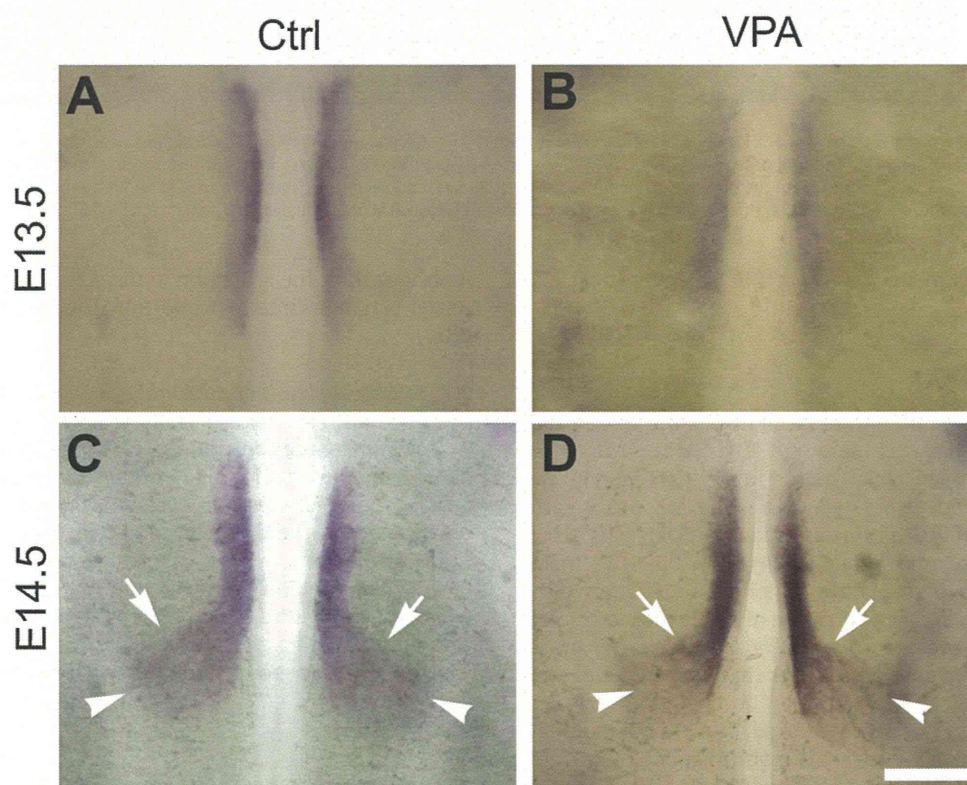


**Fig. 1.** Flat whole-mount preparation of the hindbrain. (A) Schematic diagram showing the cut site in the neural tube. Embryonic rat hindbrains were dissected and oriented with the ventricular side down (Tashiro et al., 2000). (B) Schematic diagram of a flat whole-mount preparation of the hindbrain. Figs. 2, 3 and 5 correspond to the area outlined by the red square in B. CP: cerebellar plate, Di: diencephalon, FP: floor plate, Is: isthmus, Mes: mesencephalon, Met: metencephalon, Myel: myelencephalon, Tel: telencephalon, SC: spinal cord, Vth G: trigeminal ganglion, VIIIth G: vestibular ganglion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

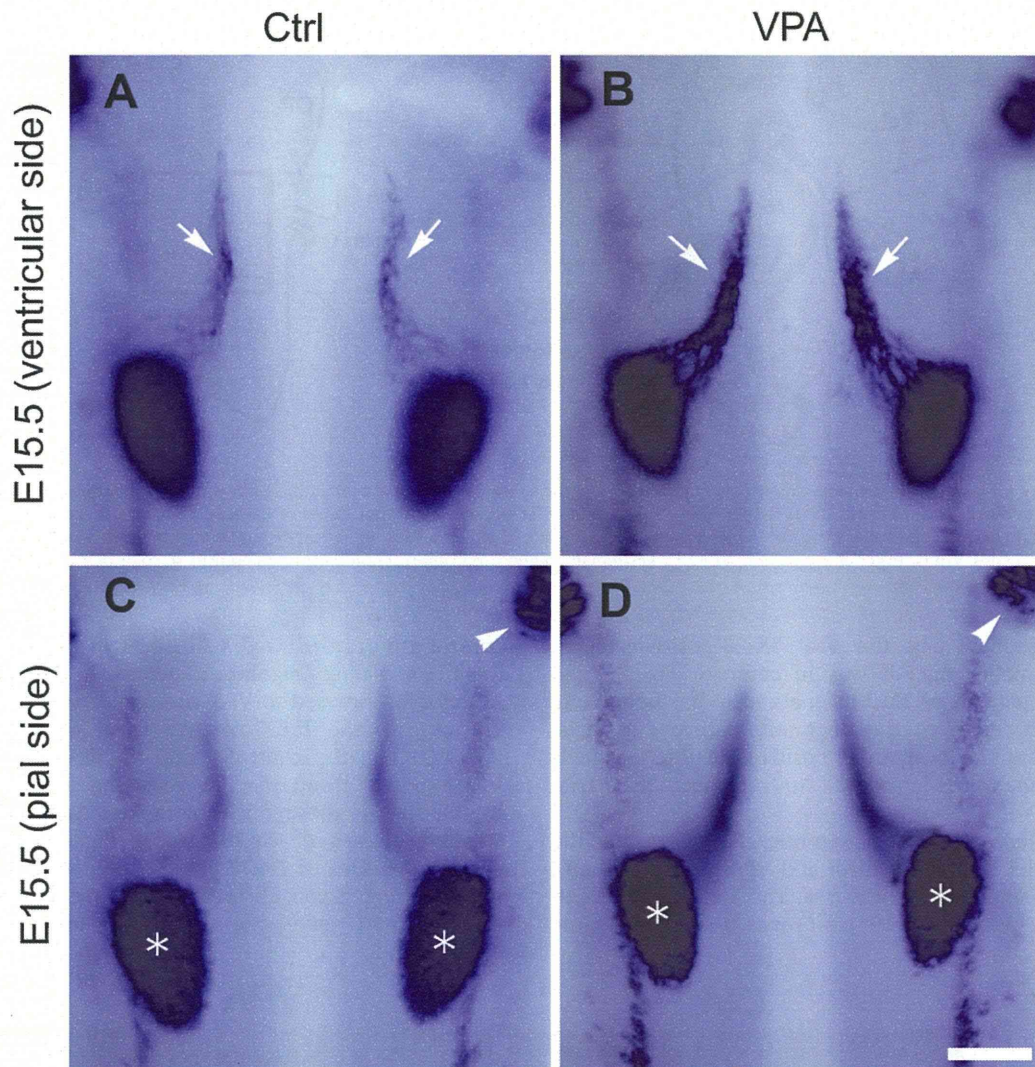
group (Fig. 3B, arrows) and the oval *Tbx20*-positive region appeared to be smaller than it was in controls (Fig. 3C and D, asterisks). The pattern of *Tbx20* signals near the floor plate was considered to represent the migratory pathway of facial motor neurons, and the oval areas positive for *Tbx20* signals at the caudal end were considered to represent the facial nuclei.

To identify differences in the migratory pathways and the sizes of facial nuclei between control and VPA-exposed groups, the *Tbx20*-positive areas were measured. The area of the *Tbx20*-positive migratory pathway ( $\times 10^4 \mu\text{m}^2$ ) was  $0.66 \pm 0.17$  in the

control group ( $n=6$ ) and  $1.37 \pm 0.31$  in the VPA-exposed group ( $n=12$ ), revealing a significant increase in the size of the pathway in embryos exposed to VPA compared with controls ( $p < 0.001$ ). The area of the *Tbx20*-positive facial nuclei ( $\times 10^4 \mu\text{m}^2$ ) was  $9.33 \pm 0.92$  in the control group ( $n=6$ ) and  $7.55 \pm 1.02$  in the VPA-exposed group ( $n=12$ ), revealing a significant reduction in the size of these nuclei compared with controls ( $p < 0.01$ ). When the sizes of *Tbx20*-positive facial nuclei were plotted against the sizes of *Tbx20*-positive migratory pathways (Fig. 4), the resulting graph showed an inverse relationship between the two, with increasing size of the migratory pathway and



**Fig. 2.** *Tbx20* expression patterns at E13.5 and E14.5. (A and B) E13.5 preparations showing *Tbx20* signals near the floor plate in the control group (A) and VPA-exposed group (B). (C and D) E14.5 preparations showing *Tbx20* signals near the floor plate in the control group (C) and VPA-exposed group (D). *Tbx20* signals extend in a caudolateral direction (arrows). Near the caudal end of the *Tbx20* expression domain, the signal fades out (arrowheads). Rostral is up. Scale bar: 250  $\mu\text{m}$ .



