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COMPARATIVE ANALYSIS OF DEVELOPMENTALLY REGULATED EXPRESSIONS OF *GADD45A*, *GADD45B*, AND *GADD45G* IN THE MOUSE AND MARMOSET CEREBRAL CORTEX

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Abstract—The cerebral cortex is an indispensable region that is involved in higher cognitive function in the mammalian brain, and is particularly evolved in the primate brain. It has been demonstrated that cortical areas are formed by both innate and activity-dependent mechanisms. However, it remains unknown what molecular changes induce cortical expansion and complexity during primate evolution. Active DNA methylation/demethylation is one of the epigenetic mechanisms that can modify gene expression via the methylation/demethylation of promoter regions. Three growth arrest and DNA damage-inducible small nuclear proteins, Gadd45 alpha, beta, and gamma, have been identified as regulators of methylation status. To understand the involvement of epigenetic factors in primate cortical evolution, we started by analyzing expression of these demethylation genes in the developing common marmoset (*Callithrix jacchus*) and mouse (*Mus musculus*) brain. In the marmoset brain, we found that cortical expression levels of *Gadd45* alpha and gamma were reduced during development, whereas there was high expression of *Gadd45 beta* in some areas of the adult brain, including the prefrontal, temporal, posterior parietal and insula cortices, which are particularly expanded in greater primates and humans. Compared to the marmoset brain, there were no clear regional differences and constant or reduced *Gadd45* expression was seen between juvenile and adult mouse brain. Double staining with a neuronal marker revealed that most *Gadd45*-expressing cells were NeuN-positive neurons. Thus, these results suggest the possibility that differential *Gadd45* expression affects neurons, contributing cortical evolution and diversity.

Key words: cerebral cortex, development, epigenetic, Gadd45, marmoset, mouse.

INTRODUCTION

One of the most interesting topics in neuroscience is how the human brain has been expanded. Many remarkable findings have been obtained from comparative studies using rodents and primates and these have demonstrated that the primate brain is not simply a larger version of the rodent brain. Primate cerebral cortices are not only huge compared to rodents but also have more areal complexities than rodent cortices at the anatomical, histological, neural connectivity, and functional level (Herculano-Houzel et al., 2007; Rilling et al., 2008; Krubitzer, 2009; Collins et al., 2010; Fietz et al., 2010; Kaas, 2012; Visel et al., 2013). Thus, for rodents and primates to show distinct cortical development, there must be genetic changes in our common ancestors that separate the primate lineage from the rodent lineage.

Mounting evidence suggests that innate genetic mechanisms control aspects of cortical development, such as neural patterning, neurogenesis, axon guidance, synapse formation, and neuron maturation, by regulating spatial and temporal expressions of transcription factors, secreted molecules, or transmembrane proteins (Rakic, 2009; Greig et al., 2013). Developmentally regulated genes have been identified as candidates responsible for primate brain development and evolution (Piao et al., 2004; Ponting and Jackson, 2005; Pollard et al., 2006; Watakabe et al., 2007; Johnson et al., 2009; Takaji et al., 2009; Bilgüvar et al., 2010; Higo et al., 2010; Yamamori, 2011; Kang et al., 2011; McLean et al., 2011; Hawrylycz et al., 2012; Mashiko et al., 2012; Graham and Fisher, 2013; Matsunaga et al., 2013; Bae et al., 2014). However, environmental factors also significantly affect brain development (Krubitzer, 2009). For example, enriched environments induce adult neurogenesis in the mouse hippocampus (Deng et al., 2010). Magnetic resonance imaging (MRI) studies have revealed that juvenile rats have larger brains when they are reared in an enriched environment (Jenks et al., 2013), and in humans and monkeys, that the size in specific cortical areas increased after learning complex cognitive tasks, even as adults (Draganski et al., 2004; Boyke et al., 2008; Driemeyer et al., 2008; Quallo et al., 2009). Thus, both innate and epigenetic mechanisms are likely to be involved in primate brain development and evolution. However, currently, less is known about how environmental factors may modulate primate cortical development.

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Abbreviations: AP, alkaline phosphatase; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxigenin; Ent, entorhinal cortex; Gadd45, growth arrest and DNA damage-inducible protein 4; GW, gestational week; Hp, hippocampus; Is, insula; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PFC, prefrontal cortex; PP, posterior parietal cortex; SSC, standard sodium citrate; SVZ, subventricular zone; VZ, ventricular zone.

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Table 1. Primers and probes

	5′ primer	3′ primer	GenBank number	Position	Probe length		References
Marmoset							
Gadd45a	CAGAAGACCGAAAGGATGG	CAGCCCCTTGACATCAGTTT	XM_002750938	134–724	591	ORF + 3′UTR	
Gadd45b	CGCCTTTTCTGGAAGGATTT*	TTTTGGCAGCAACTCAACAG*	AB863011	100–774	675**	5′UTR + ORF + 3′UTR	XM_005587469*
Gadd45g	DNA synthesis		XM_002742712	95–569	475	ORF	
Arc	ACACCCCAGAGCAGAAGCTA*	TCAGGCTGCAGACGCTAGTA*	AB863012	1868–2723	849**	3′UTR	XM_005564182*
Mouse							
Gadd45a	cDNA clone		AK144900		1219	5′UTR + ORF + 3′UTR	Carninci et al. (2005)
Gadd45b	cDNA clone		AK149895		1284	5′UTR + ORF + 3′UTR	Carninci et al. (2005)
Gadd45g	cDNA clone		AK007410		1077	5′UTR + ORF + 3′UTR	Carninci et al. (2005)
Arc	cDNA clone		AK170446		1678	5′UTR + ORF + 3′UTR	Carninci et al. (2005)

Gadd45, growth arrest and DNA damage-inducible protein 45; *Arc*, activity-regulated cytoskeleton-associated protein/activity regulated gene 3.1.

* Indicates primers designed with *Macaca fascicularis* cDNA and the reference sequence number.

** Indicates the real probe length based on marmoset cDNA.

Active DNA methylation/demethylation is one mechanism of epigenetic regulation in plants and animals (Niehrs and Schäfer, 2012). The growth arrest and DNA damage-inducible protein 45 (Gadd45) family is a major player in active demethylation. These were originally isolated as factors encoding small nuclear proteins induced by stressful growth arrest conditions or DNA damage (Niehrs and Schäfer, 2012). This family is comprised of three proteins, Gadd45 alpha, beta, and gamma (Gadd45a/b/g). Gadd45 proteins control DNA repair, cell cycle arrest, apoptosis, and differentiation by interacting with various effectors. Recently, it has been demonstrated that these proteins are also involved in active DNA demethylation that regulates gene expression through DNA repair mechanisms (Barreto et al., 2007; Ma et al., 2009; Niehrs and Schäfer, 2012).

Many studies have revealed that Gadd45 proteins play multiple roles in neural development and plasticity and that active DNA demethylation occurs during the learning process (Ma et al., 2009; Guo et al., 2011). Notably, *Gadd45a* mutant mice exhibit exencephaly (Hollander et al., 1999). RNAi knockdown of *Gadd45a* induces defects in neurite outgrowth in the embryonic neocortex (Sarkisian and Siebzehnrubl, 2012). *Gadd45b* mutant mice show impairment of activity-dependent neurogenesis and dendritic development and exhibit alterations in long-term memory and long-term potentiation in hippocampal neurons (Ma et al., 2009; Leach et al., 2012; Sultan et al., 2012). Enhanced expression of *Gadd45b* by neural activity induces brain-derived neurotrophic factor and *fibroblast growth factor-I* gene expression via active DNA demethylation of their CpG elements (Ma et al., 2009). *Gadd45b* has been identified as one of the genes responsible for human psychosis (Gavin et al., 2012). *Gadd45g* controls neuronal differentiation in fish and *Xenopus* embryos (de la Calle-Mustienes et al., 2002; Candal et al., 2004; Kaufmann and Niehrs, 2011), although no clear defects have been reported in the nervous system of *Gadd45g* knockout mice, partly due to functional redundancy (Hoffmeyer et al., 2001).

Although previous analyses of *Gadd45* gene expression have been conducted in mouse embryos (Kaufmann et al., 2011), there have been no studies to date in the primate brain. To start exploring the possible involvement of epigenetic regulations in primate cortical evolution, we examined *Gadd45a*, *Gadd45b*, and *Gadd45g* expressions in the developing cerebral cortex of the common marmoset (*Callithrix jacchus*) and compared to the mouse (*Mus musculus*).

