

phate/nitroblue tetrazolium chloride, Roche) and photographed in the same region.

**PCR**

Brain RNA was extracted from 100 mg of tissue using Trizol (GibcoBRL). From 3 µg of total RNA treated with DNase I, complementary DNAs were synthesized with oligo dT primers using SuperScript II (GibcoBRL). The sequences of the 5'- and 3'-primers used for reverse transcription (RT)-PCR are as follows: 5'-CCCAAGCTTTTCTGCTGGA-3' and 5'-TGCCTTGCCGTTCTTCCTG-3', corresponding to nucleotides 903-922 and 1672-1691 of the Fez1 cDNA, respectively. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. PCR was performed using AmpliTaq DNA polymerase (Perkin Elmer) with the following protocol: denaturation at 95 °C for 1 min; followed by 25 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s. Amplified products were analysed by electrophoresis on a 1.5% agarose gel.

Real-time quantitative RT-PCR was performed by ABI PRISM 7000 (Applied Biosystems) as described previously (Takumi *et al.*, 1999). The PCR primers were designed with Primer Express software (Applied Biosystems) and the sequences of the forward and reverse primers are as follows: 5'-CCCAGCTTCCTATCCCCATAA-3' and 5'-TGAAGGAGAACTCGGCCTTG-3', corresponding to nucleotides

965-985 and 1018-1037 of the Fez1 cDNA, respectively. Specificity of gene amplification was confirmed by measuring the size and purity of the PCR product by 10% acrylamide gel electrophoresis. For a 25-µL PCR reaction, 2.5 µL cDNA template was mixed with the forward and reverse primers to a final concentration of 300 nM each and 12.5 µL of 2 × SYBR Green PCR Master Mix (Applied Biosystems).



FIG. 1. Expression pattern of Fez1 in the adult mouse brain. Film autoradiographs of *in situ* hybridization on sagittal brain sections with <sup>35</sup>S-labeled probes. Upper panel shows the hematoxylin staining of the same section. Scale bar, 4 mm.

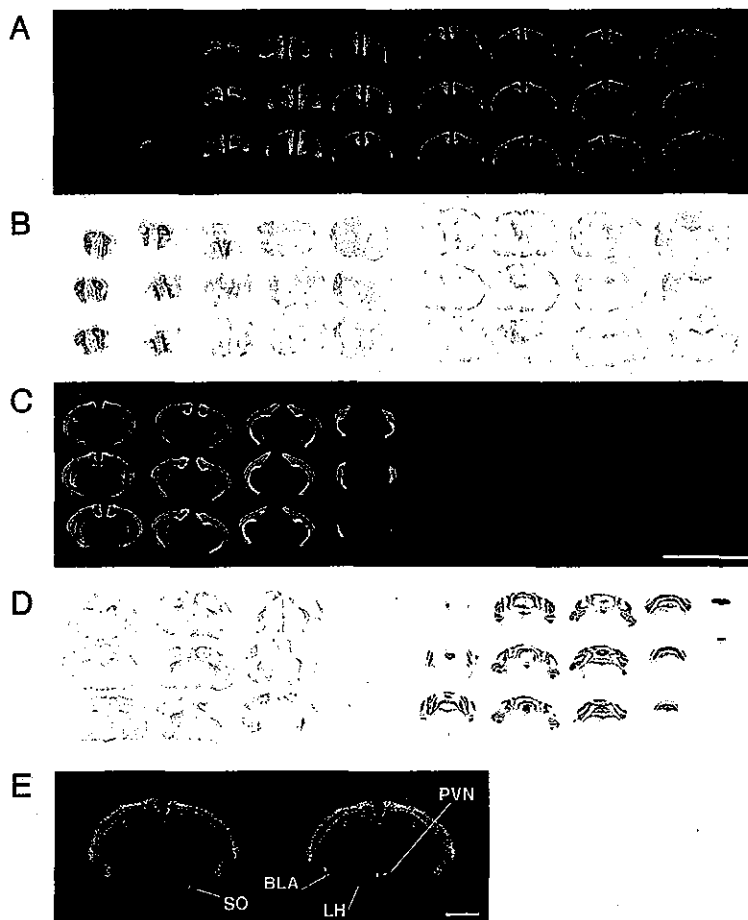


FIG. 2. Detailed expression pattern of Fez1 in the adult mouse brain. *In situ* hybridization film autoradiographs (A and C) and hematoxylin staining (B and D) of coronal sections of the adult mouse brain from the olfactory bulb to the spinal cord. A and C correspond to B and D, respectively. Expression of Fez1 mRNA in the hypothalamus and amygdala (E). BLA, basolateral amygdaloid nuclei; LH, lateral hypothalamic nuclei; PVN, paraventricular nuclei; SO, supraoptic nuclei. Scale bar, 10 mm (C) and 2 mm (E).

The reaction was first incubated at 50 °C for 2 min, then at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each gene-specific PCR was performed in triplicate. GAPDH primers were used as the control.

**Results**

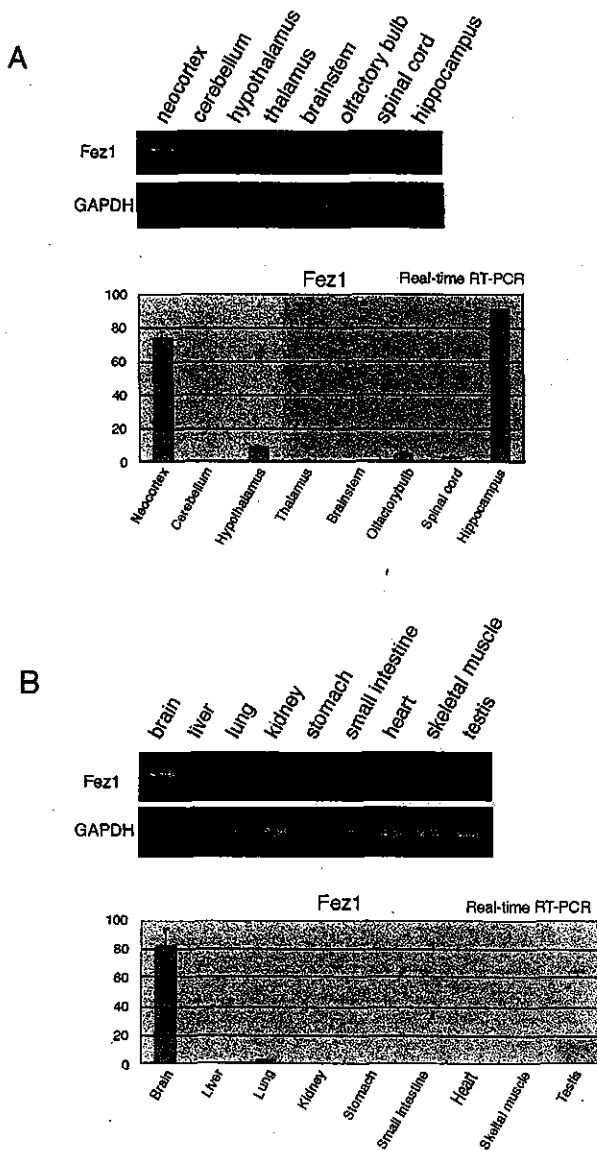
*Fez1 is predominantly expressed in the adult mouse neocortex*

Through systematic screening of genes specifically expressed in the adult neocortex, we identified an intriguing gene among several candidates (T. Takumi, A. Yamada and K. Inoue, unpublished data). Searching the NCBI (National Center for Biotechnology Information) database, this gene was identified as *Fez* (forebrain embryonic zinc-finger), the mouse homolog of *Xenopus* zinc-finger gene (Hashimoto

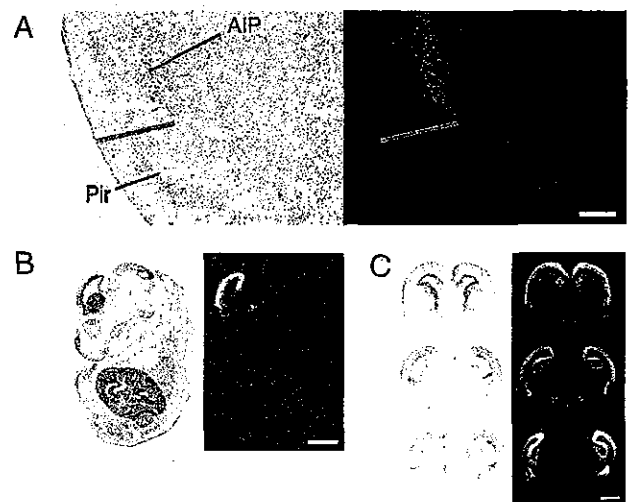
*et al.*, 2000; Matsuo-Takasaki *et al.*, 2000). *Fez* has six C<sub>2</sub>H<sub>2</sub>-type zinc-finger motifs at the C-terminus and also contains a motif similar to several transcription repressors, such as *Engrailed*, *Gooseoid*, *Dharma* and *Anf/Hes-1/Rpx1*, at the N-terminus (Hashimoto *et al.*, 2000). We named the clone we identified *Fez1*, because we found another member in the database, called *Fez2*, which is very similar to *Fez1* in primary amino acid, but has a different expression pattern (Takumi *et al.*, unpublished data). We first tested the subcellular localization of *Fez1*. Immunohistochemical analysis with an anti-*Fez1* antibody showed that *Fez1* proteins were localized in the nuclei of neurons in the adult neocortex (see Fig. 6J). EGFP-tagged *Fez1* proteins expressed in PC12 and NIH3T3 cells were also restricted to nuclei (data not shown).