**Fig. 3.** *Tbx20* expression pattern at E15.5. (A and B) E15.5 preparations, viewed from the ventricular side, showing *Tbx20* signals near the floor plate in the control group (A) and VPA-exposed group (B). Signals in the migratory pathway of the VPA-exposed preparation (B, arrows) are more intense than those in the control preparation (A, arrows). (C and D) E15.5 preparations, viewed from the pial side, showing *Tbx20*-positive facial nuclei in the control group (C) and VPA-exposed group (D). The facial nuclei are smaller in embryos exposed to VPA (D, asterisks) than in control embryos (C, asterisks). Rostral is up. Scale bar: 250  $\mu\text{m}$ .

decreasing size of facial nuclei following VPA exposure ( $p < 0.001$ ;  $R = -0.74$ ).

### 3.2. Size of facial nuclei

To determine the sizes of mature facial nuclei, the area of *cadherin 8* (*cdh8*) expression was measured (Fig. 5). The *cdh8* gene is expressed in developed facial motor nuclei (Korematsu and Redies, 1997), and is not expressed within the migratory pathway (Fig. 5). The area of the *cdh8*-positive facial nuclei ( $\times 10^4 \mu\text{m}^2$ ) was  $9.87 \pm 0.43$  in the control group ( $n = 4$ ) and  $7.06 \pm 1.31$  in the VPA-exposed group ( $n = 5$ ), revealing a significant reduction in the area of *cdh8* expression in the facial nuclei of VPA-exposed animals ( $p < 0.01$ ). Indeed, the average area of *cdh8*-positive facial nuclei in the VPA-exposed group was 28.4% less than that in the control group.

CRL of E15.5 embryos was also measured. VPA-exposed animals appeared robust and had no external malformations. CRL (mm) was  $12.80 \pm 0.37$  in the control group ( $n = 5$ ) and  $12.24 \pm 0.18$  in the VPA-exposed group ( $n = 4$ ), revealing a significant reduction in CRL in VPA-exposed embryos at E15.5 ( $p < 0.05$ ). The average CRL in VPA-exposed embryos was only 4.3% less than that in control

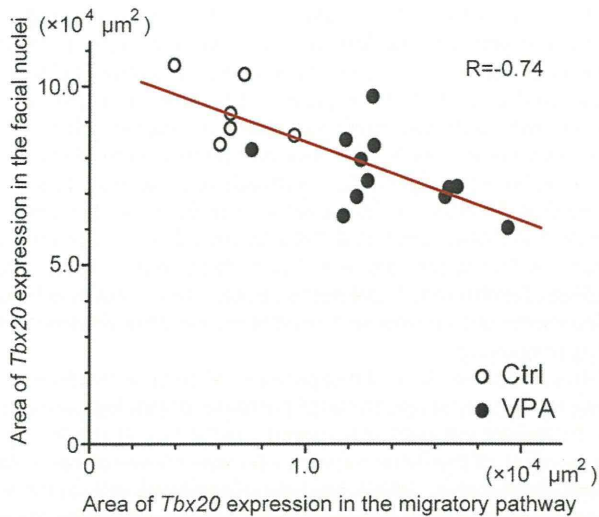
embryos; thus, the reduction in the size of the *cdh8*-positive facial nuclei was not directly proportional to the reduction in average CRL.

### 3.3. Expression levels of *cdh8* in the facial nuclei

To estimate the contribution of the cell adhesion molecule *cadherin 8* to the development of the facial nuclei, a preliminary analysis of the expression levels of *cdh8* was performed. The signal intensity in the VPA-exposed group was normalized to that in the control group, which was designated as 100%. The intensity of the *cdh8* signal in the facial nuclei was  $100.0 \pm 2.8$  in the control group ( $n = 4$ ) and  $84.5 \pm 12.2$  in the VPA-exposed group ( $n = 5$ ), revealing a significant reduction (by 15.5%) in *cdh8* expression in the VPA-exposed group compared with controls ( $p < 0.05$ ).

## 4. Discussion

We examined the development of facial nuclei in an animal model of autism, established by *in utero* exposure to VPA, using *in situ* hybridization for the molecular markers *Tbx20* and *cdh8*. Analysis of *Tbx20* expression revealed similar patterns of caudal



**Fig. 4.** Correlation between the area of the *Tbx20*-positive migratory pathway and the facial nucleus. The increase in the size of the migratory pathway is inversely proportional to the reduction in the size of the facial nucleus ( $p < 0.001$ ,  $R = -0.74$ ). Open circles: control group, filled circles: VPA-exposed group.

migration of neurons to the facial nuclei in control and VPA-exposed groups; however, this caudal migration of neurons was hindered in animals exposed to VPA, with the result that the facial nuclei were significantly smaller in these animals. Our data provide the first description of tangential migration and nucleus formation in the developing hindbrain in a rat model of autism. Moreover, this disruption during early development of the facial nuclei might contribute to the etiology of autism with facial palsy.

#### 4.1. Technical consideration

To assess the distribution of *Tbx20* and *cdh8* signals in the developing hindbrain, flat whole-mount preparations (Tashiro et al., 2000, 2007) were used. These allowed us to observe signal distributions within both the rostrocaudal and dorsoventral axes, without requiring the reconstruction of serial sections. Because later-stage whole-mount preparations are not suitable for an analysis of signal depth, we restricted our observations to the ventricular and pial sides at E15.5.

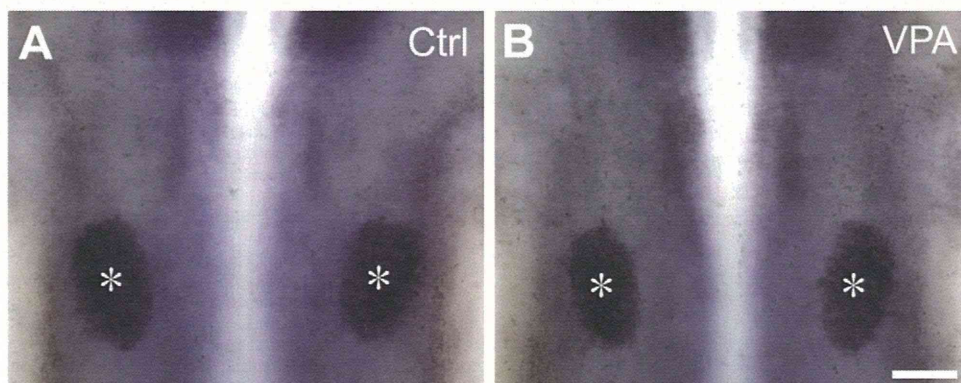
Facial nuclei were identified based on the expression of *Tbx20* and *cdh8* mRNA signals and their anatomical locations within the flat whole-mount preparations. Because *Tbx20* is expressed in all early-stage cranial motor neurons (Kraus et al., 2001; Meins et al.,

2000; Song et al., 2006), it was not possible to distinguish the facial nuclei from the abducens or trigeminal motor nuclei. However, using flat whole-mount preparations enabled us to positively identify the migratory pathways taken by neurons to reach these nuclei based on anatomical landmarks as well as the positioning of the ganglia and their roots. The trigeminal ganglion is positioned near rostrocaudal level r2, and *Tbx20* signals near the trigeminal ganglion are considered to represent the trigeminal motor nuclei (Noden, 1993). The vestibular ganglion is positioned near rostrocaudal level r4; *Tbx20* signals near the floor plate between these ganglia are considered to represent the facial motor nuclei. The neurons that comprise the facial nuclei originate in r4 and migrate caudally to r6; their migratory pathways are well-described in the literature (Chandrasekhar, 2004; Hatten, 1999; Noden, 1993; Yamamoto and Schwarting, 1991) and are consistent with the signal patterns we observed. The expansion of the area of *Tbx20*-positive signals is likely correlated to the migration of neurons to and shape of facial motor nuclei. Thus, the combination of *Tbx20* signals and positional information in flat whole-mount preparations is an appropriate tool for evaluating the development of facial nuclei.

