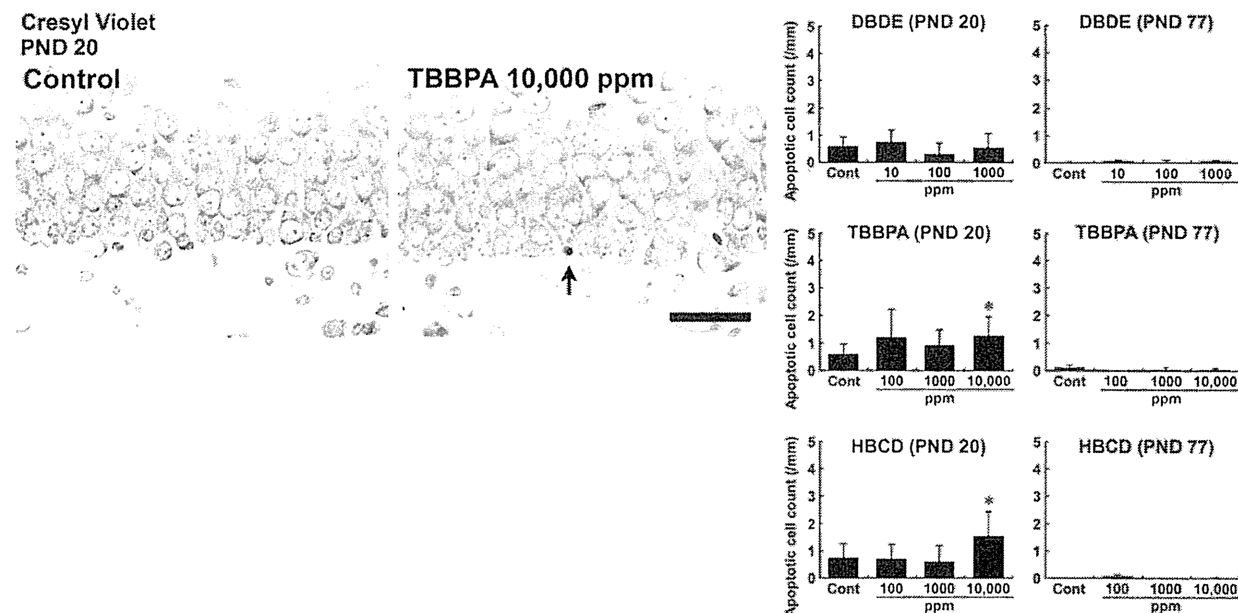

Fig. 5 Distribution of apoptotic cells in the dentate SGZ in rat offspring after maternal exposure to DBDE, TBBPA, or HBCD on both PND 20 and PND 77. Apoptotic cells were detected as apoptotic bodies with Cresyl violet staining. Note the apoptotic body (*arrow*) in an animal exposed to 10,000 ppm TBBPA (*right*) compared with the control animal without apoptotic bodies (*left*) at PND 20. *Bar* = 50 μm. The graph shows the number of apoptotic bodies/unit length (mm) of the SGZ of bilateral hemispheres at both PND 20 and PND 77. Values were expressed as mean + SD. $N = 5$–14 in each group. $^{*}P < 0.05$ versus controls (0 ppm) by Dunnett's test or Dunnett-type rank-sum test

appeared to be due to interference with the thyroid receptor-DNA binding domain. They also reported that DBDE was found to inhibit TH-induced dendrite arborization of Purkinje cells in vitro. These results suggest the potential of DBDE to suppress TH action in the brain. Interestingly, we also found development of mild white matter hypoplasia suggestive of hypothyroidism-related effect from the dose level at 100 ppm showing no significant alterations in the serum concentrations of thyroid-related hormones (Fujimoto et al. 2011).

TBBPA exposure during the gestation period has been shown to increase fetal plasma thyroid-stimulating hormone (TSH) concentration in rats, while circulating concentrations of T_3 and thyroxine (T_4) are unaltered in dams or their offspring (Meerts et al. 1999). In a recently published one-generation reproductive toxicity study of the effects of dietary TBBPA, auditory response effects, typically related to developmental hypothyroidism, were observed in offspring at the adult stage in association with serum TH changes (van der Ven et al. 2008). In the present study, however, no obvious thyrotoxic (including thyroid weight) changes were noted in the offspring except for a dose-unrelated decrease in serum T_3 concentrations at 100 and 1,000 ppm on PND 20 (Saegusa et al. 2009; Online Resource 1, Table s1). These results suggest that there was no obvious developmental hypothyroidism caused by TBBPA in the present study. With regard to the neurobehavioral effects of TBBPA, spatial learning was found to be impaired in the offspring of rats exposed to TBBPA at 250 mg/kg body weight/day (Hass and Wamberg 2002). Acute treatment of mice with TBBPA from the dose level as low as 0.1 mg/kg body weight revealed neurobehavioral effects and accumulation of TBBPA in the brain area (Nakajima et al. 2009), suggesting a possibility of direct effect of TBBPA on neuronal development. In the present study, we observed a dose-related increase in the number of reelin-expressing cells at 1,000 ppm (86.8–202.1 mg/kg body weight/day) and higher. Therefore, we suggest that TBBPA-induced brain changes may be a direct effect of TBBPA to the developing brain in the experimental conditions presented here. We have recently found a similar increase in reelin-expressing interneurons in the rat dentate hilus at the end of developmental exposure to acrylamide (Ogawa et al. 2011), a compound that has been proven to have no effects on gene expression, neurochemistry, hormone levels, or histopathology in the hypothalamus–pituitary–thyroid axis of rats (Bowyer et al. 2008).

HBCD is known to act as an inducer of cytochrome P450 (Germer et al. 2006) and uridine diphosphate-glucuronosyltransferase (van der Ven et al. 2006) in rats. This biological potential may be linked to the facilitation of TH metabolism by this enzyme to cause an increase in serum

TSH concentrations through the suppression of negative feedback by HBCD at 10,000 ppm on PND 20 in the present study. In the present study, a decrease in serum T_3 concentration and increase in serum TSH concentration on PND 20 after exposure to HBCD at 10,000 ppm suggest a development of mild hypothyroidism (Saegusa et al. 2009; Online Resource 1, Table s1). The effect was sustained until the adult stage, possibly because of the strong propensity for bioaccumulation as reported by others (Birnbaum and Staskal 2004; Chengelis 2001). As a hypothyroidism-related effect, HBCD reduced density of oligodendrocytes in the brain at 10,000 ppm (803.2–2,231.3 mg/kg body weight/day) in our previous study using the same study samples presented here (Saegusa et al. 2009). We also observed an increase in the number of NeuN-positive cells in the dentate hilus and increased apoptosis in the SGZ suggestive of the signature of impaired neuronal development after exposure to 10,000 ppm HBCD, although increased reelin-positive cells were only seen at the middle dose (1,000 ppm). Therefore, the effect of HBCD on neuronal development was observed at least at 10,000 ppm, similar to the effect on the number of oligodendrocytes in relation with developmental hypothyroidism. On the other hand, Yamada-Okabe et al. (2005) have shown a direct action of HBCD on TH receptors in a reporter gene assay. Disruption of TH-mediated neuronal development was also observed with HBCD (Ibhazehiebo et al. 2011b, c), as well as DBDE (Ibhazehiebo et al. 2011a), suggestive of the possible direct action on the developing brain.

Exposure to BFRs did not increase the number of cells expressing EphA5 or Tacr3 in the hippocampal CA1 region, which have been found to increase in number during developmental hypothyroidism (Saegusa et al. 2010a), except for a slight increase in EphA5-expressing cells on PND 20 after DBDE exposure. This suggests that these molecules could be considered to be less sensitive biomarkers for the detection of aberration in neuronal development such as neuronal mismigration compared with reelin.

In conclusion, we found that the BFRs examined here had developmental neuronal effect from doses of 100 ppm for DBDE, 1,000 ppm for TBBPA, and at least at 10,000 ppm for HBCD. A direct effect of DBDE and TBBPA on neuronal development may be considered under the present experimental conditions. However, the effect of hypothyroidism may also be operated at least at the highest dose of DBDE as well as that of HBCD. The effect of these BFRs on neurogenesis and following neuronal migration in the dentate gyrus may have occurred in the exposure period, while the biological significance of the increase in mature neuronal populations at the later stages should be further assessed. The results from this study suggest that reelin-expressing cells in the hippocampal dentate hilus may be used for the assessment of the effects of developmental neurotoxicants that can disrupt the migration and positioning of developing neurons following impairment of neurogenesis.

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References

Akaike M, Kato N, Ohno H, Kobayashi T (1991) Hyperactivity and spatial maze learning impairment of adult rats with temporary neonatal hypothyroidism. Neurotoxicol Teratol 13:317–322

Alvarez-Dolado M, Ruiz M, Del Río JA, Alcántara S, Burgaya F, Sheldon M, Nakajima K, Bernal J, Howell BW, Curran T, Soriano E, Muñoz A (1999) Thyroid hormone regulates reelin and dab1 expression during brain development. J Neurosci 19:6979–6993

Bansal R, Zoeller RT (2008) Polychlorinated biphenyls (Aroclor 1254) do not uniformly produce agonist actions on thyroid hormone responses in the developing rat brain. Endocrinology 149:4001–4008

Birnbaum LS, Staskal DF (2004) Brominated flame retardants: cause for concern? Environ Health Perspect 112:9–17

Bowyer JF, Latendresse JR, Delongchamp RR, Muskhelishvili L, Warbritton AR, Thomas M, Tareke E, McDaniel LP, Doerge DR (2008) The effects of subchronic acrylamide exposure on gene expression, neurochemistry, hormones, and histopathology in the hypothalamus-pituitary-thyroid axis of male Fischer 344 rats. Toxicol Appl Pharmacol 230:208–215

Chengelis CP (2001) A 90-day oral (gavage) toxicity study of HBCD in rats. WIL-186012. Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association, Arlington, VA

Comer CP, Norton S (1982) Effects of perinatal methimazole exposure on a developmental test battery for neurobehavioral toxicity in rats. Toxicol Appl Pharmacol 63:133–141

Cooper MA, Crockett DP, Nowakowski RS, Gale NW, Zhou R (2009) Distribution of EphA5 receptor protein in the developing and adult mouse nervous system. J Comp Neurol 514:310–328

D'Arcangelo G, Nakajima K, Miyata T, Ogawa M, Mikoshiba K, Curran T (1997) Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. J Neurosci 17:23–31

de Wit CA (2002) An overview of brominated flame retardants in the environment. Chemosphere 46:583–624

Ema M, Fujii S, Hirata-Koizumi M, Matsumoto M (2008) Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats. Reprod Toxicol 25:335–351

Eriksson P, Fischer C, Wallin M, Jakobsson E, Fredriksson A (2006) Impaired behaviour, learning and memory, in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). Environ Toxicol Pharmacol 21:317–322

Frotscher M, Haas CA, Förster E (2003) Reelin controls granule cell migration in the dentate gyrus by acting on the radial glial scaffold. Cereb Cortex 13:634–640

Fujimoto H, Woo GH, Inoue K, Takahashi M, Hirose M, Nishikawa A, Shibutani M (2011) Impaired oligodendroglial development by decabromodiphenyl ether in rat offspring after maternal exposure from mid-gestation through lactation. Reprod Toxicol 31:86–94

Germer S, Piersma AH, van der Ven L, Kamyschnikow A, Fery Y, Schmitz HJ, Schrenk D (2006) Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats. Toxicology 218:229–236

Gong C, Wang TW, Huang HS, Parent JM (2007) Reelin regulates neuronal progenitor migration in intact and epileptic hippocampus. J Neurosci 27:1803–1811

Goodman JE (2009) Neurodevelopmental effects of decabromodiphenyl ether (BDE-209) and implications for the reference dose. Regul Toxicol Pharmacol 54:91–104

Hass U, Wamberg C (2002) Developmental neurotoxicity study of the brominated flame retardant tetrabromobisphenol A in rats. Reprod Toxicol 16:412 Abstract for 30th conference of the European teratology society, Hanover, 7th–11th September 2002

Hoareau C, Hazane F, Le Pen G, Krebs MO (2006) Postnatal effect of embryonic neurogenesis disturbance on reelin level in organotypic cultures of rat hippocampus. Brain Res 1097:43–51

Hopert AC, Beyer A, Frank K, Strunck E, Wunsche W, Vollmer G (1998) Characterization of estrogenicity of phytoestrogens in an endometrial-derived experimental model. Environ Health Perspect 106:581–586

Houser CR (2007) Interneurons of the dentate gyrus: an overview of cell types, terminal fields and neurochemical identity. Prog Brain Res 163:217–232

Ibhazehiebo K, Iwasaki T, Kimura-Kuroda J, Miyazaki W, Shimokawa N, Koibuchi N (2011a) Disruption of thyroid hormone receptor-mediated transcription and thyroid hormone-induced Purkinje cell dendrite arborization by polybrominated diphenyl ethers. Environ Health Perspect 119:168–175

Ibhazehiebo K, Iwasaki T, Xu M, Shimokawa N, Koibuchi N (2011b) Brain-derived neurotrophic factor (BDNF) ameliorates the suppression of thyroid hormone-induced granule cell neurite extension by hexabromocyclododecane (HBCD). Neurosci Lett 493:1–7