*In situ* hybridization clearly showed that *Fez1* mRNA was strongly expressed specifically in the neocortex and subiculum and was more faintly expressed in a part of the hippocampus (CA1–3), but was not expressed in the olfactory bulb and dentate gyrus (Fig. 1). We further performed more detailed analysis of the expression pattern of *Fez1* by *in situ* hybridization of many areas of the brain ranging from the olfactory bulb to the spinal cord (Fig. 2). The expression pattern of *Fez1* was highly specific to the neocortex and hippocampus (CA1–3, but not the dentate gyrus), but faint expression was also seen in the supraoptic nuclei, paraventricular nuclei and lateral hypothalamic nuclei of the hypothalamus and in the basolateral nuclei of the amygdala (Fig. 2E). Furthermore, we analysed *Fez1* mRNA by RT-PCR and quantified its expression using real-time RT-PCR analysis. Consistent with the *in situ* hybridization results, the expression of *Fez1* was detected predominantly in the neocortex and hippocampus in representative brain regions, as well as very faintly in the hypothalamus (Fig. 3A). *Fez1* mRNA was specifically detected in the brain among the different peripheral tissues of the adult mouse (Fig. 3B).

Interestingly, the specificity of *Fez1* expression in the neocortex can be clearly seen by comparing its expression between the neocortex and the palaeocortex around the rhinal fissure (Fig. 4A). In contrast to its strong expression in the insular cortex of the neocortex, no *Fez1* mRNA was observed in the piriform cortex of the palaeocortex. The



**FIG. 3.** Brain regional and tissue distribution pattern of *Fez1* analysed by conventional reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR. (Upper panel) mRNA expression of *Fez1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control in the representative brain regions (A) and tissues (B) in the adult mouse. (Lower panel) Real-time quantitative RT-PCR.



**FIG. 4.** (A) *Fez1* mRNA expression around the rhinal fissure. AIP, agranular insular cortex; Pir, piriform cortex. A line shows the boundary between the neocortex and palaeocortex. Developmental expression of *Fez1* mRNA at E14.5 (B) and P0 (C). Each left panel shows the hematoxylin staining and each right panel shows *in situ* hybridization, dark-field photomicrographs (A) and film autoradiographs (B and C), respectively. Scale bar, 300 μm (A), 2 mm (B) and 1 mm (C).

developmental expression of Fez1 mRNA was also examined by *in situ* hybridization. The expression of Fez1 mRNA in the cerebral neocortex was clearly observed at E14.5, P0 and P7 stages (Fig. 4B and C), in addition to its expression in amygdala (Fig. 4C).

#### Fez1 shows a deep layer-specific pattern of expression in the adult neocortex

To date several genes have been identified as being expressed specifically in the telencephalic cortex, including *Emx1* (Simeone *et al.*, 1992). Some genes, including *CaMKII $\alpha$* , have also been identified as being forebrain specific. We compared the expression pattern of Fez1 with *Emx1* and *CaMKII $\alpha$*  in sagittal brain sections and found that Fez1 is more specifically expressed in the neocortex than these genes. Other than in the cerebral neocortex, *Emx1* was strongly expressed in the olfactory bulb and the dentate gyrus, while the hybridization signal of Fez1 was very weak or absent in these areas (Fig. 5A and B). *CaMKII $\alpha$*  expression was observed more broadly in the regions other than the cerebral cortex (Fig. 5C and F). In addition, the distribution of Fez1 in different tissues was more specific than that of *Emx1*. *Emx1* was found to be expressed strongly in the kidney, as previously reported (Briata *et al.*, 1996), while the hybridization signal of Fez1 was only detected in the whole sagittal brain sections of the postnatal 7-day mouse (Fig. 5D and E). Moreover, Fez1 has a distinct expression pattern from that of *Emx1* even in the cortex. It has been

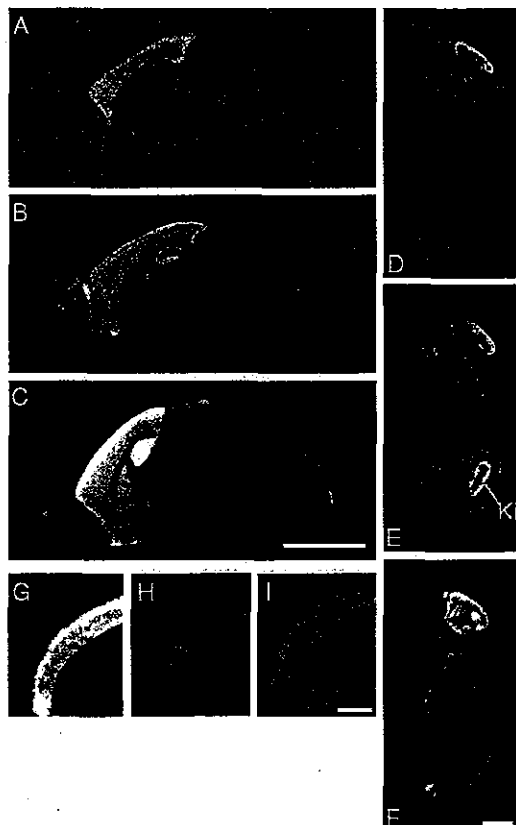


FIG. 5. Comparison of the mRNA distribution pattern of Fez1 (A, D and G), *Emx1* (B and E), *CaMKII $\alpha$*  (C and F), *Otx1* (H) and *SCIP* (I). *In situ* hybridization film autoradiographs of sagittal (A–C) or coronal (G–I) brain sections of the adult mouse and whole sagittal sections of a postnatal day 7 mouse (D–F). Ki, kidney. Scale bar, 4 mm (A–C); 5 mm (D–F) and 1 mm (G–I).

reported that *Emx1* is expressed in pyramidal cells of the cortex and the hybridization signal was observed in all layers except layer I of the cortex (Chan *et al.*, 2001), whereas the hybridization signal of Fez1 was detected only in layers V and VI of the neocortex (Fig. 6A). In contrast to Fez1, *Emx1* was widely expressed not only in the neocortex but also in the palaeocortex, such as in the piriform cortex.

In order to analyse the layer-specific expression of Fez1 in the adult neocortex, the expression of Fez1 was compared with that of *Otx1* and *SCIP*. Fez1 was clearly expressed specifically in the deeper layers of neocortex, while the expression of *Otx1* was very weak in the adult neocortex and that of *SCIP* was not specific to the deep layers but also expressed in layers II–III (Fig. 5G–I). Furthermore, immunohistochemistry using anti-Fez1 antibody showed that the immunopositive staining was localized in layers V and VI of the neocortex (Fig. 6I). Collectively these results indicate that Fez1 is expressed more specifically in the deep layers of the adult neocortex than any other genes reported so far.