In addition to *Tbx20* signals, *cdh8* signals can be used to visualize mature facial nuclei (Korematsu and Redies, 1997). Because *cdh8* is not expressed in the migratory pathways of neurons destined for the facial nuclei or other cranial motor nuclei in the hindbrain, *cdh8* signals enable us to specifically identify the area of the facial nuclei. Quantification of facial nucleus area based on *cdh8* expression is therefore more precise than measurements based on *Tbx20* expression. We have provided the first numerical quantification of the area of the developing facial nuclei following exposure to VPA *in utero*.

#### 4.2. Development of the facial nuclei in a rat model of autism

The caudal migration patterns of neurons destined for the facial nuclei in a rat model of autism induced by VPA exposure were similar to those seen in normal control rats. However, VPA exposure hindered this migration and significantly reduced the area of the facial nuclei. Although CRL was also reduced in the VPA-exposed group, the amount of decrease was less substantial than that for facial nucleus size, indicating that the reduction in the area of the facial nuclei was not due to overall shrinkage of the entire embryo after exposure to VPA. Moreover, the quantified decrease in facial nucleus area suggests an even larger percent decrease in volume: if the shape of the facial nucleus is assumed to be a sphere, then a 28.4% decrease in area equates to about a 40% decrease in volume. Thus, the total size of the facial nuclei is considerably reduced in animals exposed to VPA.



**Fig. 5.** *Cdh8* expression in the facial nuclei. (A and B) E15.5 preparation, viewed from the pial side, showing *cdh8*-positive facial nuclei in the control group (A) and VPA-exposed group (B). The facial nuclei are smaller in embryos exposed to VPA (B, asterisks) than in control embryos (A, asterisks). Note that *cdh8* signals were not detected in the migratory pathway (see also Fig. 3A and B, arrowheads). Rostral is up. Scale bar: 250 μm.

The reduction in facial nucleus size could be attributed to hindered cell migration, as we observed cells stacked in the migratory pathway, a common characteristic of failed cell migration, in addition to the observed inverse correlation between larger migratory pathway area and smaller facial nucleus size. Accumulating *in vivo* and *in vitro* studies have begun to elucidate the molecular mechanisms underlying the regulation of the caudal migration of neurons to facial motor nuclei in normal fishes and rodents (Bingham et al., 2002; McKay et al., 1997; Song et al., 2006; Studer et al., 1996; Wada et al., 2006). Some cell adhesion molecules, such as cadherin family members (LaMora and Voigt, 2009; Qu et al., 2010), could contribute to this migration by acting as scaffolds or facilitating the interactions between substrates. Therefore, a slight decrease in *cdh8* expression might lead to smaller facial nuclei by disrupting cellular migration. A decrease in *cdh8* expression may also explain the observed defasciculation and misrouting of peripheral facial nerves in the 2nd branchial arch (Tashiro et al., 2011), although the complimentary expression of other adhesion molecules would presumably regulate morphological development in peripheral and central facial nerve regions.

Another possible explanation for the reduction in facial nucleus size is a decreased number of cells within the nuclei given that the birth dates of most of these nuclei correspond to the stage at which VPA treatment was administered. VPA is known to affect cell division and the cell cycle by disturbing histone deacetylation (Chen et al., 2007; Phiel et al., 2001). The neurons of the facial nuclei are produced between days E12 and E14, with peak production at E13 (Altman and Bayer, 1982). In the present study, VPA exposure was performed at E9.5, and the short pharmacokinetic half-life of VPA in rodents (Nau et al., 1981) suggests that the effects of VPA would be limited to a narrow temporal window. Therefore, VPA treatment may reduce the number of early progenitor cells responsible for producing facial neurons. Further analysis of nucleogenesis is needed to clarify this possibility.

The mechanism by which VPA exposure affects the regulation of cell migration or differentiation remains unknown. It is possible that VPA exposure could have changed the identity of the rhombomeres. Various combinations of *Hox* gene expression are used to specify individual rhombomeres as well as to regulate the differentiation of cranial neurons (Erzurumlu et al., 2010; Noden, 1993; Trainor and Krumlauf, 2000; Yamamoto and Schwarting, 1991), and VPA exposure has been shown to alter the expression of *Hoxa1* (Stodgell et al., 2006). These reports support the hypothesis that VPA can change the identity of the rhombomeres. In addition, retinoic acid also contributes to the caudalization of the rhombencephalon (Altmann and Brivanlou, 2001; Glover et al., 2006; Yamada, 1994) and alters *Hox* gene expression (Conlon and Rossant, 1992; Langston and Gudas, 1992). Thus, VPA might affect retinoic acid signaling, which in turn regulates *Hox* gene expression.

In chicken embryos exposed to VPA *in ovo*, *Pax6* expression in the eye is diminished (Whitsel et al., 2002), indicating that VPA exposure can alter the expression of *Pax6*. Given that *Pax6* regulates the specification of ventral neuron subtypes in the hindbrain (Takahashi and Osumi, 2002), it is possible that VPA affects the differentiation of facial motor neurons *via Pax6* signaling. These reports indicate that VPA exposure affects the environment in which facial neurons differentiate and migrate within the hindbrain during development.

#### 4.3. Clinical significance

In the present study, exposure of rat embryos to VPA at E9.5 affected the development of the facial nuclei in the hindbrain. In our previous study, we showed that exposure to VPA at E9.5 also affected the development of the peripheral facial nerve (Tashiro et al., 2011). Therefore, VPA exposure at E9.5 affects the

development of the entire facial nerve. The embryonic stage E9.5 in rat corresponds to the 3rd week of human gestation. Pregnant women administered VPA, which is used as an anticonvulsant and mood-stabilizing drug, during this stage, sometimes give birth to children with fatal valproate syndrome or autism (Ehlers et al., 1992; Miller et al., 2005). The pharmacokinetic half-life of VPA in humans is 10-fold longer than it is in rodents (Nau et al., 1981), suggesting that the effects of VPA exposure on the development of the facial nerves that we observed in the rat would be more pronounced in humans. Our observations will contribute to the overall understanding of embryonic facial nerve development, and may be used to determine the appropriate conditions for drug administration during pregnancy.

Little is known about the capacity of abnormal morphogenesis during these early stages to cause postnatal physiological dysfunction. In autism patients, disordered caudal migration during the development of the facial nerve nuclei was shown to cause facial disease (Rodier et al., 1996). Analysis of eye-blink in a rat model of autism induced by VPA exposure might help to determine the level of function of the facial nerve. These and future findings may aid in the elucidation of the mechanisms underlying multiple inherent diseases.

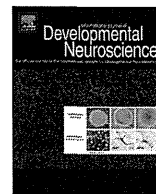
#### Acknowledgments

We thank Thaddeus Dryja for useful comments on this manuscript. This study was supported by the Ministry of Health, Labor and Welfare of the Japanese Government and a Grand-in-Aid from the School of Medicine, Mie University.

