学位論文　（要約）

Biological significance of mammalian-specific homopolymeric amino acid repeats in POU3F2: its contribution to brain development and function

（POU3F2 の哺乳類特異的単一アミノ酸リピートが有する生物学的意義：
脳の発生と機能への寄与）

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Abstract

Homopolymeric amino acid repeats represent protein regions with strings of a single amino acid residue and are abundant in eukaryotic genomes. While homopolymeric amino acid repeats exhibit diversity among species and within species, they are often strongly conserved through long spans of evolution. It has recently become clear that homopolymeric amino acid repeats play important roles in biological processes, and may be contribute to phenotypic evolution. However, their biological and evolutionary significance have not been fully demonstrated due to lack of studies with *in vivo* experiments.

POU3F2/BRN2 is a transcription factor that is mainly expressed in the central nervous system and plays an important role in brain development. POU3F2 includes multiple mammalian-specific homopolymeric amino acid repeats. Using genetically engineered mice in which all three homopolymeric amino acid repeats were deleted from the *Pou3f2* gene (*Pou3f2*$^{\Delta/\Delta}$ mice), I investigated a functional role of homopolymeric amino acid repeats.

First, *in vitro* experiment revealed that the transactivation activity of POU3F2 was down-regulated by the homopolyemeric amino acid repeats. Next, *in vivo* experiment revealed that matured and differentiating astrocytes are both decreased in the cortex of *Pou3f2*$^{\Delta/\Delta}$ mice, whereas intermediate progenitors were concomitantly increased. In addition, cortical progenitors in *Pou3f2*$^{\Delta/\Delta}$ mice less differentiated into astrocytes and more differentiated into neurons than those in control mice during astrogenesis. These results suggest that the homopolymeric amino acid repeats modulate the activity of POU3F2, and contribute to astrocyte differentiation, which may be mediated by regulation of cell fate decision in cortical progenitors. Next, cognitive function of *Pou3f2*$^{\Delta/\Delta}$ mouse was examined by two behavioral tests: object recognition test and object location test. As a result,

Pou3f2$^{\Delta/\Delta}$ mice exhibited impairements in recognition memory. Furthermore, *Pou3f2*$^{\Delta/\Delta}$ mice had lower numbers of newborn neurons in the adult hippocampus due to attenuated neural differentiation.

These results indicate that the homopolymeric amino acid repeats in POU3F2 affect cognitive function at behavioral and physiological levels, demonstrating that homopolymeric amino acid repeats are functional and beneficial in an organism.

Contents

Chapter 1

General Introduction

The forebrain is present in all vertebrates and divided into the telencephalon and diencephalon (Butler & Hodos, 2005). The former is the dorsal part of the forebrain, which contains cerebral hemispheres, olfactory bulbs, limbic system and striatum, while the latter is the caudal part of the forebrain, which contains thalamus and hypothalamus. Although the basal structure of telencephalon is conserved in vertebrates and pallium exist in fish and amphibians, the cerebral cortex appears in amniotes for the first time (Butler & Hodos, 2005). However, comparative studies have revealed that telencephalons and cerebral cortices of diapsids (reptiles and birds) are very different from those of mammals. Although the mammalian piriform cortex shows similar relative size and connectivity to those of the diapsid homolog, the hippocampus and the neocortex are much more developed in mammals. The mammalian hippocampus possesses distinct CA (*cornu ammonis*) and dentate gyrus, which are not anatomically defined in diapsids (Striedter, 2015; Goffinet, 2017). The mammalian neocortex is characterized by its thickness, large surface area and six-layered lamination, whereas the reptilian cerebral cortex has a simple three-layered structure (Naumann *et al*. 2015) and the avian pallium consists of nucleus rather than of layers (Medina & Reiner 2000).

Cytoarchitecture also varies among amniote brains. The brain is composed of neurons and glial cells, which contain astrocytes, oligodendrocytes, microglia and ependymal cells. The human brain has close to 200 billion cells in a 1:1 ratio of glia to neurons (von Bartheld *et al*. 2016). In the human cerebral cortex, 10-20 billion neurons and at most twice the number of glial cells (20-40 billion) are present (von Bartheld *et al*. 2016). Glia to neuron ratio greatly varies among species. One of the most noticeable features of vertebrate nervous system evolution is difference in the distribution and the

number of astrocytes. In lampreys, sharks, bony fish and amphibians, ependymoglia are predominant and star-shaped astrocytes have yet to be identified (Appel, 2013). In reptiles, a small number of astrocytes are intermingled with numerous ependymoglia. Turtles have a few astrocytes only in the spinal cords (Lazzari & Franceschini 2006); however squamata and crocodiles have astrocytes in several areas in the brains (Lôrincz & Kálmán 2015). In contrast, mammals have relatively few ependymoglia and quite a large number of astrocytes (Appel, 2013). Therefore, abundant astrocytes are one of the mammalian traits as well as highly developed cerebral cortex. Astrocytes have long been considered to be passive cells that physically support neurons. Recent works, however, have demonstrated that astrocytes play critical roles in the development and function of the CNS. Astrocytes contribute to homeostasis in the CNS by maintaining appropriate levels of pH, ions and fluid, and support development and function of the neural network through synaptogenesis, metabolic and energy regulation, producing and recycling neurotransmitters, and blood brain barrier formation (reviewed in Simard & Nedergaard 2004; Allen & Barres 2009; Clarke & Barres 2013). Furthermore, astrocytes respond neural activity via various neurotransmitter receptors and release neurotransmitter-like molecules that influence the activity of neurons following intracellular calcium elevation, which suggest that astrocytes are directly involved in information processing in the brain (Cornell-Bell *et al*. 1990; Fiacco *et al*. 2009; Halassa *et al*. 2009; Santello *et al*. 2012; Verkhratsky *et al*. 2012). Recently, it has been reported that astrocytes contribute to cognitive functions, including memory and learning, and their dysfunction is implicated neurologic and psychiatric disorders (reviewed in Cornell-Bell *et al*. 1990; Fiacco *et al*. 2009; Halassa *et al*. 2009; Santello *et al*. 2012; Verkhratsky *et al*. 2012). Interestingly, human astrocytes display larger and more complex morphology and higher electrophysiological performance than those of rodents (Oberheim *et al*. 2006; Oberheim *et al*. 2009), and are supposed to underlie human brain evolution (Robertson 2014; Oberheim Bush & Nedergaard 2017). Indeed, Han *et al*. (2013) reported that the

engraftment of human astrocytes enhanced synaptic plasticity and learning ability in mice, suggesting the evolutionary changes in human astrocytes contribute to higher cognitive function of the human brain. However, molecular basis of astrocyte evolution remains largely unknown.

The divergence in gene expression and regulation among species has been believed to be one of the primary sources of phenotypic variation and play a critical role in evolution (King & Wilson 1975; Shapiro *et al*. 2004; Ihmels *et al*. 2005; Prud'homme *et al*. 2006; Wray 2007; Reed *et al*. 2011; Arnoult *et al*. 2013). Changes in transcriptional regulation can fall into two major classifications: mutations in DNA binding sites proximal to target genes (so-called *cis* mutations) or mutations in transcriptional regulators quantitatively and qualitatively (so-called *trans* mutations) (Voordeckers *et al*. 2015). There are a lot of variations in *cis*-regulatory sequences between species (Borneman *et al*. 2007; Odom *et al*. 2007; Bradley *et al*. 2010) and it has been argued for a long time that *cis*-regulatory changes underlie most of the variations in transcriptional regulation (Carroll 2005; Prud'homme *et al*. 2007; Wray 2007). Mutations in protein-coding region of transcription factors were considered to be an unlikely source of changes in transcriptional regulation, because such mutations may simultaneously affect multiple target genes and evoke negative pleiotropic effects (Hsia & McGinnis 2003; Carroll 2005), although *cis*-regulatory changes would only cause changes in the expression pattern of this particular gene and might be relatively tolerated. However, recent studies have indicated that trans-mutations are more common than previously supposed (Brem *et al*. 2002; Yvert *et al*. 2003). Moreover, they can play an important role in regulatory network evolution through modulating abundance, protein-protein interactions and post-translational modifications of transcription factors (Grove *et al*. 2009; Lynch *et al*. 2011; Reece-Hoyes *et al*. 2013; Sayou *et al*. 2014). Therefore, trans-regulatory changes are attracting considerable interest in terms of molecular evolution.

Homopolymeric amino acid repeats, also called as tandem amino acid repeats,

homopeptide repeats, single amino acid repeats or amino acid runs, represent protein regions with strings of a single amino acid residue. Until recently, the function of protein regions with homopolymeric amino acid repeats was poorly understood and they were often considered as the protein equivalent of junk DNA sequences (Haerty & G. Brian Golding 2010), because these regions can interfere with protein crystallization and are difficult to identify three-dimensional structure (Bannen *et al*. 2007). Indeed, protein data banks provide with insufficient representation of protein regions with homopolymeric amino acid repeats (Wootton & Federhen 1993; Huntley & Golding 2002; Bannen *et al*. 2007; Gall *et al*. 2007). However, homopolymeric amino acid repeats within protein coding regions have drawn attention due to the association with diseases (Almeida *et al*. 2013; Budworth & McMurray 2013). Since over 20 years ago, it has been reported that the expansion of trinuleotide tracts was the underlying mutation for pathogenic activity; CAG expansion in the androgen receptor (*AR*) gene of spinobulbar muscular atrophy (SBMA) (La Spada *et al*. 1991), CAG expansion in the huntingtin (*HTT*) gene of Huntington's disease (HD) (The Huntington's Disease Collaborative Research Group 1993), GCG expansion in the poly-(A) binding protein nuclear 1 (*PABPN1*) gene of oculopharyngeal muscular dystrophy (OPMD) (Messaed *et al*. 2007). Generally, expanded triplet repeats in a coding region of a gene introduce self-assembly, aggregation and abnormal function into the produced protein, suggesting that homopolymeric amino acid repeats are able to participate in protein functions. Recently, it has become clear that homopolymeric amino acid repeats play important roles in biological processes. Within a protein, homopolymeric amino acid repeats are considered to often constitute intrinsically disordered regions (Simon & Hancock 2009; Gojobori & Ueda 2011) that are unstructured in normal condition but can undergo a disorder-to-order transition upon binding with an interacting partner (Uversky *et al*. 2000; Dyson & Wright 2002; Dyson & Wright 2005). There flexibilities provide proteins with capacity to bind multiple partners. In multiple species, "hub proteins" that interact with a large number of other proteins contain significantly longer and more

frequent repeats or disordered regions (Dosztányi *et al*. 2006). In fact, it has been demonstrated that homopolymeric amino acid repeats influence protein-protein interaction of repeat-containing proteins (Emili *et al*. 1994; Imafuku *et al*. 1998; Alvarez *et al*. 2003; Salichs *et al*. 2009), and modulate transactivation activity of transcription factors (Gerber *et al*. 1994; Lanz *et al*. 1995; Janody *et al*. 2001; Galant & Carroll 2002; Buchanan *et al*. 2004; Brown *et al*. 2005).