EXPERIMENTAL PROCEDURES

Ethics

Research protocols were approved by the Animal Care and Use Committee of RIKEN (# H25-EP018) and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and sample preparation

Common marmosets were purchased from the Research Resource Center of the RIKEN Institute. Prior to the

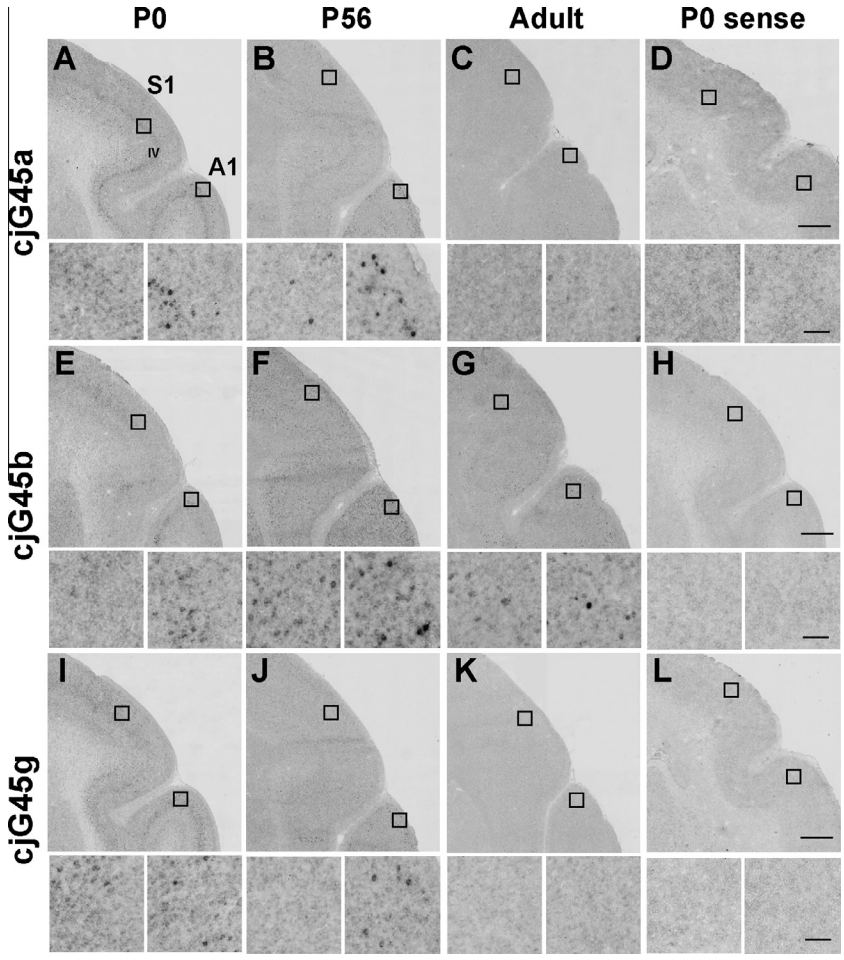

Fig. 1. *Gadd45a* (A–C), *Gadd45b* (E–G) and *Gadd45g* (I–K) expressions and staining with each sense probe (D, H, L) at postnatal day 0 (P0; A, E, I), P56 (B, F, J), and adulthood (C, G, K) in S1 and A1 of the marmoset brain. The small panels are magnified views of the S1 (left) and A1 (right) regions, respectively. *Gadd45a* expressions decreased in S1 and A1 during postnatal development (A–C). *Gadd45b* expression was observed (G), whereas *Gadd45g* expression disappeared by the adult stage (K). A1, primary auditory cortex; *Gadd45*, growth arrest and DNA damage-inducible protein 45; cjG45a, marmoset Gadd45a; cjG45b, marmoset Gadd45b; cjG45g, marmoset Gadd45g; S1, primary somatosensory cortex. Scale bars = 1 mm and 100 μm.

experiments, all juvenile marmosets were housed with their parents, while adult marmosets were housed individually in a breeding room. Animals were kept on a 12-h light–dark cycle, at 27 °C with 50% humidity; they had *ad libitum* access to water and a standard marmoset diet of CMS-1 (Clea, Tokyo, Japan) with supplements. For postnatal marmoset analysis, we used two postnatal day 0 (P0) males, one P0 female, two P56 males, one P56 female, one adult male, and two adult females (adults were > 36 months old). We did not detect any significant differences in *Gadd45* expression between male and female marmosets. For embryonic analysis, around 11–12 gestational week (GW) old marmoset embryos, from two independent mothers, were used. GW was estimated based on apparent forelimb morphology (Hikishima et al., 2013). Mice were purchased from Nihon SLC Inc. (Shizuoka, Japan) and bred in our laboratory facilities. We used three P0, three P14, and three P56 mice.

Marmosets and mice were anesthetized with an intramuscular injection of sodium pentobarbital (75 and 150 mg/kg, respectively; Dainihon Seiyaku, Osaka, Japan), followed by perfusion with ice-cold phosphate-buffered saline (PBS, pH 7.4) and immediate dissection. For embryonic marmosets, dams were anesthetized with ketamine (15 mg/kg) followed by isoflurane, and embryos were collected by cesarian section. For embryonic mice, dams were sacrificed using sodium pentobarbital followed by cervical dislocation, and embryos were collected. Marmoset and mouse embryos were fixed in PBS solution with 4% paraformaldehyde (PFA) and immersed in PBS with 20% sucrose solution after dissection. Brains were embedded in a Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Fine Technical Co. Ltd., Tokyo, Japan) and frozen on dry ice. Frozen sections were cut serially at a thickness of 14–20 μm using a cryostat (Leica Microsystems Inc., Wetzlar, Germany). These sections were then used for either *in situ* hybridization or thionine staining for neuroanatomical references. Total RNA was extracted by dissecting brain tissue and placing it in Qiazol lysis reagent (QIAGEN, Venlo, The Netherlands), and the RNA was purified using an RNeasy Lipid Tissue Mini Kit (QIAGEN).

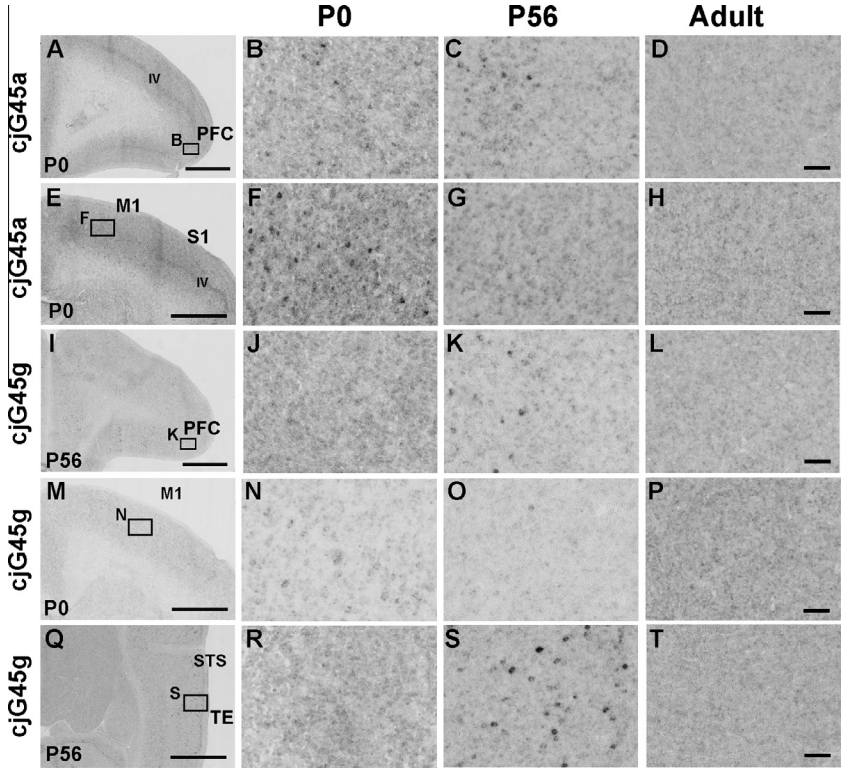

Fig. 2. *Gadd45a* (A–H) and *Gadd45g* (I–T) expression at P0 (A, B, E, F, J, M, N, R), P56 (C, G, I, K, O, Q, S) and adulthood (D, H, L, P, T) in the PFC (A–D, I–L), M1 (E–H, M–P), and Area TE (Q–T) of the marmoset brain. Note that transient increases of *Gadd45g* expression were seen in the PFC and Area TE (K, S). B–D, F–H, J–L, N–P and R–T show high-magnification views of each brain area. M1, primary motor cortex; PFC, prefrontal cortex. Scale bars = 1 mm (A, E, I, M, Q) and 100 μm (D, H, L, P, T).