#### References

- Altman, J., Bayer, S.A., 1982. Development of the cranial nerve ganglia and related nuclei in the rat. *Advances in Anatomy, Embryology and Cell Biology* 74, 1–90.
- Altmann, C.R., Brivanlou, A.H., 2001. Neural patterning in the vertebrate embryo. *International Review of Cytology* 203, 447–482.
- Bingham, S., Higashijima, S., Okamoto, H., Chandrasekhar, A., 2002. The zebrafish trilobite gene is essential for tangential migration of branchiomotor neurons. *Developmental Biology* 242, 149–160.
- Bloch-Gallego, E., Causeret, F., Ezan, F., Backer, S., Hidalgo-Sánchez, M., 2005. Development of precerebellar nuclei: instructive factors and intracellular mediators in neuronal migration, survival and axon pathfinding. *Brain Research. Brain Research Reviews* 49, 253–266.
- Chandrasekhar, A., 2004. Turning heads: development of vertebrate branchiomotor neurons. *Developmental Dynamics* 229, 143–161.
- Charman, T., Baird, G., 2002. Practitioner review: diagnosis of autism spectrum disorder in 2- and 3-year-old children. *Journal of Child Psychology and Psychiatry* 43, 289–305.
- Chen, P.S., Wang, C.C., Bortner, C.D., Peng, G.S., Wu, X., Pang, H., Lu, R.B., Gean, P.W., Chuang, D.M., Hong, J.S., 2007. Valproic acid and other histone deacetylase inhibitors induce microglial apoptosis and attenuate lipopolysaccharide-induced dopaminergic neurotoxicity. *Neuroscience* 149, 203–212.
- Chédotal, A., Rijli, F.M., 2009. Transcriptional regulation of tangential neuronal migration in the developing forebrain. *Current Opinion in Neurobiology* 19, 139–145.
- Chédotal, A., 2010. Should I stay or should I go? Becoming a granule cell. *Trends in Neurosciences* 33, 163–172.
- Conlon, R.A., Rossant, J., 1992. Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes *in vivo*. *Development* 116, 357–368.
- Ehlers, K., Sturje, H., Merker, H.J., Nau, H., 1992. Valproic acid-induced spina bifida: a mouse model. *Teratology* 45, 145–154.
- Erzurumlu, R.S., Murakami, Y., Rijli, F.M., 2010. Mapping the face in the somatosensory brainstem. *Nature Reviews Neuroscience* 11, 252–263.
- Filipek, P.A., Accardo, P.J., Baranek, G.T., Cook, E.H., Dawson, G., Gordon, B., Gravel, J.S., Johnson, C.P., Kallen, R.J., Levy, S.E., Minshew, N.J., Ozonoff, S., Prizant, B.M., Rapin, I., Rogers, S.J., Stone, W.L., Teplin, S., Tuchman, R.F., Volkmar, F.R., 1999. The screening and diagnosis of autistic spectrum disorders. *Journal of Autism and Developmental Disorders* 29, 439–484.
- Gillberg, C., Winnergård, L., 1984. Childhood psychosis in a case of Moebius syndrome. *Neuropediatrics* 15, 147–149.
- Glover, J.C., Renaud, J.S., Rijli, F.M., 2006. Retinoic acid and hindbrain patterning. *Journal of Neurobiology* 66, 705–725.
- Hatten, M.E., 1999. Central nervous system neuronal migration. *Annual Review of Neuroscience* 22, 511–539.
- Huang, Z., 2009. Molecular regulation of neuronal migration during neocortical development. *Molecular and Cellular Neurosciences* 42, 11–22.

- Ingram, J.L., Peckham, S.M., Tisdale, B., Rodier, P.M., 2000. Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicology and Teratology* 22, 319–324.
- Korematsu, K., Redies, C., 1997. Expression of *cadherin-8* mRNA in the developing mouse central nervous system. *Journal of Comparative Neurology* 387, 291–306.
- Kraus, F., Haenig, B., Kispert, A., 2001. Cloning and expression analysis of the mouse T-box gene *Tbx20*. *Mechanisms of Development* 100, 87–91.
- LaMora, A., Voigt, M.M., 2009. Cranial sensory ganglia neurons require intrinsic N-cadherin function for guidance of afferent fibers to their final targets. *Neuroscience* 159, 1175–1184.
- Langston, A.W., Gudas, L.J., 1992. Identification of a retinoic acid responsive enhancer 3' of the murine homeobox gene *Hox-1.6*. *Mechanisms of Development* 38, 217–228.
- McKay, I.J., Lewis, J., Lumsden, A., 1997. Organization and development of facial motor neurons in the Kreisler mutant mouse. *European Journal of Neuroscience* 9, 1499–1506.
- Meins, M., Henderson, D.J., Bhattacharya, S.S., Sowden, J.C., 2000. Characterization of the human *Tbx20* gene, a new member of the T-box gene family closely related to the *Drosophila* H15 gene. *Genomics* 67, 317–332.
- Miller, M.T., Strömland, K., Ventura, L., Johansson, M., Bandim, J.M., Gillberg, C., 2005. Autism associated with conditions characterized by developmental errors in early embryogenesis: a mini review. *International Journal of Developmental Neuroscience* 23, 201–219.
- Miyazaki, K., Narita, N., Narita, M., 2005. Maternal administration of thalidomide or valproic acid causes abnormal serotonergic neurons in the offspring: implication for pathogenesis of autism. *International Journal of Developmental Neuroscience* 23, 287–297.
- Narita, M., Oyabu, A., Imura, Y., Kamada, N., Yokoyama, T., Tano, K., Uchida, A., Narita, N., 2010. Nonexploratory movement and behavioral alterations in a thalidomide or valproic acid-induced autism model rat. *Neuroscience Research* 66, 2–6.
- Narita, N., Kato, M., Tazoe, M., Miyazaki, K., Narita, M., Okado, N., 2002. Increased monoamine concentration in the brain and blood of fetal thalidomide- and valproic acid-exposed rat: putative animal models for autism. *Pediatric Research* 52, 576–579.
- Nau, H., Zierer, R., Spielmann, H., Neubert, D., Gansau, C., 1981. A new model for embryotoxicity testing: teratogenicity and pharmacokinetics of valproic acid following constant-rate administration in the mouse using human therapeutic drug and metabolite concentrations. *Life Sciences* 29, 2803–2814.
- Nieto, M.A., Patel, K., Wilkinson, D.G., 1996. In situ hybridization analysis of chick embryos in whole mount and tissue sections. *Methods in Cell Biology* 51, 219–235.
- Noden, D.M., 1993. Spatial integration among cells forming the cranial peripheral nervous system. *Journal of Neurobiology* 24, 248–261.
- Ornitz, E.M., Guthrie, D., Farley, A.H., 1977. The early development of autistic children. *Journal of Autism and Childhood Schizophrenia* 7, 207–229.
- Ornoy, A., 2009. Valproic acid in pregnancy: how much are we endangering the embryo and fetus? *Reproductive Toxicology* 28, 1–10.
- Phiel, C.J., Zhang, F., Huang, E.Y., Guenther, M.G., Lazar, M.A., Klein, P.S., 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *Journal of Biological Chemistry* 276, 36734–36741.
- Qu, Y.B., Glasco, D.M., Zhou, L.B., Sawant, A., Ravni, A., Fritzsche, B., Damrau, C., Murdoch, J.N., Evans, S., Pfaff, S.L., Formstone, C., Goffinet, A.M., Chandrasekhar, A., Tissir, F., 2010. Atypical cadherins *Celsr1–3* differentially regulate migration of facial branchiomotor neurons in mice. *Journal of Neuroscience* 30, 9392–9401.
- Rodier, P.M., Ingram, J.L., Tisdale, B., Nelson, S., Romano, J., 1996. Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. *Journal of Comparative Neurology* 370, 247–261.
- Rodier, P.M., Bryson, S.E., Welch, J.P., 1997. Minor malformations and physical measurements in autism: data from Nova Scotia. *Teratology* 55, 319–325.
- Song, M.R., Shirasaki, R., Cai, C.L., Ruiz, E.C., Evans, S.M., Lee, S.K., Pfaff, S.L., 2006. T-Box transcription factor *Tbx20* regulates a genetic program for cranial motor neuron cell body migration. *Development* 133, 4945–4955.
- Stodgell, C.J., Ingram, J.L., O'Bara, M., Tisdale, B.K., Nau, H., Rodier, P.M., 2006. Induction of the homeotic gene *Hoxa1* through valproic acid's teratogenic mechanism of action. *Neurotoxicology and Teratology* 28, 617–624.
- Strömland, K., Nordin, V., Miller, M., Akerström, B., Gillberg, C., 1994. Autism in thalidomide embryopathy: a population study. *Developmental Medicine and Child Neurology* 36, 351–356.
- Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., Krumlauf, R., 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* 384, 630–634.
- Takahashi, M., Osumi, N., 2002. *Pax6* regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. *Development* 129, 1327–1338.
- Tashiro, Y., Endo, T., Shirasaki, R., Miyahara, M., Heizmann, C.W., Murakami, F., 2000. Afferents of cranial sensory ganglia pathfind to their target independent of the site of entry into the hindbrain. *Journal of Comparative Neurology* 417, 491–500.
- Tashiro, Y., Yanagawa, Y., Obata, K., Murakami, F., 2007. Development and migration of GABAergic neurons in the mouse myelencephalon. *Journal of Comparative Neurology* 503, 260–269.
- Tashiro, Y., Oyabu, A., Imura, Y., Uchida, A., Narita, N., Narita, M., 2011. Morphological abnormalities of embryonic cranial nerves after in utero exposure to valproic acid: implications for the pathogenesis of autism with multiple developmental anomalies. *International Journal of Developmental Neuroscience* 29, 359–364.
- Trainor, P.A., Krumlauf, R., 2000. Patterning the cranial neural crest: hindbrain segmentation and *Hox* gene plasticity. *Nature Reviews Neuroscience* 1, 116–124.
- Wada, H., Tanaka, H., Nakayama, S., Iwasaki, M., Okamoto, H., 2006. *Frizzled3a* and *Celsr2* function in the neuroepithelium to regulate migration of facial motor neurons in the developing zebrafish hindbrain. *Development* 133, 4749–4759.
- Whitsel, A.I., Johnson, C.B., Forehand, C.J., 2002. An in ovo chicken model to study the systemic and localized teratogenic effects of valproic acid. *Teratology* 66, 153–163.
- Williams, G., King, J., Cunningham, M., Stephan, M., Kerr, B., Hersh, J.H., 2001. Fetal valproate syndrome and autism: additional evidence of an association. *Developmental Medicine and Child Neurology* 43, 202–206.
- Yamada, T., 1994. Caudalization by the amphibian organizer: brachyury, convergent extension and retinoic acid. *Development* 120, 3051–3062.
- Yamamoto, M., Schwarting, G., 1991. The formation of axonal pathways in developing cranial nerves. *Neuroscience Research* 11, 229–260.