Ibhazehiebo K, Iwasaki T, Shimokawa N, Koibuchi N (2011c) 1,2,5,6,9,10-αHexabromocyclododecane (HBCD) impairs thyroid hormone-induced dendrite arborization of Purkinje cells and suppresses thyroid hormone receptor-mediated transcription. Cerebellum 10:22–31

Kitamura S, Kato T, Iida M, Jinno N, Suzuki T, Ohta S, Fujimoto N, Hanada H, Kashiwagi K, Kashiwagi A (2005) Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. Life Sci 76:1589–1601

Kundakovic M, Chen Y, Guidotti A, Grayson DR (2009) The reelin and GAD67 promoters are activated by epigenetic drugs that facilitate the disruption of local repressor complexes. Mol Pharmacol 75:342–354

Lavado-Autric R, Ausó E, García-Velasco JV, Arufe Mdel C, Escobar del Rey F, Berbel P, Morreale de Escobar G (2003) Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. J Clin Invest 111:1073–1082

Lilienthal H, Verwer CM, van der Ven LT, Piersma AH, Vos JG (2008) Exposure to tetrabromobisphenol A (TBBPA) in Wistar rats: neurobehavioral effects in offspring from a one-generation reproduction study. Toxicology 246:45–54

Lussier AL, Caruncho HJ, Kalynchuk LE (2009) Repeated exposure to corticosterone, but not restraint, decreases the number of reelin-positive cells in the adult rat hippocampus. Neurosci Lett 460:170–174

Masutomi N, Shibutani M, Takagi H, Uneyama C, Takahashi N, Hirose M (2003) Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. Toxicology 192:149–170

Meerts IATM, Assink Y, Cenijn PH, Weijers BM, van den Berg JHJ, Bergman Å, Koeman JH, Brouwer A (1999) Distribution of the flame retardant tetrabromobisphenol A in pregnant and fetal rats and effect on thyroid hormone homeostasis. Organohalogen Compd 40:375–378

Nakajima A, Saigusa D, Tetsu N, Yamakuni T, Tomioka Y, Hishinuma T (2009) Neurobehavioral effects of tetrabromobisphenol A, a brominated flame retardant, in mice. Toxicol Lett 189:78–83

Numachi Y, Yoshida S, Yamashita M, Fujiyama K, Toda S, Matsuoka H, Kajii Y, Nishikawa T (2007) Altered EphA5 mRNA expression in rat brain with a single methamphetamine treatment. Neurosci Lett 424:116–121

Nuñez JL, McCarthy MM (2004) Cell death in the rat hippocampus in a model of prenatal brain injury: time course and expression of death-related proteins. Neuroscience 129:393–402

Ogawa B, Ohishi T, Wang L, Takahashi M, Taniai E, Hayashi H, Mitsumori K, Shibutani M (2011) Disruptive neuronal development by acrylamide in the hippocampal dentate hilus after developmental exposure in rats. Arch Toxicol 85:987–994

Porterfield SP (2000) Thyroidal dysfunction and environmental chemicals—potential impact on brain development. Environ Health Perspect 108(Suppl 3):433–438

Rice DC, Reeve EA, Herlihy A, Zoeller RT, Thompson WD, Markowski VP (2007) Developmental delays and locomotor activity in the C57BL6/J mouse following neonatal exposure to the fully-brominated PBDE, decabromodiphenyl ether. Neurotoxicol Teratol 29:511–520

Saegusa Y, Fujimoto H, Woo GH, Inoue K, Takahashi M, Mitsumori K, Hirose M, Nishikawa A, Shibutani M (2009) Developmental toxicity of brominated flame retardants, tetrabromobisphenol A and 1,2,5,6,9,10-hexabromocyclododecane, in rat offspring after maternal exposure from mid-gestation through lactation. Reprod Toxicol 28:456–467

Saegusa Y, Woo G-H, Fujimoto H, Inoue K, Takahashi M, Hirose M, Igarashi K, Kanno J, Mitsumori K, Nishikawa A, Shibutani M (2010a) Gene expression profiling and cellular distribution of molecules with altered expression in the hippocampal CA1 region after developmental exposure to anti-thyroid agents in rats. J Vet Med Sci 72:187–195

Saegusa Y, Woo G-H, Fujimoto H, Kemmochi S, Shimamoto K, Hirose M, Mitsumori K, Nishikawa A, Shibutani M (2010b) Sustained production of Reelin-expressing interneurons in the hippocampal dentate hilus after developmental exposure to anti-thyroid agents in rats. Reprod Toxicol 29:407–414

Salthun-Lassalle B, Traver S, Hirsch EC, Michel PP (2005) Substance P, neurokinins A and B, and synthetic tachykinin peptides protect mesencephalic dopaminergic neurons in culture via an activity-dependent mechanism. Mol Pharmacol 68:1214–1224

Schoonover CM, Seibel MM, Jolson DM, Stack MJ, Rahman RJ, Jones SA, Mariash CN, Anderson GW (2004) Thyroid hormone regulates oligodendrocyte accumulation in developing rat brain white matter tracts. Endocrinology 145:5013–5020

Shibutani M, Woo G-H, Fujimoto H, Saegusa Y, Takahashi M, Inoue K, Hirose M, Nishikawa A (2009) Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents. Reprod Toxicol 28:297–307

Smith PW, Dawson LA (2008) Neurokinin 3 (NK3) receptor modulators for the treatment of psychiatric disorders. Recent Pat CNS Drug Discov 3:1–15

Stein T, Cosimo E, Yu X, Smith PR, Simon R, Cottrell L, Pringle MA, Bell AK, Lattanzio L, Sauter G, Lo Nigro C, Crook T, Machesk LM, Gusterson BA (2010) Loss of reelin expression in breast cancer is epigenetically controlled and associated with poor prognosis. Am J Pathol 177:2323–2333

Tseng LH, Li MH, Tsai SS, Lee CW, Pan MH, Yao WJ, Hsu PC (2008) Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and hepatic enzyme activity in male mouse offspring. Chemosphere 70:640–647

van der Ven LT, Verhoef A, van de Kuil T, Slob W, Leonards PE, Visser TJ, Hamers T, Herlin M, Håkansson H, Olausson H, Piersma AH, Vos JG (2006) A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats. Toxicol Sci 94:281–292

van der Ven LT, Van de Kuil T, Verhoef A, Verwer CM, Lilienthal H, Leonards PE, Schauer UM, Cantón RF, Litens S, De Jong FH, Visser TJ, Dekant W, Stern N, Håkansson H, Slob W, Van den Berg M, Vos JG, Piersma AH (2008) Endocrine effects of tetrabromobisphenol-A (TBBPA) in Wistar rats as tested in a one-generation reproduction study and a subacute toxicity study. Toxicology 245:76–89

Viberg H, Fredriksson A, Jakobsson E, Orn U, Eriksson P (2003) Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. Toxicol Sci 76:112–120

Viberg H, Fredriksson A, Eriksson P (2007) Changes in spontaneous behaviour and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabrominated diphenyl ether (PBDE 209). Neurotoxicology 28:136–142

Vijverberg HP, van den Berg M (2004) Re: Viberg H et al. (2003) Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. Toxicol Sci 76, 112–120. Toxicol Sci 79:205–206

Yamada-Okabe T, Sakai H, Kashima Y, Yamada-Okabe H (2005) Modulation at a cellular level of the thyroid hormone receptor-mediated gene expression by 1,2,5,6,9,10-hexabromocyclododecane (HBCD), 4,4′-diiodobiphenyl (DIB), and nitrofen (NIP). Toxicol Lett 155:127–133

Zhang L, Blomgren K, Kuhn HG, Cooper-Kuhn CM (2009) Effects of postnatal thyroid hormone deficiency on neurogenesis in the juvenile and adult rat. Neurobiol Dis 34:366–374

Zhang W, Cai Y, Sheng G, Chen D, Fu J (2011) Tissue distribution of decabrominated diphenyl ether (BDE-209) and its metabolites in sucking rat pups after prenatal and/or postnatal exposure. Toxicology 283:49–54

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Developmental Exposure to Manganese Chloride Induces Sustained Aberration of Neurogenesis in the Hippocampal Dentate Gyrus of Mice

Liyun Wang,* Takumi Ohishi,* Ayako Shiraki,* Reiko Morita,*,† Hirotoshi Akane,*,† Yoshiaki Ikarashi,‡ Kunitoshi Mitsumori,* and Makoto Shibutani*,[1]

*Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, Fuchu-shi, Tokyo 183-8509, Japan; †Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, Gifu-shi, Gifu 501-1193, Japan; and ‡Division of Environmental Chemistry, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan

[1]To whom correspondence should be addressed at Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan. Fax: +81-42-367-5771. E-mail: mshibuta@cc.tuat.ac.jp.

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The effect of exogenously administered manganese (Mn) on developmental neurogenesis in the hippocampal dentate gyrus was examined in male mice after maternal exposure to $MnCl_2$ (0, 32, 160, or 800 ppm as Mn in diet) from gestational day 10 to day 21 after delivery on weaning. Immunohistochemistry was performed to monitor neurogenesis and interneuron subpopulations on postnatal days (PNDs) 21 and 77 (adult stage). Reelin-synthesizing γ-aminobutyric acid (GABA)ergic interneurons increased in the hilus with ≥ 160 ppm on weaning to sustain to PND 77 at 800 ppm. Apoptosis in the neuroblast-producing subgranular zone increased with 800 ppm and TUC4-expressing immature granule cells decreased with 800 ppm on weaning, whereas at the adult stage, immature granule cells increased. On PND 21, transcript levels increased with *Reln* and its receptor gene *Lrp8* and decreased with *Dpysl3* coding TUC4 in the dentate gyrus, confirming immunohistochemical results. Double immunohistochemistry revealed a sustained increase of reelin-expressing and NeuN-lacking or weakly positive immature interneurons and NeuN-expressing mature neurons in the hilus through to the adult stage as examined at 800 ppm. Brain Mn concentrations increased at both PNDs 21 and 77 in all $MnCl_2$-exposed groups. These results suggest that Mn targets immature granule cells causing apoptosis and neuronal mismigration. Sustained increases in immature reelin-synthesizing GABAergic interneurons may represent continued aberration in neurogenesis and following migration to cause an excessive response for overproduction of immature granule cells through to the adult stage. Sustained high concentration of Mn in the brain may be responsible for these changes.

Key Words: manganese chloride ($MnCl_2$); hippocampal dentate gyrus; γ-aminobutyric acid (GABA)ergic interneurons; neurogenesis; reelin.

Manganese (Mn) is a trace essential element necessary for immune function, regulation of blood sugar, cellular energy, reproduction, digestion and bone growth, which also aids in the defense against free radicals (Aschner and Aschner, 2005). Excess Mn can exert serious neurotoxic effects on both humans and experimental animals (Dobson *et al.*, 2004). Exposure of mature animals and humans to Mn produces neurological disorders, also known as manganism, with symptoms similar to those of Parkinsonism (Soldin and Aschner, 2007). Experimentally, neuronal death of the nigrostriatal dopaminergic system is a typical histological feature of manganism (Kitazawa *et al.*, 2005). Estimated safe and adequate daily dietary intake (ESADDI) of Mn has been estimated to be approximately 0.6 mg/day at 7–12 months of age, 1.2 mg/day at 1–3 years of age, 1.5 mg/day at 4–8 years of age and 2–5 mg/day for adults (Aschner and Aschner, 2005). On the other hand, ESADDI for newborns has been estimated to be 0.003 mg/day, less than that for adults or children (Aschner and Aschner, 2005). Several reports have documented that neonates are at an increased risk of Mn-induced neurotoxicity compared with adults due to their ability to achieve higher brain Mn levels and altered brain dopamine concentrations following Mn exposure (Chandra and Shukla, 1978; Dorman *et al.*, 2000; Kontur and Fechter, 1988). However, the risk of Mn-induced neurotoxicity during brain development, both pre- and postnatally, has received relatively little attention.

The hippocampal dentate gyrus, a unique structure that can continue neurogenesis during postnatal life, is crucial for higher brain functions such as learning, memory, and motivation (Montaron *et al.*, 2004). Postnatal neurogenesis (so-called adult neurogenesis) occurs in the neuroblast-producing subgranular zone (SGZ) of the dentate gyrus from type 1 progenitor cells and produces intermediate generations in the order of type 2a, type 2b, and type 3 cells in the SGZ. The type 3 cells undergo final mitosis to differentiate into immature granule cells and then to mature granule cells (Hodge *et al.*, 2008). In addition, within the dentate gyrus, γ-aminobutyric acid (GABA)ergic interneurons play a role in

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the neuronal development and maintenance of granule cells. Reelin, an extracellular matrix glycoprotein, is expressed in these interneurons and aids in neuronal migration and ensures correct positioning (Ramos-Moreno *et al.*, 2006). Altered reelin-expression in the dentate gyrus has been reported to be a cause of some neurological diseases, such as depression and epilepsy (Gong *et al.*, 2007; Lussier *et al.*, 2009). Experimentally, developmental hypothyroidism leads to impaired neuronal migration as well as white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation (Goodman and Gilbert, 2007; Lavado-Autric *et al.*, 2003; Schoonover *et al.*, 2004). We recently found sustained aberrant increases in immature GABAergic interneurons synthesizing reelin in the hilus of the dentate gyrus until the adult stage following developmental exposure to antithyroid agents, suggestive of the reflection of disrupted neuronal migration and positioning by these reagents (Saegusa *et al.*, 2010).