Homopolymeric amino acid repeats are abundant in eukaryotic genomes (Golding 1999; Huntley & Golding 2000; Faux *et al*. 2005). These repeats evolve rapidly (Newfeld *et al*. 1994), and the frequency and size of repeats vary greatly among species (Karlin *et al*. 2002; Sim & Creamer 2004; Faux *et al*. 2005). In vertebrates, mammals tend to have high amino acid repeat content (Faux *et al*. 2005), although green anole (*Anolis carolinensis*) is the species with the largest number of amino acid repeats among the vertebrates studied in the past (Wu *et al*. 2016). The variety of repeat contents may be related to the GC content bias of different species (Sumiyama *et al*. 1996, Nakachi *et al*. 1997, Cocquet *et al*. 2003, Veitia 2004). On the other hand, differences in replication slippage that underlie the formation and excision of repeats may also affect amino acid repeat frequency. The distribution of homopolymeric amino acid repeats in genome is not random and these repeats are frequently found in proteins related to transcription, DNA/RNA binding, or development (Gerber *et al*. 1994; Albà *et al*. 1999; Albà & Guigó 2004; Hancock & Simon 2005; Faux *et al*. 2005). While homopolymeric amino acid repeats can differ widely among species and within species, they are often strongly conserved through long spans of evolution. Nonconserved repeats tend to be encoded by runs of identical codons, suggesting that they have been generated recently since replication slippage play a predominant role in the formation of new repeats, whereas a mixture of synonymous codons encodes conserved repeats (Albà *et al*. 1999; Albà & Guigó 2004; Mularoni *et al*. 2007). It has been reported that conserved repeats are found more often in highly constrained protein regions than in fast-evolve protein regions, suggesting that homopolymeric amino acid repeats

associate with selection (Mularoni *et al*. 2007). Indeed, Mularoni *et al*. (2010) performed comparative analysis of homopolymeric amino acid repeats conservation among vertebrates and indicated that natural selection has played an important role in evolution of homopolymeric amino acid repeats. It has also been observed that alterative splicing exons, which evolve faster than constitutively spliced exons as a consequence of relaxation of selective constraint, are enriched with homopolymeric amino acid repeats (Haerty & G Brian Golding 2010). Furthermore, homopolymeric amino acid repeats may have been acted as a driving force of evolutionary changes in genes duplicated through two rounds of whole genome duplication of vertebrates (Rado-Trilla *et al*. 2015). These suggest that homopolymeric amino acid repeats are under selective constraints and may have important functions for adaptation. Recently, it has been indicated that homopolymeric amino acid repeats associate with phenotypic differences between species and can be adaptive (Kashi & King 2006). For example, a polyalanine tract conserved in the insect ultrabithorax protein (*Ubx*) is a repressor domain and may have contributed to the diversification of posterior thoracic and anterior abdominal segments in modern insects (Galant & Carroll 2002). A polyalanine repeat is also present in mammalian *Hoxd13*. The deletion of the *Hoxd13* polyalanine repeat affects mammalian limb formation (Anan *et al*. 2007) and, in cetaceans, a novel expansion of the repeat is supposed to be involved in acquiring of flippers (Wang *et al*. 2009). Fondon & Garner (2004) proposed homopolymeric amino acid repeats enable rapid morphological evolution, following their findings that length variation of amino acid repeats in the aristaless-like 4 (*Alx4*) and runt-related transcription factor 2 (*Runx2*) genes were associated with the differences in limb and skull morphologies among different breeds of dogs. Although these studies strongly suggest the association between phenotypic variation and homopolymeric amino acid repeats, their biological and evolutional significance have not been demonstrated due to very few experimental studies conducted *in vivo*.

POU domain transcription factors serve a variety of biological functions

including organ development and endocrine system function including neuroendocrine function (Wegner *et al*. 1993). POU domain transcription factors can be classified into at least six different classes; of these, class III POU domain transcription factors (POU3F1/OCT6, POU3F2/BRN2, POU3F3/BRN1, and POU3F4/BRN4) are mainly expressed in the developing and adult central nervous system (CNS). The onset of *Pou3f2* expression is in ventricular zone progenitor cells of the early developing brain, and this expression pattern transitions to particular brain regions or nerve nuclei with developmental progression (He *et al*. 1989; Dominguez *et al*. 2012). POU3F2 interacts cooperatively with other transcription factors to regulate a number of neurodevelopmental genes such as *Delta1*, which encodes a Notch ligand (Castro *et al*. 2006). *Pou3f2* knockout mice die soon after birth and exhibit hypothalamic and pituitary deficiencies (Nakai *et al*. 1995; Schonemann *et al*. 1995). Another study used a double-knockout paradigm to reveal that *Pou3f2* and *Pou3f3*, which are co-expressed in the developing neocortex, redundantly regulate cortical neuron migration and layer production (McEvilly *et al*. 2002; Sugitani *et al*. 2002). It has also been reported that fibroblasts can be directly differentiated into neural precursor cells or functional neurons by forced expression of *Pou3f2* with a combination of *Ascl1* and *Myt1l* (Vierbuchen *et al*. 2010; Pang *et al*. 2011; Pfisterer *et al*. 2011). Collectively, these findings suggest that *Pou3f2* plays an important role in neural differentiation.

POU3F2 has an evolutionarily well-conserved domain in its C-terminus known as the POU domain that consists of POU-specific domain, POU-homeo domain, and a linker sequence. The POU domain binds specific DNA sequences such as the ATGCAAAT octamer sequence. In contrast, the N-terminus of POU3F2 shows significant variation among species: mammalian POU3F2 includes three homopolymeric amino acid repeats consisted with glycine, glutamine and proline, respectively, whereas these repeats are absent in non-mammalian vertebrate orthologs (Sumiyama *et al*. 1996; Nakachi *et al*. 1997). Nasu *et al*. (2014) previously reported that homopolymeric amino acid repeats in POU3F2 affect murine behavior. They

generated two types of knock-in mice: *Pou3f2*$^{tro/tro}$ mice, in which the entire coding region of *Pou3f2* is replaced with the amphibian (*Xenopus. tropicalis*) ortholog (lacking homopolymeric repeats), and *Pou3f2*$^{\Delta/\Delta}$ mice, in which all three homopolymeric amino acid repeats are deleted from the transactivation domain (Figure 2.1b). Both types of the mice displayed phenotypic defects in maternal behaviors such as pup retrieval. In addition, they found that *Pou3f2*$^{tro/tro}$ and *Pou3f2*$^{\Delta/\Delta}$ mice exhibited decreased brain expression of the rate-limiting enzymes for serotonin and dopamine synthesis (i.e., tryptophan hydroxylase and tyrosine hydroxylase, respectively). Despite these insights, the functional significance of homopolymeric amino acid repeats in POU3F2 remains unclear.

The aim of this thesis is to increase our understanding of biological and evolutional significance of homopolymeric amino acid repeats by investigating a role of the repeats in POU3F2 using *Pou3f2*$^{\Delta/\Delta}$ mice. Chapter 2 describes the role of these repeats in the regulation of POU3F2 activity and cortical development with special reference to astrocyte generation. The amino acid repeats down-regulated the transactivation activity of POU3F2 and affect astrocyte differentiation of neural progenitor cells (NPCs) in the developing cortex. Chapter 3 describes the effect of the repeats in POU3F2 on the cognitive ability, which has been linked to *Pou3f2*. Cognitive performances in behavioral tasks were impaired in *Pou3f2*$^{\Delta/\Delta}$ mice. Consistently, adult hippocampal neurogenesis was decreased in *Pou3f2*$^{\Delta/\Delta}$ mice. Chapter 4 presents summary and general discussion of the results from Chapter 2 and Chapter 3.

Chapter 2

Functional Role of Homopolymeric Amino Acid Repeats in POU3F2 with Regard to Astrogenesis

2-1. Introduction

Mammalian brain development starts with forming the neural tube consist of neuroepithelial cells. The dorso-anterior part of the neural tube later gives rise to the cortex. Initially, neuroepithelial cells undergo rounds of symmetric divisions and expand the progenitor cell pool. At the onset of neurogenesis, neuroepithelial cells start expressing glial cell markers, change the morphology and called as apical radial glial cells (aRGCs). During neurogenesis in the mammalian cortex, aRGCs are located around lateral ventricles called as the ventricular zone (VZ), and divide both symmetrically and asymmetrically; along with expansion own pools, aRGCs produce neurons or more differentiated progenitor cells. Newborn neurons migrate along the radially extended process (basal process) of aRGCs and basal radial glial cells (bRGCs) to the surface of cortex. Initially, pioneer-neurons form the horizontal plexus called as the preplate, and subsequently newborn neurons constitute the cortical plate. Neurons migrate into the cortical plate in an inside-first, outside-last manner. Layers 6 and 5 (populated deep layer neurons) are formed at first, followed by the construction of layers 4, 3 and 2 (upper layer neurons). Excitatory cortical neurons, which are predominant in cortex are generated from cortical NPCs as described above, whereas GABAergic interneurons tangentially migrate into the cortical plate from the ganglionic eminences.

In the late gestation to perinatal periods, aRGCs acquire the potential to differentiate astrocytes and oligodendrocytes. Neurogenesis-to-gliogenesis switching is regulated rigorously because it is critical for the generation of a proper number of each

cell type, underlying correct formation of neural circuits and information processing in synapses. In the cortex, this switch occurs around E16-18 in mice. Once specified, astrocyte lineages express a set of marker genes such as *Glast*, *Fabp7*/*Blbp* and *Fgfr3* (Owada *et al*. 1996; Shibata *et al*. 1997; Pringle *et al*. 2003; Anthony *et al*. 2004), and migrate along aRGCs processes. After asymmetric production of astrocyte precursors from aRGCs and migration to their final position, astrocytes divide symmetrically to produce more astrocytes.

