is correlated with the neurogenic effect of fluoxetine within the SGZ of the DG. To address this hypothesis, I examined the expression level of the mature GC marker, calbindin, since the calbindin level was used as an index of GC dematuration (Kobayashi et al., 2010). Chronic fluoxetine treatment significantly reduced calbindin-IR in both the GC layer and molecular layer of the DG in WT mice, but not in 5-HT4R KO mice. To examine the correlation between the neurogenic effect and the dematuration effect of fluoxetine, I compared the number of proliferating cells and calbindin-IR in the DG of the same animals, and found that the number of proliferating cells was inversely correlated with calbindin-IR. It is known that antidepressant treatment increases expression of neurotrophic factors, including *Bdnf*, in the DG (Nibuya et al., 1995; Sillaber et al., 2008), which has been implicated in enhanced neurogenesis (Scharfman et al., 2005). I therefore examined whether there is a correlation between the increased expression of these factors and the reduced expression of calbindin mRNA (*Calb1*) by RT-qPCR in the individual fluoxetine-treated DG in comparison with the average expression levels in the control DG. I found that the change in the *Bdnf* expression was inversely correlated with that of *Calb1*. These results demonstrate that the neurogenic effect of fluoxetine in the SGZ is closely associated with the progression of GC dematuration in the DG. Altogether, these results suggest that 5-HT_4 receptor-mediated dematuration and accompanying changes in mature GCs underlie the neurogenic effects of SSRI treatment in the DG, and provide a new hypothesis that dematured GCs enhance adult neurogenesis in the DG during chronic SSRI treatment.

CONCLUSION

The summary of the results presented in this study as follows:

Chapter 1

1. Neural activation by ECS transiently reverses mature phenotypes of GCs in the DG to immature-like phenotypes.
2. Repeated ECS stabilizes immature-like phenotypes of GCs.

These results suggest that neural activation reverses terminal maturation of GCs to a immature-like state; the reversal process is called as dematuration.

Chapter 2

1. Glutamatergic signals via the NMDA receptor is critical for ECS-induced dematuration of GCs in the DG.
2. Augmentation of synaptic inhibition by diazepam induces rematuration of dematured GCs.

These results suggest that the altered excitatory/inhibitory balance bidirectionally regulates terminal maturation of GCs.

Chapter 3

1. Chronic treatment with SSRI induces enhanced adult neurogenesis in the DG and dematuration of GCs via the 5-HT_4 receptor.
2. The 5-HT_4 receptor is expressed in mature GCs, but not in neural progenitor cells or immature GCs.
3. Enhanced adult neurogenesis and progression of dematuration are significantly correlated with each other during chronic SSRI treatment.

These results suggest a new hypothesis that dematured GCs enhance adult neurogenesis in the DG during chronic SSRI treatment.

The present study demonstrated a novel regulatory mechanism of terminal maturation of GCs via the altered E/I balance in the DG and suggested a unique interaction between

newborn neurons and pre-existing neurons. This study will provide a new insight into the physiological basis underlying neuropsychiatric disorders, and contribute to a possible fundamental remedy for these disorders.

ACKNOWLEDGEMENTS

I would like to appreciate gratefully to Professor Kazuhisa Nakayama, Graduate School of Pharmaceutical Sciences, Kyoto University and Associate Professor Eri Segi-Nishida, Graduate School of Pharmaceutical Sciences, Kyoto University for their supports through the course of this work.

I would like to express my gratitude to Professor Hidenori Suzuki and Associate Professor Katsunori Kobayashi, Graduate School of Medicin, Nippon Medical School for electrophysiological analysis and helpful discussion.

I would like to express my appreciation to Dr. Shuji Kaneko, Dr. Takayuki Nakagawa, Dr. Kazuki Nagayasu, Mr. Naoya Nishitani Graduate School of Pharmaceutical Sciences, Kyoto University for assays of monoamine measurements and helpful discussion.

I would like to thank Dr. Yukihiko Sugimoto, Dr. Yasushi Okuno, Dr. Hye-Won Shin, Dr. Yohei Katoh, Dr. Hiroyuki Takatsu, Dr. Senye Takahashi, Dr. Kazushi Morimoto, Dr. Tomoaki Inazumi, Ms. Mamiko Sukeno, Ms. Mari Sakaida, Mr. Toshihiko Kira, for their technical advice and helpful discussion.

I also thank members of the Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University for their continuous encouragements, helpful discussions and technical supports.

Finally, I would like to thank my family for persistent understanding and support for many things.

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