Experimental Mn exposure results in a high concentration of Mn in the dentate gyrus in neonatal rats (Takeda *et al.*, 1999) and has been shown to induce neurological, cognitive, and neuropsychological effects in children (Khan *et al.*, 2011); however, histopathological evidence of the impaired brain development by excess exposure to Mn is not clear. Because neonates are at an increased risk of Mn-induced neurotoxicity, a dose-response study of developmental neurotoxicity of Mn employing brain development parameters should be conducted. In the present study, to elucidate whether exogenously administered Mn targets developing neurons causing permanent disruption of neuronal differentiation, we examined neurogenesis in terms of cell proliferation, apoptosis, and differentiation in the dentate gyrus using mice following developmental $MnCl_2$ exposure. We also analyzed distribution changes of interneurons expressing reelin or glutamic acid decarboxylase (GAD) 67 in the dentate hilus. At the end of exposure at the weaning and adult stage, serum concentrations of thyroid-related hormones and brain concentrations of Mn were also analyzed. Because lactational Mn exposure has been shown to alter the iron (Fe) status of offspring in an inverse fashion (García *et al.*, 2006), brain Fe concentrations were also measured.

MATERIALS AND METHODS

Chemicals and Animals

Manganese chloride hydrate ($MnCl_2{\cdot}xH_2O$; CAS No. 73913-06-1) containing 27.8% Mn was purchased from Sigma Chemical Co (St Louis, MO). A total of 60 pregnant Slc:ICR mice were purchased from Japan SLC Inc. (Hamamatsu, Japan) at gestation day (GD) 1 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in polycarbonate cages with wood chip bedding, maintained in an air-conditioned animal room (temperature: 24 ± 1°C; relative humidity: 55 ± 5%) with a 12-h light/dark cycle and allowed access to food and tap water (Mn concentration: 0.000 mg/l) *ad libitum*. A regular MF basal diet (Oriental Yeast Co. Ltd., Tokyo, Japan) (Mn concentration 4.84 mg/100 g basal diet) and water were given *ad libitum* throughout the experimental period. All offspring consumed a regular MF basal diet and water *ad libitum* from postnatal day (PND) 21 onwards.

Experimental Design

All procedures of this study were conducted in compliance with the "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Experiment 1. Maternal animals were randomly divided into four groups including untreated controls (Fig. 1). Fifteen dams per group were treated with 0, 32, 160, or 800 ppm of Mn in the form of $MnCl_2{\cdot}xH_2O$ mixed in the powdered basal diet from GD 10 to PND 21 (PND 0: the day of delivery). This exposure period was determined because the formation of hippocampus starts from GD 11 and neurogenesis in the SGZ is active during postnatal period from PNDs 3 to 14 in mice (Reznikov, 1991). At PND 4, the 10 dams that delivered more than eight male offspring per group were selected to cull litters randomly, leaving eight male and two female offspring. Remaining dams were sacrificed under deep ether anesthesia, and the remaining offspring were sacrificed by rapid decapitation under ether anesthesia. At PND 21, 10 dams and 30 male and 20 female offspring (three male and two female offspring per dam) per group were subjected to prepubertal necropsy. The remaining male offspring were maintained until PND 77. At PND 77, all pups were subjected to adult stage autopsy. The body weights (BWs) and food consumptions of dams and pups were determined every 3–7 days. Mortality of pups was examined daily after birth (PND 2) until PND 77.

On PNDs 21 and 77, animals were weighed and sacrificed by exsanguination from the abdominal aorta under deep anesthesia with ether. Dams were examined for uterine implantation sites at autopsy on weaning. Brain, liver, and kidneys were collected at autopsy in 10 male and 10 female offspring per group. Because neurogenesis is influenced by circulating levels of steroid hormones during the estrous cycle (Pawluski *et al.*, 2009), male offspring were selected for immunohistochemical assays, and brains from 10 males per group (one male per dam) were prepared for this purpose. Brains from 10 male offspring per group and six dams per group were subjected to estimation of Mn and Fe concentrations in the cerebellum. Remaining brains from other offspring were used for other experimental purposes.

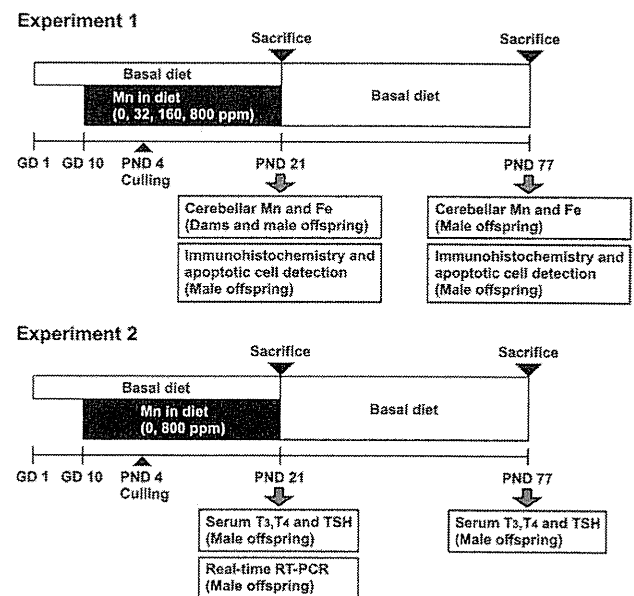

FIG. 1. Experimental design.

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At necropsies of offspring on PNDs 21 and 77, weights of the brain, liver, and kidneys were measured.

Experiment 2. To evaluate the effects of developmental exposure to $MnCl_2 \cdot xH_2O$ on the serum concentrations of thyroid-related hormones and messenger RNA (mRNA) expression in the dentate gyrus, an additional $MnCl_2$ exposure study was performed (Fig. 1). Dams were randomly divided into two groups. Fifteen dams per group were treated with 0 (untreated controls) or 800 ppm Mn in the form of $MnCl_2 \cdot xH_2O$ in the MF basal diet from GD 10 to PND 21. On PND 4, the 10 dams that delivered more than 10 male offspring per group were selected to cull litters randomly, leaving 10 male offspring. Remaining dams were sacrificed under deep ether anesthesia, and the remaining offspring were sacrificed by rapid decapitation under ether anesthesia. On PND 21, all dams and 40 male offspring per group (four males per dam) were sacrificed under ether anesthesia, and blood samples were collected from the abdominal aorta. Serum was prepared and stored at −80°C to measure thyroid-stimulating hormone (TSH), triiodothyronine (T_3), and thyroxine (T_4) concentrations at Mitsubishi Chemical Medience (Tokyo, Japan). Remaining males were kept to PND 77 and subjected to blood sample collection for hormone analysis.

On PNDs 21 and 77, brains of all offspring were collected at autopsy. Five males per group (one male per dam) on PND 21 were subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) assay. Remaining brains were used for other experimental purposes.

Determination of Mn and Fe Concentrations in the Cerebellum

To measure concentrations of Mn and Fe in brain tissue, frozen cerebellar tissue of dams and male offspring at PNDs 21 and 77 (dams: n = 6/group at PND 21; offspring: n = 10/group of each stage) were digested using a microwave oven (MARS5; CEM Corporation, Matthews, NC); the digested samples were analyzed using inductively coupled plasma mass spectrometry (HP-7500; Hewlett-Packard Co., Palo Alto, CA) with the monitoring mass of m/z as 55 for Mn and 56 for Fe. Measured Mn and Fe concentrations were normalized to total protein determined according to the micro bicinchoninic acid method (Pierce Biotechnologies, Rockford, IL) using bovine serum albumin as a standard.

Immunohistochemistry and Apoptotic Cell Detection

For immunohistochemical analysis, brains in the subgroups of male offspring sacrificed at PNDs 21 and 77 were fixed in Bouin's solution at room temperature overnight. Coronal slices embedded in paraffin at the positions of −2.2 mm from the bregma were prepared for immunohistochemical staining (3 μm in thickness).

Sections were incubated overnight at 4°C with antibodies listed in Supplementary table 1. To quench endogenous peroxidase, slides were incubated in 0.3% (vol/vol) hydrogen peroxide in absolute methanol for 30 min. Immunodetection was carried out using a VECTASTAIN Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) with 3,3′-diaminobenzidine (DAB)/hydrogen peroxide (H_2O_2) as the chromogen, as previously described (Shibutani *et al.*, 2007). The sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

For double staining of reelin and NeuN, a postmitotic neuron marker (Mullen *et al.*, 1992), DAB-Cobalt was used to visualize reelin and Vector Red Alkaline Phosphate Substrate Kit I (Vector Laboratories Inc.) to visualize NeuN.

For evaluation of apoptosis in the SGZ of the dentate gyrus, the terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay was applied to brain sections. Detection of apoptotic bodies was carried out using the Apop Tag *in situ* apoptosis detection kit S7100 (Millipore Corporation, Billerica, MA) according to the instructions provided by the manufacturer with DAB/H_2O_2 as the chromogen.

Morphometry of Immunolocalized Cells and Apoptotic Cells

Reelin-, NeuN-, or GAD67-positive cells distributed in the hilus of the dentate gyrus were bilaterally counted and normalized for the number per unit area of the hilar area (polymorphic layer). In the SGZ of the dentate gyrus, apoptotic bodies as detected by the TUNEL assay, proliferating cells as detected by nuclear immunoreactivity of proliferating cell nuclear antigen (PCNA), and the expression of neuronal progenitor markers including Pax6, Tbr2, NeuroD1, and TUC4 were bilaterally counted and normalized to the length of the granule cell layer measured as previously described (Saegusa *et al.*, 2010). For quantitative measurements of each immunoreactive cellular component, digital photomicrographs at 200- or 400-fold magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co., Ltd.), and quantitative measurements were performed using the WinROOF image analysis software package (version 5.7, Mitani Corporation, Fukui, Japan).

Real-Time RT-PCR

For real-time RT-PCR analyses, the cerebrum of male offspring was removed at prepubertal necropsy on PND 21 (n = 5/group) and was fixed with methacarn solution for 8 h, then dehydrated by ice-cold absolute ethanol overnight at 4°C (Shibutani *et al.*, 2000). A coronal brain slice at the position between −2.2 and −2.8 mm from bregma was prepared, and portions of the dentate gyrus were collected using a biopsy punch (Φ1.0 mm, Kai Industries Co., Ltd., Seki, Japan) and stored in ethanol at −80°C until extraction. Total RNA was extracted with the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Analysis of mRNA levels for molecules listed in Supplementary table 2 was performed with real-time RT-PCR in the dentate gyrus tissue. *Reln*, *Lrp8*, and *Vldlr* are genes encoding reelin and its receptors (Hack *et al.*, 2007). *Gad1* encodes GAD67 (Wierońska *et al.*, 2010). *Pax6*, *Eomes* (also known as *Tbr2*), and *Dpysl3* (also known as *Tuc4*) are genes encoding neuronal-stage defining marker molecules (Breunig *et al.*, 2007; Hodge *et al.*, 2008; Knoth *et al.*, 2010). First-strand complementary DNA was synthesized from 2 μg of total RNA in the presence of dithiothreitol, deoxynucleotide triphosphates, random primers, RNaseOUT, and SuperScript III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA) in a 20 μl total reaction mixture. Real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) and the Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems Japan Ltd.) according to the manufacturer's protocol. The PCR primers shown in Supplementary table 2 were designed using Primer Express software (Version 3.0; Applied Biosystems Japan Ltd.). The relative differences in gene expression were calculated using threshold cycle (C_T) values that were first normalized to those of housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and then relative to a control C_T value by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Maternal data from experiment 1 regarding BW, food consumption, and Mn and Fe concentrations in the brain were analyzed using individual animals as the experimental unit. Data for offspring regarding the BW during the experiment, BWs, and organ weights at necropsy, Mn and Fe concentrations in the brain, immunohistochemical analysis, TUNEL assay, as well as real-time RT-PCR and serum hormonal measurements in Experiment 1 or 2 were analyzed using the litter as the experimental unit. For comparison of the numerical data between the control- and Mn-dosed groups, Bartlett's test for equal variance was used to determine whether the variance was homogenous between the groups. If the variance was homogenous, numerical data were assessed using Dunnett's test to compare between the control and each treated groups. If a significant difference in variance was observed, the Steel's test (Steel, 1959) was used instead.

In the case of data consisting of two sample groups, numerical data were assessed using the F-test for homogeneity of variance and Student's t-test was applied when the variance was homogenous between the groups using a test for equal variance. If a significant difference in variance was observed, Aspin-Welch's t-test was then performed.

All analyses were performed using the Excel Statistics 2008 software package (Social Survey Research Information Co. Ltd., Tokyo, Japan).