POU3F2 has been widely used as a marker for upper-layer neurons in the cortex (Hagino-Yamagishi *et al*. 1997). A series of studies have indicated that POU3F2 and POU3F3, paralog of POU3F2, redundantly regulate the radial migration of postmitotic cortical neurons (McEvilly *et al*. 2002), upper-layer production (Sugitani *et al*. 2002) and neurogenesis in the cortex (Castro *et al*. 2006; Dominguez *et al*. 2012). Castro *et al*. (2006) reported that POU3F2 cooperatively binds to ASCL1/MASH1 and regulates *Delta1*, which encodes a Notch ligand, and may regulate other genes involved in division of progenitors and differentiation and migration of neurons. Dominguez *et al*. (2013) demonstrated that *Ngn1*, *Tbr1*, *Tbr2* and *Hes5*, which are proneural genes encoding basic helix-loop-helix (bHLH) transcription factors, are downstream of *Pou3f2* gene. Overexpression of POU3F2 by electroporation promotes neural differentiation in the chick neural tube (Castro *et al*. 2006) and the developing mouse cortex (Dominguez *et al*. 2012). Recently, Nagao *et al*. (2016) demonstrated that Zinc finger- and BTB domain-containing protein 20 (Zbtb20) directly repress the expression of POU3F2 and play an important role in generation of cortical astrocytes. They have reported that overexpression of POU3F2 attenuated astrocyte differentiation and promoted neural differentiation in cortical NPCs, suggesting that POU3F2 obstructs astrogenesis.

Nasu *et al*. (2014) have demonstrated that the homopolymeric amino acid repeats in POU3F2 are involved in murine behavior, suggesting the repeats function in the brain. However, the molecular and cellular mechanism has not been elucidated. In

this chapter, I investigated whether the homopolymeric amino acid repeats in POU3F2 influence the property and functional role of POU3F2. Using an *in vitro* model system, I found that repeat-deleted POU3F2 showed higher transactivation activity than wild-type POU3F2. On the other hand, the expression pattern of POU3F2 in the developing embryonic cortex unchanged in the presence or absence of the repeats. Next, I investigated the effect of the repeats deletion from POU3F2 on the murine cerebral cortex. I found that non-neural cells were decreased in the cortex of *Pou3f2*$^{\Delta/\Delta}$ mice accompanied with reductions in the brain weight. Immunohistochemical analysis revealed that cortical astrocytes and astrocyte progenitors were decreased in *Pou3f2*$^{\Delta/\Delta}$ mice, whereas neuronal progenitors were increased. Although proliferative activity of cortical progenitors were not different between wild-type mice and *Pou3f2*$^{\Delta/\Delta}$ mice, cortical astrogenesis were impaired in *Pou3f2*$^{\Delta/\Delta}$ mice. Taken together, these results suggest that the repeats in POU3F2 are involved in cortical astrocyte differentiation.

2-2. Materials and Methods

Animals

Pou3f2$^{\Delta/\Delta}$ mouse strain was generated as described previously (Nasu *et al*. 2014). The mice used in this study had been backcrossed to C57BL/6J for more than 16 generations. The mice were housed under constant temperature and humidity with a 12 h light/dark cycle and with access to food and water ad libitum. All experiments involving animals were approved by The University of Tokyo, and conducted in accordance with the guidelines.

Plasmid Construction

To express POU3F2 fused in-frame to the DNA-binding domain of yeast Gal4 (amino acids 1 to 147; Gal4DBD) (Gal4DBD-POU3F2), pFN11A (BIND) Flexi® vector (Promega, USA), which expresses Gal4DBD under the control of the CMV promoter, was used. The full-length POU3F2 cDNA fragments were amplified by PCR using genomic DNA of wild-type mouse. The resultant fragments were digested with SgfI and PmeI, and inserted into the SgfI - PmeI sites of pFN11A vectors. The expression vectors encoding repeat deleted-POU3F2 fused to the Gal4DBD (Gal4DBD-POU3F2ΔGQP) were generated similarly. All constructs were confirmed by sequencing.

Cell Culture and One-Hybrid Reporter Assay

Neuro-2a cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) containing 10% FBS (Life Technologies, USA), 2mM L-Glutamine (Sigma) and penicilline-streptomycyn (Sigma), and used for one-hybrid reporter assay. At first, 5×10^4 cells were plated into 24 well plate. Reaching 50–70% confluent, the cells were transfected with pGL4.31 reporter vectors (Promega, USA), in which firefly luciferase expression is driven by five repeats of yeast Gal4-binding sites introduced upstream of

the minimal TATA promoter, together with the plasmids to express unfused Gal4DBD or different Gal4DBD fusion proteins as indicated in the figure legends (Figure 2.2a) by Lipofectamine 3000 (Life Technologies); 0.75µl of Lipofectamine 3000 was diluted by Opti-MEM (Gibco, USA), and mixed with 0.5µg of reporter plasmid and 0.5µg of fusion protein expression plasmid followed by incubation for 10 minutes. DNA-lipid complexes were then added to cells. Forty-eight hours after the transfection, a luciferase assay was performed using a Dual-Glo™ Luciferase Assay System (Promega) and a Lumat LB9501 (Berthold Technologies, Germany) according to the manufacturer's instructions. Transfections were done in triplicates, and the experiments repeated independently four times.

Bromodeoxyuridine (BrdU) Administration

Pregnant females were injected intraperitoneally with 50 mg/kg BrdU (Sigma-Aldrich, USA) in normal saline. For analysis of progenitor cell proliferation, BrdU was given at E17.5 and embryos were removed 24 h later. To examine the neurogenesis and astrogenesis, BrdU was given at E16.5 and embryos were removed 48 h later.

Tissue Preparation

Dissected embryonic brains were fixed overnight in 4% paraformaldehyde at 4 °C. Postnatal mice were deeply anesthetized with intraperitoneal pentobarbital and transcardially perfused with 15–20 ml of phosphate-buffered saline (PBS; pH 7.4) followed by 50 ml of 4% paraformaldehyde/0.2% glutaraldehyde. After perfusion, brains were harvested and postfixed with the same fixative for 2 h at 4°C. Brains were washed in PBS, immersed in 25% buffered sucrose overnight at 4°C, embedded in Tissue-Tek OCT Compound (Sakura Finetek Japan Co., Ltd, Japan), frozen in liquid nitrogen, and stored at −70°C until use. Frozen brains were sectioned serially (thickness, 14 µm for embryos and 20 µm for postnatal mice) with a cryostat HM505E (Carl Zeiss, Germany) at −20°C and mounted on 0.5% gelatin-coated glass slides.

Antibodies

Primary and secondary antibodies used for immunohistochemistry were as follows: rabbit anti-POU3F2 (1:400; abcam, UK), rabbit anti-S100β (1:800; abcam), rabbit anti-GLAST (1:200; FRONTIER INSTITUTE, Japan), rabbit anti-TBR2 (1:400; abcam), rabbit anti-Ki67 (1:800; abcam), rat anti-BrdU (1:800; abcam), rabbit anti-NeuN (1:800; abcam), goat anti-rat IgG H&L (DyLight 488) (1:400; abcam), goat anti-rabbit IgG H&L (DyLight 594) (1:400; abcam).

Immunohistochemistry

For staining with POU3F2, S100β, GLAST and TBR2, sections were washed 3 times with PBS (10 min per wash) and subsequently blocked in PBS containing 2% goat serum (Wako, Japan) and 0.1% Triton-X100 (Roche, Switzerland) for 1 h at room temperature. Sections were incubated with primary antibody for 12 h at 4°C. Thereafter, the sections were washed 4 times with PBS (15 min per wash), incubated in secondary antibody for 3 h at room temperature, and washed 4 times with PBS (15 min per wash). Sections were then incubated with 2.5 μM TO-PRO-3 iodide (Invitrogen, Italy) and 50 μg/ml RNase in PBS for 20 min at room temperature for nuclear DNA staining and subsequently washed 3 times with PBS (5 min per wash). Finally, sections were embedded in 10% glycerin in PBS, and stored in a moist environment at 4°C. For BrdU/GLAST or BrdU/NeuN double staining, sections were incubated with 1 M HCl for 30 min at 45°C followed by wash with PBS (4 times, 15 min per wash) before blocking. For BrdU/Ki67 double staining, a step of formaldehyde fixation was added to protect immunoreactivity of Ki67 from the denaturation step according to Kee *et al*. (2002) with slight modifications. In detail, sections were washed 3 times with PBS (10 min per wash) and subsequently blocked in PBS containing 2% goat serum (Wako, Japan) and 0.1% Triton-X100 (Roche, Switzerland) for 1 h at room temperature. Sections were incubated with anti-Ki67 antibody for 12 h at 4°C. Thereafter the

sections were washed 4 times with PBS (15 min per wash), incubated in secondary antibody for 3 h at room temperature, and washed 4 times with PBS (15 min per wash). Sections were incubated with 8% paraformaldehyde at room temperature, and washed 3 times with PBS (10 min per wash). Sections were incubated with 1 M HCl for 30 min at 45°C followed by wash with PBS (4 times, 15 min per wash). Sections were incubated in anti-BrdU antibody for 12h at 4°C and subsequently washed with PBS (4 times, 15 min per wash). Thereafter sections were incubated in secondary antibody for 3 h at room temperature followed by wash with PBS (4 times, 15 min per wash). Sections were then incubated with 2.5 μM TO-PRO-3 iodide and 50 μg/ml RNase in PBS for 20 min at room temperature for nuclear DNA staining and subsequently washed 3 times with PBS (5 min per wash). Finally, sections were embedded in 10% glycerin in PBS, and stored in a moist environment at 4°C.

Isotropic Fractionator

Isotropic fractionator was performed in accordance with the methods of Herculano-Houzel & Lent (2005). Ten weeks of age female mice were deeply anesthetized with intraperitoneal pentobarbital and transcardially perfused with 15–20 ml of PBS followed by 50 ml of 4% paraformaldehyde. After perfusion, brains were harvested and postfixed with the same fixative overnight at 4°C. The meninges were removed and the tissue was dissected into four parts: cerebral cortex (includes hippocampus), cerebellum, olfactory bulb, and the rest of the brain (Figure 2.5a). Each tissue parts were chopped into small pieces with a scalpel and then homogenized in a hypotonic solution (40 mm sodium citrate and 1% Triton X-100) with a 7 ml glass homogenizer until the smallest visible fragments were dissolved. The homogenate was then transferred to a 15 ml centrifuge tube and centrifuged for 10 min at 4000 × *g* at 4°C. The pellet was resuspended with PBS. Aliquots from the nuclear suspension were stained with 1 μg/ml DAPI and counted under a fluorescent microscope using a hemocytometer. To count neurons, aliquots from each sample were collected by the

centrifugation for 10 min at 4000 × *g* at 4°C and resuspended with 0.2M solution of boric acid (pH 9.0). The suspension was incubated at 75°C for 1h and collected by the centrifugation. The pellet was resuspended and incubated with anti-NeuN antibody (1:200, ABN78; Millipore, Germany) overnight at 4°C. Thereafter, the nuclear suspension was washed with PBS, incubated with secondary antibody (1:200, ab96901; abcam) for 2h at room temperature, and washed with PBS. The nuclei were resuspended with PBS, stained with 1 μg/ml DAPI and counted under a fluorescent microscope using a hemocytometer.