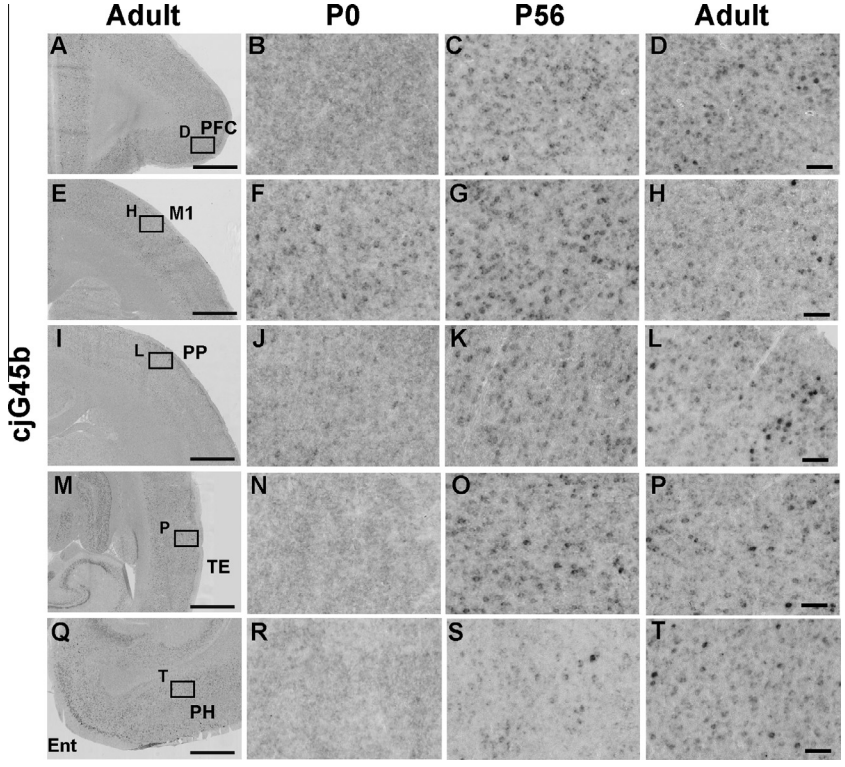

Fig. 3. *Gadd45b* expression at P0 (B, F, J, N, R), P56 (C, G, K, O, S), and in adulthood (A, D, E, H, I, L, M, P, Q, T) in the PFC (A–D), M1 (E–H), PP (I–L), Area TE (M–P), and PH (Q–T) of the marmoset brain. Note that increased *Gadd45b* expressions were seen in the PFC, PP, TE, and PH (D, L, P, T). B–D, F–H, J–L, N–P and R–T show high-magnification views of each brain area. PH, parahippocampal area; PP, posterior parietal cortex. Scale bars = 1 mm (A, E, I, M, Q) and 100 μm (D, H, L, P, T).

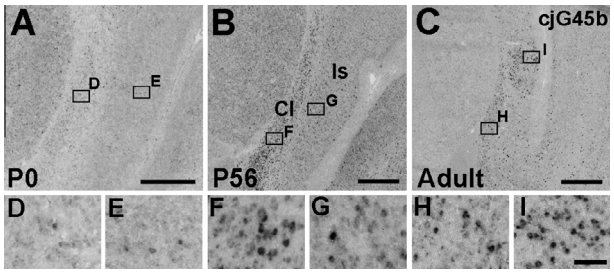

Fig. 4. *Gadd45b* expressions at P0 (A, D, E), P56 (B, F, G) and adulthood (C, H, I) in the Is and Cl of the marmoset brain. D, F, H and E, G, I are high-magnification views of the Cl and Is, respectively. Cl, claustrum; Is, insula. Scale bars = 1 mm (A–C) and 100 μm (I).

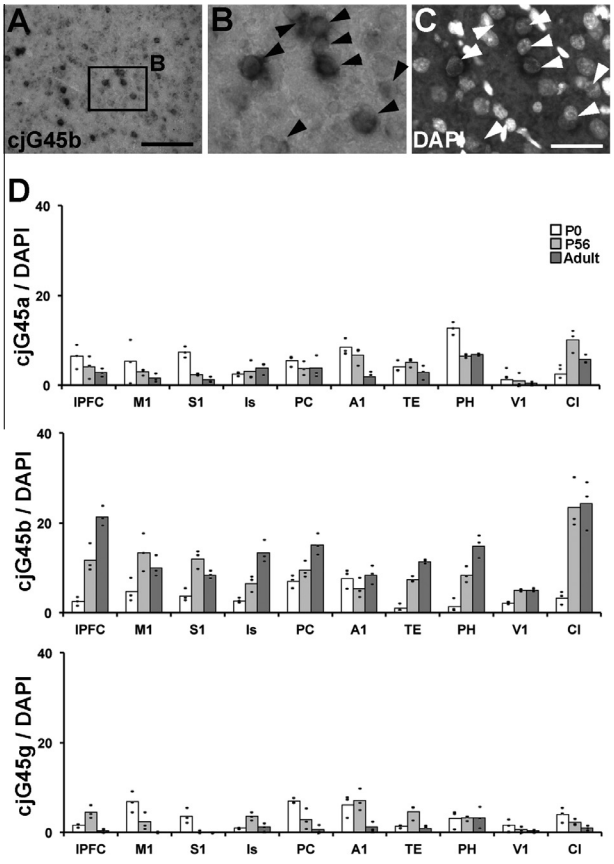

Fig. 5. Cell counting analysis of *Gadd45*-expressing cells in the developing marmoset brain. A sample image of *in situ* hybridization with *Gadd45b* probe for cell counting (A–C). (A) The S1 region of the adult marmoset brain under the microscope, (B) a high magnification view of A and (C) photo of tissue counterstained with DAPI. Arrowheads indicate cells expressing *Gadd45b*. (D) Relative ratios of *Gadd45*-expressing cells in the developing marmoset brain. Data are shown as the mean (n = 3) and each value is plotted on the graph. The vertical axis represents the percentage of *Gadd45*-expressing cells to DAPI-positive cells. lPFC, lateral PFC; V1, primary visual area. Scale bars = 200 μm (A) and 50 μm (C).

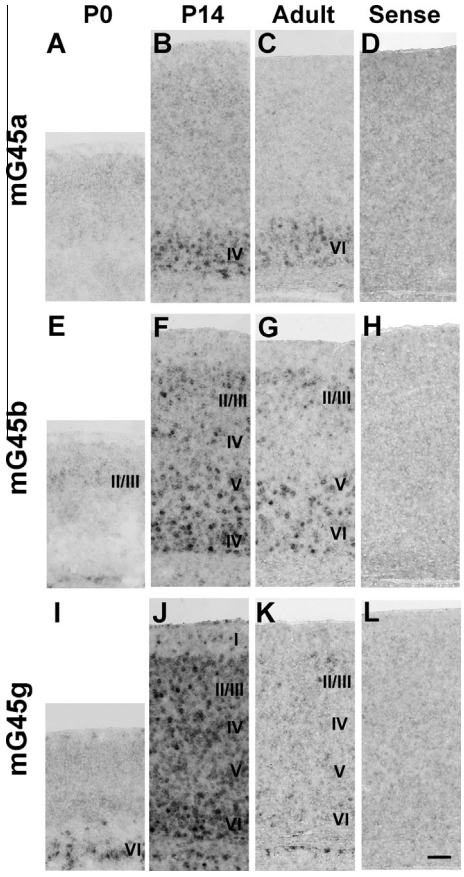

Fig. 6. *Gadd45a* (A–C), *Gadd45b* (E–G) and *Gadd45g* (I–K) expressions and staining with each sense probe (D, H, L) in the mouse primary somatosensory cortex (S1) at P0 (A, E, I), P14 (B, F, J) and adulthood (C, D, G, H, K, L). Note that *Gadd45a* expression was only seen in the deep layer of the neocortex and persisted in the adult brain. Staining levels of *Gadd45b* expression decreased from P14 to adulthood. *Gadd45g* expression declined but was still detectable in the adult brain. mG45a, mouse Gadd45a; mG45b, mouse Gadd45b; mG45g, mouse Gadd45g. Scale bar = 100 μm.