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RESULTS

Maternal Data (Experiment 1)

During the gestation and lactation periods, there were no significant differences in maternal BW following $MnCl_2$ exposure (Fig. 2A). Dams in the 800 ppm group showed statistically significant decreases in food consumption from PNDs 8 to 21 (Fig. 2B). Mean daily intake of $MnCl_2$ as Mn by dams during the gestation period were 8.6 ± 0.7, 10.6 ± 1.1, 18.3 ± 7.0, and 54.2 ± 6.5 mg/kg BW/day at 0, 32, 160, and 800 ppm, respectively; during the lactation period daily intakes were 17.3 ± 4.5, 22.3 ± 1.2, 42.5 ± 8.0, and 140.9 ± 6.4 mg/kg BW/day at 0, 32, 160, and 800 ppm, respectively. $MnCl_2$ treatment did not affect the length of gestation, number of implantations in the uterus, and live births (Supplementary table 3); however, at PND 21, one dam treated with 800 ppm was found dead.

Offspring Growth and Survival (Experiment 1)

Male pups showed significant decreases in BW at 160 ppm from PNDs 21 to 70 and at 800 ppm from PNDs 17 to 63 (Fig. 3). In contrast, female pups showed no changes in BW from PNDs 2 to 77 (data not shown).

With regard to clinical signs of offspring, all male pups in the 160 and 800 ppm groups exhibited somewhat higher sensitivity against handling stimuli when compared with untreated controls. During home cage observations, both groups, particularly the 160 ppm group, showed more hyperactive and aggressive behavior, and sometimes raced around to bump into the cage wall. Until the end of Mn exposure (PND 21), one male pup for each of the 32 and 160 ppm groups and two male pups from the 800 ppm group died. After weaning, 31 male pups died through to necropsy at PND 77; however, this was not related to the dose of Mn. Six male pups died in the 32 ppm group, 14 in the 160 ppm group, and 11 from the 800 ppm group.

Offspring Body and Organ Weights at PNDs 21 and 77 (Experiment 1)

On necropsy at PND 21, BW of male pups significantly decreased following 160 ppm exposure and continued to

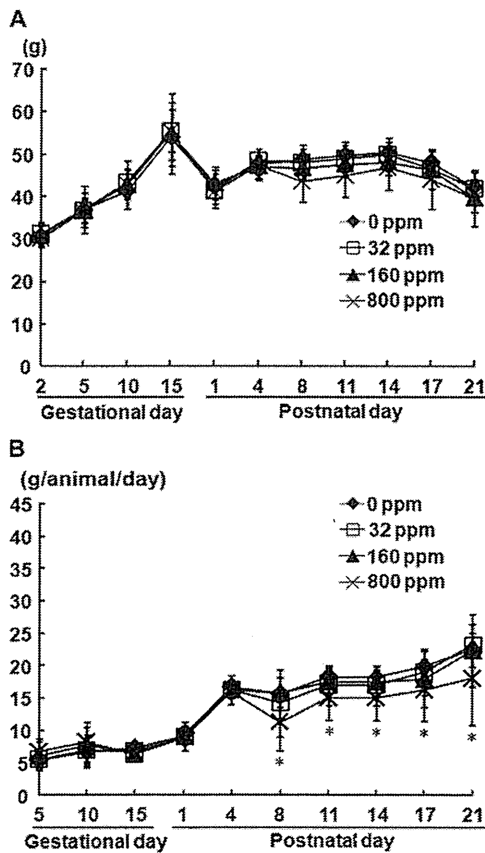

FIG. 2. BW and food consumption of dams exposed to $MnCl_2$ from GD 10 to PND 21. (A) Maternal BW. (B) Maternal food consumption. Values are expressed as the mean ± SD. *Significantly different from the untreated controls by Dunnett's test or Steel's test ($p < 0.05$).

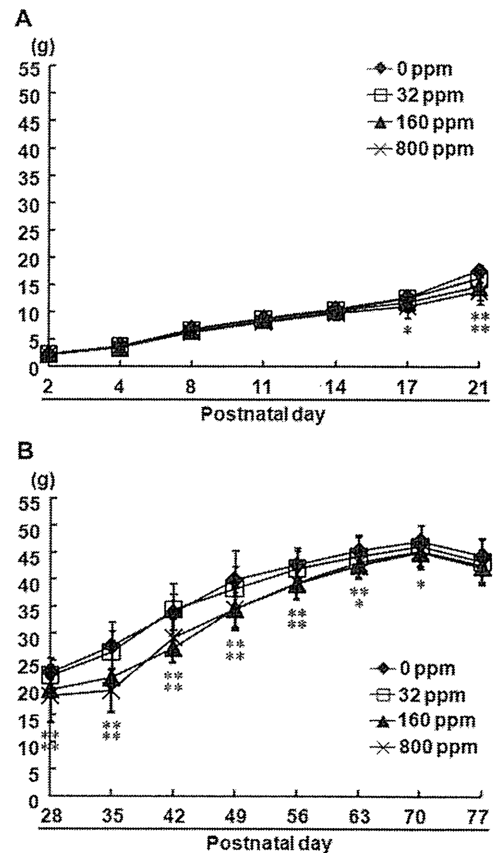

FIG. 3. BW of male offspring exposed maternally to $MnCl_2$ from GD 10 to PND 21. (A) Lactation period. (B) After lactation period. Values are expressed as the mean ± SD. * and **Significantly different from the untreated controls by Dunnett's test or Steel's test (* $p < 0.05$ and ** $p < 0.01$).

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TABLE 1

Body and Organ Weights at Prepubertal and Terminal Necropsies in Male Offspring Exposed Maternally to $MnCl_2$ in the Diet From Midgestation to the End of Lactation

	Mn in diet (ppm)[a]			
	0	32	160	800
PND 21				
Number of male offspring examined	10	10	10	10
BW	17.72 ± 1.10[b]	16.23 ± 1.72	14.76 ± 2.37**	13.76 ± 2.02**
Brain (g)	0.45 ± 0.02	0.44 ± 0.03	0.43 ± 0.02	0.42 ± 0.03
g/100 g BW	2.52 ± 0.32	2.71 ± 0.23	2.96 ± 0.20**	3.11 ± 0.30**
Liver (g)	1.03 ± 0.09	0.85 ± 0.13	0.78 ± 0.22**	0.70 ± 0.10**
g/100 g BW	5.81 ± 0.25	5.24 ± 0.32*	5.16 ± 0.35**	5.11 ± 0.32**
Kidneys (g)	0.24 ± 0.02	0.22 ± 0.02	0.20 ± 0.03**	0.19 ± 0.03**
g/100 g BW	1.36 ± 0.12	1.38 ± 0.07	1.36 ± 0.08	1.40 ± 0.17
PND 77				
Number of male offspring examined	10	10	10	10
BW	44.43 ± 3.20	43.29 ± 4.26	42.22 ± 3.22	42.46 ± 2.99
Brain (g)	0.49 ± 0.02	0.50 ± 0.03	0.48 ± 0.02	0.49 ± 0.02
g/100 g BW	1.14 ± 0.11	1.14 ± 0.10	1.17 ± 0.08	1.20 ± 0.07
Liver (g)	1.81 ± 0.22	1.75 ± 0.18	1.70 ± 0.17	1.81 ± 0.15
g/100 g BW	4.15 ± 0.32	3.94 ± 0.31	4.15 ± 0.35	4.41 ± 0.33
Kidneys (g)	0.73 ± 0.14	0.70 ± 0.07	0.66 ± 0.03	0.70 ± 0.09
g/100 g BW	1.68 ± 0.25	1.59 ± 0.16	1.62 ± 0.09	1.71 ± 0.21

[a]Dietary administration of Mn was performed in the form of $MnCl_2 \cdot xH_2O$.
[b]Mean ± SD.
* and ** Significantly different from the untreated controls by Dunnett's test or Steel's test (* $p < 0.05$ and ** $p < 0.01$).

decrease in a dose-dependent manner (Table 1). The relative brain weight increased significantly from 160 ppm. With regard to liver weight, significant decreases were observed in the absolute weight from 160 ppm and in the relative weight from 32 ppm. Absolute kidney weight significantly decreased from 160 ppm. There were no significant changes in the body and organ weights following $MnCl_2$ exposure in female pups at PND 21 (data not shown).

On PND 77, there were no statistically significant differences in body, brain, liver, and kidney weights.

Serum Concentrations of Thyroid-Related Hormones in Male Offspring (Experiment 2)

Maternal $MnCl_2$ exposure at 800 ppm significantly decreased serum T_4 concentration as compared with the untreated controls, whereas serum concentrations of T_3 and TSH did not significantly change at 800 ppm (Table 2). On PND 77, serum concentrations of T_3, T_4, and TSH in offspring at 800 ppm did not show significant changes as compared with untreated controls.

Brain Concentrations of Manganese and Iron in Dams and Male Offspring (Experiment 1)

Cerebellar concentrations of Mn and Fe from dams in the $MnCl_2$ treatment groups remained unchanged at all Mn doses at PND 21. With regard to offspring, cerebellar Mn concentration showed significant dose-related increases from 32 ppm at PND 21. Although the magnitude of accumulation was rather mild, cerebellar Mn concentration increased in all dosed groups at PND 77 (Table 3). On the other hand,

TABLE 2

Serum Concentrations of Thyroid-Related Hormones

	Mn in diet (ppm)[a]	
	0	800
PND 21		
Number of offspring examined	40 (10)[b]	40 (10)
T_3 (ng/dl)	53.67 ± 6.97[c]	48.91 ± 6.85
T_4 (μg/dl)	3.62 ± 0.70	3.16 ± 0.48*
TSH (pg/ml)	11.12 ± 6.02	13.11 ± 4.09
PND 77		
Number of offspring examined	40 (10)	40 (10)
T_3 (ng/dl)	78.20 ± 10.51	80.90 ± 8.23
T_4 (μg/dl)	4.56 ± 0.56	4.87 ± 0.44
TSH (pg/ml)	48.12 ± 10.03	58.01 ± 23.02

[a]Dietary administration of Mn was performed in the form of MnCl2·xH2O.
[b]Number in parenthesis represents the total number of pooled samples: 3–4 cases/sample.
[c]Mean ± SD.
*Significantly different from the untreated controls by Student's or Aspin-Welch's *t*-test (* $p < 0.05$).

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TABLE 3
Cerebellar Manganese and Iron Concentrations

	Mn in diet (ppm)[a]			
	0	32	160	800
Dams				
Number of dams examined	6	6	6	6
Mn (μg/mg protein)	0.021 ± 0.005[b]	0.022 ± 0.005	0.023 ± 0.001	0.024 ± 0.004
Fe (μg/mg protein)	0.657 ± 0.015	0.655 ± 0.012	0.654 ± 0.011	0.659 ± 0.012
Offspring				
PND 21				
Number of offspring examined	10	10	10	10
Mn (μg/mg protein)	0.022 ± 0.003	0.026 ± 0.005*	0.032 ± 0.003**	0.038 ± 0.002**
Fe (μg/mg protein)	0.452 ± 0.015	0.454 ± 0.017	0.457 ± 0.015	0.452 ± 0.012
PND 77				
Number of offspring examined	10	10	10	10
Mn (μg/mg protein)	0.013 ± 0.002	0.017 ± 0.004*	0.021 ± 0.002*	0.021 ± 0.005*
Fe (μg/mg protein)	0.545 ± 0.017	0.543 ± 0.016	0.540 ± 0.013	0.550 ± 0.018

[a]Dietary administration of Mn was performed in the form of MnCl2·xH2O.
[b]Mean ± SD.
* and ** Significantly different from the untreated controls by Dunnett's test or Steel's test (* $p < 0.05$ and ** $p < 0.01$).

cerebellar Fe concentration was unchanged in all dosed groups at both PNDs 21 and 77 in offspring.

Neuron Distribution in the Dentate Hilus of Male Offspring (Experiment 1)

In the hilus of the dentate gyrus, reelin- or GAD67-positive cells indicative of GABAergic interneurons sparsely distributed as described previously in rats (Ogawa *et al.*, 2011; Saegusa *et al.*, 2010). Statistically significant increases in the number of reelin-positive cells were observed in the 160 ppm group and increases in NeuN- and GAD67-positive cells were observed in the 800 ppm group in the hilus when compared with untreated controls at PND 21 (Figs. 4A–C). On PND 77, neurons positive for reelin or NeuN increased in the hilus at 800 ppm as compared with the untreated controls (Figs. 5A and B). GAD67 did not show any significant changes between the untreated controls and any of the $MnCl_2$-treated groups (Fig. 5C). With regard to double immunohistochemistry for reelin and NeuN, the number of neurons expressing reelin but lacking or weakly expressing NeuN increased with an Mn dose of 800 ppm at PND 21 (Fig. 4D). Neuron populations lacking reelin expression but expressing NeuN were also increased at this dose. On PND 77, a similar distribution was observed with 800 ppm Mn (Fig. 5D).

Apoptosis and Proliferating Cells in the SGZ of Male Offspring (Experiment 1)

Both TUNEL-positive apoptotic cells and PCNA-positive proliferating cells in the SGZ were sparsely observed in the SGZ on PNDs 21 and 77 (Fig. 6). Apoptotic cells significantly increased at 800 ppm on PND 21 as compared with the untreated controls (Fig. 6A); however, there was no difference with any dose of Mn at PND 77 when compared with untreated controls (Fig. 6B). The number of proliferating cells did not fluctuate with Mn at any dose on PNDs 21 and 77 as compared with the corresponding untreated controls (Figs. 6C and D).