#### Fez1 is expressed in glutamatergic neurons but not GABAergic neurons

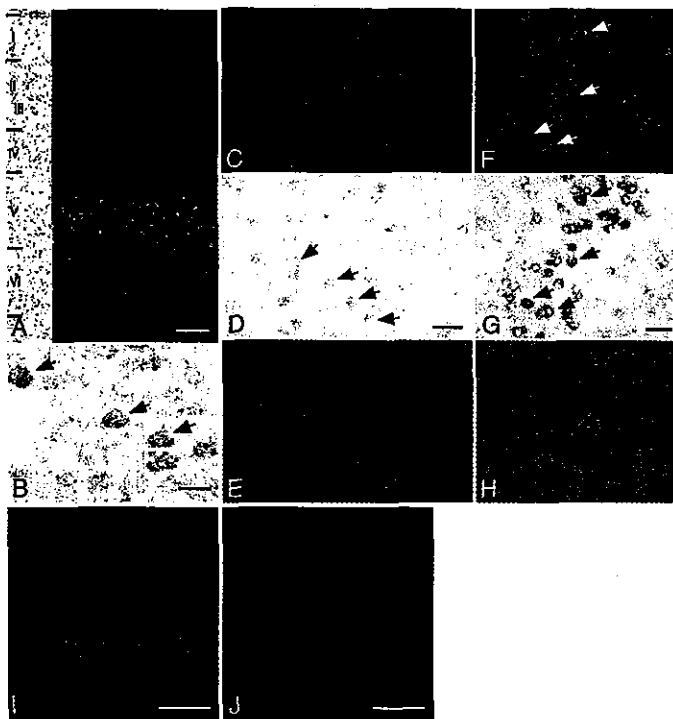
We then investigated which cells in the neocortex express Fez1 by two-color double-labeling *in situ* hybridization using DIG- and biotin-labeled probes, and analysed whether Fez1 is expressed in GABAergic neurons in the neocortex. Fez1-expressing cells do not express glutamic acid decarboxylase 67 (*GAD67*), a marker for GABAergic neurons (Fig. 6B–D), but *CaMKII $\alpha$* , a marker for glutamatergic neurons (Fig. 6E–G). This, combined with morphological analysis, clearly showed that Fez1 is expressed in non-GABAergic neurons, namely in excitatory pyramidal neurons (Fig. 6B).

#### Fez1 expression is restricted to the deep layers of the neocortex at embryonic and postnatal stages

It has been reported that *Otx1* is specifically expressed in the deep layers and the ventricular zone of the cortex during early development (Frantz *et al.*, 1994b). We compared the expression pattern of *Otx1* and Fez1 in the cortex during the developmental stages (Fig. 7). In addition to the cortical layer, *Otx1* was expressed strongly in the ventricular zone from the early to late embryonic stages, as reported previously (Frantz *et al.*, 1994b). Interestingly, the strong signal of Fez1 was first detected in the superficial thin layers at about E13, corresponding to the time when the cortical plate is just beginning to be constructed at the superficial layer (Fig. 7A). This expression of Fez1 in the cortical plate was detected at all embryonic stages, while no signal was detected at the ventricular and intermediate zone. At E14.5, a weak hybridization signal was detected in the subplate neurons in addition to the cortical plate. At the postnatal stages, the signal of Fez1 in neurons of the deep cortical layers remains strong, while that of *Otx1* appears to weaken or disappear (Fig. 7B).

#### Fez1 expression in the cortex of the reeler mouse

In the neurological mutant mouse, *reeler*, a defect in the migration of cortical plate neurons prevents splitting of the preplate, resulting in a reversed laminar structure of the neocortex (Caviness, 1982). To confirm which neuronal layers express Fez1, we examined the distribution pattern of Fez1 in the adult *reeler* mouse cortex (Fig. 8). In the cerebral cortex of wild-type mouse, most Fez1-labeled cells in layer V were strongly labeled, whereas most labeled cells in layer VI are weakly labeled (Fig. 8A and B). In the cerebral cortex of the *reeler*

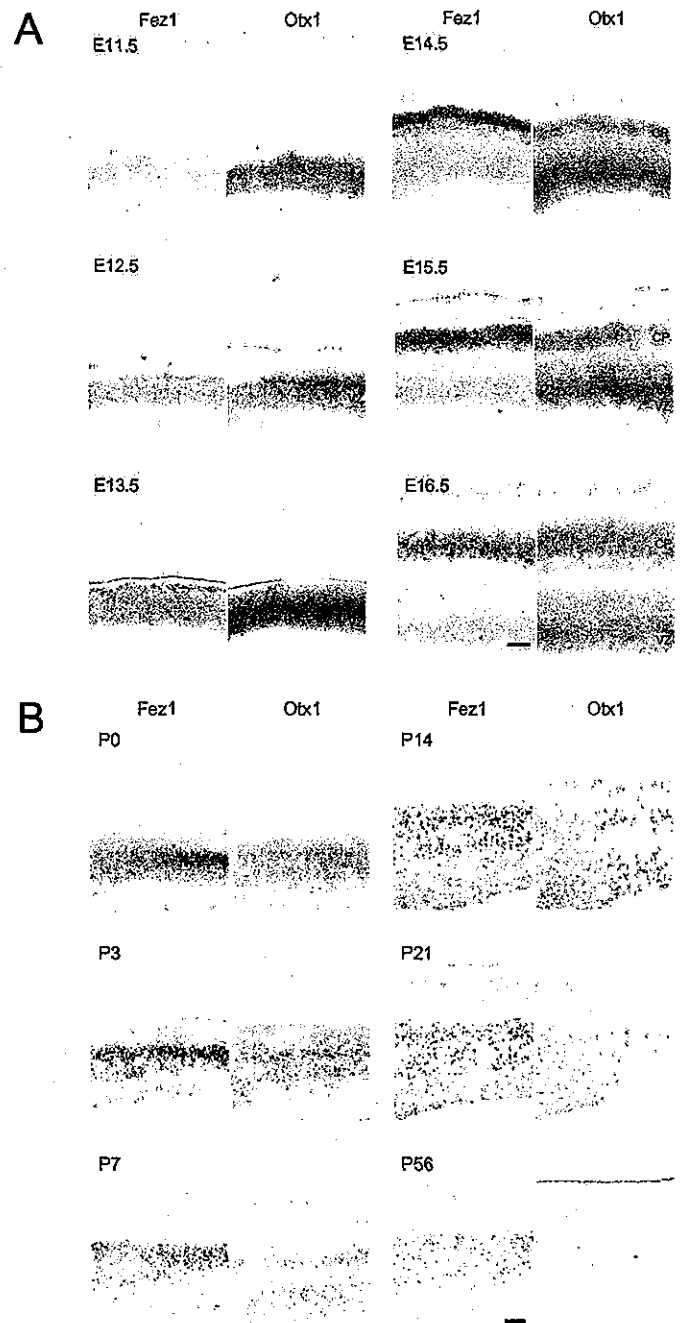


**FIG. 6.** Cellular distribution of *Fez1* mRNA in the adult mouse brain. (A) *In situ* hybridization emulsion autoradiograph of a  $^{35}\text{S}$ -labeled *Fez1* probe in the neocortex performed on coronal brain sections of the adult mouse. (B) *In situ* hybridization signal of a DIG-labeled *Fez1* probe in layer V of the cerebral neocortex in a sagittal brain section of the adult mouse. Arrows indicate cells with the typical soma shape of pyramidal cells. (C and D) Two-color double-labeling *in situ* hybridization with a biotin-labeled *GAD67* probe or *CaMKII $\alpha$*  and DIG-labeled *Fez1* probe. Hybridization signals were visualized with HNPP/FastRed TR for the biotin-labeled *GAD67* probe (C) or *CaMKII $\alpha$*  (F), and with BCIP/NBT for the DIG-labeled *Fez1* probe (D and G). (E and H) Overlay of the images shown in C and F, and D and G. Immunohistochemical staining of the mouse cortex with *Fez1* antibody (I and J). Scale bar, 100  $\mu\text{m}$  (A), 25  $\mu\text{m}$  (B), 50  $\mu\text{m}$  (C–H), 200  $\mu\text{m}$  (I) and 50  $\mu\text{m}$  (J).

mouse, on the other hand, strongly labeled cells were scattered widely throughout all layers of the cortex, whereas weakly labeled cells were mainly distributed just beneath the pial surface (Fig. 8C and D). This distribution pattern is very similar to the pattern of retrograde labeled cells after injection of dye into the spinal cord and thalamus of adult wild-type and *reeler* mice (Yamamoto *et al.*, 2003).

## Discussion

The mammalian neocortex, which contains over 10 billion neurons in humans, is an associative center of our sensory, motor and cognitive functions. Recent studies using molecular biology and genetics have identified various genes involved in brain development, but even now that the human genome sequence is available, our understanding of the adult neocortex at the molecular level is still at a primitive stage despite its functional importance. Using a global screening approach, we identified *Fez1* as a gene predominantly expressed in the adult mouse neocortex. *Fez1* will be valuable not only for exploring the developmental contribution or physiological function of the adult neocortex, but also for using its promoter in gene-manipulated animals. A promoter that controls the expression of a transgene specifically in the neocortex could be used for conditional strategies.