Isolation and cloning of cDNA

Marmoset *Gadd45a*, *Gadd45b* (GenBank No. AB863011), and *activity-regulated cytoskeleton-associated protein/activity regulated gene 3.1* (*Arc/Arg3.1*) (AB863012) cDNA fragments were isolated from the adult brain of a female marmoset by reverse transcription-polymerase chain reaction (RT-PCR). Primers for *Gadd45b* and *Arc* were designed according to macaque cDNAs. For *Gadd45g*, we synthesized a 475 base pair DNA fragment (Eurofins MWG Operon, Huntsville, LA, USA) corresponding to the N-terminal region of the marmoset Gadd45-like protein. Each cDNA fragment was inserted into pGEM-T Easy vectors (Promega, Madison, WI, USA). For mouse probes, we used cDNA fragments obtained from the Fantom cDNA project (Carninci et al., 2005). The cDNAs employed for making probes are summarized in Table 1. Sense and antisense probes were synthesized using SP6 or T7 RNA polymerase (Roche Diagnostics, Basel, Switzerland) with digoxigenin (DIG)-labeling mix (Roche Diagnostics).

Histochemical analysis of tissue sections

In situ hybridization of all tissue sections was performed using a previously described method (Matsunaga et al., 2013). Briefly, the sections were post-fixed for 10 min with PBS solution with 4% PFA and then washed three times

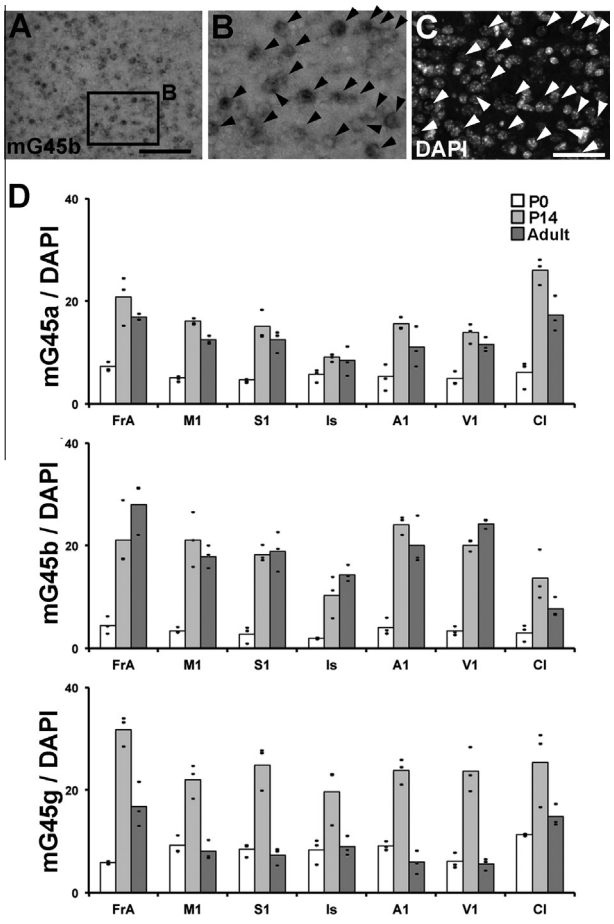

Fig. 7. Cell counting analysis of *Gadd45*-expressing cells in developing mouse brain. A sample image of *in situ* hybridization with *Gadd45b* probe for cell counting (A–C). (A) The S1 region of P14 mouse brain under the microscope, (B) high magnification view of A, and (C) photo of tissue counterstained with DAPI. Arrowheads indicate cells expressing *Gadd45b*. (D) Relative ratios of *Gadd45*-expressing cells in the developing mouse brain. Note that we show the ratio of *Gadd45a*-expressing cells in the layer VI (not in all cortical layers), because clear *Gadd45a* expression was only seen in the layer VI (A). Data are shown as the mean (n = 3) and each value is plotted on the graph. The vertical axis represents the percentage of *Gadd45*-expressing cells to DAPI-positive cells. FrA, frontal association area. Scale bars = 200 μm (A) and 50 μm (C).

for 3 min in PBS. The slides were delipidated with acetone (only postnatal tissues), acetylated, and washed in PBS with 1% Triton-X100 (Wako Pure Chemical, Osaka, Japan). Slides were then incubated at room temperature with hybridization buffer containing 50% formamide (Wako Pure Chemical), 5 × standard sodium citrate (SSC), 5 × Denharlt's solution (Sigma, St. Louis, MO, USA), 250 μg/mL yeast tRNA (Roche Diagnostics), and 500 μg/mL DNA (Roche Diagnostics). Sections were hybridized overnight at 72 °C in hybridization buffer with RNA probes. They were then rinsed in 0.2 × SSC for 2 h and then blocked for 2 h in a solution of 0.1 M Tris (pH 7.5) and 0.15 M NaCl with 10% sheep serum. The slides were incubated overnight with alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche Diagnostics). After washing three times in a solution of 0.1 M Tris (pH 7.5) and 0.15 M NaCl with 0.1% Polysorbate 20 (Wako Pure Chemical), AP activity was detected by adding nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics), and subsequently counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Antisense probes exhibited region-specific staining, whereas no clear signals above the background were detected with the sense probes. Some slides were used for immunohistochemical analysis as previously described (Matsunaga et al., 2011). We used an anti-NeuN mouse monoclonal antibody (Merck Millipore, Billerica, MA, USA, 1:200) or anti-Tbr2 rabbit polyclonal antibody (Abcam, Cambridge, UK, 1:200) and a Cy3-conjugated anti-mouse or rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA, 1:400). All images were captured by a NanoZoomer 2.0 slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) or an ORCA-Flash2.8 digital camera (Hamamatsu Photonics) under a BX-50 microscope (Olympus, Tokyo, Japan). Adobe Photoshop software (ver. CS5; Adobe Systems, Mountain View, CA, USA) was used to convert color images to black and white, crop unnecessary areas, and juxtapose panels. To quantify numbers of positive cells, we used the Cell Counter plug-in for ImageJ software (NIH, Bethesda, MD, USA). Cells were counted regardless of their expression level (i.e., both strongly and weakly positive cells were counted). The numbers of *in situ* hybridization-positive cells and DAPI-positive

Table 2. Brief summary of *Gadd45* expression in the isocortex (neocortex) and allocortex (hippocampus and entorhinal cortex)

		Marmoset		Mouse	
		Time course	Spatial pattern	Time course	Spatial pattern
Gadd45a	Neocortex	↓	Disperse	→	Layer VI
	Entorhinal cortex	↓	Layer II–VI	↓	Layer II–VI
	Hippocampus	↓	CA1–3, DG	↓	CA1–3, DG
Gadd45b	Neocortex	↑	Areal difference	→	Similar
	Entorhinal cortex	→	Layer II–VI	→	Layer II–VI
	Hippocampus	→	CA1–3, DG	→	CA1–3, DG
Gadd45g	Neocortex	↓	Disperse	↓	Disperse
	Entorhinal cortex	↓	Layer II–V	↓	Layer II–VI
	Hippocampus	↓	CA1–3, DG	↓	CA1–3, DG

Gray columns indicate remarkable differences between mice and marmosets. Note that *Gadd45* expression differs more prominently in the neocortex compared to the allocortex. DG, dentate gyrus; *Gadd45*, growth arrest and DNA damage-inducible protein 45.