Neuronal Progenitor Distribution in the SGZ of Male Offspring (Experiment 1)

On PND 21, the number of Pax6-, Tbr2- or NeuroD1-positive cells did not fluctuate with 800 ppm Mn (Figs. 7A–C). In contrast, a statistically significant decrease in the number of TUC4-positive cells was found at 800 ppm when compared with the untreated controls (Fig. 7D).

On PND 77, the number of progenitor cells immunoreactive for the neuronal-stage defining markers examined here decreased in the SGZ (Figs. 8A–D). TUC4-positive cells significantly increased with 800 ppm Mn treatment, whereas the number of Pax6-, Tbr2- or NeuroD1-positive cells did not fluctuate at 800 ppm.

Transcript Expression in the Dentate Gyrus of Male Offspring at PND 21 (Experiment 2)

Transcript expression levels of *Reln*, *Lrp8*, and *Gad1* after normalization with the level of *Hprt* and *Gapdh* significantly increased following exposure to 800 ppm Mn on PND 21 in comparison with the levels from untreated controls (Table 4). In contrast, transcript levels of *Dpysl3* significantly decreased at 800 ppm.

DISCUSSION

With regard to distribution changes in the neuronal progenitor cells in the SGZ, we revealed a decrease in TUC4-positive cells

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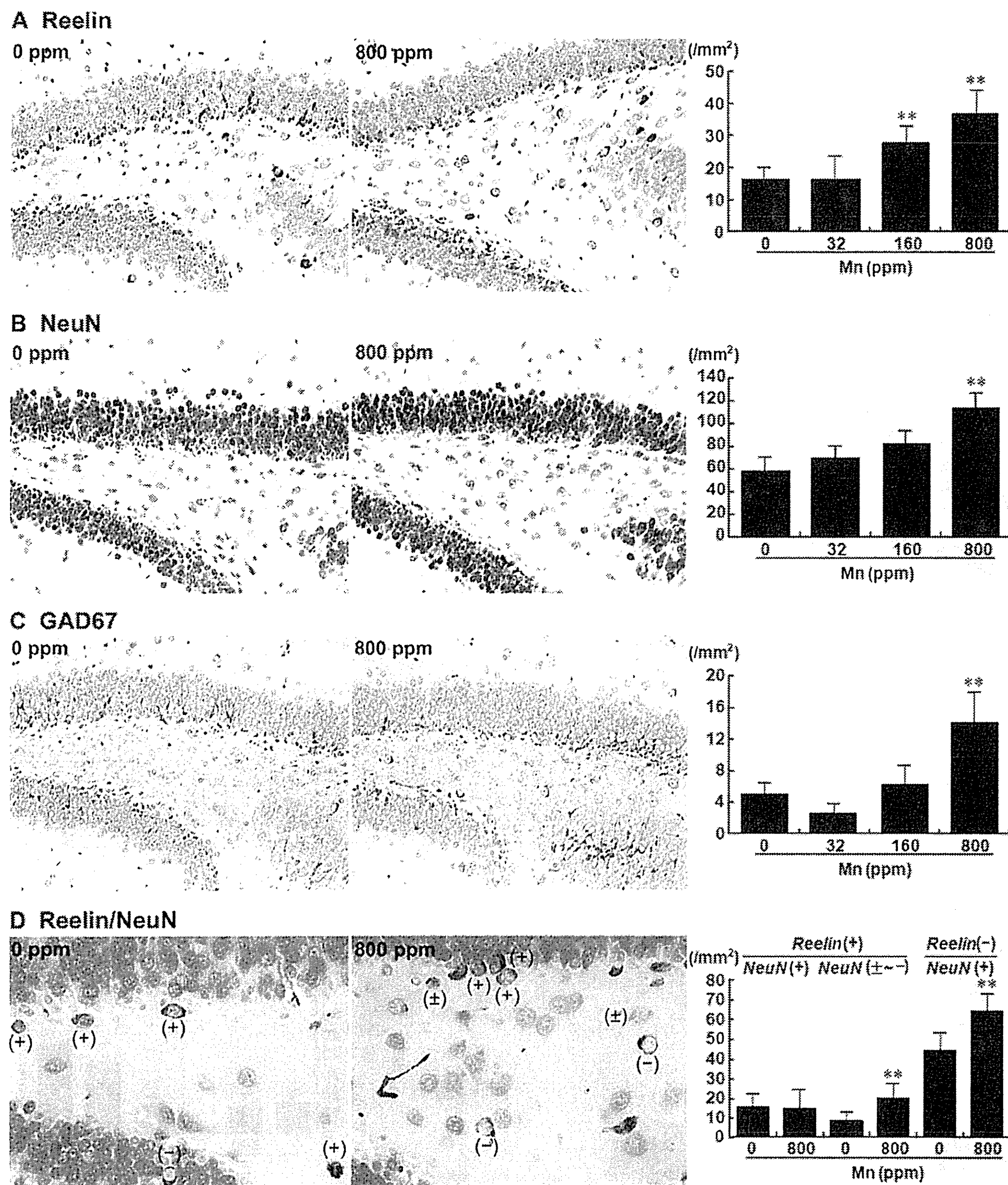

FIG. 4. Distribution of immunoreactive cells for reelin, NeuN, and GAD67 and double staining of reelin and NeuN in the dentate hilus of male offspring at PND 21 exposed maternally to $MnCl_2$ from GD 10 to PND 21. (A) Reelin. (B) NeuN. (C) GAD67. (D) Double staining of reelin (brown) and NeuN (red; for full color figures, please see online version). Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 21. (A), (B), and (C) Magnification, 200×. (D) Magnification, 400×. Graphs show the number of immunoreactive cells for each antigen/unit area (mm^2) of the hilus of the bilateral hemispheres at PND 21. Values are expressed as the mean + SD. ** Significantly different from the untreated controls by Dunnett's test or Steel's test ($p < 0.01$).

following 800 ppm Mn treatment at PND 21, whereas the distribution of progenitor cells expressing Pax6, Tbr2, and NeuroD1 was unchanged with Mn exposure. Pax6 and Tbr2 are markers for early-stage progenitor cells (Breunig *et al.*, 2007; Hodge *et al.*, 2008). In contrast, NeuroD1 is not a specific marker of neurogenesis and is expressed in cells ranging from type 2b progenitor cells to immature granule cells, with peak expression in type 3 cells (Breunig *et al.*, 2007; Hodge *et al.*, 2008; Knoth *et al.*, 2010). On the other hand, TUC4 is an early postmitotic neuronal marker of immature granule cells (Knoth *et al.*, 2010). Considering the increase in TUNEL-positive apoptotic cells in the SGZ in 800 ppm Mn-exposed offspring,

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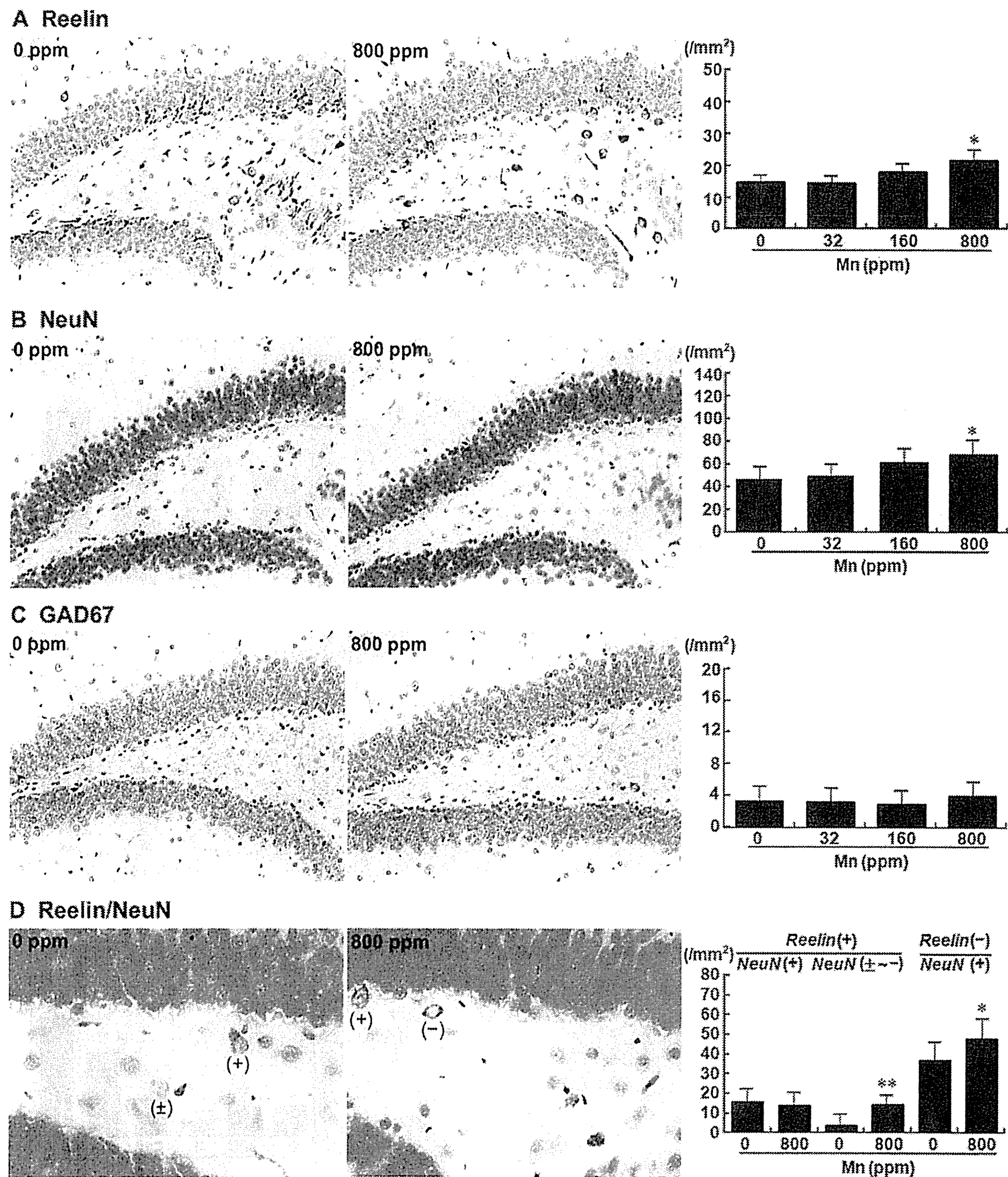

FIG. 5. Distribution of immunoreactive cells for reelin, NeuN, and GAD67 and double staining of reelin and NeuN in the dentate hilus of male offspring at PND 77 exposed maternally to $MnCl_2$ from GD 10 to PND 21. (A) Reelin. (B) NeuN. (C) GAD67. (D) Double staining of reelin (brown) and NeuN (red; for full color figures, please see online version). Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 77. (A), (B), and (C) Magnification, 200×. (D) Magnification, 400×. Graphs show the number of immunoreactive cells for each antigen/unit area (mm^2) of the hilus of the bilateral hemispheres at PND 77. Values are expressed as the mean + SD. * and ** Significantly different from the untreated controls by Dunnett's test or Steel's test (* $p < 0.05$ and ** $p < 0.01$).

developmental exposure to Mn may target immature granule cells leading to apoptosis during Mn exposure in the present study.