**FIG. 7.** *Fez1* and *Otx1* mRNA expression in the cortex during prenatal (A) and postnatal (B) stages. Hybridization signals of the DIG-labeled probes were visualized with BCIP/NBT (purple). Prenatal sections were counterstained with eosin (pink). CP, cortical plate; VZ, ventricular zone. Scale bar, 100  $\mu\text{m}$  (A) and 200  $\mu\text{m}$  (B).

### Cerebral cortex-specific promoters

As described above, through systematic screening, we identified *Fez1*, a gene expressed primarily in the neocortex, and not in the palaeocortex. Up to date, the promoters of *Emx1* and *CaMKII $\alpha$*  have been used for regulating gene expression in the adult cerebral cortex (Tsien *et al.*, 1996; Iwasato *et al.*, 2000). However, regional specificity of these two genes is lower than that of *Fez1*. Besides the neocortex, our results showed that *Emx1* is strongly expressed in the olfactory bulb and the hippocampus and weakly expressed in the brainstem.

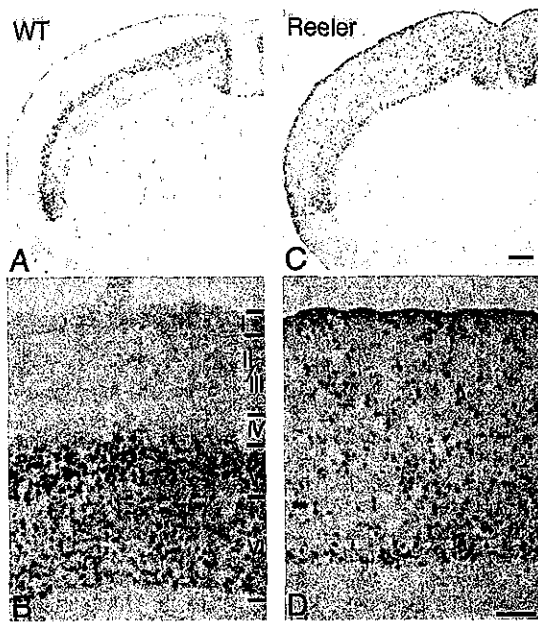


FIG. 8. mRNA expression pattern of Fez1 in the adult wild-type and *reeler* mouse cortex. Upper panels show *in situ* hybridization signals of the DIG-labeled Fez1 probe in coronal wild-type (A) and *reeler* (C) mouse brain sections. The lower panels show high-magnification views of wild-type (B) and *reeler* (D) sections. Scale bar, 500  $\mu$ m (A and C) and 200  $\mu$ m (B and D).

Furthermore, strong expression was also observed in the kidney. The specificity of CaMKII $\alpha$  expression was even lower than that of Emx1. CaMKII $\alpha$  was strongly expressed in the hippocampus and striatum, and a weak to intermediate level of expression was observed in the olfactory bulb, septum, thalamus, inferior colliculus, spinal cord and Purkinje cells in the cerebellum (Eröndü & Kennedy, 1985; Burgin *et al.*, 1990). Therefore, the use of the Fez1 promoter would be more beneficial for neocortex-specific gene manipulation in adult mice.

It has been reported that Fez1 is also expressed in the ventral forebrain during the embryonic stages of the zebrafish and mouse (Hashimoto *et al.*, 2000; Matsuo-Takasaki *et al.*, 2000). Furthermore, it has been shown that mutation of zebrafish Fez leads to loss of diencephalic monoaminergic neurons in a non-cell-autonomous manner (Levkowitz *et al.*, 2003). Our results showed that Fez1 is also expressed in the embryonic hypothalamus. However, this expression in the hypothalamus was reduced in the postnatal mouse brain and only a faint signal was observed in the adult hypothalamus, whereas the strong expression of Fez1 in the neocortex is maintained into adulthood. Therefore, the different expression pattern of Fez1 in the hypothalamus and neocortex indicates that Fez1 may play distinct roles in brain development and maintenance of adult brain functions.

#### A marker for pyramidal cells of the cerebral neocortex in layers V and VI

The Fez family is highly conserved among many species from worms to humans. In zebrafish and *Xenopus*, Fez is expressed mainly in the forebrain and is thought to play a role in forebrain development (Hashimoto *et al.*, 2000; Matsuo-Takasaki *et al.*, 2000). In zebrafish, Fez is reported to induce *dlx2* and *dlx6* expression (Yang *et al.*, 2001). These homeobox genes are also highly conserved between species and are involved in the development of the brain. Recently,

*Dlx* genes were reported to induce GADs in slice cultures of the mouse embryonic cerebral cortex (Stuhmer *et al.*, 2002). These reports imply that Fez may regulate GABAergic specification through regulation of *Dlx* expression. However, our results showed that Fez1 is not expressed in GABAergic neurons but in pyramidal cells, at least in some populations of the adult mouse cerebral neocortex.

The vertebrate cerebral neocortex is organized into six layers (I–VI). Each layer shows a characteristic feature of the constituent cell type, density and axonal connections. Although the pyramidal cells are present in layers II–VI, Fez1 is specifically expressed in layers V and VI of the adult mouse cerebral neocortex. Layers V and VI contain pyramidal cells that project their axons to the subcortical areas, whereas pyramidal cells of the superficial layers send their axons to other cortical areas. Several genes have been reported to show layer-specific patterns of expression (Hevner *et al.*, 2003). Genes reported to be specifically expressed in layers V–VI of neocortex include the POUIII domain transcription factor SCIP/Oct-6/*tst-1* (He *et al.*, 1989; Frantz *et al.*, 1994a), the homeodomain protein Otx1 (Frantz *et al.*, 1994b), the ETS transcription factor er81 (Weimann *et al.*, 1999) and the receptor-like protein-tyrosine phosphatase PTP $\lambda$  (Cheng *et al.*, 1997). SCIP/Oct-6/*tst-1* is a multifunctional transcription factor, and its involvement in peripheral myelination (Jaegle *et al.*, 1996) and differentiation of the epidermis (Andersen *et al.*, 1997) are well characterized; however, its contribution to neural development in the CNS remains to be elucidated. Analysis of Otx1 mutant mice revealed that Otx1 is required for the refinement of exuberant axonal projections of cortical efferent neurons (Weimann *et al.*, 1999). Er81 has been reported to regulate the development of selective connections between sensory and motor neurons in the spinal cord as well as to be involved in carcinogenesis (Arber *et al.*, 2000; Goel & Janknecht, 2003). A comparison of these molecular markers including Fez1 identified here is as follows. All genes described above are expressed in the forebrain from the early stages of corticogenesis. However, among them, Otx1 expression in the neocortex is dramatically reduced in the adult mouse. The expression of SCIP/Oct-6/*tst-1* in the adult neocortex is seen in layers II–III in addition to layers V–VI, and it is also expressed in a variety of brain regions in the adult, such as the CA1 area of the hippocampus, subiculum, striatum, tenia tecta and indusium griseum, the peripheral nervous system (PNS) and even in the testis (He *et al.*, 1989; Frantz *et al.*, 1994a). Er81 is expressed in the spinal cord and PNS, as well as various peripheral tissues including kidney, heart and lung (Brown & McKnight, 1992; Arber *et al.*, 2000). PTP $\lambda$  expression in the adult brain appears to be downregulated, and other areas of prominent expression include the piriform cortex, endopiriform nucleus, amygdaloid nuclei, subiculum, and CA1 and CA2 of the hippocampal formation as well as peripheral tissues such as lung and kidney (Cheng *et al.*, 1997). Thus, Fez1 may serve as an ideal molecular marker that is specifically expressed in layers V–VI of the adult neocortex. Furthermore, the expression of Fez1 in adults suggests that it is necessary not only for the establishment but also for the maintenance of layer-related cell fates.