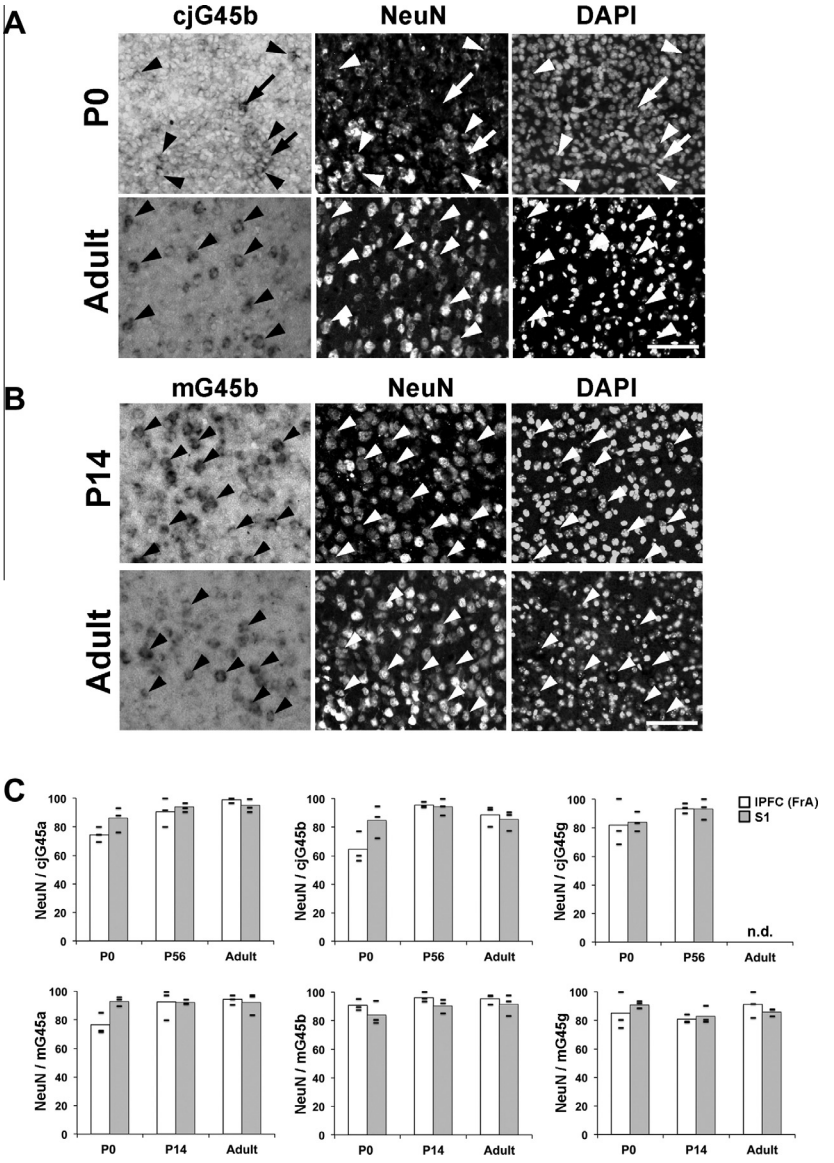

Fig. 8. Relative ratios of NeuN-positive neurons to *Gadd45*-positive cells. Examples of *in situ* hybridization for *Gadd45b* and immunostaining for NeuN in the lateral prefrontal area of P0 and adult marmoset brains (A) and in the frontal association area of P14 and adult mouse brain (B). Arrowheads indicate NeuN-positive *Gadd45b*-expressing cells. Arrows indicate NeuN-negative *Gadd45b*-expressing cells. (C) Relative ratio of NeuN-positive neurons to *Gadd45*-positive cells in lateral PFC (or mouse frontal association area) and S1 region. Data are shown as the mean ($n = 3$) and each value is plotted on the graph. The vertical axis represents the percentage of NeuN-positive cells to *Gadd45*-expressing cells. Most *Gadd45* positive cells were NeuN-positive neurons, and that there was no clear difference between marmoset and mouse brains. Note that ratio of neurons to *Gadd45*-positive cells tend to be underestimated than the real, because immature neurons at neonatal stage are still NeuN-negative and strong NBT/BCIP chromogenic stain of *in situ* hybridization quenches immunofluorescence of NeuN-positive cells. FrA, frontal association area; lPFC, lateral PFC; n.d. no clearly expressing cells. Scale bar = 100 μm.

cells were counted in three different randomly selected areas on each slide, and calculated as a ratio of *in situ* hybridization-positive cells to DAPI-positive cells. We analyzed three individual brains for each area, probe and stage. Relative ratio of NeuN-positive cells to Gadd45-positive cells was calculated similarly.

RESULTS

In this study, we mainly examined *Gadd45a*, *Gadd45b and Gadd45g* expression in the developing postnatal neocortex (Figs. 1–7). To precisely analyze spatial and temporal expression patterns, we also counted the number of cells expressing each *Gadd45* gene (Figs. 5 and 7). Our findings are summarized in Table 2.

Developmental reduction in *Gadd45a* and *Gadd45g* expression and increase in *Gadd45b* expression in the postnatal marmoset neocortex

Gadd45a expression was evident in the P0 marmoset neocortex. At the neonatal stage, *Gadd45a* expression was sparsely distributed and weak expression in layer IV was noted. However, expression levels decreased in adulthood (Figs. 1A–C, 2A–H and 5D).

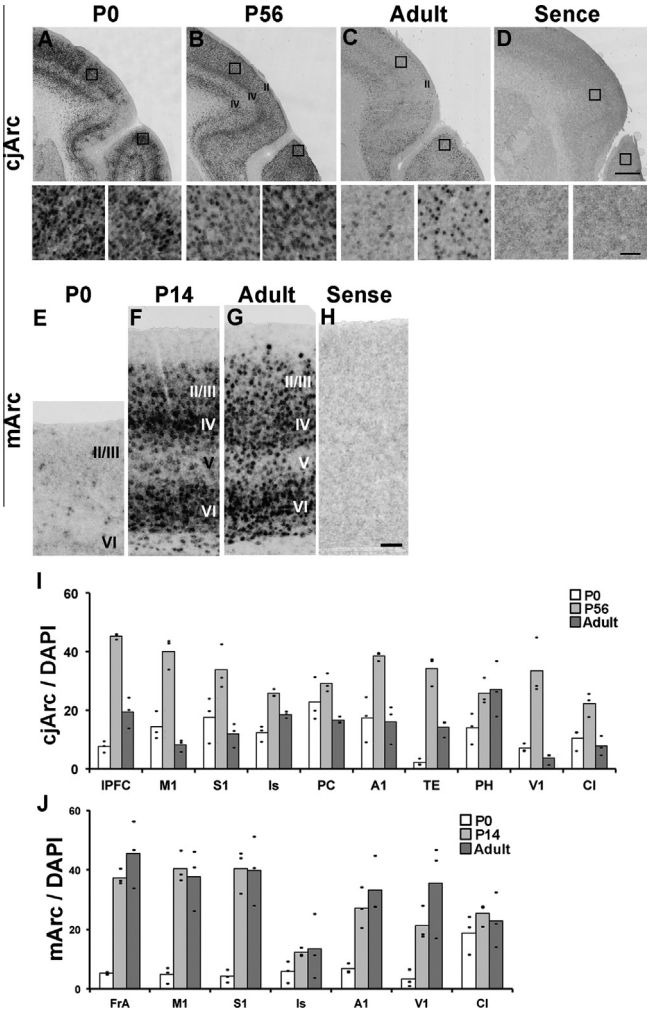

Fig. 9. *Arc* expression in marmoset and mouse neocortex. *Arc* expression in marmoset S1 and A1 (A–C) and staining with sense probe (D). *Arc* expression in mouse S1 (E–G) and staining with sense probe (H). The small panels are magnified views of the S1 (left) and A1 (right) regions, respectively. Relative ratios of *Arc*-expressing cells in the developing marmoset (I) and mouse (J) brain. Data are shown as the mean (n = 3) and each value is plotted on the graph. The vertical axis represents the percentage of *Arc*-expressing cells to DAPI-positive cells. *Arc*, activity-regulated cytoskeleton-associated protein/activity regulated gene 3.1; cjArc, marmoset Arc; mArc, mouse Arc. Scale bars = 1 mm (D) and 100 μm (D, H).

Gadd45g expression was also reduced during postnatal development (Figs. 1I–K, 2I–T and 5D). In some brain areas, such as the prefrontal cortex (PFC) and area TE, *Gadd45g* expression transiently increased during the early postnatal stage (Fig. 2K, S), possibly because these brain areas may be less mature than other cortical areas (Matsunaga et al., 2013). In these areas, *Gadd45g* expression had decreased by adulthood.

During the neonatal stage, *Gadd45b* expression was weak; however, it increased during development (Figs. 1E–G, 3, 4 and 5D). Although *Gadd45b* expression was seen throughout the neocortex, regional differences were observed for *Gadd45b* expression (Figs. 1E–G, 3, 4 and 5D). In contrast to *Gadd45a* and *Gadd45g*, many *Gadd45b*-positive cells were seen in the PFC, posterior parietal cortex (PP), and temporal cortex, even in the adult brain (Fig. 3). Many *Gadd45b*-positive cells were also seen in the insula (Is) and claustrum (Cl) (Fig. 4). These brain areas correspond to regions that are highly evolved in the primate brain (Kowian, 1999; Bauernfeind et al., 2013).