Similar to the developmental exposure study of antithyroid agents in rats (Saegusa *et al.*, 2010; Shibutani *et al.*, 2009), we observed a sustained increase of reelin-expressing and NeuN-lacking or weakly expressing neurons in the dentate hilus from PND 21 through to PND 77 as well as an increase of reelin-lacking and NeuN-expressing mature neurons in our $MnCl_2$-exposed cases. Reelin is a secreted extracellular matrix glycoprotein that plays a critical role in neuronal migration and positioning during brain development (D'Arcangelo *et al.*,

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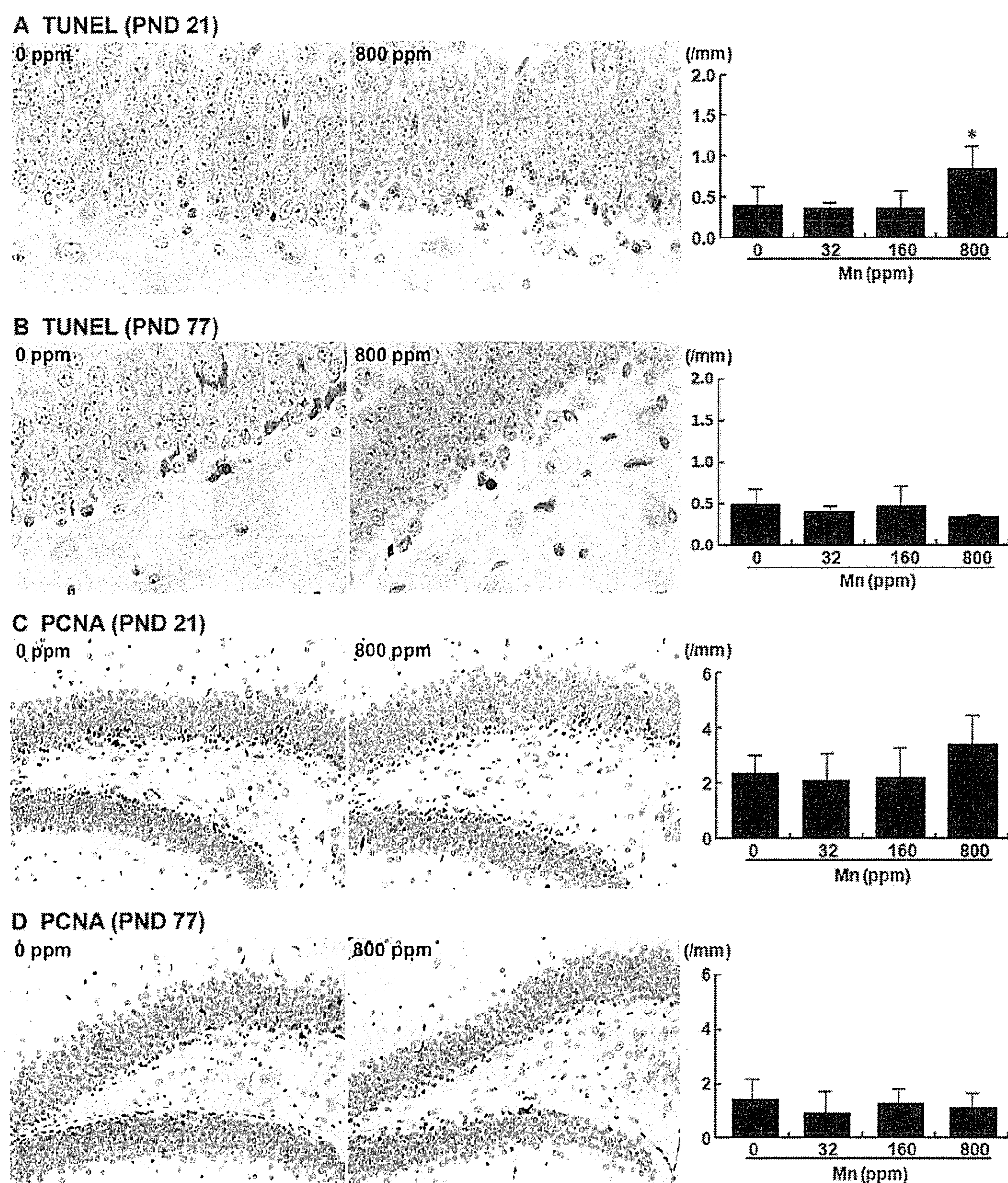

FIG. 6. Distribution of apoptotic cells and proliferating cells in the dentate SGZ of male offspring at both PNDs 21 and 77 exposed maternally to $MnCl_2$ from GD 10 to PND 21. (A) and (B) TUNEL-positive apoptotic cells at PNDs 21 and 77. (C) and (D) PCNA-positive cells at PNDs 21 and 77. Representative images from 0 ppm group (left) and from 800 ppm group (right) at PNDs 21 and 77. (A) and (B) Magnification, 400×. (C) and (D) Magnification, 200×. Graphs show the number of immunoreactive cells/unit length (mm) of the SGZ of bilateral hemispheres at PNDs 21 and 77. Values are expressed as the mean + SD. *Significantly different from the untreated controls by Dunnett's test or Steel's test ($p < 0.05$). For full color figures, please see online version.

1997). Also, it has been suggested that GABAergic interneurons in the hilar region release reelin and regulate the migration and maturation of newborn granule cells in adults (Lussier *et al.*, 2009). Interestingly, faint expression of mature neuronal markers, such as NeuN and microtubule-associated protein-2, has been reported to reflect the immature nature of neurons (Seki, 2002). An increase of GAD67-expressing cells at PND 21 after maternal $MnCl_2$ exposure was found in the present study, confirming the increase of GABAergic interneurons (Saegusa *et al.*, 2010). Therefore, the sustained increase of reelin-expressing and NeuN-lacking or weakly expressing neurons were considered immature GABAergic interneurons

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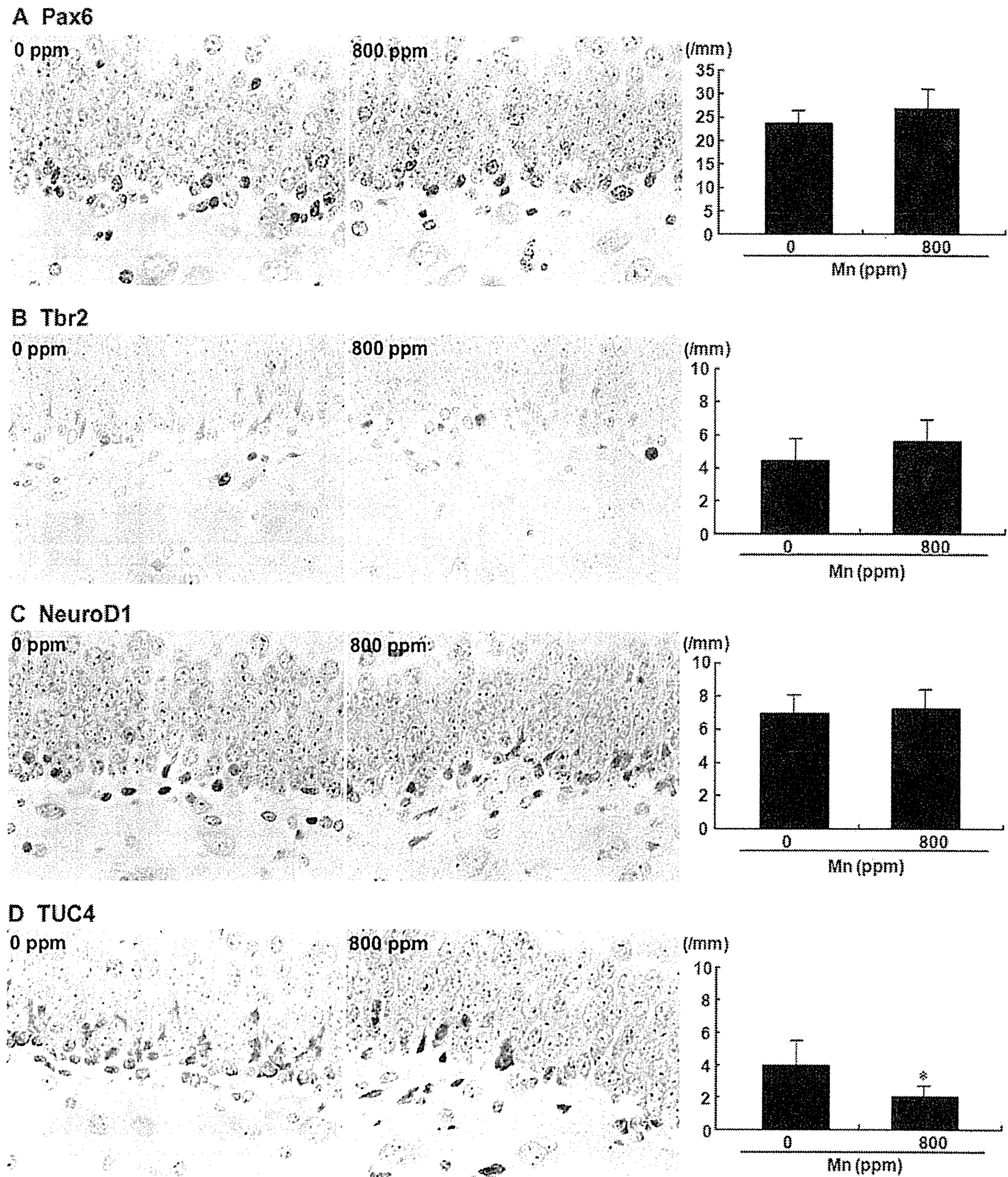

FIG. 7. Distribution of immunoreactive cells for Pax6, Tbr2, NeuroD1, and TUC4 in the dentate SGZ of male offspring at PND 21 exposed maternally to $MnCl_2$ from GD 10 to PND 21. (A) Pax6. (B) Tbr2. (C) NeuroD1. (D) TUC4. Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 21. Magnification, 400×. Graphs show the number of immunoreactive cells for each antigen/unit length (mm) of the SGZ of bilateral hemispheres at PND 21. Values are expressed as the mean + SD. * Significantly different from the untreated controls by Student's or Aspin-Welch's *t*-test ($p < 0.05$). For full color figures, please see online version.

responding to neuronal mismigration due to impaired neurogenesis of dentate granule cells even at the adult stage. A decrease of TUC4-expressing immature granule cells at PND 21 may be responsible for the initial increase of immature reelin-expressing interneurons in the hilus. The sustained increase of this population of cells may be a sign of continued aberration in neurogenesis and migration that causes the overproduction of immature granule cells at the adult stage. On the other hand, it has been reported that GABAergic interneurons provide direct neural inputs toward type 2 progenitor cells in the SGZ and promote neural differentiation (Tozuka *et al.*, 2005). Thus, sustained increases of NeuN-expressing neuron populations in the hilus with 800 ppm Mn through to the adult stage may represent mature interneurons that stop producing reelin but

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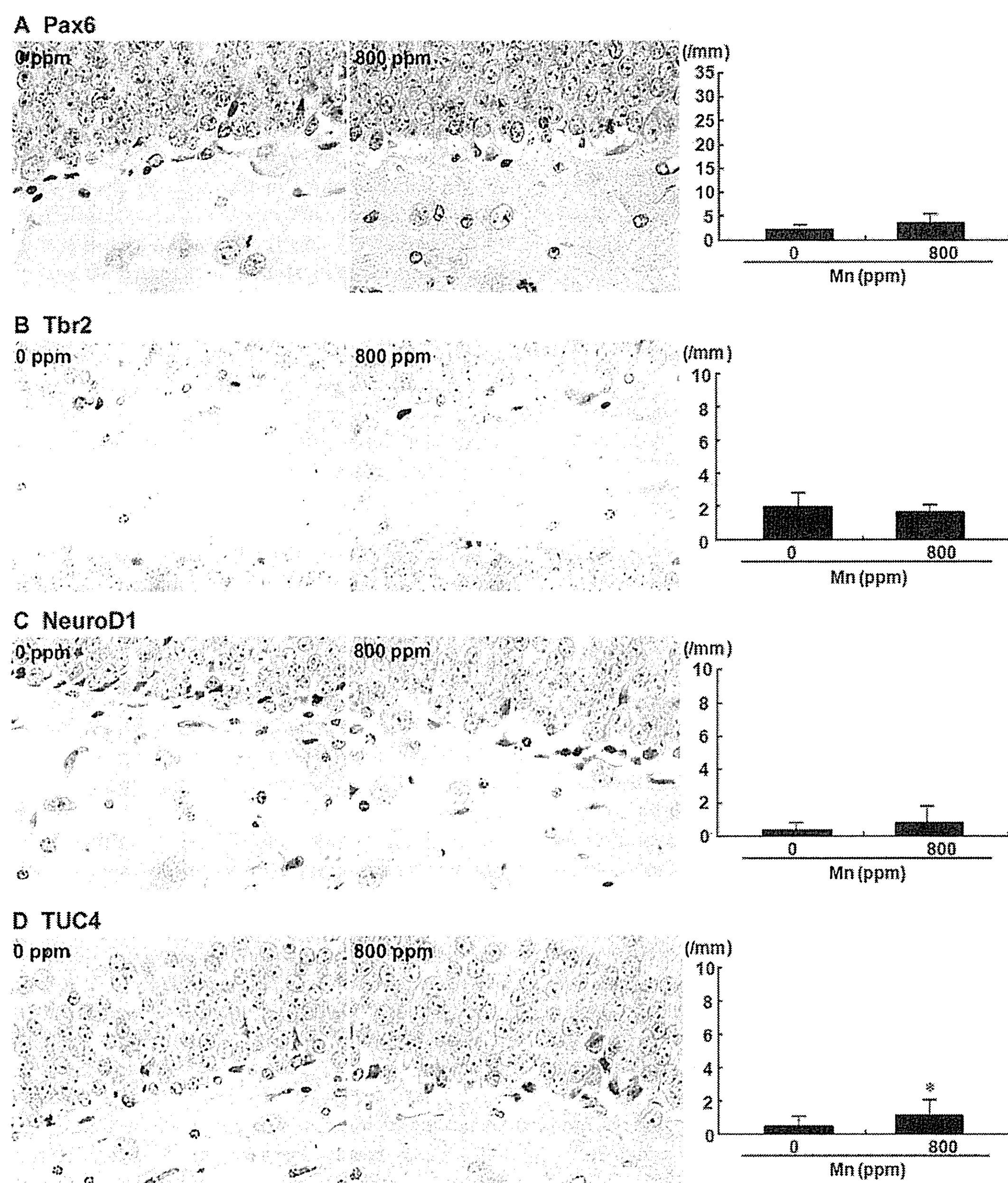

FIG. 8. Distribution of immunoreactive cells for Pax6, Tbr2, NeuroD1, and TUC4 in the dentate SGZ of male offspring at PND 77 exposed maternally to $MnCl_2$ from GD 10 to PND 21. (A) Pax6. (B) Tbr2. (C) NeuroD1. (D) TUC4. Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 77. Magnification, 400×. Graphs show the number of immunoreactive cells for each antigen/unit length (mm) of the SGZ of bilateral hemispheres at PND 77. Values are expressed as the mean + SD. * Significantly different from the untreated controls by Student's or Aspin-Welch's *t*-test ($p < 0.05$). For full color figures, please see online version.

facilitate progenitor cells to undergo differentiation to immature granule cells.