2-3. Results and Discussion

第２章

本章については、５年以内に雑誌等で刊行予定のため、非公開。

2-4. Table and Figures

第２章

本章については、５年以内に雑誌等で刊行予定のため、非公開。

Chapter 3

Phenotypic Effect of Homopolymeric Amino Acid Repeats in POU3F2 with Regard to Cognition

3-1. Introduction

In mammals, an ability to remember unique experience promises adaptive capability for dynamic environment and phylogenetic success. This ability, called as recognition memory or recognition, is one of the most important cognitive functions. Recognition could be explained as the function to distinguish whether a particular event has been previously encountered or not. Recognition is often associated with episodic memory, which was described as "a system that receives and stores information about temporally dated episodes or events, and temporal-spatial relations among them" by Tulving (1984). Recognition provides mammals with several properties such as memory-based prediction, planning for the distant future and building social relationship and networks (Allen & Fortin 2013).

Hippocampus is one of the regions where recognition memory involves. In mammals, neurons are continuously generated in the adult dentate gyrus of the hippocampus throughout life (reviewed in Gage 2000; Gould & Gross 2002; Zhao *et al*. 2008; Deng *et al*. 2010). Compared to mature neurons, immature neurons in the adult hippocampus exhibit increased excitability and plasticity (Schmidt-Hieber *et al*. 2004; Ge *et al*. 2007), and these properties underlie the unique function of newborn neurons in the dentate gyrus. Newborn neurons in the adult dentate gyrus are functionally integrated into existing circuits and contribute to cognitive functions, in particular learning and memory. The dentate gyrus receives inputs from the cortex and acts as a preprocessor of incoming information, assisting information processing in the hippocampus. Especially, it has been suggested that the dentate gyrus and adult

hippocampal neurogenesis play a role in pattern separation, a process by which overlapping or similar input signals (representations) are transformed into less similar output signals (Hvoslef-Eide & Oomen 2016). Pattern separation is considered to support storage of unique representations underlying appropriate memory recall and play an important role in recognition memory.

Pou3f2 is recently associated with cognitive performance and bipolar disorder (Mühleisen *et al*. 2014; Rietveld *et al*. 2014; Okbay *et al*. 2016) as well as autism-like behaviors in mice (Belinson *et al*. 2016). Given that POU3F2 plays a critical role in cortical development, proper regulation of POU3F2 must be important for cognitive functions. Nasu *et al*. (2014) indicated that the homopolymeric amino acid repeats in POU3F2 are involved in the capacity of dams to discriminate unfamiliar pups from familiar ones. Therefore, POU3F2 may contribute to recognition memory via the homopolymeric amino acid repeats.

In this chapter, I examined cognitive function and hippocampal neurogenesis in *Pou3f2*$^{\Delta/\Delta}$ mice. *Pou3f2*$^{\Delta/\Delta}$ mice exhibited impairment in recognition memory in object recognition and object location tests. Immunohistochemistry for doublecortin, a marker of immature neurons, revealed a lower number of newborn neurons in the dentate gyrus of adult *Pou3f2*$^{\Delta/\Delta}$ mice compared to wild-type mice. Moreover, the decrease in newborn neurons was related to impairment in neuronal differentiation of NPCs in the adult dentate gyrus. Consistent with this observation, adult *Pou3f2*$^{\Delta/\Delta}$ mice had lower numbers of BrdU and NeuN double-positive cells at 4 weeks after BrdU injection compared to control mice, indicating the decreased generation of mature granule cells in *Pou3f2*$^{\Delta/\Delta}$ mice. However, NPCs derived from the adult dentate gyrus of *Pou3f2*$^{\Delta/\Delta}$ mice exhibited normal neuronal differentiation, suggesting that the homopolymeric amino acid repeats in POU3F2 may affect NPCs in the adult dentate gyrus through an extrinsic signaling pathway.

3-2. Materials and Methods

Behavioral Test

A white plastic chamber (40 × 40 × 40 cm) with one black striped wall and an opposing wall with black circles was utilized for behavioral test. The chamber was illuminated with a fluorescent light and a video camera was fixed above the chamber to record animal behavior. Object recognition test was performed in accordance with the methods of Leger *et al*. (2013) with slight modifications (Figure 3.1a). Briefly, mice were habituated to the chamber for 5 min twice per day (separated by a 6-h interval) on 2–3 consecutive days. Twenty-four hours after the final habituation session, mice were placed into the chamber and allowed to explore two identical objects that were placed in adjacent corners of the test arena for 10 min (the familiarization phase). Mice were reintroduced to the test arena 1 h after familiarization; during this phase (the test phase), mice were allowed to explore one object that was the same as that in the previous section and one novel object placed in adjacent corners for 10 min.

Subsequently, 24 h after the object recognition test, the mice were subjected to object location test in accordance with the methods of Murai *et al*. (2007) with slight modifications (Figure 3.1d). Similar to the protocol for object recognition test, mice were first allowed to explore two identical objects in adjacent corners of the test arena for 10 min (the familiarization phase). Mice were reintroduced to the test arena 1 h after familiarization; in this phase (the test phase), the placement of one of the objects was changed to a different corner. The objects used in the behavioral tests were a set of wooden blocks of similar size and different shape (cubic, cylinder, and cone). Exploration time was defined as the time spent with the nose directed towards the object within a distance of 2 cm. In calculation of the discrimination index, exploration time was scored until the total exploration time was equal to 15 s. The discrimination index for the object recognition test was calculated as follows: (novel object exploration time − familiar object exploration time)/(total exploration time). Similarly,

a recognition index for the object location test was calculated as follows: (novel object location exploration time – same object location exploration time)/(total exploration time). Objects and object locations were selected at random. Mice were 8–11 weeks of age at the time of behavioral testing.

BrdU Administration

To label newborn neurons in the dentate gyrus, mice were administered intraperitoneal BrdU (Sigma-Aldrich, US) dissolved in normal saline. For long-term BrdU labeling, mice received 50 mg/kg BrdU once per day for 5 consecutive days and were sacrificed at 4 weeks after the first injection. For short-term BrdU labeling, mice received 200 mg/kg BrdU 3 times at 2-h intervals and were sacrificed 24 h after the first injection. Mice were 8–11 weeks of age at the time of BrdU administration.

Tissue Preparation

Mice were deeply anesthetized with intraperitoneal pentobarbital and transcardially perfused with 15–20 ml of phosphate-buffered saline (PBS; pH 7.4) followed by 50 ml of 4% paraformaldehyde/0.2% glutaraldehyde. After perfusion, brains were harvested and postfixed with the same fixative for 2 h at 4°C. Brains were washed in PBS, immersed in 5% buffered sucrose overnight at 4°C, embedded in Tissue-Tek OCT Compound (Sakura Finetek Japan Co., Ltd, Japan), frozen in liquid nitrogen, and stored at −70°C until use. Frozen brains were sectioned serially (thickness, 20 μm) with a cryostat HM505E (Carl Zeiss, Germany) at − 20°C and mounted on 0.5% gelatin-coated glass slides.

Immunohistochemistry

For doublecortin staining, sections were washed 3 times with PBS (10 min per wash) and subsequently blocked in PBS containing 2% goat serum (Wako) and 0.1% Triton-X100 (Roche, Switzerland) for 1 h at room temperature. Sections were

incubated with rabbit anti-doublecortin antibody (1:2000; Abcam) for 12 h at 4°C. Thereafter, the sections were washed 4 times with PBS (15 min per wash), incubated in goat anti-rabbit IgG (Abcam) for 3 h at room temperature, and washed again with PBS 4 times for 15, 30, 45, and 60 min, respectively. Sections were then incubated with 2.5 μM TO-PRO-3 iodide (Invitrogen) and 50 μg/ml RNase in PBS for 20 min at room temperature for nuclear DNA staining and subsequently washed 3 times with PBS (5 min per wash). Mice were 8–10 weeks of age at the time of immunohistochemical staining for doublecortin. For BrdU/NeuN double staining or BrdU staining, sections were washed 3 times with PBS (10 min per wash). Sections were incubated with 1 M HCl for 30 min at 45°C, washed 4 times with PBS (15 min per wash), blocked, and incubated in a mixture of rat anti-BrdU (1:400; Abcam) and rabbit anti-NeuN (1:400; Millipore) or rat-anti-BrdU (1:400; Abcam) alone for 12 h at 4°C. Thereafter, the sections were washed 4 times with PBS (15 min per wash) and incubated in a mixture of goat anti-rat IgG (Abcam) and goat anti-rabbit IgG (Abcam) or goat anti-rat IgG (Abcam) alone for 3 h at room temperature. Sections were then washed 4 times with PBS for 15, 30, 45 and 60 min, respectively, embedded in 10% glycerin in PBS, and stored in a moist environment at 4°C. Doublecortin-, BrdU/NeuN-, and BrdU-expressing cells in the granule cell layer of the dentate gyrus were counted using a FV1000D confocal microscope (Olympus, Japan) and ImageJ software. Left or right hemisphere was used for the doublecortin-positive cell count, and both hemispheres were used for BrdU/NeuN- and BrdU-positive cell count due to acquire the enough number of cells. The number of doublecortin-positive cells in the granule cell layer and the subgranular zone of the dentate gyrus were counted in every tenth coronal section of the hippocampus of each hemisphere, and the area of the granule cell layer used for count were measured using ImageJ software. The density of doublecortin-positive cells in the granule cell layer is estimated by dividing the number of immunopositive cells by the area of the granule cell layer. To estimate the volume of entire granule cell layer, the areas were summed and multiplied by the distance between sections sampled. To

estimate the total number of doublecortin-positive cells in the dentate gyrus, immunopositive cells counted in every tenth coronal section of the hippocampus were multiplied by 20. Similarly, BrdU/NeuN- and BrdU-positive cells in the granule cell layer and subgranular zone of the dentate gyrus were counted in every tenth coronal section of the hippocampus and multiplied by 10. To analyze the maturation stages of newborn neurons, we categorized doublecortin-expressing cells in the subgranular zone into 3 stages depending on the dendritic morphology according to Plümpe *et al*. (2006). Cells with no or very short processes were categorized as "proliferative" neuroblasts. Cells with processes of intermediate length and immature morphology were categorized as "intermediate" cells. Intermediate neuroblasts include cells showing dendrites without branch reaching the molecular layer. More mature neurons with dendrites branching in the molecular layer were categorized as "postmitotic" neurons.