## Thyroid hormone-dependent development of the GABAergic pre- and post-synaptic components in the rat hippocampus

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### ARTICLE INFO

#### Article history:

Received 18 March 2013

Received in revised form 30 August 2013

Accepted 17 September 2013

#### Keywords:

Thyroid hormone  
Brain development  
Hippocampus  
GABAergic system  
GAD65  
GAD67  
KCC2

### ABSTRACT

Thyroid hormone (TH) plays essential roles in normal brain development mainly by regulating gene expression through binding to specific nuclear receptors which serve as transcription factors. Previous studies showed that perinatal deficiency of TH or impairment of its signaling severely affect brain development, especially the development of the  $\gamma$ -aminobutyric acid (GABA) system, but cellular and molecular targets of the hormone are only partly uncovered. In the present study, we focused on the developing rat hippocampus which was confirmed to be one of the regions highly sensitive to TH status, and found two new targets of the hormone among the pre- and post-synaptic components of the GABAergic system. One was glutamic acid decarboxylase 65 (GAD65), the protein level of which was reduced to less than 50% of control in the hippocampus of hypothyroid rats (obtained by administering 0.025% methimazole in drinking water to pregnant dams from gestational day 15 until 4 weeks postpartum) and recovered to control levels by daily thyroxine-replacement after birth. Reduction in GAD65 protein was correlated immunohistochemically with a 37% reduction in the number of GAD65-positive cells as well as a reduction in GAD65-positive processes. In contrast, the other GAD isotype, GAD67, was not affected by TH status. A subpopulation of GABAergic neurons containing parvalbumin was also confirmed to be highly dependent on TH status. The second target of thyroid hormone was neuron-specific  $K^+/Cl^-$  co-transporter, KCC2, which is responsible for switching of GABA action from excitatory to inhibitory. In the euthyroid hippocampus, a sharp rise of *kcc2* expression was observed at postnatal day (PND)10 which was followed by a large increase in KCC2 protein at PND15. This transient rise in *kcc2* expression was completely suppressed by hypothyroidism, resulting in nearly 80% reduction in KCC2 protein at PND15. These results indicate that the development of GABAergic terminals and the excitatory to inhibitory maturation of GABA signaling are strongly dependent on TH.

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### 1. Introduction

Thyroid hormone (TH) is essential for proper brain development. Its insufficiency during the perinatal period results in a syndrome known as cretinism in humans which consists of mental retardation, ataxia, and deafness together with impairment of body growth. At the cellular level, relatively later processes of central nervous system (CNS) development such as neurogenesis, cell migration, axonal and dendritic proliferation, synaptogenesis, and myelination are affected by hypothyroidism (Bernal and Nunez,

1995; Oppenheimer and Schwartz, 1997; Koibuchi and Chin, 2000; Thompson and Potter, 2000; Williams, 2008).

As TH exerts its effects mainly through binding to the specific nuclear receptors of the steroid-retinoic acid-TH receptor superfamily which function as ligand-regulated transcription factors, one of the essential steps in understanding its mechanism of action is to identify genes regulated by TH. Many previous studies have focused on the rodent cerebellum as a model system since it develops postnatally during the critical time window of TH action and is severely affected by hypothyroidism. Microarray-based global gene expression analyses of the developing cerebellum from animals with altered TH status (Quignodon et al., 2007; Takahashi et al., 2008) or of cultured cerebellar neurons after addition of the transcriptionally active TH, 3,5,5'-triiodothyronine (T3) (Quignodon et al., 2007; Chatonnet et al., 2012) resulted in the identification of several new candidate genes, and chromatin immunoprecipitation assays have been carried out to directly identify TH target genes

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(Quignodon et al., 2007; Dong et al., 2009), but the total number of genes confirmed to be directly regulated by TH is still limited.

Hypothyroidism has a strong negative effect on the development of the  $\gamma$ -aminobutyric acid (GABA) system *in vivo* and *in vitro* (reviewed in Wiens and Trudeau, 2006), leading to impairments in GABAergic circuit formation and inhibitory neurotransmission in the cerebral cortex and the hippocampus (Gilbert et al., 2007; Friauf et al., 2008a) as well as the differentiation and development of GABAergic neurons (Manzano et al., 2007; Westerholz et al., 2010). Reduced TH signaling in TH receptor  $\alpha 1$  (TR $\alpha 1$ ) knockout mice (Guadaño-Ferraz et al., 2003) or mice expressing mutant TR $\alpha 1$  with 10-fold lower affinity for TH (Venero et al., 2005; Wallis et al., 2008) results in significant reductions in GABAergic terminals in the hippocampus correlated with behavioral abnormalities. However, molecular targets of the hormone in the GABAergic system are only partly uncovered.

Since previous studies have been carried out under different experimental conditions and have focused on different regions of the brain, we first examined the differential sensitivity of the developing rat brain regions (cerebral cortex, hippocampus, striatum, midbrain, and cerebellum) toward TH status in the present study by measuring the mRNA expressions of the known TH-dependent genes. For this purpose, three genes were selected (neurogranin, hairless, myelin basic protein) which are widely expressed in the brain and well established as direct targets of T3 *in vivo*. In the hippocampus which was confirmed to be one of the highly sensitive regions to TH under the present experimental protocol, TH-dependent alterations in the amount of both pre- and post-synaptic components of the GABAergic system were further investigated.

GABA is the main inhibitory neurotransmitter in the mature CNS, but it mainly acts as an excitatory neurotransmitter during early stages of development (Ben-Ari, 2002; Payne et al., 2003; Ben-Ari et al., 2007; Farrant and Kaila, 2007). In addition to the differentiation of GABAergic neurons and establishment of the GABAergic synapses, this switching in the action of GABA is another key event in the development of the GABAergic system. It takes place around the 2nd postnatal week in rats through reduction of intracellular  $\text{Cl}^-$  concentration (Rivera et al., 1999). Since this period overlaps with the critical time window of TH-dependent growth in the CNS (Oppenheimer and Schwartz, 1997; Thompson and Potter, 2000), we further examined the TH-dependence of the expression of the neuron-specific  $\text{K}^+/\text{Cl}^-$  co-transporter (KCC2) which is responsible for excluding intracellular  $\text{Cl}^-$  and establishing low intracellular  $\text{Cl}^-$  concentration (Rivera et al., 1999; Payne et al., 2003; Fiumelli and Woodin, 2007). The results indicate that TH is essential both for the differentiation of the presynaptic GABAergic neurons and the maturation of the postsynaptic components of the GABAergic system in the developing hippocampus.