In the present study, we found systemic growth retardation in offspring after weaning following treatment with $\geq$ 160 ppm Mn, but we did not detect alterations in absolute brain weights in any $MnCl_2$-exposed groups as compared with untreated controls. In our previous study, maternal protein restriction causing progressive systematic growth retardation involving decreased brain weight did not affect neurogenesis and the distribution of GABAergic interneurons expressing reelin in offspring (Ohishi *et al.*, 2010). Therefore, we judge that Mn-induced systemic growth retardation observed here was weaker

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TABLE 4
Real-Time RT-PCR Analysis in the Hippocampal Dentate Gyrus on PND 21

	0 ppm Mn		800 ppm Mn	
	Relative Transcript Level Normalized To		Relative Transcript Level Normalized To	
	Hprt	*Gapdh*	*Hprt*	*Gapdh*
Reln	1.02 ± 0.23[a]	1.23 ± 0.75	2.76 ± 0.35**	4.09 ± 0.97**
Lrp8	1.01 ± 0.14	1.08 ± 0.47	1.63 ± 0.29**	2.41 ± 0.39**
Vldlr	1.00 ± 0.05	1.10 ± 0.52	0.93 ± 0.09	1.38 ± 0.10
Gad1	1.00 ± 0.04	1.13 ± 0.58	1.86 ± 0.06**	2.76 ± 0.45**
Pax6	1.05 ± 0.40	1.03 ± 0.26	1.13 ± 0.40	1.66 ± 0.54
Eomes	1.06 ± 0.47	1.02 ± 0.21	1.22 ± 0.48	1.80 ± 0.66
Dpysl3	1.00 ± 0.06	1.12 ± 0.06	0.47 ± 0.04**	0.69 ± 0.03**

Notes. Hprt, hypoxanthine-guanine phosphoribosyltransferase; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Reln*, reelin; *Lrp8*, low density lipoprotein receptor-related protein 8; *Vldlr*, very-low-density lipoprotein receptor; *Gad1*, glutamic acid decarboxylase 1 (glutamic acid decarboxylase 67); *Pax6*, paired box 6; *Eomes*, eomesodermin homolog (T-box brain protein 2); *Dpysl3*, dihydropyrimidinase-like 3.

[a]Mean ± SD ($n = 5$) relative to the expression levels in untreated controls. Real-time PCR analysis of *Hprt* and *Gapdh* was performed for the analysis of each target gene.

Significantly different from the untreated controls by Student's or Aspin-Welch's t-test ($p < 0.01$).

than that induced by maternal protein restriction, and therefore, it did not affect neurogenesis and interneuron distribution. On the other hand, we found offspring deaths during and after Mn exposure in all dosed groups without relation to the dose of Mn, irrespective of the lack of apparent general toxicity in dams and offspring except for the systemic growth retardation of offspring. The largest numbers of deaths were observed at the middle dose showing more hyperactive and aggressive behavior as compared with the highest dose. Although offspring deaths have not been reported due to excess Mn exposure in experimental animals, we previously found offspring deaths showing similar hyperactive and aggressive behavior as well as sustained aberration of neurogenesis after developmental hypothyroidism in rats (Saegusa *et al.*, 2010; Shibutani *et al.*, 2009). Therefore, developmental effect of Mn on the brain may be related to offspring deaths. Of note, mouse pups may gradually start to consume diet from around PND 10, and therefore, mg-test-substance per kg-BW basis of pups may actually be consuming a higher dose than adult case during their second week of the lactation period.

Mn exposure via maternal milk from PNDs 4 to 21 caused Mn accumulation in the cerebellum, midbrain, and striatum in rat offspring (Garcia *et al.*, 2006). In the present study, we examined cerebellar tissue to represent brain concentration of Mn. We observed increased Mn concentrations in the cerebellum in male offspring with $\geq$ 32 ppm Mn at PND 21 to sustain higher Mn concentrations at PND 77 when compared with time-matched untreated controls, despite no concentration changes in dams. Exposure *in utero* and during lactation to inhaled $MnSO_4$ has been reported to increase Mn concentrations in the striatum and cerebellum of offspring at doses that do not change Mn concentrations in dams (Dorman *et al.*, 2005). Studies on experimental exposure to $MnCl_2$ revealed that Mn concentrations in the brain in developing rats were higher than that in adult rats at the same dose (Dorman *et al.*, 2000; Takeda *et al.*, 1999). The increase in offspring brain Mn concentrations may be related to increased Mn absorption from the juvenile gastrointestinal tract as well as an incompletely formed neonatal blood-brain barrier and a virtual absence of excretory mechanisms until weaning (Dorman *et al.*, 2000). These results suggest that offspring are rather unprotected against developmental exposure to Mn, in contrast to adult animals that have protective functions against ingested Mn even at high doses. Therefore, only offspring are at risk of neurotoxicity, which includes impaired neuronal differentiation by maternal Mn exposure. Interestingly, an *in vitro* study using astrocyte-neuron cocultures has shown that $MnCl_2$ inhibited the ability of astrocytes to promote neurite outgrowth of hippocampal neuronal precursor cells (Giordano *et al.*, 2009). This result may be related to the decrease of immature granule cells in the SGZ at the end of exposure in the present study. Because immature granule cells already have dendritic growth cones and recurrent basal dendrites (Ribak *et al.*, 2004), immature granule cells may be the target of Mn, which suppresses differentiation into mature granule cells causing apoptosis.

In the present study, we detected a mild reduction in the serum concentration of T_4 in male offspring on weaning after developmental Mn exposure at 800 ppm, whereas there were no changes in the serum concentrations of T_3 and TSH. This T_4 reduction did not last into the adult stage. In contrast, a dietary study of rats treated with 10 mg/kg/day of $MnSO_4$ (3.6 mg/kg/day as Mn) for 5 weeks resulted in a significant decrease in serum T_3, T_4, and TSH concentrations, and a 2-year study of Mn in mice with a dose of 584 mg/kg/day $MnSO_4$ (213 mg/kg/day as Mn) increased the incidence of hyperplasia and dilatation of thyroid follicles (reviewed in Soldin and Aschner, 2007). These results suggest an antithyroid effect of Mn; however, our present study indicates a weak antithyroid effect. Interestingly, lactational Mn exposure study reporting Mn accumulation in the brain of rat offspring at a dietary dose of 100 ppm Mn showed a slightly reduced brain Fe-concentration (Garcia *et al.*, 2006). Because perinatal Fe deficiency has shown to cause reduced neonatal rat circulating and brain thyroid hormone concentrations as well as brain Fe concentration (Bastian *et al.*, 2010), thyroid hormone status may be affected by excess Mn exposure. However, we did not observe any changes in cerebellar Fe concentrations by Mn exposure even at 800 ppm in offspring in the present study, whereas the reason for the discrepancy between the above mentioned study and ours was not clear. On the other hand, we previously

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observed similar hippocampal interneuron changes during developmental hypothyroidism, accompanied with striking antithyroid effects on serum thyroid–related hormones using rats (Saegusa *et al.*, 2010). However, the magnitude of brain changes was similar to the Mn-exposed cases observed here. Therefore, hypothyroidism may not be the major cause of developmental neuronal effects by Mn.

In the present study, the rodent chow contained high concentrations of Mn (4.84 mg/100 g MF basal diet). This dietary level provides a daily Mn intake of 8.6–17.3 mg/kg BW during gestation and lactation periods in dams of untreated controls. As mentioned earlier, the ESADDI of Mn has been estimated to be approximately 0.6 mg/day at 7–12 months of age, 1.2 mg/day at 1–3 years of age, 1.5 mg/day at 4–8 years of age, and 2–5 mg/day for adults. For newborns, the ESADDI has been estimated to be 0.003 mg/day, less than that for adults or children (Aschner and Aschner, 2005). Therefore, developmental exposure studies in rodents are performed with extremely high-basal Mn-intake levels as compared with human counterparts. In the present study, we found an increase of reelin-synthesizing interneurons in the dentate gyrus following treatment with 160 ppm Mn at PND 21. Of note, this Mn exposure level provides only a 2.1- to 2.5-fold increase in the daily intake of Mn when compared with untreated control animals, which is a small difference in exposure levels.

In conclusion, developmental Mn exposure in the form of $MnCl_2 \cdot xH_2O$ in mice induced an increase in apoptosis, which targeted immature granule cells during development. To this effect, the relationship to developmental hypothyroidism may be small. The sustained increase in immature reelin-synthesizing GABAergic interneurons may be a sign of continued aberration in neurogenesis and following neuronal migration, which causes the overproduction of immature granule cells at the adult stage. Increased NeuN-expressing neurons in the hilus at the adult stage may represent mature interneurons that stop producing reelin but facilitate differentiation of progenitor cells to immature granule cells. Sustained high concentrations of Mn in the brain may be responsible for these changes. Because neurogenesis in the hippocampal dentate gyrus continues during postnatal life, there is a possibility that excess Mn affects neurogenesis even at the adult stages.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Aschner, J. L., and Aschner, M. (2005). Nutritional aspects of manganese homeostasis. *Mol. Aspects Med.* **26,** 353–362.

Bastian, T. W., Prohaska, J. R., Georgieff, M. K., and Anderson, G. W. (2010). Perinatal iron and copper deficiencies alter neonatal rat circulating and brain thyroid hormone concentrations. *Endocrinology* **151,** 4055–4065.

Breunig, J. J., Silbereis, J., Vaccarino, F. M., Sestan, N., and Rakic, P. (2007). Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 20558–20563.

Chandra, S. V., and Shukla, G. S. (1978). Manganese encephalopathy in growing rats. *Environ. Res.* **15,** 28–37.

D'Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. (1997). Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J. Neurosci.* **17,** 23–31.

Dobson, A. W., Erikson, K. M., and Aschner, M. (2004). Manganese neurotoxicity. *Ann. N. Y. Acad. Sci.* **1012,** 115–128.

Dorman, D. C., McElveen, A. M., Marshall, M. W., Parkinson, C. U., James, R. A., Struve, M. F., and Wong, B. A. (2005). Tissue manganese concentrations in lactating rats and their offspring following combined in utero and lactation exposure to inhaled manganese sulfate. *Toxicol. Sci.* **84,** 12–21.

Dorman, D. C., Struve, M. F., Vitarella, D., Byerly, F. L., Goetz, J., and Miller, R. (2000). Neurotoxicity of manganese chloride in neonatal and adult CD rats following subchronic (21-day) high-dose oral exposure. *J. Appl. Toxicol.* **20,** 179–187.

Garcia, S. J., Gellein, K., Syversen, T., and Aschner, M. (2006). A manganese-enhanced diet alters brain metals and transporters in the developing rat. *Toxicol. Sci.* **92,** 516–525.

Giordano, G., Pizzurro, D., VanDeMark, K., Guizzetti, M., and Costa, L. G. (2009). Manganese inhibits the ability of astrocytes to promote neuronal differentiation. *Toxicol. Appl. Pharmacol.* **240,** 226–235.

Gong, C., Wang, T. W., Huang, H. S., and Parent, J. M. (2007). Reelin regulates neuronal progenitor migration in intact and epileptic hippocampus. *J. Neurosci.* **27,** 1803–1811.

Goodman, J. H., and Gilbert, M. E. (2007). Modest thyroid hormone insufficiency during development induces a cellular malformation in the corpus callosum: A model of cortical dysplasia. *Endocrinology* **148,** 2593–2597.

Hack, I., Hellwig, S., Junghans, D., Brunne, B., Bock, H. H., Zhao, S., and Frotscher, M. (2007). Divergent roles of ApoER2 and Vldlr in the migration of cortical neurons. *Development* **134,** 3883–3891.

Hodge, R. D., Kowalczyk, T. D., Wolf, S. A., Encinas, J. M., Rippey, C., Enikolopov, G., Kempermann, G., and Hevner, R. F. (2008). Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. *J. Neurosci.* **28,** 3707–3717.

Khan, K., Factor-Litvak, P., Wasserman, G. A., Liu, X., Ahmed, E., Parvez, F., Slavkovich, V., Levy, D., Mey, J., van Geen, A., *et al.* (2011). Manganese exposure from drinking water and children's classroom behavior in Bangladesh. *Environ. Health Perspect.* **119,** 1501–1506.

Kitazawa, M., Anantharam, V., Yang, Y., Hirata, Y., Kanthasamy, A., and Kanthasamy, A. G. (2005). Activation of protein kinase C delta by

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proteolytic cleavage contributes to manganese-induced apoptosis in dopaminergic cells: Protective role of Bcl-2. *Biochem. Pharmacol.* **69,** 133–146.

Knoth, R., Singec, I., Ditter, M., Pantazis, G., Capetian, P., Meyer, R. P., Horvat, V., Volk, B., and Kempermann, G. (2010). Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PLoS One* **5,** e8809.