Neural Precursor Cells Isolation, Propagation and Differentiation

NPCs in adult hippocampus were isolated as previously described (Babu *et al*., 2007, 2011). Briefly, dentate gyri of hippocampus were removed from animals (n = 4-5) under a stereomicroscope. The tissues were dissolved into single-cell suspension using enzymes and a physical digestion. Neural precursor cells are separated from differentiated cells and debris using density gradient separation by a centrifugation with 22% Percoll. Pelleted NPCs are separated from supernatant, washed, resuspended in growth medium (Neurobasal A with supplements B27 and Glutamax-1 and growth factors EGF and FGF2) and plated on plastic dishes coated with poly-D-lysine and laminin. Cells were passaged using Accutase every 3-4 days. To induce differentiation, the growth medium was changed to differentiation medium (Neurobasal A with supplements B27, Glutamax-1, retinoic acid and BDNF), and replacing 50% of the media was performed every 2 days.

Immunocytochemistry

Cells were washed with PBS and incubated in 4% paraformaldehyde for 20 min at room temperature. The cells were then washed with PBS 3 times (5 min per wash). The cells were incubated in blocking solution consisting of PBS with 3% goat serum and 0.1% Triton-X100 for 30 min at room temperature. Following blocking, the cells were incubated in PBS with 3% goat serum and rabbit anti-Map2ab antibody (1:500; Sigma) and mouse anti-GFAP antibody (1:500; Dako) overnight at 4 °C. Thereafter, the cells were washed with PBS 3 times (5 min per wash). The cells were incubated in PBS with 3% goat serum, goat anti-rabbit IgG antibody (1:500; abcam) and goat anti-mouse IgG antibody (1:500; abcam) for 1 h at room temperature. The cells were washed twice with PBS for 5 min each, and incubated in nuclear stain solution (PBS with 1uM TO-PRO-3 iodide and 50ug/ml RNaseA) for 30 min at room temperature. The cells washed twice with PBS for 10 min each. The cells were then embedded in 90% glycerin in PBS. The number and morphology of neurons were analyzed using a FV1000D confocal microscope (Olympus, Japan) and ImageJ software.

3-3. Results and Discussion

Pou3f2$^{\Delta/\Delta}$ Mice Exhibit Cognitive Impairment

I first performed object recognition test to investigate cognitive function. This test is based on the innate preference of mice for novel rather than familiar objects or environments (Leger *et al*. 2013). During the familiarization phase, wild-type and *Pou3f2$^{\Delta/\Delta}$* mice did not spend significantly different amounts of time exploring the two identical objects (p = 0.506 and p = 0.971 for male and female mice, respectively, student's t-test; Figure 3.1b), suggesting no differences in motivation, curiosity, and sensory functions between wild-type and *Pou3f2$^{\Delta/\Delta}$* mice. In the test phase, both male and female wild-type mice showed preferences for the novel object based on discrimination indices (t_{18} = 2.726, $p < 0.05$ and t_{12} = 5.243, $p < 0.001$ for male and female mice, respectively, one-sample t-test; Figure 3.1c). Alternatively, *Pou3f2$^{\Delta/\Delta}$* mice did not spend significantly different amounts of time exploring the novel object versus the familiar object (t_{10} = 0.333, p = 0.747 and t_{11} = 0.974, p = 0.353 for male and female mice, respectively, one-sample t-test; Figure 3.1c). Female *Pou3f2$^{\Delta/\Delta}$* mice had significantly lower discrimination indices compared to female wild-type mice ($p < 0.05$, student's t-test; Figure 3.1c), and a non-significant but similar trend was observed in male *Pou3f2$^{\Delta/\Delta}$* mice compared to male wild-type mice (p = 0.121, student's t-test; Fig. 3.1c).

Next, I performed object location test to evaluate spatial recognition (Murai *et al*. 2007). During the familiarization phase, there were no statistically significant differences in total sniffing time between wild-type and *Pou3f2$^{\Delta/\Delta}$* mice (p = 0.404 and p = 0.262 for male and female mice, respectively, student's t-test; Figure 3.1e), as is the case in the object recognition test. Male and female wild-type mice showed significant preferences for the novel location object versus the familiar location object (t_{18} = 5.444, $p < 0.001$ and t_{12} = 5.057, $p < 0.001$ for male and female mice, respectively, one-sample t-test; Figure 3.1f). Alternatively, *Pou3f2$^{\Delta/\Delta}$* mice did not show significant

preference for the novel location object (t_{10} = 0.707, p = 0.497 and t_{11} = 1.514, p = 0.161 for male and female mice, respectively, one-sample *t*-test; Fig. 3.1f). Female *Pou3f2*$^{\Delta/\Delta}$ mice had significantly lower discrimination indices compared to female wild-type mice ($p < 0.001$, student's *t*-test; Figure 3.1f) and a non-significant but similar trend was observed in male *Pou3f2*$^{\Delta/\Delta}$ mice compared to male wild-type mice ($p = 0.087$, student's *t*-test; Figure 3.1f).

These results show that *Pou3f2*$^{\Delta/\Delta}$ mice exhibit impairments in object and spatial recognition, indicating the homopolymeric amino acid repeats in POU3F2 contribute to cognitive function. In the both recognition test, male and female *Pou3f2*$^{\Delta/\Delta}$ mice had discrimination indices close to 0, indicating that *Pou3f2*$^{\Delta/\Delta}$ mice were unable to discriminate novel objects from familiar objects; yet, in within-sex comparisons, there was only a statistical difference between the performances of female *Pou3f2*$^{\Delta/\Delta}$ and wild-type mice. Previous studies have indicated that male mice have a weaker ability to identify novel objects compared to female mice (Ghi *et al*. 1999; Frick & Gresack 2003; Sutcliffe *et al*. 2007), consistent with my observation. My present observation is also consistent with the previous finding that the repeats in POU3F2 affect pup recognition of dams (Nasu *et al*. 2014). In addition, these results offer crucial evidence for involvement of POU3F2 in cognitive function, confirming previous genome-wide analyses, in which *Pou3f2* was associated with cognitive performance and bipolar disorder (Mühleisen *et al*. 2014; Rietvelda *et al*. 2014; Okbay *et al*. 2016).

Pou3f2$^{\Delta/\Delta}$ *Mice Have Lower Numbers of Newborn Neurons in the Adult Hippocampus*

I assessed adult hippocampal neurogenesis in female *Pou3f2*$^{\Delta/\Delta}$ mice. Doublecortin is a microtubule-binding protein that is expressed in cell types ranging from early neuroblasts to 28-day-old neurons in the adult dentate gyrus (Rao & Shetty 2004; Karten *et al*. 2006; Plümpe *et al*. 2006). The density of doublecortin-positive cells in the dentate gyrus was 17.7% lower in *Pou3f2*$^{\Delta/\Delta}$ mice compared to wild-type mice ($p <$

0.05, Welch's *t*-test; Figure 3.2b). Similarly, the total number of doublecortin-positive cells in the dentate gyrus was 21.7% lower in *Pou3f2*$^{\Delta/\Delta}$ mice compared to wild-type mice ($p < 0.01$, Welch's *t*-test; Figure 3.2c). There was no difference in size of the granule cell layer between wild-type mice and *Pou3f2*$^{\Delta/\Delta}$ mice ($p = 0.623$, student's *t*-test; Figure 3.2d). These results indicated that newborn neurons in the adult dentate gyrus were decreased in *Pou3f2*$^{\Delta/\Delta}$ mice. Doublecortin-positive cells can be categorized into three stages of development based on cell morphology (Plümpe *et al*. 2006): proliferative-stage cells, intermediate-stage cells and postmitotic-stage cells. *Pou3f2*$^{\Delta/\Delta}$ mice showed significantly fewer numbers of intermediate-stage and postmitotic-stage cells compared to wild-type mice ($p < 0.05$ and $p < 0.01$ for intermediate-stage and postmitotic-stage cells, respectively, student's *t*-test; Figure 3.2e). *Pou3f2*$^{\Delta/\Delta}$ mice also showed a significantly lower percentage of postmitotic-stage doublecortin-positive cells (23.3 ± 2.6% $p < 0.05$, student's *t*-test; Figure 3.2f) and a significantly higher percentage of proliferative-stage doublecortin-positive cells (58.0 ± 2.1%, $p < 0.05$, student's *t*-test; Figure 3.2f), compared to wild-type mice (28.6 ± 4.7% and 52.4 ± 4.2% for postmitotic-stage and proliferative-stage cells, respectively; Figure 3.2f). These findings indicated that decreased adult hippocampal neurogenesis was specifically related to impaired cell maturation rather than decreased neuronal precursor proliferation.

Next, I analyzed the expression of NeuN, a marker for mature neurons, in BrdU-labeled cells in the dentate gyrus. NeuN and BrdU co-localization was examined at 4-weeks after BrdU injection, when a majority of BrdU-labeled newborn cells were expected to have differentiated into mature granule cells. Female *Pou3f2*$^{\Delta/\Delta}$ mice had 29.9% fewer numbers of BrdU/NeuN-positive cells in the adult dentate gyrus compared to wild-type mice ($p < 0.05$, student's *t*-test; Figure 3.3b). This result is consistent with the above result that maturation of newborn neurons is impaired in adult dentate gyrus in *Pou3f2*$^{\Delta/\Delta}$ mice. In addition, male *Pou3f2*$^{\Delta/\Delta}$ mice similarly showed 32.2% fewer number of BrdU/NeuN-positive cells in the adult dentate gyrus

compared to wild-type mice ($p < 0.01$, student's *t*-test; Figure 3.3b), suggesting impaired adult hippocampal neurogenesis in *Pou3f2*$^{\Delta/\Delta}$ mice independent of sex.

To investigate whether the reduced proliferation of neural precursor cells led to impaired adult hippocampal neurogenesis in *Pou3f2*$^{\Delta/\Delta}$ mice, I analyzed the cell proliferation in the subgranular zone of the dentate gyrus by examining BrdU labeling 24 h after BrdU injection. *Pou3f2*$^{\Delta/\Delta}$ mice showed significantly higher numbers of BrdU-labeled cells in the subgranular zone of the dentate gyrus compared to wild type mice ($p < 0.05$; Figure 3.3c). This result is consistent with the result that impaired adult hippocampal neurogenesis in *Pou3f2*$^{\Delta/\Delta}$ mice was due to decreased differentiation rather than reduced cell proliferation.