Differential *Gadd45* expression in the rodent and primate neocortex

In the mouse brain, region-specific *Gadd45* expression became evident after the postnatal stage and expression increased during the first 2 weeks of postnatal life (Figs. 6 and 7). Since P0 marmosets are more mature than P0 mice, and the neonatal marmoset cortex roughly corresponds to the cortex of P14 mice (Mashiko et al., 2012) and P0 mouse cortex roughly corresponds to embryonic marmoset cortex, we only mention P14 and adult mouse brains for comparison with developing postnatal marmoset brains below.

Gadd45 expression in the marmoset differed to *Gadd45* expression in the mouse. In the mouse neocortex (Figs. 6 and 7), clear *Gadd45a* expression was only seen in the deep layer, and its expression continued in the adult brain (Fig. 6B, C). Conversely, *Gadd45a*-positive cells were sparsely distributed in layers II–VI of the marmoset neocortex (Figs. 1A, B and 2A–C, E, F). *Gadd45g* expression reduced over time in

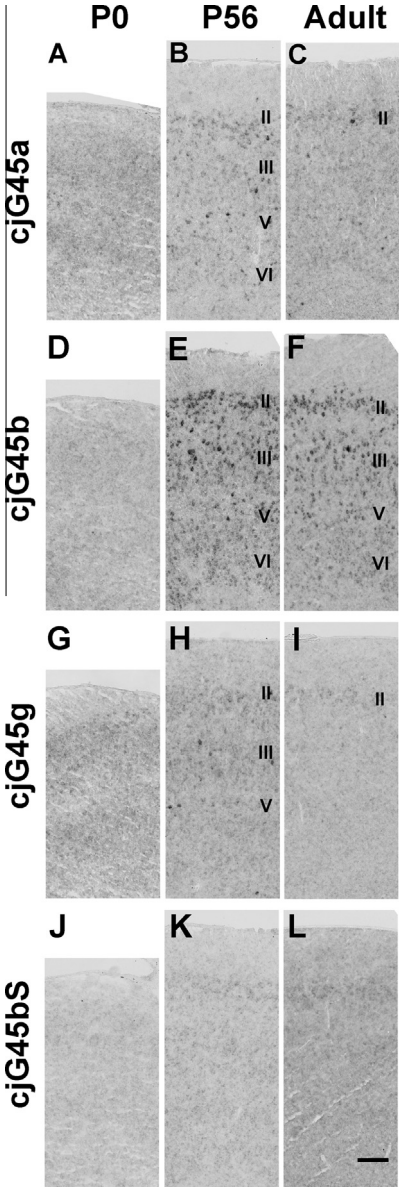

Fig. 10. *In situ* hybridization of the marmoset entorhinal cortex with antisense probes for *Gadd45*a (A–C), *Gadd45b* (D–F) and *Gadd45g* (G–I) and a sense probe for *Gadd45b* (J–L) at P0 (A, D, G, J), P56 (B, E, H, K) and adulthood (C, F, I, L). Scale bar = 500 μm.

the marmoset (Fig. 1I–K), but its expression persisted in the adult mouse brain (Fig. 6J, K). Although the number of *Gadd45b* expressing cells increased in the marmoset cortex, numbers were constant, or decreased, during development in the mouse and there were no significant differences in *Gadd45b* expression between cortical areas (Figs. 6F, G and 7D).

To verify whether expression differences between the mouse and marmoset brain were due to differences in cell types expressing *Gadd45*, we performed double staining using *in situ* hybridization and immunohistochemical analysis with NeuN, a neuronal marker. Although the expression patterns of *Gadd45* in marmoset and mouse brains were diverse, there were no significant differences in the relative ratio of NeuN-positive neurons to *Gadd45*-positive cells in the marmoset and mouse brains (Fig. 8). Thus, it seems that NeuN-positive neurons are the major *Gadd45*-expressing cells in both the mouse and marmoset brain, and that diverse *Gadd45* expression is not due to the differences in the proportion of neurons and glia.

Transiently strong *Arc* expression in the developing marmoset neocortex

Developmental regional differences in *Gadd45b* expression in the neocortex were only seen in the marmoset brain, not the mouse brain, suggesting the possibility that diverse *Gadd45b* expression results in areal differences in the neocortex. However, it has been shown that *Gadd45* expression may be altered by neural activity (Ma et al., 2009), therefore, we suspected that these temporal and regional differences in *Gadd45b* expression may be caused by fluctuations in neuronal activity, not by intrinsic areal diversity. To address this possibility we examined expression patterns of an immediate early gene, Arc, as a marker for neuronal activity (Guzowski et al., 1999) and compared *Arc* expression to *Gadd45b* expression.

Arc expression was observed in the entire neocortex of the marmoset brain during the neonatal stage (Fig. 9A–C). In general, its expression increased postnatally, reached a maximum during the juvenile stage, and decreased during adulthood (Fig. 9I). In contrast to *Gadd45b*, there was no clear developmental increase in *Arc* expression in the marmoset neocortex, suggesting that region-specific *Gadd45b* expression is not due to neuronal activity. In the mouse neocortex, *Arc* and *Gadd45b* expression was relatively constant between the juvenile and adult stage (Fig. 9J).

Less diverse *Gadd45* expression in hippocampal areas in the postnatal marmoset and mouse brain

In contrast to the neocortex, *Gadd45* expression appeared to be more conserved in the hippocampal areas in the marmoset and mouse brain.

In the postnatal marmoset brain, *Gadd45a* showed weak expressions in layer II–VI of the entorhinal cortex (Ent) and hippocampus (Hp), and this expression was reduced but maintained during adulthood (Figs. 10B, C, 11A–C and 12A–C). Weak *Gadd45a* expression was also seen in the mouse Ent (Fig. 13B, C) and Hp (Fig. 14B, C). In the mouse Ent, *Gadd45a* expression was seen in both deep and upper layers of the cortex.

Strong *Gadd45b* expression was seen in both marmoset and mouse Ent (Figs. 10E, F and 13E, F) and Hp (Figs. 11D–F, 12D–F and 14E, F).

Although expression levels were different, developmental reduction in *Gadd45g* expression was also seen in both marmoset and mouse Ent (Figs. 10H, I and 13H, I) and Hp (Figs. 11G–I, 12G–I and 14H, I).

Arc expression was broadly seen in both marmoset and mouse Ent and Hp (Fig. 15).

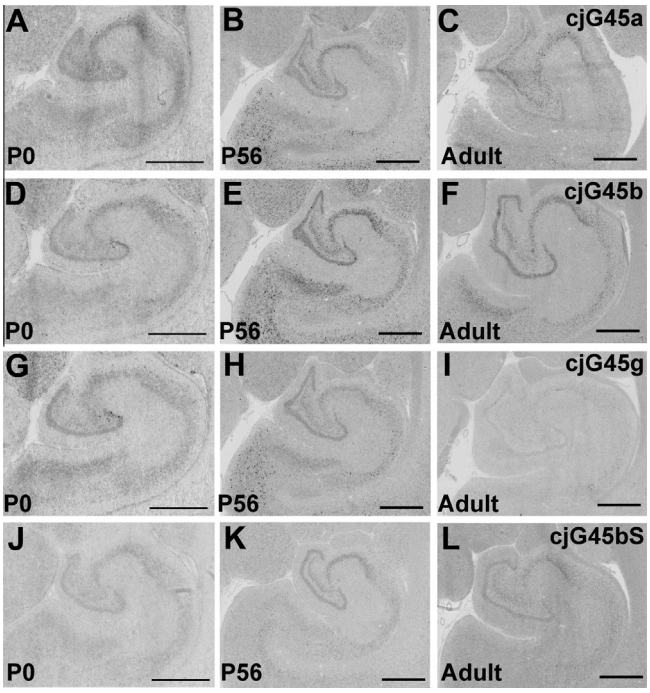

Fig. 11. *In situ* hybridization of marmoset hippocampal areas with antisense probes for *Gadd45*a (A–C), *Gadd45b* (D–F), and *Gadd45g* (G–I) and a sense probe for *Gadd45b* (J–L) at P0 (A, D, G, J), P56 (B, E, H, K) and adulthood (C, F, I, L). Scale bar = 500 μm.