Kontur, P. J., and Fechter, L. D. (1988). Brain regional manganese levels and monoamine metabolism in manganese-treated neonatal rats. *Neurotoxicol. Teratol.* **10,** 295–303.

Lavado-Autric, R., Ausó, E., García-Velasco, J. V., Arufe Mdel, C., del Rey, F. E., Berbel, P., and de Escobar, G. M. (2003). Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. *J. Clin. Invest.* **111,** 954–957.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25,** 402–408.

Lussier, A. L., Caruncho, H. J., and Kalynchuk, L. E. (2009). Repeated exposure to corticosterone, but not restraint, decreases the number of reelin-positive cells in the adult rat hippocampus. *Neurosci. Lett.* **460,** 170–174.

Montaron, M. F., Koehl, M., Lemaire, V., Drapeau, E., Abrous, D. N., and Le Moal, M. (2004). Environmentally induced long-term structural changes: Cues for functional orientation and vulnerabilities. *Neurotox. Res.* **6,** 571–580.

Mullen, R. J., Buck, C. R., and Smith, A. M. (1992). NeuN a neuronal specific nuclear protein in vertebrates. *Development* **116,** 201–211.

Ogawa, B., Ohishi, T., Wang, L., Takahashi, M., Taniai, E., Hayashi, H., Mitsumori, K., and Shibutani, M. (2011). Disruptive neuronal development by acrylamide in the hippocampal dentate hilus after developmental exposure in rats. *Arch. Toxicol.* **85,** 987–994.

Ohishi, T., Wang, L., Ogawa, B., Fujisawa, K., Taniai, E., Hayashi, H., Mitsumori, K., and Shibutani, M. (2010). No effect of sustained systemic growth retardation on the distribution of reelin-expressing interneurons in the neuron-producing hippocampal dentate gyrus in rats. *Reprod. Toxicol.* **30,** 591–599.

Pawluski, J. L., Brummelte, S., Barha, C. K., Crozier, T. M., and Galea, L. A. (2009). Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. *Front. Neuroendocrinol.* **30,** 343–357.

Ramos-Moreno, T., Galazo, M. J., Porrero, C., Martínez-Cerdeño, V., and Clascá, F. (2006). Extracellular matrix molecules and synaptic plasticity: Immunomapping of intracellular and secreted Reelin in the adult rat brain. *Eur. J. Neurosci.* **23,** 401–422.

Reznikov, K. Y. (1991). Cell proliferation and cytogenesis in the mouse hippocampus. *Adv. Anat. Embryol. Cell Biol.* **122,** 1–74.

Ribak, C. E., Korn, M. J., Shan, Z., and Obenaus, A. (2004). Dendritic growth cones and recurrent basal dendrites are typical features of newly generated dentate granule cells in the adult hippocampus. *Brain Res.* **1000,** 195–199.

Saegusa, Y., Woo, G.-H., Fujimoto, H., Kemmochi, S., Shimamoto, K., Hirose, M., Mitsumori, K., Nishikawa, A., and Shibutani, M. (2010). Sustained production of Reelin-expressing interneurons in the hippocampal dentate hilus after developmental exposure to anti-thyroid agents in rats. *Reprod. Toxicol.* **29,** 407–414.

Schoonover, C. M., Seibel, M. M., Jolson, D. M., Stack, M. J., Rahman, R. J., Jones, S. A., Mariash, C. N., and Anderson, G. W. (2004). Thyroid hormone regulates oligodendrocyte accumulation in developing rat brain white matter tracts. *Endocrinology* **145,** 5013–5020.

Seki, T. (2002). Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. *J. Neurosci. Res.* **70,** 327–334.

Shibutani, M., Lee, K.-Y., Igarashi, K., Woo, G.-H., Inoue, K., Nishimura, T., and Hirose, M. (2007). Hypothalamus region-specific global gene expression profiling in early stages of central endocrine disruption in rat neonates injected with estradiol benzoate or flutamide. *Dev. Neurobiol.* **67,** 253–269.

Shibutani, M., Uneyama, C., Miyazaki, K., Toyoda, K., and Hirose, M. (2000). Methacarn fixation: A novel tool for analysis of gene expressions in paraffin-embedded tissue specimens. *Lab. Invest.* **80,** 199–208.

Shibutani, M., Woo, G.-H., Fujimoto, H., Saegusa, Y., Takahashi, M., Inoue, K., Hirose, M., and Nishikawa, A. (2009). Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents. *Reprod. Toxicol.* **28,** 297–307.

Soldin, O. P., and Aschner, M. (2007). Effects of manganese on thyroid hormone homeostasis: Potential links. *Neurotoxicology* **28,** 951–956.

Steel, R. G. D. (1959). A multiple comparison rank sum test: Treatments versus control. *Biometrics* **15,** 560–572.

Takeda, A., Ishiwatari, S., and Okada, S. (1999). Manganese uptake into rat brain during development and aging. *J. Neurosci. Res.* **56,** 93–98.

Tozuka, Y., Fukuda, S., Namba, T., Seki, T., and Hisatsune, T. (2005). GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. *Neuron* **47,** 803–815.

Wierońska, J. M., Brański, P., Siwek, A., Dybala, M., Nowak, G., and Pilc, A. (2010). GABAergic dysfunction in mGlu7 receptor-deficient mice as reflected by decreased levels of glutamic acid decarboxylase 65 and 67kDa and increased reelin proteins in the hippocampus. *Brain Res.* **1334,** 12–24.

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REPRODUCTIVE TOXICOLOGY

Similar distribution changes of GABAergic interneuron subpopulations in contrast to the different impact on neurogenesis between developmental and adult-stage hypothyroidism in the hippocampal dentate gyrus in rats

Ayako Shiraki · Hirotoshi Akane · Takumi Ohishi · Liyun Wang · Reiko Morita · Kazuhiko Suzuki · Kunitoshi Mitsumori · Makoto Shibutani

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Abstract Hypothyroidism affects neurogenesis. The present study was performed to clarify the sensitivity of neurogenesis-related cellular responses in the hippocampal dentate gyrus between developmental and adult-stage hypothyroidism. An exposure study of methimazole (MMI) as an anti-thyroid agent at 0, 50, 200 ppm in the drinking water was performed using pregnant rats from gestation day 10 to postnatal day (PND) 21 (developmental hypothyroidism) and adult male rats by setting an identical exposure period from PND 46 through to PND 77 (adult-stage hypothyroidism). Offspring with developmental hypothyroidism were killed at PND 21 or PND 77, and animals with adult-stage hypothyroidism were killed at PND 77. Proliferation and apoptosis were unchanged in the dentate subgranular zone by either developmental or adult-stage hypothyroidism. With regard to precursor granule cells, a sustained reduction of paired box 6-positive stem or early progenitor cells and a transient reduction of doublecortin-positive late-stage progenitor cells were observed after developmental hypothyroidism with MMI at 50 and 200 ppm. These cells were unchanged by adult-stage hypothyroidism. With regard to γ-aminobutyric acid (GABA) ergic interneuron subpopulations in the dentate hilus, the number of parvalbumin-positive cells was decreased and the number of calretinin-positive cells was increased after both developmental and adult-stage hypothyroidism with MMI at 50 and 200 ppm. Fluctuations in GABAergic interneuron numbers with developmental hypothyroidism continued through to PND 77 with 200 ppm MMI. Considering the roles of GABAergic interneuron subpopulations in neurogenesis and neuronal differentiation, subpopulation changes in GABAergic interneurons by hypothyroidism may be the signature of aberrant neurogenesis even at the adult stage.

Keywords Hypothyroidism · Hippocampal dentate gyrus · Impaired neurogenesis · γ-Aminobutyric acid (GABA) ergic interneurons · Parvalbumin (Pvalb)

Abbreviations

ADHD	Attention deficit and hyperactivity disorder
Calb2	Calretinin
C_T	Threshold cycle
Dcx	Doublecortin
GABA	γ-Aminobutyric acid
GD	Gestational day
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
MMI	Methimazole
Pax6	Paired box 6
PCNA	Proliferating cell nuclear antigen
PND	Postnatal day
Pvalb	Parvalbumin
PTU	6-propyl-2-thiouracil
SD	Sprague–Dawley
SGZ	Subgranular zone
T_3	Triiodothyronine
T_4	Thyroxine
TH	Thyroid hormone
TSH	Thyroid-stimulating hormone

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A. Shiraki · H. Akane · T. Ohishi · L. Wang · R. Morita · K. Suzuki · K. Mitsumori · M. Shibutani (✉)
Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
e-mail: mshibuta@cc.tuat.ac.jp

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TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

Introduction

Thyroid hormones (THs) play a crucial role in normal brain development during fetal and neonatal periods. They have many effects relating to neural proliferation, cell migration, neuronal differentiation, and synaptogenesis (Bernal and Nunez 1995). In humans, fetal and postnatal TH deficiency may cause mental retardation and attention deficit and hyperactivity disorder (ADHD) (Delange 2000; Vermiglio et al. 2004). TH is also essential for brain function in adulthood. Adult-stage hypothyroidism is linked to anxiety and depression, as well as impaired learning and memory (Almeida et al. 2007; Sait Gönen et al. 2004).

Experimentally, developmental hypothyroidism induces neurological deficits, learning impairments, memory dysfunction (Pineda-Reynoso et al. 2010; Akaike et al. 1991), and ADHD-like behaviors (Negishi et al. 2005). Rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) and methimazole (MMI) show aberrant brain growth, resulting in an impairment of neuronal migration as well as white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation (Lavado-Autric et al. 2003; Schoonover et al. 2004; Goodman and Gilbert 2007). In addition, experimental induction of hypothyroidism in adult rats affects cell morphology and brain function, leading to depression-like behaviors (Koromilas et al. 2010, Montero-Pedrazuela et al. 2006). Thus, pharmacological induction of hypothyroidism in rats during the developmental stage and the adult stage may provide experimental models for ADHD and anxiety, respectively.

The hippocampus is involved in crucial neuronal networks responsible for cognitive, emotional and memory function. In the hippocampal formation, the subgranular zone (SGZ) of the dentate gyrus retains the capacity to produce new neurons throughout adult life. This process is called adult neurogenesis (Eriksson et al. 1998) and raises the possibility that developmental susceptibility of the dentate gyrus to neurotoxicants may affect neurogenesis in adulthood as well as during development. There are increasing numbers of chemicals that have recently been revealed to affect proliferation and differentiation of progenitor cells in the SGZ through exposure in mice or rats during postnatal life (Choi et al. 2011; Hwang et al. 2011; Nam et al. 2011; Yan et al. 2011; Yoo et al. 2011).

In neurogenesis in the SGZ of the hippocampal dentate gyrus, type-1 stem cells undergo self-renewal to produce intermediate generations, in order: type-2a, type-2b, and type-3 cells. Type-3 cells undergo final mitosis to differentiate into immature granule cells, then into mature granule cells (Hodge et al. 2008). There are a number of neuronal stage-defining markers to define these cell types. Among them, paired box 6 (Pax6) is a marker for type-1 and type-2a progenitor cells (Breunig et al. 2007; Hodge et al. 2008). In contrast, doublecortin (Dcx) is expressed from type-2b progenitor cells to immature granule cells with peak expression in immature granule cells (Breunig et al. 2007; Knoth et al. 2010). Therefore, chemical effect on neurogenesis could be roughly evaluated using these two markers.

It is well known that there is interplay between interneurons and neurogenesis. The major interneuron populations of the dentate gyrus use γ-aminobutyric acid (GABA) as their major neurotransmitter. Basket cells and chandelier cells are a subpopulation of these interneurons that synthesize a calcium-binding protein, parvalbumin (Pvalb), and synapse directly onto the soma or initial axonal segment of principal cells of the hippocampus (Houser 2007). In addition, Pvalb-expressing cells are the most active interneurons. They express high levels of cytochrome *c* in the mitochondria, which represents high activity for GABAergic transmission (Gulyás et al. 2006). There also are calretinin (Calb2)-expressing neurons distributed in the dentate gyrus as another major interneuron population (Gulyás et al. 1996). Our previous studies have revealed that developmental hypothyroidism by maternal exposure to MMI or PTU in rats causes a sustained increase in immature interneurons in the hilar region of the dentate gyrus, reflecting aberration in neurogenesis and following migration of granule cell lineage (Saegusa et al. 2010). This result may suggest that the dentate gyrus is a useful region for evaluation of developmental neurotoxicity affecting neurogenesis in combination with analysis of interneuron subpopulations.

Understanding of the cellular and molecular mechanisms governing adult neurogenesis may provide an alternative tool to establish in vivo system for screening developmental neurotoxicants affecting neurogenesis, such as in the 28-day repeated-oral dose-toxicity study scheme. For this purpose, it is reasonable to compare the sensitivity of neurogenesis-related cellular responses against developmental neurotoxicants between developmental and adult-stage exposures. The present study thereby aimed to elucidate the sensitivity of cellular responses involved in neurogenesis of the hippocampal dentate gyrus by exposure of rats to MMI, using Pax6 and Dcx as neuronal stage-defining markers and Pvalb and Calb2 as interneuron markers. A young adult exposure model was compared with a developmental exposure model by setting an identical exposure period.