## 2. Materials and methods

### 2.1. Animal treatment

Pregnant Wistar/ST rats were purchased from SLC (Shizuoka, Japan). They were housed individually under controlled temperature ( $24 \pm 1^\circ\text{C}$ ) on a 12-h-light (06:00–18:00 h): 12-h-dark (18:00–06:00 h) cycle. Food and water were available *ad libitum*. Three pregnant dams were used to obtain pups in each of the following three treatment groups; control, hypothyroid, hypothyroid with thyroxine (T4) replacement. Hypothyroid and T4-replacement groups were rendered hypothyroid by administering 0.025% (w/v) 2-mercapto-1-methylimidazole (MMI) in drinking water to their mothers from the 15th day of conception. From the day of birth, pups from the T4-replacement group received daily subcutaneous injection of T4 (20 ng/g body weight/day) dissolved in saline while control and hypothyroid pups received saline until sacrifice 6 h after the last injection. For one series of experiments, five pups each of control, hypothyroid and T4-replacement groups were used at postnatal day (PND) 4, 10, 15 and 28. They were sacrificed under deep ether anesthesia and blood samples were drawn from the heart and collected with 5 mM EDTA as anticoagulant. After perfusion with phosphate-buffered saline (PBS), the brains were

removed and separated sagittally into halves. In total, four series of animals were used to obtain tissue samples for mRNA and protein extractions and immunohistochemical observation. Five brain regions (cerebral cortex, hippocampus, striatum, midbrain and cerebellum) were excised for mRNA extractions and hippocampi were excised for protein extractions, immediately frozen in liquid  $\text{N}_2$ , and kept at  $-80^\circ\text{C}$  until use. For immunohistochemistry, brain halves were immediately fixed in 4% paraformaldehyde (PFA).

All animal treatments have been approved by the Animal Experimentation Committee of Aoyama-Gakuin University and were carried out under veterinary supervision and in accordance with the 'Guidelines for the Use of Animals in Neuroscience Research'.

### 2.2. Measurement of plasma T4 levels

Blood samples were centrifuged at  $1500 \times g$  for 20 min at room temperature to obtain the plasma as the supernatant. T4 in the plasma from control ( $n=5-12$ ), hypothyroid ( $n=5-12$ ) and T4-replacement groups ( $n=5-11$ ) were measured by competitive enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Automation Inc.) according to the protocols recommended by the manufacturer.

### 2.3. Real-time quantitative fluorescence-based PCR

Total RNA was prepared individually from five brain regions obtained from five pups of each treatment group at PND4, 10, 15 and 28, using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized from  $2 \mu\text{g}$  total RNA from one animal using SuperScript II reverse transcription kit (Invitrogen Life Technologies). Expression levels of the following 12 genes in cDNA samples were quantified by fluorescence-based real-time PCR using Smart Cycler System (Cepheid, Sunnyvale, CA, USA) with SYBR Premix ExTaq (Takara, Shiga, Japan): cyclophilin-A (*ppia*), neurogranin (*rc3*), hairless (*hr*), reelin (*reln*), myelin basic protein (*mnp*), glutamic acid decarboxylase 65 (*gad65*), GABA(A) receptor subunit  $\alpha 1$  (*gabra1*), GABA(A) receptor subunit  $\beta 2$  (*gabrb2*), GABA(A) receptor subunit  $\gamma 2$  (*gabrg2*), gephyrin (*gphn*), collybistin (*arhgef9*),  $\text{K}^+/\text{Cl}^-$  cotransporter (*kcc2*). Data were analyzed by  $2^{-\Delta\Delta\text{Ct}}$  method using cyclophilin-A (*ppia*) as an internal standard gene, as its expression level was not affected by thyroid status (Muñoz et al., 1991) or during postnatal development (Takahashi et al., 2005). PCR primers were designed in Oligo 6.0 primer analysis software (Molecular BiologyInsights). Sequences of the PCR primers are listed in Table 1.

### 2.4. Western blotting

Each hippocampal sample obtained from each group at PND4, 10, 15 and 28 ( $n=5$  for either group at each time point) was homogenized in 10 volumes of Cel-Lytic MT (Sigma, St. Louis, MO, USA) containing protease inhibitor cocktail (Sigma), centrifuged at  $12,000 \times g$  for 11 min at  $4^\circ\text{C}$  and supernatant was collected. Concentrations of extracted proteins in the supernatant were determined by Protein Quantification Kit – Wide Range (Dojindo Molecular Technologies, Kumamoto, Japan) before proceeding with Western blotting. Each protein extract was combined with 4 $\times$  sample buffer (0.25 M Tris-HCl, 40% glycerol, 8% SDS, 0.04% bromophenol blue, 8%  $\beta$ -mercaptoethanol) and boiled for 5 min. Samples were separated by SDS-PAGE on Tris-HCl gels followed by electrophoretic transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The blots were blocked for 1 h at RT with 2% BSA or 2.5% skimmed milk in PBS containing 0.1% Tween 20 (PBS-T) and 0.02% sodium azide, and incubated overnight at  $4^\circ\text{C}$  with one of the following primary antibodies in 2% BSA and 0.02% sodium azide-containing PBS; monoclonal anti-glutamic acid decarboxylase 65 (GAD65; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-GAD67 (1:10,000; Abcam, Cambridge, United Kingdom), rabbit polyclonal anti- $\text{K}^+/\text{Cl}^-$  cotransporter (KCC2; 1:5000; Millipore) and monoclonal anti- $\beta$ -actin (1:10,000; Sigma, St. Louis, MO). After being rinsed in PBS-T, the blots were incubated for 1 h at RT with either of the following horseradish peroxidase (HRP)-conjugated secondary antibodies in 2% BSA with 0.02% sodium azide; anti-rabbit IgG (1:5000) and anti-mouse IgG (1:10,000) (both from Jackson ImmunoResearch, West Grove, PA). The blots were rinsed several times in PBS-T and visualized by exposure to Hyperfilm ECL (GE Healthcare, Buckinghamshire, United Kingdom) using an Immobilon Western Chemiluminescent HRP Substrate (Millipore). For quantification, the films were scanned, and the density of each band was measured in ImageJ (National Institutes of Health), and the results normalized using  $\beta$ -actin as standard.

### 2.5. Immunohistochemistry

For immunohistochemical analysis, brain samples were fixed in 4% PFA for 48 h at  $4^\circ\text{C}$ , dehydrated, embedded in paraffin, and cut into 8- $\mu\text{m}$ -thick coronal sections. Three consecutive sections per animal taken from identical locations in the frontal part of lateral hippocampus were used for immunohistochemical staining with three different antibodies; the first section with anti-GAD65 antibody, the second section with anti-GAD67 antibody, and the third section with anti-parvalbumin (PV) antibody. After deparaffinization and rehydration, the sections were incubated in 0.5% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 20 min at RT to quench endogenous peroxidase activity, and boiled with 0.01 M citrate buffer for 20 min or incubated in 0.3% Triton

**Table 1**  
Primers for real-time quantitative PCR.

	Forward	Reverse
<i>ppia</i>	GCAAGACCAGCAAGAAGATCACC	CTTCAGTGAGAGCAGAGATTACAG
<i>rc3</i>	GACTCCCTACTGTGTTTGTGAG	CTACGCCACGCGAAGCCAGC
<i>hr</i>	GCTGACCCCTCCCTCATGG	GCAGTTGAGATACACAGAGGAAG
<i>mbp</i>	GGCACAGAGACACGGGCATCC	GCGACTTCTGGGCGAGGGAGC
<i>gabra1</i>	GGGTAAGGTGAGGCTGTCATTGT	TCTGTGTTAGGCTGGCTCATCTC
<i>gabbr2</i>	CGATGACCACAATCAATACCCATCT	GCCATAAAGACAAAGACAAAGCACC
<i>gabrg2</i>	GAGTGTGTTGGATGGCAAGGACTG	AAGCAGAAGGCGGTAGGGAAGAA
<i>gphn</i>	TGGAACCTCACAAGGCGGAG	AAAAGTAGCTGTGCCGTTGC
<i>arhgef9</i>	GAAGGACAGCCGCTATCAAC	ATCACTGTGCTCTGGGCAG
<i>kcc2</i>	CCATCCAGGTGACTTTACTCTGA	CCTCTCATTATACAAGGGTTTG

X-100 for 15 min at RT to retrieve the antigen. They were rinsed in PBS and incubated in 4% BSA for 30 min at RT to block nonspecific binding sites. Rabbit polyclonal anti-GAD65 antibody (1:500; Sigma), monoclonal anti-GAD67 antibody (1:2000; Abcam) or monoclonal anti-PV antibody (1:4000; Sigma) was then applied to each section, and the sections were incubated overnight at 4 °C. They were further rinsed in PBS and incubated for 1 h at RT with either of the following biotinylated secondary antibodies in BSA/PBS; anti-mouse IgG (1:1000) and anti-rabbit IgG (1:1000) (both from Chemicon/Millipore). The sections were incubated in biotin-conjugated horseradish peroxidase with streptavidin (ABC Elite Kit; Vector, Burlingame, CA) at a dilution of 1:1000 for 30 min at RT and then visualized with nickel-intensified diaminobenzidine. The sections were dehydrated and mounted in Entellan New (Merck, Darmstadt, Germany), and observed with a microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany). The images were recorded with a color CCD camera (ProgRes; Genoptik, Jena, Germany).