In the *reeler* mouse, Fez1-positive neurons, which are confined to the deep layers of the normal neocortex, are radially intermingled in the adult neocortex (Fig. 8). This distribution pattern of Fez1-positive neurons is very similar to that of corticospinal tract (CST) and corticothalamic tract (CTT) neurons identified by retrograde labeling (Yamamoto *et al.*, 2003). Strongly labeled neurons that are likely to be CST neurons were located in layer V of wild-type mouse and were widely scattered throughout all depths of the cortex in the *reeler* mouse, while weakly labeled neurons that are likely to be CTT neurons were expressed in layer VI of wild-type mouse and were mainly distributed just beneath the pia mater in the *reeler* mouse. Moreover, as observed in

the wild-type mouse cortex, the hybridization signal in the insular cortex was stronger than the other areas of the cortex of the *reeler* mouse, suggesting that tangential organization is not impaired in the *reeler* mouse (Fig. 8). These data suggest that *Fez1* is expressed at least in CTT and CST neurons even in the *reeler* mouse. Combined with the finding that *Fez1* is induced in these neurons before they extend their axons to the subcortical areas at E13.5 (Fig. 7), this observation suggests that *Fez1* might play a role in axonal outgrowth and targeting to these areas. In fact, our preliminary results of experiments using knockout mice of the *Fez* family are compatible with this possibility. Further detailed examination will uncover the function of *Fez1* in the cerebral cortex.

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## Abbreviations

CaMKII $\alpha$ , alpha subunit of the calcium/calmodulin-dependent protein kinase II; CST, corticospinal tract; CTT, corticothalamic tract; DIG, digoxigenin; GABA,  $\gamma$ -aminobutyric acid; GAD67, glutamic acid decarboxylase 67; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNPP, 2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate; NBT, nitroblue tetrazolium chloride; PB, phosphate buffer; PCR, polymerase chain reaction; RT, reverse transcriptase; SSC, standard sodium citrate.

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## Effects of psychotomimetic and antipsychotic agents on neocortical and striatal concentrations of various amino acids in the rat

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### Abstract

A subcutaneous injection of small and moderate doses (1.6, 3.2, 4.0 and 4.8 mg/kg) of the schizophrenomimetic methamphetamine caused a dose-related increase in the tissue content (the net content) of L-Arg and L-Asn in the neocortex and striatum at 60 min, but not at 360 min, after injection. The methamphetamine-induced (4.8 mg/kg) increases in levels of these amino acids were significantly attenuated by pretreatment with an antipsychotic drug, haloperidol (1 mg/kg) or clozapine (10 mg/kg). In the neocortex, a clozapine-reversible increase in the level of L-Thr was also observed 60 min after methamphetamine administration. Striatal concentrations of L-Glu, L-Ser, L-Thr, Gly and L-Ala were augmented by the same regimen in a haloperidol- and clozapine-sensitive fashion. A moderate dose of another schizophrenomimetic

phencyclidine (7.5 mg/kg) given subcutaneously induced robust abnormal behavior, a diminution in the neocortical and striatal levels of L-Asp and an increase in the striatal L-Ala content without significant effects on the other amino acids studied. These results suggest that neocortical and striatal L-Arg, L-Asn, L-Thr, Gly, L-Ala or L-Ser may be implicated in the psychotomimetic effects of methamphetamine and might display mutual interaction with cerebral dopaminergic transmission. The differential effects of methamphetamine and phencyclidine on the net neocortical and striatal concentrations of various amino acids might, at least in part, underlie the distinct features of psychoses induced by these two drugs.

**Keywords:** antipsychotics, L-arginine, L-asparagine, methamphetamine, neocortex, striatum.

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Intrinsic amino acids in the mammalian brain have been found to play a variety of crucial roles in excitatory and inhibitory neurotransmission and their modulation. It is well established that Glu and Asp cause an excitatory postsynaptic potential of the neuronal membrane (Hollmann and Heinemann 1994), whereas GABA, Gly and taurine (Tau) induce an inhibitory postsynaptic potential (Huxtable 1992; Olsen 2002). More recently, D-Ser and Gly have been shown to act as co-agonists for the NMDA-type excitatory amino acid receptor (Danysz and Parsons 1998).

These amino acids appear to be involved in the regulation and pathophysiology of higher brain functions such as cognition, thought, emotion and perception in humans. Thus, agonists for the benzodiazepine site of the GABA<sub>A</sub> receptor ameliorate anxiety and panic attack (Biggio *et al.* 1995; Olsen 2002). Inverse agonists for this site, in turn, cause or augment anxiety (Braestrup *et al.* 1982; Bonetti *et al.* 1988). Interruption of NMDA receptor-mediated neurotransmission results in memory disturbance,

schizophrenia-like psychosis or cerebellar ataxia (Javitt 2002; Tanii *et al.* 1994; Ogawa *et al.* 2003). In support of these observations, the facilitation of excitatory signaling by Gly, D-Ser or D-cycloserine has been reported to improve schizophrenic symptoms (Contreras 1990; Tanii *et al.* 1991, 1994; Hashimoto *et al.* 1991; Javitt 2002) or ataxia in patients with spino-cerebellar degeneration (Ogawa *et al.* 2003). Moreover, altered indices of GABA and Glu systems, including tissue concentrations (which are defined as 'net concentrations' in this study), and binding, release and uptake activities have been found in the CSF,

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*Abbreviations used:* CLZ, clozapine; HAL, haloperidol; MAP, methamphetamine; NO, nitric oxide; PCP, phencyclidine.



blood, post-mortem brain tissues or by brain imaging of neuropsychiatric patients and in animal models of neuropsychiatric disorders (Carlsson *et al.* 2001). However, there are only a few reports of a systematic approach to the possible pathogenetic or pathophysiological roles of various amino acids in brain dysfunction (Kubota *et al.* 2002).

In the present study, to obtain an insight into the hypothetical disturbance of NMDA receptor function in schizophrenia and the unknown target systems for schizophrenomimetic and antipsychotic drugs, we have studied the effects of these psychotropic agents on the net content of rat neocortical and striatal amino acids that are postulated to participate directly or indirectly in control of the excitatory amino acid receptor. To this end, we have quantified three groups of amino acids: (1) excitatory amino acids acting at the Glu site of the NMDA receptor and their potential metabolites (L-Glu, L-Gln, L-Asp and L-Asn) (Reubi *et al.* 1980; Hollmann and Heinemann 1994; Dieterich *et al.* 2003; Waagepetersen *et al.* 2003); (2) amino acids acting at the co-agonist (Gly) site of the NMDA receptor (Gly and D-Ser) (Danysz and Parsons 1998) and L-Ser, a potential precursor for these NMDA co-agonists (Takahashi *et al.* 1997); and (3) a precursor of nitric oxide (NO), a diffusible gas regulator of the NMDA receptor, L-Arg (Kuriyama and Ohkuma 1995). The net concentrations of Tau, a putative inhibitory neurotransmitter, and L-Thr and L-Ala, neutral amino acids with undefined effects on neural activity, have also been measured in the two brain areas for comparison because interactions between these amino acids and psychotropic drugs are largely unknown.

For these pharmacological experiments, we have chosen different types of schizophrenia-related drugs: dopaminergic psychotomimetic methamphetamine (MAP) (Nishikawa *et al.* 1983; Seiden *et al.* 1993), glutamatergic psychotomimetic phencyclidine (PCP) (Tanii *et al.* 1994; Javitt 2002) as a comparison, typical antipsychotic haloperidol (HAL) and atypical antipsychotic clozapine (CLZ) (Miyamoto *et al.* 2002). The neocortex and striatum were selected for this quantitative examination, because some neocortico-striatal pathways have been shown to be involved in the pathophysiology of psychotogen-induced abnormal behavior (Hashimoto *et al.* 1998) and of schizophrenia (Carlsson *et al.* 2001; Tekin and Cummings 2002).

## Materials and methods

### Animals

The present animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University and were approved by the Animal Investigation Committee of the Institution. Male Wistar rats (ST strain; Clea Japan, Inc., Tokyo, Japan) at postnatal day 56, weighing 200–230 g, were used. The

animals were housed at  $22.0 \pm 0.5^\circ\text{C}$  in a humidity-controlled room under a 12-h light–dark cycle and had free access to food and water.

### Chemicals

PCP hydrochloride was kindly synthesized and donated by Yamanouchi Pharmaceutical, Co., Ltd. (Tsukuba, Japan). MAP hydrochloride was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) with official permission of the Tokyo Metropolitan Bureau of Public Health. The other chemicals used were of ultrapure quality and commercially available.

### Drug administration

MAP hydrochloride and PCP hydrochloride were dissolved in physiological saline for subcutaneous (s.c.) injection. HAL and CLZ were dissolved in 0.15% tartaric acid (Kusumi *et al.* 1995) and titrated with 0.05 M NaOH to approximately pH 5.0. HAL, CPZ and vehicle (0.15% tartaric acid, titrated as above) were administered intraperitoneally (i.p.). All injections were made immediately before administration at a volume of 1.0 mL/kg. Some animals were pretreated with HAL (1.0 mg/kg) or CLZ (10.0 mg/kg) 30 min before MAP (4.8 mg/kg) administration. Control animals were injected with saline or the vehicle instead.