Homopolymeric Amino Acid Repeats in POU3F2 do not Affect Neural Differentiation of Adult Hippocampal NPCs in vitro

To investigate the differentiation of adult hippocampal NPCs under *in vitro* condition, I isolated NPCs from adult hippocampal dentate gyrus as adherent monolayer cell culture. Under differentiation condition, this cell culture develops into neurons with properties of hippocampal granule cells. NPCs were induced to differentiate into neurons for 10 days in the absent of growth factors (Figure 3.4a). There were no statistically significant differences in density between NPCs of wild-type mice and those of *Pou3f2*$^{\Delta/\Delta}$ mice after 10 days under the differentiation condition ($p = 0.795$, student's *t*-test; Figure 3.4b). Next, I examined the expression of Map2ab, which is a mature neuron marker. There were no statistically significant differences in ratio of Map2ab-positive cells ($p = 0.950$, student's *t*-test; Figure 3.4c). Moreover, there were no differences in the neuronal morphologies between wild-type neurons and *Pou3f2*$^{\Delta/\Delta}$ neurons (Figure 3.4d, e). These results indicate that the deletion of amino acid repeats in POU3F2 does not intrinsically affect neural differentiation of adult hippocampal NPCs. This is consistent with the result that the repeats in POU3F2 decrease the transactivation activity of POU3F2 *in vitro* (Figure 2.2b) and do not change embryonic

neurogenesis (Figure 2.5b). The homopolymeric amino acid repeats in POU3F2 may regulate neuronal differentiation in the adult hippocampal dentate gyrus through an extrinsic signaling pathway.

Astrocytes constitute of neurogenic niche and contribute to the differentiation of NPCs in the adult hippocampal dentate gyrus as well as in the developing brain. Song *et al*. (2002) have demonstrated that astrocytes promote neuronal fate commitment of NPCs derived from adult hippocampal using cell culture systems. Furthermore, a series of *in vivo* studies have reported that astrocytes regulate the differentiation of newborn neurons in the adult hippocampus through Wnt signaling (Lie *et al*. 2005), ephrin-B signaling (Ashton *et al*. 2012) and D-serine secretion (Sultan *et al*. 2015). In order to quantify the astrocyte density in the adult hippocampal dentate gyrus, I examined the immunofluorescent labeling of GFAP, which is a marker for mature astrocytes. The density of GFAP-positive cells in the dentate gyrus was 13.0% lower in *Pou3f2*$^{\Delta/\Delta}$ mice compared to wild-type mice ($p < 0.01$, student's *t*-test; Figure 3.4f, g), indicating that astrocytes are decreased in the dentate gyrus of *Pou3f2*$^{\Delta/\Delta}$ mice as well as in the cortex. Therefore, the impairment of neuronal differentiation in the adult dentate gyrus of *Pou3f2*$^{\Delta/\Delta}$ mice may be attributed in part to the decrease in astrocytes in the adult dentate gyrus. In addition, decreased expression of the rate-limiting enzymes for serotonin and dopamine synthesis in *Pou3f2*$^{\Delta/\Delta}$ mice might explain the results in this study, because serotonin and dopamine are considered to regulate adult hippocampal neurogenesis. However, the roles of serotonin and dopamine in neuronal differentiation in the adult dentate gyrus have been debated. Pharmacological studies of serotonin suggest a positive effect of serotonin on the maturation of newborn neurons (Jha *et al*. 2006; Wang *et al*. 2008; Klempin *et al*. 2010; Mendez-David *et al*. 2014). In contrast, genetically modified mice with life-long or transient depletion of brain serotonin enhanced the survival of the newborn neurons (Diaz *et al*. 2013; Sachs *et al*. 2013; Song *et al*. 2016), as well as chronic pharmacological serotonin depletion did (Diaz *et al*. 2013). These conflicting reports

may be due to the diverse roles of serotonin according to the responsible receptor subtype as well as differences in experimental design. Similar conflicting reports have been published regarding the role of dopamine in regulating the maturation of newborn neurons in the adult dentate gyrus. Treatment with a dopamine D2 receptor antagonist was reported to increase the maturation of newborn neurons in the dentate gyrus and to have no effect in separate studies (Halim *et al*. 2004; Wang *et al*. 2004; Keilhoff *et al*. 2010). Pharmacological dopamine depletion reduced or had no effect on the survival of newborn neurons in the hippocampus (Park & Enikolopov 2010; Khaindrava *et al*. 2011). Furthermore, the chronic inhibition of dopamine reuptake was reported to have no effect on neuronal precursor differentiation and survival (Domínguez-Escribà *et al*. 2006). Future studies are required to determine the exact involvement of serotonin and dopamine depletion in the decreased maturation of newborn neurons in the adult dentate gyrus of *Pou3f2*$^{\Delta/\Delta}$ mice.

In summary, the homopolymeric amino acid repeats in POU3F2 contribute to recognition memory and adult hippocampal neurogenesis. These results confirm that the homopolymeric amino acid repeats in POU3F2 play a role at phenotypic level. Given that recognition memory plays a critical role in cognitive function and is necessary for an animal's survival and social interaction between individuals, the homopolymeric amino acid repeats in POU3F2 can be greatly important and, therefore, highly conserved in mammals. Many studies have indicated that adult hippocampal neurogenesis plays an important role in learning, memory, and cognition. Additionally, it has been reported that newborn neurons in the adult dentate gyrus contribute to performance in the object recognition (Bruel-Jungerman *et al*. 2005; Jessberger *et al*. 2009) and object location tests (Goodman *et al*. 2010). Therefore, it is likely that deficits in novel object and novel location recognition in *Pou3f2*$^{\Delta/\Delta}$ mice were related to decreased adult hippocampal neurogenesis. Adult hippocampal neurogenesis contributes to not only memory and learning but also affective behavior and stress response (Sahay & Hen 2008; Levone *et al*. 2015), suggesting the repeats in POU3F2

can affect a broad range of behavior such as maternal behavior (Nasu *et al*. 2014).

3-4. Figures

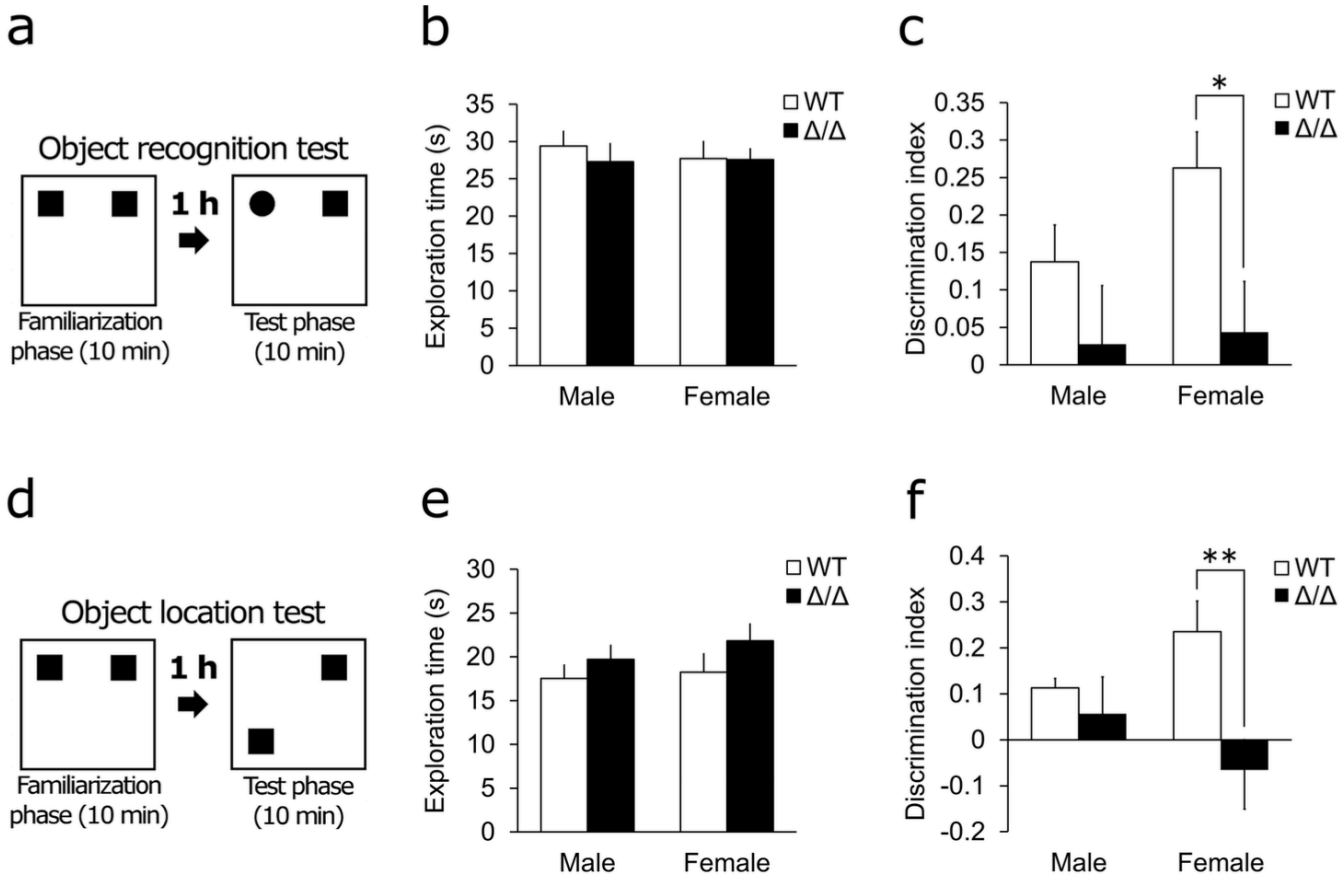

Figure 3.1 Impaired object and spatial recognition in *Pou3f2*$^{\Delta/\Delta}$ mice.