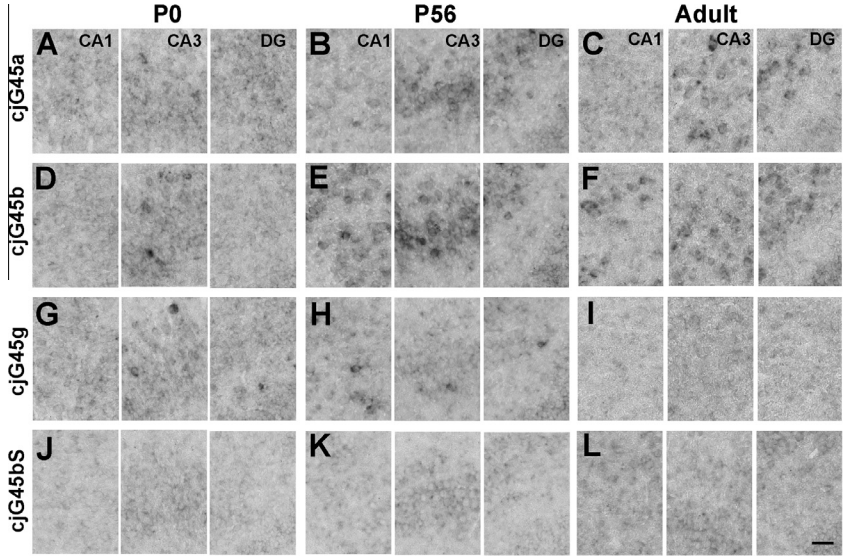

Fig. 12. High-magnification views of the developing marmoset hippocampus. *In situ* hybridization of marmoset hippocampal areas with antisense probes for *Gadd45*a (A–C), *Gadd45b* (D–F), and *Gadd45g* (G–I) and a sense probe for *Gadd45b* (J–L) at P0 (A, D, G, J), P56 (B, E, H, K) and adulthood (C, F, I, L). Scale bar = 50 μm. DG, dentate gyrus.

Conserved *Gadd45* expression in the embryonic mouse and marmoset brain

To conclude our investigation, we examined *Gadd45* expression in the embryonic marmoset brain. Although no clear *Gadd45a* or *Gadd45b* expression was observed in GW11–12 marmoset neocortex, strong *Gadd45g* expression was seen in the ventricular zone (VZ) and subventricular zone (SVZ) of the developing neocortex (Fig. 16A–F). *Gadd45g*-expressing cells were sparsely distributed in these layers. Some *Gadd45g*-positive cells were Tbr2-positive (Fig. 16D), indicating that some populations of *Gadd45g*-positive cells were intermediate neuronal progenitors (Kelava et al., 2012).

As in the marmoset brain, strong *Gadd45g* expression was seen in the VZ of the mouse neocortex, which was consistent with a previous report (Kaufmann et al., 2011). Cells strongly expressing *Gadd45g* were sparsely

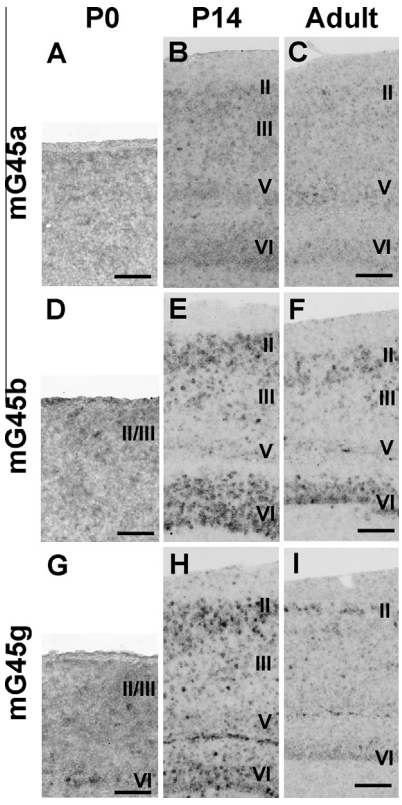

Fig. 13. *Gadd45a* (A–C), *Gadd45b* (D–F), and *Gadd45g* (G–I) expressions in mouse entorhinal cortex at P0 (A, D, G), P14 (B, E, H) and adulthood (C, F, I). Scale bars = 200 μm (A, D, G) and 500 μm (C, F, I).

distributed in SVZ of the embryonic mouse neocortex (Fig. 16I). In contrast, no clear region-specific *Gadd45a* or *Gadd45b* expression was seen in the mouse neocortex (Fig. 16G, H), a finding that was similar to what we observed in the marmoset brain.

DISCUSSION

Temporal differences in *Gadd45* expression

Each *Gadd45* gene exhibited distinct spatial and temporal patterns of expression in the marmoset brain. *Gadd45g* was strongly expressed in the VZ and SVZ during the embryonic stage, but its expression rapidly decreased in the postnatal brain. In contrast to *Gadd45g*, neither *Gadd45a* nor *Gadd45b* was clearly expressed in marmoset embryos. *Gadd45a* expression was detected during the neonatal stage, but its expression was reduced during subsequent development. In contrast, *Gadd45b* expression increased during postnatal development. In some cortical areas, high *Gadd45b* expression was maintained in the adult brain. Co-staining with NeuN revealed that the majority of postnatal *Gadd45*-expressing cells were mature neurons. Thus, it appears that *Gadd45g*, *Gadd45a*, and *Gadd45b* expression is associated with distinct stages of neuronal development in the marmoset brain.

Although the molecular mechanisms controlling cell growth arrest or active DNA demethylation remain unknown, it has been suggested that there is some functional redundancy in the Gadd45 family. Functional blocking studies in zebrafish embryos revealed that knockdown of all Gadd45 members is necessary to reduce DNA demethylation of an injected artificially-methylated DNA fragment, suggesting that Gadd45 members have redundant roles in DNA demethylation (Rai et al., 2008). Knockdown of *Gadd45a* or *Gadd45g* expression in *Xenopus* embryos blocked neural differentiation, and knockdown of both genes synergistically enhanced the phenotype (Kaufmann and Niehrs, 2011). Loss-of-function analyses in mice revealed that both *Gadd45a* and *Gadd45b* are involved in neurite outgrowth (Ma et al., 2009; Sarkisian and Siebzehnrubl, 2012). Thus, it seems that all Gadd45 members have some mutual functions in growth arrest and active demethylation. On the other hand, members of the Gadd45 family play multiple roles not only in active DNA demethylation (Barreto et al., 2007; Niehrs and Schäfer, 2012) but also in mitogen-activated protein kinase (MAPK) signaling (Chi et al., 2004; Gierl et al., 2012; Warr et al., 2012). Single knockout of the *Gadd45b* gene is sufficient to induce abnormal phenotypes of hippocampal-dependent long-term memory (Leach et al., 2012; Sultan et al., 2012), even though *Gadd45a* and *Gadd45g* are also expressed in the mouse hippocampus. This finding suggests the

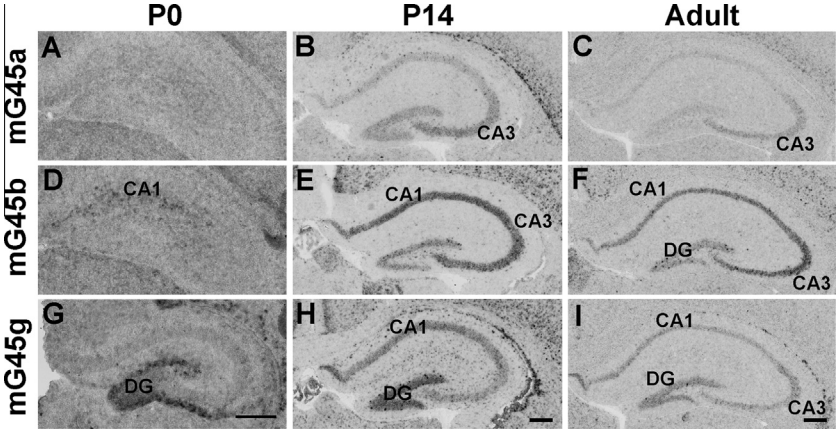

Fig. 14. *Gadd45a* (A–C), *Gadd45b* (D–F), and *Gadd45g* (G–I) expressions in mouse hippocampus at P0 (A, D, G), P14 (B, E, H) and adulthood (C, F, I). Scale bars = 500 μm (H, I) and 200 μm (G).