Doses always refer to the free bases and were selected for the following reasons: (1) the smaller dose 1.6 mg/kg and larger doses 4.0 and 4.8 mg/kg of MAP induce different behavioral changes, hyperlocomotion and stereotypy respectively (Nishikawa *et al.* 1983); (2) PCP at 7.5 mg/kg causes robust hyperactivity, stereotyped behavior and ataxia (Tanii *et al.* 1994); (3) 1.0 mg/kg HAL completely blocks the behavioral effects of the larger doses of MAP (Ujike *et al.* 1988; Shirayama *et al.* 2000); and (4) 10.0 mg/kg CLZ is generally accepted to show the pharmacological properties as an atypical antipsychotic in animal models of schizophrenia (e.g. anti-PCP effects) (Qiao *et al.* 2001; Sams-Dodd 1996). The effects of MAP, HAL and CLZ on various amino acids in the brain were examined at 60 min and also 360 min after their administration because changes in neocortical D-Ser levels have been found to peak at 360 min after systemic administration of Gly or L-Ser (Takahashi *et al.* 1997).

### Tissue preparation

Rats were killed by cervical dislocation 60 or 360 min after acute injection of MAP or PCP. According to the atlas of Paxinos and Watson (1986), the neocortex (the dorsal part of the cerebral cortex divided along the rhinal fissure) and striatum were rapidly removed in the cold, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until use.

### HPLC analysis of amino acids

The simultaneous determination of amino acid enantiomers and non-chiral amino acids in the human cortex was accomplished by HPLC with fluorometric detection as described previously but with minor modifications (Hashimoto *et al.* 1992b). Briefly, the brain sample was homogenized in 10 volumes of 4% trichloroacetic acid after the addition of D-homocysteic acid, and the homogenate was centrifuged at 14 500 g for 20 min at  $4^\circ\text{C}$ . The supernatant was stored at  $-80^\circ\text{C}$  until derivatization. The resulting sample was derivatized with *N*-tert-butylloxycarbonyl-L-cystein and *o*-phthalaldehyde for 2 min at room temperature. The amino acid derivative was immediately applied to the HPLC system and separated on a



4- $\mu$ m Nova-Pak C18 column (300  $\times$  3.9 mm internal diameter; Waters, Tokyo, Japan). The column was operated at a constant flow rate of 0.9 mL/min at 30°C. Mobile phase A was 0.1 M acetate buffer (pH 6.0) containing 12% acetonitrile; mobile phase B was 0.1 M acetate buffer containing 20% acetonitrile. The separation of amino acid derivatives was performed with a linear gradient from mobile phase A to B in 50 min. Fluorescent amino acid derivatives were detected in an FP-2025 spectrofluorometer (Jasco International Co. Ltd, Tokyo, Japan). Excitation and emission wavelengths were 344 and 443 nm respectively. The reliability of the quantitative analysis for D-, L- and non-chiral amino acids was established by comparing its result with those obtained by gas chromatography (GC) or GC-mass spectrometric assay methods (Hashimoto *et al.* 1992a,b).

#### Statistical analysis

The statistical significance of the data was evaluated using one-way ANOVA followed by the Scheffé post-hoc test for the quantitative analysis of the amino acid concentrations.

## Results

### Effects of acute injection of MAP, PCP, HAL and CLZ on the net content of various amino acids in rat neocortex and striatum at 60 min after injection

In the neocortex, acute administration of MAP (1.6, 3.2 or 4.0 mg/kg, s.c.) caused a dose-related and marked increase in

**Table 1** Effects of acute injection of psychostimulant or antipsychotic on the net concentrations of various amino acids in the rat neocortex at 60 min post-injection

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu$ mol/g of wet weight)				
	NMDA receptor-related amino acids				
	Glutamate site				Nitric oxide
	L-Glu	L-Gln	L-Asp	L-Asn	L-Arg
Saline	13.6 $\pm$ 0.1	5.06 $\pm$ 0.06	3.96 $\pm$ 0.08	0.113 $\pm$ 0.002	0.090 $\pm$ 0.002
MAP (1.6)	14.5 $\pm$ 0.1	4.90 $\pm$ 0.07	3.51 $\pm$ 0.03*	0.118 $\pm$ 0.002	0.115 $\pm$ 0.006*
MAP (3.2)	14.8 $\pm$ 0.2*	4.96 $\pm$ 0.07	3.60 $\pm$ 0.09	0.159 $\pm$ 0.010**	0.129 $\pm$ 0.011**
MAP (4.0)	14.2 $\pm$ 0.4	4.68 $\pm$ 0.11*	4.02 $\pm$ 0.09	0.172 $\pm$ 0.005**	0.163 $\pm$ 0.007**
PCP (7.5)	14.1 $\pm$ 0.3	5.22 $\pm$ 0.08	3.57 $\pm$ 0.04*	0.115 $\pm$ 0.003	0.099 $\pm$ 0.003
HAL (1.0)	12.8 $\pm$ 0.5	5.28 $\pm$ 0.12	4.26 $\pm$ 0.14	0.114 $\pm$ 0.002	0.087 $\pm$ 0.003
CLZ (10.0)	12.9 $\pm$ 0.2	5.22 $\pm$ 0.13	4.30 $\pm$ 0.04	0.108 $\pm$ 0.004	0.080 $\pm$ 0.004

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu$ mol/g of wet weight)					
	NMDA receptor-related amino acids					
	Glycine site			Other amino acids		
	Gly	D-Ser	L-Ser	Tau	L-Thr	L-Ala
Saline	0.94 $\pm$ 0.02	0.251 $\pm$ 0.002	1.05 $\pm$ 0.02	5.71 $\pm$ 0.07	0.599 $\pm$ 0.008	0.685 $\pm$ 0.010
MAP (1.6)	0.94 $\pm$ 0.02	0.263 $\pm$ 0.003	1.00 $\pm$ 0.02	5.76 $\pm$ 0.06	0.612 $\pm$ 0.017	0.712 $\pm$ 0.005
MAP (3.2)	1.01 $\pm$ 0.03	0.251 $\pm$ 0.003	1.09 $\pm$ 0.03	5.64 $\pm$ 0.05	0.627 $\pm$ 0.021	0.787 $\pm$ 0.010**
MAP (4.0)	1.08 $\pm$ 0.03**	0.255 $\pm$ 0.002	1.22 $\pm$ 0.03**	5.68 $\pm$ 0.06	0.722 $\pm$ 0.018**	0.734 $\pm$ 0.017
PCP (7.5)	0.94 $\pm$ 0.03	0.250 $\pm$ 0.002	0.99 $\pm$ 0.01	5.62 $\pm$ 0.08	0.632 $\pm$ 0.009	0.732 $\pm$ 0.019
HAL (1.0)	0.95 $\pm$ 0.01	0.258 $\pm$ 0.002	1.06 $\pm$ 0.01	5.81 $\pm$ 0.04	0.604 $\pm$ 0.020	0.650 $\pm$ 0.004
CLZ (10.0)	0.94 $\pm$ 0.02	0.255 $\pm$ 0.003	1.04 $\pm$ 0.01	5.84 $\pm$ 0.08	0.588 $\pm$ 0.012	0.663 $\pm$ 0.011

Rats were treated with a subcutaneous injection of saline, MAP or PCP, or an intraperitoneal injection of HAL or CLZ acutely, and were killed 60 min thereafter. The amino acids quantified in this study are divided into the following four groups that are defined in view of the relations to the NMDA receptor: (1) excitatory amino acids acting at the glutamate site of the NMDA receptor and their potential metabolites, L-glutamate, L-glutamine, L-aspartate, L-asparagine, (2) a precursor of nitric oxide, a diffusible gas regulator of the NMDA receptor, L-arginine, (3) amino acids acting at the glycine site of the NMDA receptor, glycine and D-serine, and a potential precursor for these NMDA co-agonists, L-serine, and (4) other amino acids including a putative inhibitory neurotransmitter, taurine, and neutral amino acids, L-threonine and L-alanine, whose interactions with the NMDA receptor are not unknown. Results represent the mean with SEM of the data obtained from 6-17 animals. \* $p$  < 0.05, \*\* $p$  < 0.01 as compared to respective saline-treated controls.