### 2.6. Statistical analysis

All numerical data were expressed as mean  $\pm$  SEM and analyzed by one-way analysis of variance (ANOVA). When ANOVA revealed significant differences, it was followed by two-tailed Student's *t*-test. Level of significance was set at  $p < 0.05$ .

## 3. Results

### 3.1. Confirmation of the effect of maternal MMI-treatment and postnatal T4-replacement on the plasma thyroxine level of neonatal rats

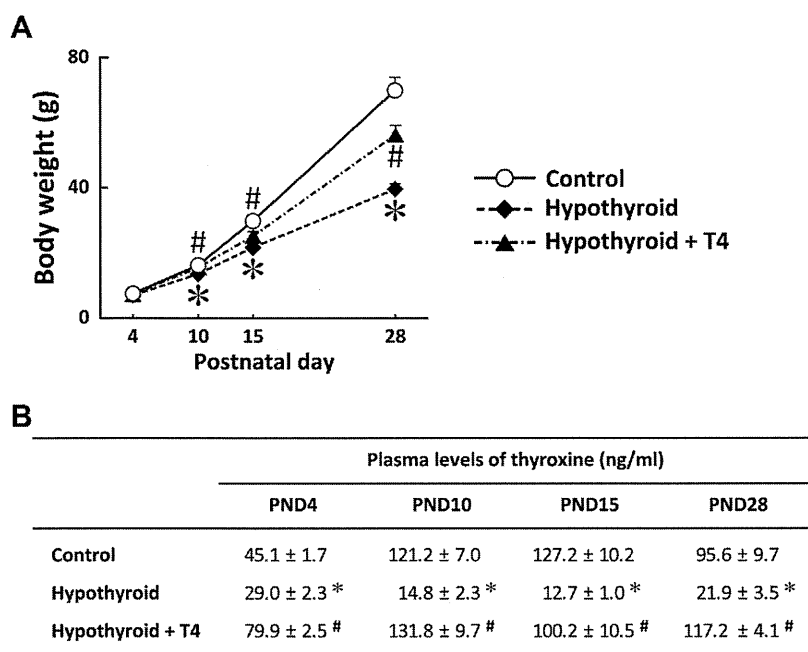
Among the three experimental groups of control, hypothyroid (MMI-treated), and hypothyroid with T4-replacement, there was

no significant difference in dam's body weights, litter size, sex ratio of the offspring or survival of pups to weaning. Hypothyroid pups had body weights comparable to control pups at postnatal day 4 (PND4), but started to show reductions in body weight gain around PND7 (Fig. 1A). They also showed retardations in development such as a delay in eye opening and hair growth. Pups in the T4-replacement group showed a significant recovery of the growth curve compared with the hypothyroid group.

To confirm the hypothyroid state of the MMI-treated group and its rescue by T4-replacement, plasma T4 level was determined in animals from the three groups at PND 4, 10, 15 and 28. As shown in Fig. 1B, plasma T4 level of control pups showed a 2.7-fold increase between PND4 and 10, was maintained at this high level until PND15, and started to decline at PND28. Hypothyroid pups had a severe reduction in plasma T4 level at all time points whereas the pups in T4-replacement group had normal or higher plasma level of T4 measured 6 h after T4 injection. In particular, T4-replacement resulted in the plasma T4 level significantly higher than that of the control at PND4 when normal T4 level was still low.

### 3.2. Differences in sensitivities toward TH status among the developing brain regions

Since previous studies have been carried under different experimental conditions to induce hypothyroidism (such as timing



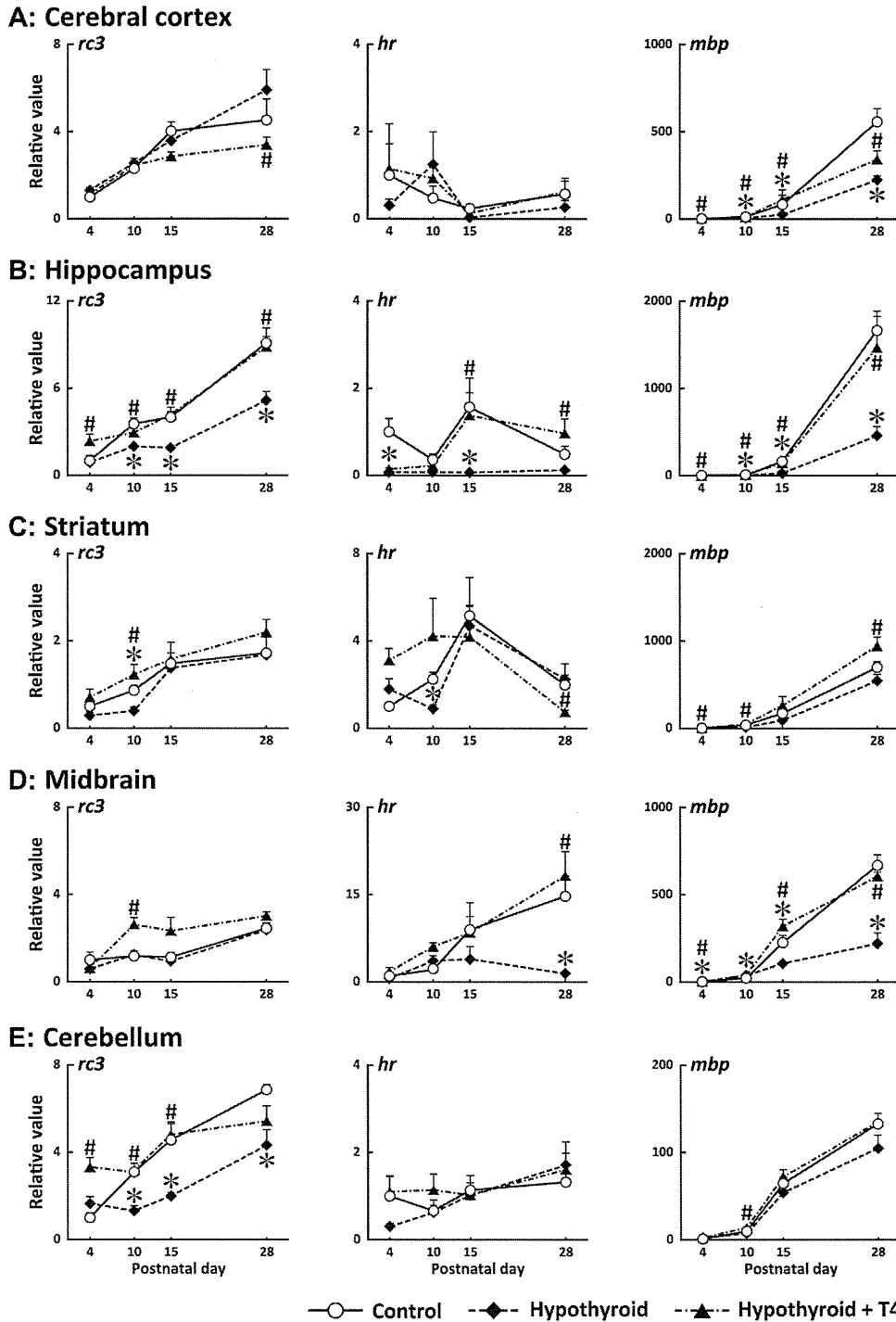
**Fig. 1.** Body weights and plasma thyroxine (T4) levels of pups during development. (A) Body weights of control (open circles), hypothyroid (solid squares) and T4-replacement groups (solid triangles) of pups at PND4, 10, 15 and 28. Results are expressed as means  $\pm$  SEM.  $n = 5-25$  (control group),  $n = 6-30$  (hypothyroid group),  $n = 10-31$  (T4-replacement group). (B) Plasma levels of T4 in the three experimental groups at PND4, 10, 15 and 28. Results are expressed as means  $\pm$  SEM.  $n = 5-12$  (control group, hypothyroid group, T4-replacement group). \* $P < 0.05$  between control and hypothyroid groups. # $P < 0.05$  between hypothyroid and T4-replacement groups.