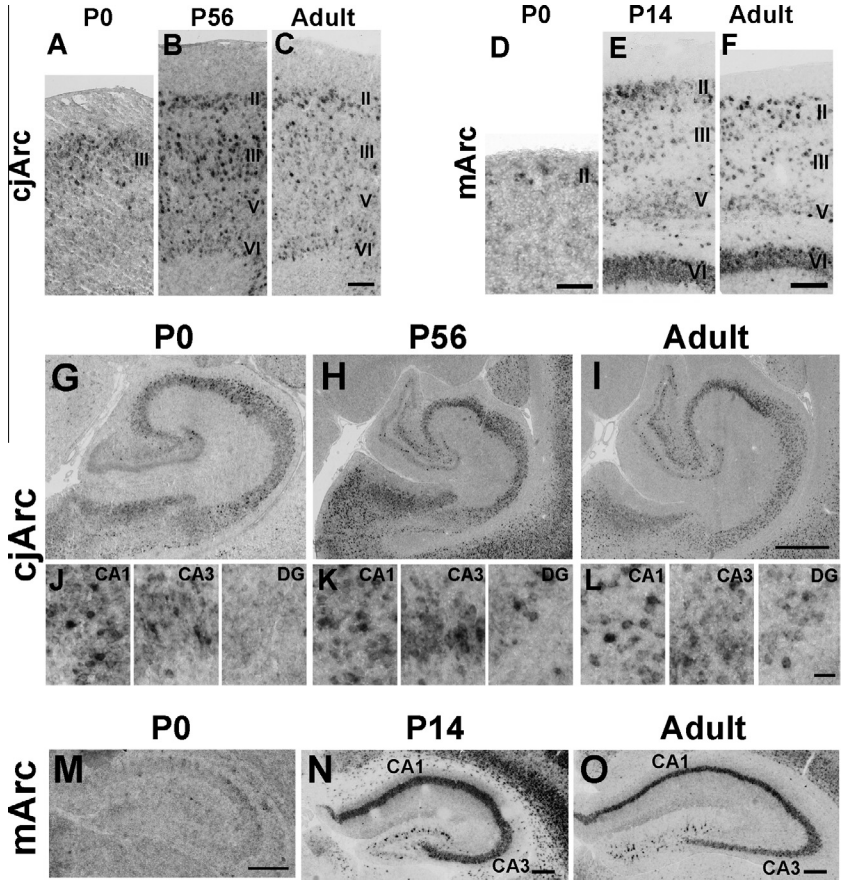

Fig. 15. *Arc* expression in marmoset entorhinal cortex (A–C) and hippocampus (G–L), and mouse entorhinal cortex (D–F) and hippocampus (M–O). J, K and L are high-magnification views of G, H and I, respectively. Scale bars = 500 μm (I, N, O), 200 μm (F, M), 100 μm (C, D), and 10 μm (L).

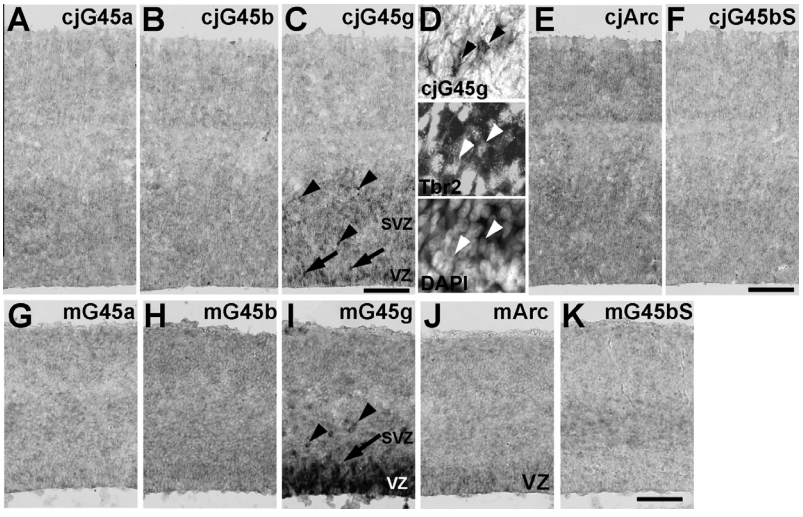

Fig. 16. *Gadd45s* and *Arc* expressions in embryonic brains from gestational week 12 marmosets (A–F) and embryonic day 16 mice (G–K) and co-stained with *Gadd45g* probe and specific antibody for Tbr2 (D). Arrows and arrowheads indicate *Gadd45g*-expressing cells in the VZ and SVZ, respectively (C, I). *Gadd45g*-positive cells co-express Tbr2, an intermediate neuronal progenitor marker (D, arrowheads). cjG45bS, marmoset Gadd45b sense probe; mG45bS, mouse Gadd45b sense probe. SVZ, subventricular zone; VZ, ventricular zone. Scale bars = 200 μm (F) and 100 μm (K).

possibility of functional differences among *Gadd45* forms. Thus, further analysis is needed to explore the functional similarities and differences between Gadd45 family members: for example, in their interactions with other components or signaling cascades. Such studies could promote our understanding of the biological significance of temporal changes in *Gadd45* expression.

Cortical areal differences in *Gadd45* expression

Gadd45b expressions increased during postnatal development, and high *Gadd45b* expression persisted in the adulthood in some cortical areas: the PFC, Is, PP, TE, and PH maintained high levels of expression compared to primary areas, such as the M1, S1 and V1. Interestingly, a comparative imaging study between monkeys and humans revealed that these association areas corresponded to cortical areas that are particularly evolved in the primate brain i.e., the prefrontal, parietal, and temporal lobes (Hill et al., 2010). Although there is no evidence indicating region-specific methylation status in primate neocortex yet, it may be possible that methylation states differ even within the neocortex, and that diverse methylation states result in different levels of plasticity in various cortical areas.

As the immediate early gene *Arc* showed a different time course of expression than *Gadd45b* (*Arc* expression peaked in the juvenile stage and decreased in adulthood) and there were no clear regional differences, *Gadd45b* expression in the adult brain may be induced by an activity-independent internal state of cortical plasticity, rather than by cortical neuronal activity-dependent mechanisms. Thus, it may be possible that some brain areas are better able to induce plastic changes by responding to environmental factors and that Gadd45b induces this plasticity by actively demethylating DNA, resulting in the modulation of dendritic spines or axonal branch formation. However, comprehensive studies analyzing methylation status of promoter regions of various genes or overall methylation in brain tissues in each cortical area should be necessary to verify the hypothesis.

Species differences in *Gadd45* expression

In this study, we identified several differences in *Gadd45* expression between rodents and primates. Although the overall percentage of *Gadd45*-expressing cells was higher in mice, the ratio of *Gadd45*-expressing cells decreased during mouse development. In contrast, the number of *Gadd45b*-expressing cells increased during marmoset development, suggesting the possibility that the primate brain may have more *Gadd45b*-dependent functional plasticity than the rodent brain. *Gadd45a* expression was seen in layer VI of the mouse neocortex, whereas weakly *Gadd45a*-positive cells were observed in layer IV, and were only sparsely distributed in all layers of the neocortex. Considering that Gadd45 family members have some functional redundancy, these species differences in *Gadd45a* expression may be partly compensated for by *Gadd45b* expression. Alternatively, diverse *Gadd45a* expression may have specific functions in primate cortical development. Differential *Gadd45* expression was more diverse in the neocortex than the allocortex, suggesting the possibility that *Gadd45* expression is related to areal diversity and evolution of the primate neocortex. Although no significant difference was detected in embryonic *Gadd45g* expression between mouse and marmoset, it has been recently suggested that differential *Gadd45g* expression in the SVZ underlines evolutionary expansion of the human neocortex (LaMonica et al., 2012; Geschwind and Rakic, 2013; Sun and Hevner, 2014). Functional analysis using electroporation or a viral vector system could elucidate the functional significance of these species differences in *Gadd45* expression.

CONCLUSION

Gene expression analysis revealed that there are distinct spatial and temporal patterns of *Gadd45a*, *Gadd45b* and *Gadd45g* expression in the developing marmoset brain. In particular, *Gadd45b* showed area-specific high-level expression in the associated cortical areas of the adult marmoset brain, in contrast to the mouse brain. Most *Gadd45*-expressing cells were NeuN-positive neurons. Thus, these results suggest the possibility that differential *Gadd45* expression affects neurons, contributing cortical evolution and diversity.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest.

ROLE OF AUTHORS

Conceived and designed the experiments: EM, AI. Performed the experiments: EM, SN, MO. Analyzed the data: EM, SN. Wrote the paper: EM. Study supervision: AI.

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