**Table 2** Effects of acute injection of psychostimulant or antipsychotic on the net concentrations of various amino acids in the rat striatum at 60 min post-injection

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)				
	NMDA receptor-related amino acids				
	Glutamate site				Nitric oxide
	L-Glu	L-Gln	L-Asp	L-Asn	L-Arg
Saline	12.2 $\pm$ 0.1	5.86 $\pm$ 0.07	2.57 $\pm$ 0.05	0.107 $\pm$ 0.001	0.141 $\pm$ 0.003
MAP (1.6)	12.5 $\pm$ 0.1	5.68 $\pm$ 0.07	2.26 $\pm$ 0.03	0.117 $\pm$ 0.001	0.146 $\pm$ 0.003
MAP (3.2)	12.7 $\pm$ 0.2*	5.52 $\pm$ 0.04**	2.25 $\pm$ 0.21	0.134 $\pm$ 0.009**	0.190 $\pm$ 0.014**
MAP (4.0)	13.2 $\pm$ 0.1**	5.33 $\pm$ 0.08**	2.40 $\pm$ 0.05	0.165 $\pm$ 0.005**	0.236 $\pm$ 0.009**
PCP (7.5)	12.4 $\pm$ 0.1	5.66 $\pm$ 0.07*	2.26 $\pm$ 0.03*	0.109 $\pm$ 0.002	0.160 $\pm$ 0.005
HAL (1.0)	11.5 $\pm$ 0.1**	6.14 $\pm$ 0.14*	2.31 $\pm$ 0.03	0.109 $\pm$ 0.001	0.136 $\pm$ 0.004
CLZ (10.0)	11.2 $\pm$ 0.1**	6.02 $\pm$ 0.09	2.54 $\pm$ 0.03	0.105 $\pm$ 0.002	0.133 $\pm$ 0.005

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)					
	NMDA receptor-related amino acids					
	Glycine site			Other amino acids		
	Gly	D-Ser	L-Ser	Tau	L-Thr	L-Ala
Saline	0.825 $\pm$ 0.009	0.236 $\pm$ 0.002	0.697 $\pm$ 0.007	8.97 $\pm$ 0.08	0.620 $\pm$ 0.006	0.695 $\pm$ 0.013
MAP (1.6)	0.850 $\pm$ 0.014	0.244 $\pm$ 0.003	0.734 $\pm$ 0.008	8.95 $\pm$ 0.07	0.661 $\pm$ 0.015	0.879 $\pm$ 0.020**
MAP (3.2)	0.877 $\pm$ 0.025	0.230 $\pm$ 0.002	0.754 $\pm$ 0.023	8.99 $\pm$ 0.08	0.644 $\pm$ 0.021	0.943 $\pm$ 0.020**
MAP (4.0)	0.964 $\pm$ 0.012**	0.237 $\pm$ 0.003	0.815 $\pm$ 0.019**	8.68 $\pm$ 0.06**	0.700 $\pm$ 0.020**	0.926 $\pm$ 0.016**
PCP (7.5)	0.832 $\pm$ 0.007	0.232 $\pm$ 0.002	0.692 $\pm$ 0.014	9.03 $\pm$ 0.07	0.641 $\pm$ 0.009	0.769 $\pm$ 0.014*
HAL (1.0)	0.828 $\pm$ 0.012	0.226 $\pm$ 0.003	0.705 $\pm$ 0.007	8.74 $\pm$ 0.09	0.631 $\pm$ 0.017	0.729 $\pm$ 0.014
CLZ (10.0)	0.841 $\pm$ 0.018	0.221 $\pm$ 0.002*	0.690 $\pm$ 0.007	8.92 $\pm$ 0.06	0.600 $\pm$ 0.010	0.653 $\pm$ 0.014

Rats were treated with a subcutaneous injection of saline, MAP or PCP, or an intraperitoneal injection of HAL or CLZ acutely, and were killed 60 min thereafter. The amino acids quantified in this study are divided into the following four groups that are defined as indicated in the subheading of Table 1. Results represent the mean with SEM of the data from 6-15 animals.

\* $p < 0.05$ , \*\* $p < 0.01$  as compared to respective saline-treated controls.

**Table 3** Effects of acute injection of psychostimulant or antipsychotic on the ratios of L-Asn/L-Asp and L-Gln/L-Glu in the rat brain at 60 min post-injection

Injection (mg/kg, s.c. or i.p.)	Neocortex		Striatum	
	L-Asn/L-Asp	L-Gln/L-Glu	L-Asn/L-Asp	L-Gln/L-Glu
Saline	0.032 $\pm$ 0.001	0.358 $\pm$ 0.003	0.041 $\pm$ 0.001	0.478 $\pm$ 0.007
MAP (1.6)	0.038 $\pm$ 0.001	0.321 $\pm$ 0.003*	0.051 $\pm$ 0.001	0.441 $\pm$ 0.006
MAP (3.2)	0.048 $\pm$ 0.002**	0.323 $\pm$ 0.005*	0.058 $\pm$ 0.008**	0.428 $\pm$ 0.006**
MAP (4.0)	0.048 $\pm$ 0.001**	0.316 $\pm$ 0.010**	0.067 $\pm$ 0.002**	0.389 $\pm$ 0.003**
PCP (7.5)	0.036 $\pm$ 0.001	0.352 $\pm$ 0.008	0.048 $\pm$ 0.001	0.449 $\pm$ 0.005
HAL (1.0)	0.030 $\pm$ 0.001	0.389 $\pm$ 0.014	0.046 $\pm$ 0.001	0.534 $\pm$ 0.012
CLZ (10.0)	0.028 $\pm$ 0.001	0.394 $\pm$ 0.012*	0.041 $\pm$ 0.001	0.520 $\pm$ 0.009

Rats were treated with a subcutaneous injection of saline, MAP or PCP, or an intraperitoneal injection of HAL or CLZ acutely, and were killed 60 min thereafter. Results represent the mean with SEM of the data obtained from 6-17 animals.

\* $p < 0.05$ , \*\* $p < 0.01$  as compared to respective saline-treated controls.

**Table 4** Effects of acute injection of psychostimulant or antipsychotic on the net concentrations of various amino acids in the rat neocortex at 360 min post-injection

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)				
	NMDA receptor-related amino acids				
	Glutamate site				Nitric oxide
	L-Glu	L-Gln	L-Asp	L-Asn	L-Arg
Vehicle	10.5 $\pm$ 0.1	5.19 $\pm$ 0.07	3.06 $\pm$ 0.05	0.106 $\pm$ 0.003	0.110 $\pm$ 0.003
MAP (4.0)	10.6 $\pm$ 0.1	5.33 $\pm$ 0.07	3.16 $\pm$ 0.09	0.110 $\pm$ 0.003	0.101 $\pm$ 0.002*
HAL (1.0)	10.2 $\pm$ 0.1	5.24 $\pm$ 0.18	3.18 $\pm$ 0.03	0.109 $\pm$ 0.003	0.111 $\pm$ 0.003
CLZ (10.0)	10.4 $\pm$ 0.1	5.14 $\pm$ 0.10	3.24 $\pm$ 0.06	0.108 $\pm$ 0.002	0.109 $\pm$ 0.003

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)					
	NMDA receptor-related amino acids					
	Glycine site			Other amino acids		
	Gly	D-Ser	L-Ser	Tau	L-Thr	L-Ala
Vehicle	0.722 $\pm$ 0.020	0.242 $\pm$ 0.003	0.740 $\pm$ 0.010	5.82 $\pm$ 0.07	0.564 $\pm$ 0.010	0.628 $\pm$ 0.020
MAP (4.0)	0.732 $\pm$ 0.038	0.239 $\pm$ 0.003	0.794 $\pm$ 0.044	5.60 $\pm$ 0.08	0.527 $\pm$ 0.009*	0.651 $\pm$ 0.020
HAL (1.0)	0.740 $\pm$ 0.023	0.241 $\pm$ 0.004	0.774 $\pm$ 0.017	5.66 $\pm$ 0.10	0.561 $\pm$ 0.013	0.606 $\pm$ 0.011
CLZ (10.0)	0.735 $\pm$ 0.020	0.237 $\pm$ 0.004	0.729 $\pm$ 0.017	5.72 $\pm$ 0.08	0.531 $\pm$ 0.015	0.601 $\pm$ 0.016

Rats were treated with a subcutaneous injection of vehicle or MAP, or an intraperitoneal injection of HAL or CLZ acutely, and were killed 360 min thereafter. The amino acids quantified in this study are divided into the following four groups that are defined as indicated in the subheading of Table 1. Results represent the mean with SEM of the data obtained from 6-14 animals. \* $p < 0.05$  as compared to respective saline-treated controls.

the content of L-Asn and L-Arg up to 152 and 181% respectively, at 60 min after injection (Table 1). The levels of L-Glu, L-Ser, Gly, L-Ala and L-Thr were also raised, and those of L-Gln were reduced by the MAP treatment, although the magnitude of these changes was small (Table 1). There were no MAP-induced alterations in the concentrations of D-Ser and Tau. An acute subcutaneous injection of PCP induced a significant decrease in L-Asp content without any effects on the other amino acids studied 60 min thereafter (Table 1).