(a) Diagram of the object recognition test protocol. (b) Exploration time in wild-type (WT) mice and *Pou3f2*$^{\Delta/\Delta}$ (Δ/Δ) mice in the familiarization phase of object recognition test (WT male, n = 18; WT female, n = 12; Δ/Δ male, n = 10; Δ/Δ female, n = 11). (c) Discrimination indices in the object recognition test for each genotype according to sex (WT male, n = 18; WT female, n = 12; Δ/Δ male, n = 10; Δ/Δ female, n = 11). (d) Diagram of the object location test protocol. (e) Exploration time in wild-type (WT) mice and *Pou3f2*$^{\Delta/\Delta}$ (Δ/Δ) mice in the familiarization phase of object location test (WT male, n = 18; WT female, n = 12; Δ/Δ male, n = 10; Δ/Δ female, n = 11). (f) Discrimination indices in the object location test for each genotype according to sex (WT male, n = 18; WT female, n = 12; Δ/Δ male, n = 10; Δ/Δ female, n = 11). *p < 0.05, **p < 0.01 (student's t test). Data are presented as means ± SEM.

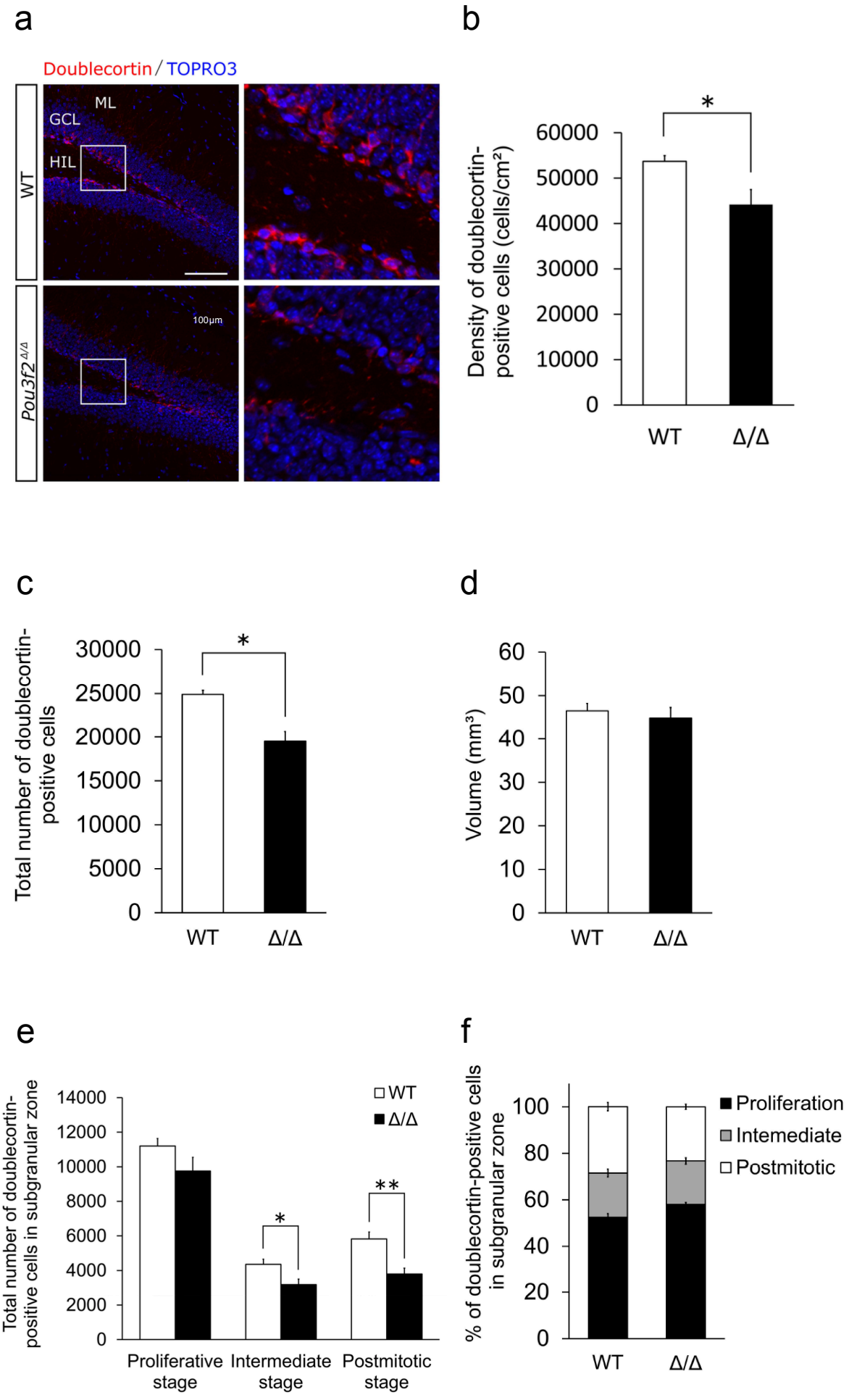
a
Doublecortin / TOPRO3
ML
GCL
HIL
WT
Pou3f2Δ/Δ
100μm
b
Density of doublecortin-positive cells (cells/cm²)
60000
50000
40000
30000
20000
10000
0
*
WT
Δ/Δ
c
Total number of doublecortin-positive cells
30000
25000
20000
15000
10000
5000
0
*
WT
Δ/Δ
d
Volume (mm³)
60
50
40
30
20
10
0
WT
Δ/Δ
e
Total number of doublecortin-positive cells in subgranular zone
14000
12000
10000
8000
6000
4000
2000
0
WT
Δ/Δ
*
**
Proliferative stage
Intermediate stage
Postmitotic stage
f
% of doublecortin-positive cells in subgranular zone
100
80
60
40
20
0
Proliferation
Intemediate
Postmitotic
WT
Δ/Δ

Figure 3.2 Impaired adult hippocampal neurogenesis in *Pou3f2*$^{\Delta/\Delta}$ mice

(a) Doublecortin-positive cells (red) in the dentate gyrus of wild-type mice (upper) and *Pou3f2*$^{\Delta/\Delta}$ mice (lower). (b) The density of doublecortin-positive cells in the dentate gyrus of female wild-type and *Pou3f2*$^{\Delta/\Delta}$ mice (WT female, $n = 7$; Δ/Δ female, $n = 6$). (c) The number of doublecortin-positive cells in the adult dentate gyrus of female wild-type and *Pou3f2*$^{\Delta/\Delta}$ mice (WT female, $n = 7$; Δ/Δ female, $n = 6$). (d) The volume of granule cell layer of female wild-type and *Pou3f2*$^{\Delta/\Delta}$ mice (WT female, $n = 7$; Δ/Δ female, $n = 6$). (e) Doublecortin-positive cells in the subgranular zone of the dentate gyrus in each genotype categorized according to staging based on dendritic morphology (WT female, $n = 7$; Δ/Δ female, $n = 6$). (f) The percentage of each stage of doublecortin posive cells in the subgranular zone of female wild-type and *Pou3f2*$^{\Delta/\Delta}$ mice. GCL, granule cell layer; HIL, hilus; and ML, molecular layer. *$p < 0.05$, **$p < 0.01$ (student's *t* test). Data are presented as means ± SEM.

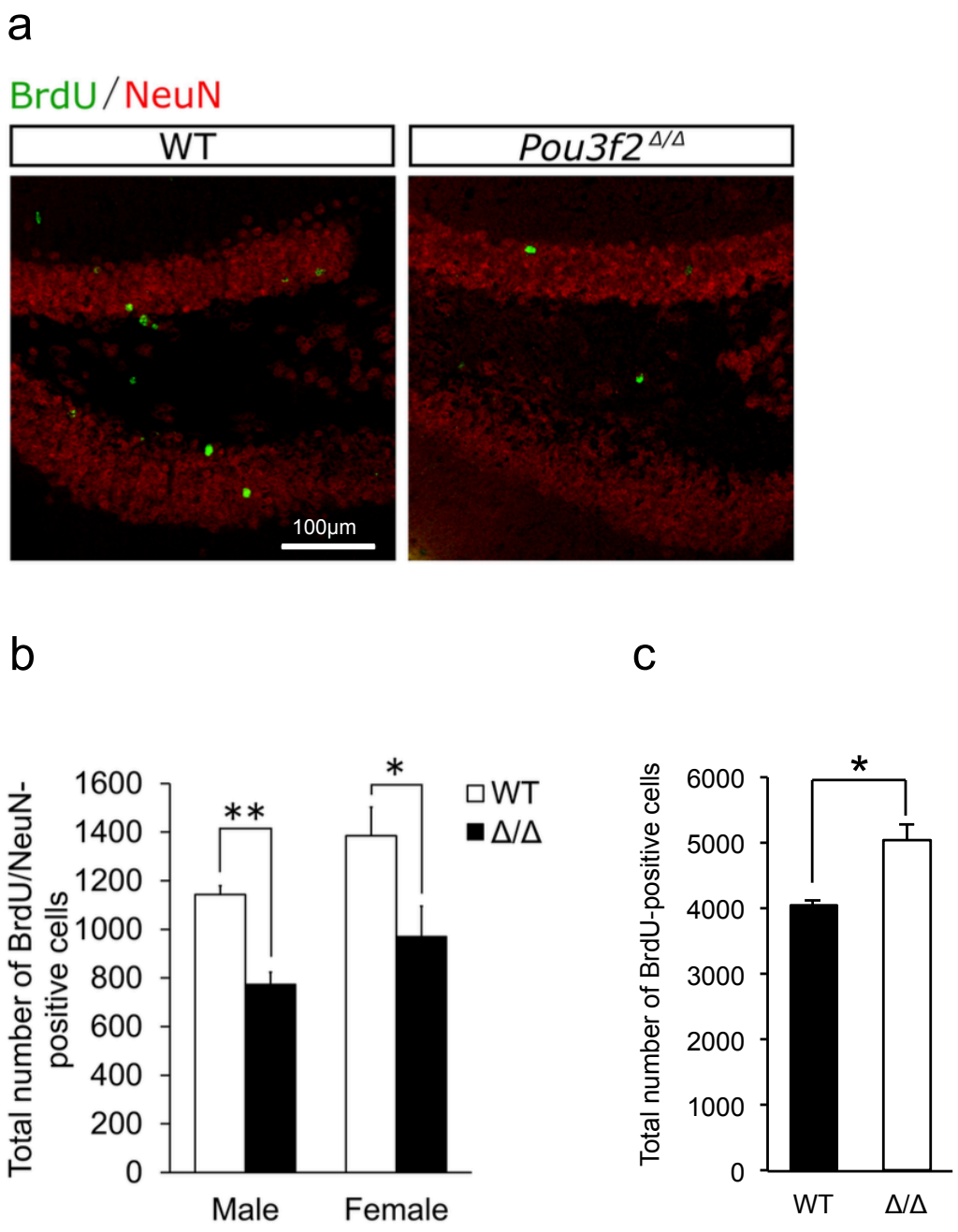

Figure 3.3 Impaired neuronal maturation in the adult hippocampus of *Pou3f2*$^{\Delta/\Delta}$ mice

(a) BrdU-positive cells (green) and NeuN-positive cells (red) in the dentate gyrus of wild-type (left) and *Pou3f2*$^{\Delta/\Delta}$ (right) mice. (b) NeuN/BrdU double-positive cells in the dentate gyrus for each genotype according to sex (WT male, n = 9; Δ/Δ male, n = 8; WT female, n = 7; Δ/Δ female, n = 6). (c) Neural precursor proliferation in the dentate gyrus for each genotype according to sex (WT male, n = 3; Δ/Δ male, n = 3; WT female, n = 6; Δ/Δ female, n = 6). Scale bar = 100 μm. *p < 0.05, **p < 0.01 (student's t test). Data are presented as means ± SEM.