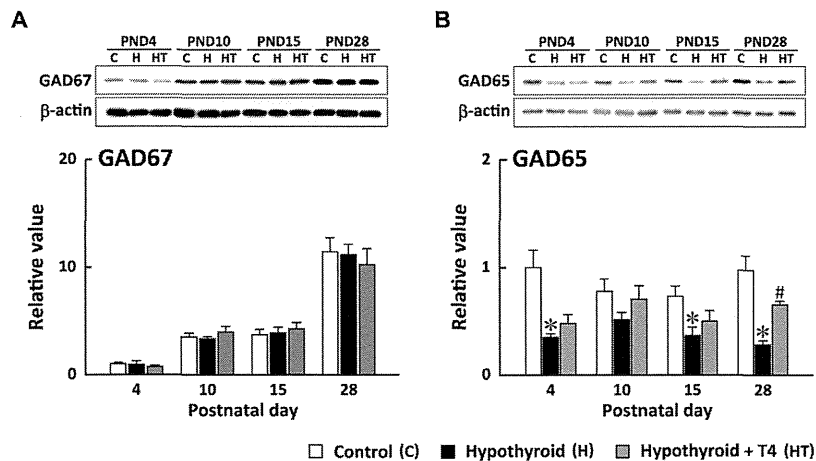
of anti-thyroid drug treatment) and focused on different brain regions, we attempted to evaluate the differences in sensitivities toward TH status among the developing rat brain regions under the present experimental protocol by examining mRNA expression of the three known TH-responsive genes, neurogranin (*rc3*), myelin basic protein (*mbp*), and hairless (*hr*) by real-time PCR in five brain regions (cerebral cortex, hippocampus, striatum, midbrain and cerebellum) in the three groups of animals (Fig. 2). We chose these genes because they are widely expressed in the brain and

their gene structures have been well characterized and shown to possess TH-responsive elements (TREs) which are functional *in vivo* (Farsetti et al., 1991; Martínez de Arrieta et al., 1999; Thompson and Bottcher, 1997).

Expression of each gene in different regions at different time points was compared pairwise between hypothyroid and control groups as well as between hypothyroid and T4-replacement groups. When the expression of a gene was significantly down-regulated in the hypothyroid group compared with the control



**Fig. 2.** Comparison of expression profiles of TH-responsive genes in the five brain regions of control, hypothyroid and T4-replacement groups during development. The mRNA expression of *rc3*, *hr* and *mbp* in the five brain regions (A: cerebral cortex, B: hippocampus, C: striatum, D: midbrain, E: cerebellum) of pups in the control (open circles), hypothyroid (solid squares) and T4-replacement groups (solid triangles) was quantified by real-time PCR at PND4, 10, 15 and 28 using *ppia* as internal standard. Each mRNA level is expressed relative to that of the control group at PND4 (means + SEM of 5 rats/group/age). #*P* < 0.05 between hypothyroid and T4-replacement groups.



**Fig. 3.** GAD67 and GAD65 protein levels in the hippocampus of control, hypothyroid and T4-replacement groups during development. GAD67 (A) and GAD65 (B) protein levels were quantified by Western blotting with anti-GAD67 and anti-GAD65 antibodies, respectively. Immunoreactive bands of GAD67 and GAD65 (examples shown in upper panels) in the hippocampus of control (open bars), hypothyroid (solid bars) and T4-replacement groups (shaded bars) at different ages were quantified using  $\beta$ -actin as standard and expressed relative to that of the control group at PND4 (means  $\pm$  SEM of 5 rats/group/age). \* $P < 0.05$  between control and hypothyroid groups. # $P < 0.05$  between hypothyroid and T4-replacement groups.

(marked with asterisks in Fig. 2) and significantly up-regulated in the T4-replacement group compared with the hypothyroid group (marked with # in Fig. 2) at the same time, it was judged as TH-responsive expression. In the cerebral cortex (Fig. 2A), only *mbp* at PND10, 15, and 28 showed TH-responsive expression. In contrast, TH-responsive expression was observed with *rc3* and *mbp* at three time points as well as *hr* at PND15 in the hippocampus (Fig. 2B). In the striatum (Fig. 2C) and the cerebellum (Fig. 2E), only *rc3* at one (PND10) or two (PND10, 15) time points was TH-responsive, while *hr* at PND28 and *mbp* at three time points were responsive in the midbrain (Fig. 2D). The results thus showed that the hippocampus had TH-sensitivity comparable to or higher than that of the well-studied TH-sensitive regions such as the cerebellum under our experimental protocol.

### 3.3. Effect of TH status on the development of GABAergic neurons in the hippocampus

To examine the role of TH in the development and maturation of the GABAergic system in the hippocampus, protein expressions of the GABA-synthesizing enzymes, GAD67 and GAD65, were first compared in the three experimental groups.

The two GAD isoforms showed sharp differences in protein expression both developmentally and in response to altered TH status (Fig. 3). In the control hippocampus, the amount of GAD67 increased more than 10-fold in a biphasic manner between PND4 and 28 (Fig. 3A) whereas that of GAD65 did not change significantly during the same time (Fig. 3B). On the other hand, the amount of GAD65 responded to TH status with significant reductions in the hypothyroid compared with the control at PND4, 15, and 28. At PND28, GAD65 protein in hypothyroid pups was decreased to 29% of that in control pups, which was recovered significantly by continuous T4-replacement (Fig. 3B). In contrast, differences in the amount of GAD67 were not observed among the three experimental groups between PND4 and 28 (Fig. 3A).

To determine whether the decrease of GAD65 protein in the hypothyroid hippocampus was caused by a decrease in the number of GABAergic neurons or not, GAD67- and GAD65-positive cells in matching sections from 6 animals per experimental group were numerically evaluated by immunohistochemistry at PND28 (Figs. 4 and 5). In the control hippocampus, GAD67- or GAD65-positive cells were distributed throughout the hippocampus proper and the dentate gyrus (DG), both in the

principal cell layer and other layers. The number of positive cells was largest in the CA1 subregion, followed by comparable numbers in CA3 and DG subregions. There were consistently more somata positive for GAD67 than for GAD65 as described previously (Fukuda et al., 1997). In addition to staining of GABAergic cell bodies, perisomatic staining of pyramidal cells was observed with both GAD antibodies in CA1–CA3. Staining of dendritic layers (stratum radiatum and stratum oriens) was stronger with GAD65 antibody compared with GAD67, confirming the preferential synaptic localization of GAD65 (Esclapez et al., 1994; Fukuda et al., 1998). Strong staining only with GAD67-antibody was observed along mossy fiber pathways (stratum lucidum of CA3 and hilus of the DG) as described previously (Sloviter et al., 1996; Fukuda et al., 1997; Gutiérrez et al., 2003).

There were no significant differences in the GAD67 staining or in the number of GAD67-positive cells among the three experimental groups (Fig. 4). In contrast, there was a 37% reduction in the number of GAD65-positive cells in the hypothyroid hippocampus compared to the control, which was completely recovered by T4-replacement (Fig. 5B). At the same time, GAD65 immunostaining in the principal cell layer was also reduced by hypothyroidism and recovered by T4-replacement (Fig. 5B).

A subpopulation of GABAergic neurons containing parvalbumin (PV) was also affected by perinatal hypothyroidism (Fig. 6), as previously shown in the neocortex and the hippocampus (Gilbert et al., 2007). PV-positive cells were located mainly in the pyramidal cell layer of CA1–3 and the hilar edge of the granule cell layer in DG. Their number was significantly decreased in all four subregions of the hippocampus in the hypothyroid group, resulting in a 33% reduction in total compared with the control (Fig. 6B). The number of PV-positive cells was completely recovered by T4-replacement. In addition to staining of the GABAergic cell bodies, prominent perisomatic staining of principal cells as well as clear staining of processes both in the principal cell layer and extending in the dendritic layers (stratum radiatum and stratum oriens) were observed with PV-antibody (Fig. 6A). Such non-somatic staining was also reduced by hypothyroidism and recovered by T4-replacement.

### 3.4. Effect of the TH status on the postsynaptic components of the GABAergic system

Paralleling the decrease in the number of pre-synaptic GABAergic neurons and their processes due to hypothyroidism, alterations