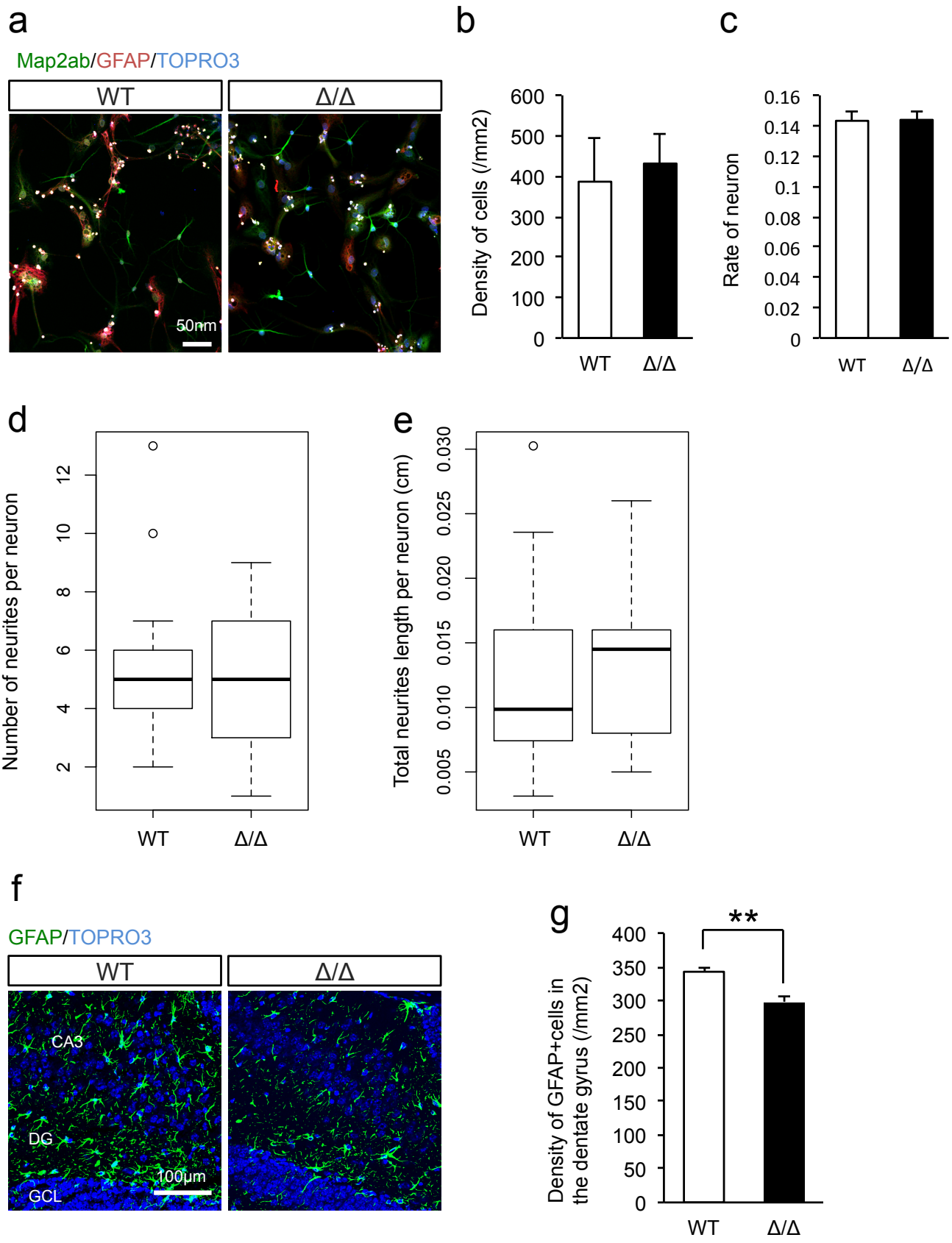
a
Map2ab/GFAP/TOPRO3
WT
Δ/Δ
50nm
b
Density of cells (/mm2)
600
500
400
300
200
100
0
WT
Δ/Δ
c
Rate of neuron
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
WT
Δ/Δ
d
Number of neurites per neuron
12
10
8
6
4
2
WT
Δ/Δ
e
Total neurites length per neuron (cm)
0.030
0.025
0.020
0.015
0.010
0.005
WT
Δ/Δ
f
GFAP/TOPRO3
WT
Δ/Δ
CA3
DG
GCL
100μm
g
**
Density of GFAP+cells in the dentate gyrus (/mm2)
400
350
300
250
200
150
100
50
0
WT
Δ/Δ

Figure 3.4 No effect of POU3F2ΔGQP on neuronal differentiation *in vitro*

(a) Immunostaining of Map2ab (green) and GFAP (red) in cultured adult hippocampal NPCs derived from wild-type (left) and *Pou3f2*$^{\Delta/\Delta}$ (right) mice after 10 days under the differentiation condition. Nuclei were stained with To-PRO-3 iodide. (b) The nuclear density of cultured adult hippocampal NPCs derived from wild-type and *Pou3f2*$^{\Delta/\Delta}$ mice upon 10 days of differentiation (n = 3 experiments). (c) The ratio of Map2ab-positive cells in total cells after 10 days under the differentiation condition for each genotype (n = 3 experiments). (d) The number of neurites per neuron after 10 days under the differentiation condition (n = 12 neurons for each genotype). (e) The total length of neurites per neuron after 10 days under the differentiation condition (n = 12 neurons for each genotype). (f) GFAP positive cells (green) in the dentate gyrus of wild-type (left) and *Pou3f2*$^{\Delta/\Delta}$ (right) mice. (g) The density of GFAP-positive cells in the dentate gyrus of wild-type and *Pou3f2*$^{\Delta/\Delta}$ mice (WT female, n = 6; Δ/Δ female, n = 5). GCL, granule cell layer; DG, dentate gyrus. *$p < 0.05$, **$p < 0.01$ (student's t-test). Data are presented as means ± SEM.

Chapter 4

Conclusion

Although it has been believed that *cis*-regulatory changes underlie most of the variations in transcriptional regulation and mainly contribute to the phenotypic evolution, the importance of trans-regulatory changes has recently been reassessed. In this study, I focused on homopolymeric amino acid repeats. It has become clear that homopolymeric amino acid repeats are involved in many biological processes and associate with phenotypic variation. However, their biological and evolutional significances remain to be elucidated because there are few experimental studies to demonstrate them.

In order to investigate the biological function of homopolymeric amino acid repeats, I investigated a role of three mammalian-specific amino acid repeats contained in POU3F2, a transcription factor involved in brain development. Using *Pou3f2*$^{\Delta/\Delta}$ mice, I examined whether the deletion of the repeats in POU3F2 affect on cortical development and cognitive function of mice. In chapter 2, an *in vitro* experiment revealed that the transactivation activity of POU3F2 was decreased by the homopolyemeric amino acid repeats. In addition, *in vivo* experiments revealed that glial cells were decreased in the cortex of *Pou3f2*$^{\Delta/\Delta}$ mice. Based on these results, I hypothesized that generation of cortical astrocytes is impaired in *Pou3f2*$^{\Delta/\Delta}$ mice. Indeed, matured and differentiating astrocytes are both decreased in the cortex of *Pou3f2*$^{\Delta/\Delta}$ mice, whereas IPs were increased in *Pou3f2*$^{\Delta/\Delta}$ mice. Although the proliferation rate of cortical progenitors was not changed in *Pou3f2*$^{\Delta/\Delta}$ mice, astrogenesis was decreased and neurogenesis was increased in the cortex of *Pou3f2*$^{\Delta/\Delta}$ mice. Taken together, these results suggest that neuronal versus glial fate decision in cortical progenitors was impaired in *Pou3f2*$^{\Delta/\Delta}$ mice. In chapter 3, cognitive function was examined by two behavioral tests: object recognition test and object location test.

As a result, *Pou3f2*$^{\Delta/\Delta}$ mice exhibited impairment in object recognition and spatial recognition. Furthermore, *Pou3f2*$^{\Delta/\Delta}$ mice have lower numbers of newborn neurons in the adult hippocampus due to impairment in neural differentiation. *In vitro* experiments suggested that the repeats in POU3F2 are indirectly involved in neural differentiation of NPCs in the adult hippocampus.

In summary, this work has revealed that homopolymeric amino acid repeats serve a particular function in an organism with significant phenotypic effect. Given that recognition memory is necessary for mammalian survival and reproduction, the homopolymeric amino acid repeats in POU3F2 can be considered as greatly significant and influential. This seems to be due to the functional importance of POU3F2 in brain development. Generally, highly constrained proteins contain large number of homopolymeric amino acid repeats (Mularoni *et al*. 2007), suggesting that functionally significant proteins prefer homopolymeric amino acid repeats. Therefore, similarly to the repeats in POU3F2, other homopolymeric amino acid repeats can perform a crucial role by inserting into key genes involved in vital biological processes such as development. In this study, astrogenesis was affected by the homopolymeric amino acid repeats in POU3F2, indicating that taxon-specific amino acid repeats can contribute to phenotypic evolution. Taken together, these findings support the idea that homopolymeric amino acid repeats are advantageous trans-regulatory changes in evolution.

I have employed a loss-of-function approach, in which all of the homopolymeric amino acid repeats were just deleted from POU3F2. Further experiments using other approaches, for example a stepwise deletion of the repeats or a gain-of-function approach, should be performed to confirm the implication of this study. In addition, investigating other genes that contain homopolymeric amino acid repeats would help us to understand the general significance of them. Interestingly, homopolymeric amino acid repeats are frequently found in genes involved in transcription, signaling and neuronal development in mammals (Faux *et al*. 2005;

Mularoni *et al*. 2010). Future research should therefore investigate how much homopolymeric amino acid repeats have contributed to brain development, function and evolution.

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