In the striatum (Table 2), the MAP regimen produced a prominent increase in the content of L-Asn, L-Arg and L-Ala by 54, 67 and 33% respectively, and modest changes occurred in the levels of L-Glu (+ 9%), L-Gln (- 9%), L-Ser (+ 17%), L-Thr (+ 13%), Gly (+ 17%) and Tau (- 3%). No significant alterations were observed in L-Asp and D-Ser content following the three doses of MAP (Table 2). PCP at a subcutaneous dose of 7.5 mg/kg diminished L-Asp and L-Gln concentrations, raised L-Ala levels and failed to affect the other amino acid contents examined.

As an index of excitatory amino acid metabolism, we calculated the ratio of L-Asn to L-Asp concentration and of L-Gln to L-Glu concentration. In the neocortex and striatum,

acute MAP injection markedly increased the L-Asn/L-Asp ratio and decreased the L-Gln/L-Glu ratio 60 min thereafter, whereas PCP did not have significant effects on these ratios (Table 3).

Neither HAL nor CLZ altered the net neocortical concentrations of the amino acids studied (Table 1). However, these antipsychotics slightly reduced the striatal L-Glu content (Table 2), CLZ produced a very limited modification in the neocortical L-Gln/L-Glu ratio (Table 3) and in the striatal D-Ser content (Table 2), and HAL slightly increased striatal L-Gln levels.

#### Effects of acute injection of MAP, HAL and CLZ on the net contents of various amino acids in the rat neocortex and striatum at 360 min after injection

As indicated in Tables 4 and 5, 360 min after administration of MAP (4.0 mg/kg, s.c.), HAL (1.0 mg/kg, i.p.) or CLZ (10.0 mg/kg, i.p.) only minimal changes were observed in the net amino acid levels in the neocortex and striatum: (1) MAP decreased neocortical L-Thr (- 7%) and L-Arg (- 8%), and striatal L-Thr (- 7%) content with no significant effects on the other amino acid concentrations in the two brain areas; (2) HAL induced a slight but significant reduction in striatal L-Asp

**Table 5** Effects of acute injection of psychostimulant or antipsychotic on the net concentrations of various amino acids in the rat striatum at 360 min post-injection

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)				
	NMDA receptor-related amino acids				
	Glutamate site				Nitric oxide
	L-Glu	L-Gln	L-Asp	L-Asn	L-Arg
Vehicle	9.71 $\pm$ 0.15	5.54 $\pm$ 0.11	2.06 $\pm$ 0.05	0.098 $\pm$ 0.003	0.131 $\pm$ 0.004
MAP (4.0)	9.68 $\pm$ 0.18	5.53 $\pm$ 0.17	2.10 $\pm$ 0.06	0.095 $\pm$ 0.002	0.126 $\pm$ 0.003
HAL (1.0)	9.54 $\pm$ 0.12	5.95 $\pm$ 0.16	1.85 $\pm$ 0.05**	0.103 $\pm$ 0.001	0.133 $\pm$ 0.004
CLZ (10.0)	9.55 $\pm$ 0.13	5.39 $\pm$ 0.08	2.16 $\pm$ 0.03	0.099 $\pm$ 0.002	0.135 $\pm$ 0.003

Injection (mg/kg, s.c. or i.p.)	Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)					
	NMDA receptor-related amino acids					
	Glycine site			Other amino acids		
	Gly	D-Ser	L-Ser	Tau	L-Thr	L-Ala
Vehicle	0.655 $\pm$ 0.018	0.210 $\pm$ 0.003	0.605 $\pm$ 0.010	8.07 $\pm$ 0.07	0.566 $\pm$ 0.013	0.625 $\pm$ 0.010
MAP (4.0)	0.637 $\pm$ 0.014	0.209 $\pm$ 0.005	0.604 $\pm$ 0.012	7.74 $\pm$ 0.20	0.526 $\pm$ 0.012*	0.656 $\pm$ 0.032
HAL (1.0)	0.650 $\pm$ 0.015	0.214 $\pm$ 0.007	0.618 $\pm$ 0.016	8.03 $\pm$ 0.19	0.572 $\pm$ 0.008	0.676 $\pm$ 0.016
CLZ (10.0)	0.658 $\pm$ 0.012	0.204 $\pm$ 0.005	0.603 $\pm$ 0.021	8.04 $\pm$ 0.19	0.523 $\pm$ 0.010*	0.606 $\pm$ 0.012

Rats were treated with a subcutaneous injection of vehicle or MAP, or an intraperitoneal injection of HAL or CLZ acutely, and were killed 360 min thereafter. The amino acids quantified in this study are divided into the following four groups that are defined as indicated in the subheading of Table 1. Results represent the mean with SEM of the data obtained from 6-14 animals. \* $p < 0.05$ , \*\* $p < 0.01$  as compared to respective saline-treated controls.

**Table 6** Effects of acute injection of psychostimulant or antipsychotic on the ratios of L-Asn/L-Asp and L-Gln/L-Glu in the rat brain at 360 min post-injection

Injection (mg/kg, s.c. or i.p.)	Neocortex		Striatum	
	L-Asn/L-Asp	L-Gln/L-Glu	L-Asn/L-Asp	L-Gln/L-Glu
Vehicle	0.030 $\pm$ 0.001	0.545 $\pm$ 0.006	0.050 $\pm$ 0.001	0.623 $\pm$ 0.009
MAP (4.0)	0.031 $\pm$ 0.001	0.559 $\pm$ 0.009	0.049 $\pm$ 0.002	0.614 $\pm$ 0.016
HAL (1.0)	0.029 $\pm$ 0.001	0.584 $\pm$ 0.024	0.059 $\pm$ 0.002**	0.686 $\pm$ 0.030
CLZ (10.0)	0.028 $\pm$ 0.001	0.545 $\pm$ 0.008	0.048 $\pm$ 0.001	0.620 $\pm$ 0.017

Rats were treated with a subcutaneous injection of vehicle, MAP or PCP, or an intraperitoneal injection of HAL or CLZ acutely, and were killed 360 min thereafter. Results represent the mean with SEM of the data obtained from 6-14 animals.

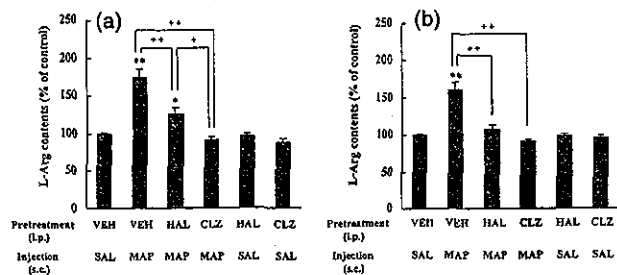
\*\* $p < 0.01$  as compared to respective saline-treated controls.

levels without affecting the levels of other amino acids in the neocortex and striatum; and (3) CLZ produced no changes except for a reduction in striatal L-Thr levels.

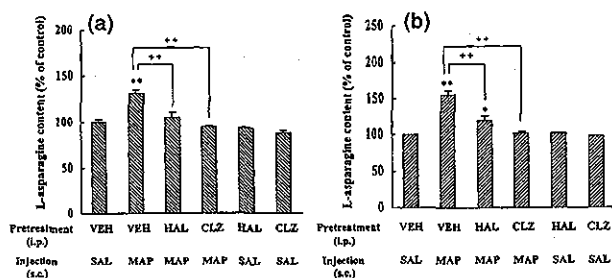
There were no significant changes in the L-Asn/L-Asp and L-Gln/L-Glu ratios in the neocortex and striatum 360 min after systemic MAP, HAL or CLZ application, except HAL-induced augmentation in striatal L-Asn/L-Asp ratios (Table 6).

#### Effects of pretreatment with HAL or CLZ on the net content of various amino acids in the rat neocortex and striatum 60 min after acute injection of MAP

In the neocortex and the striatum, pretreatment with HAL (1.0 mg/kg, i.p.) and CLZ (10.0 mg/kg, i.p.) attenuated the ability of MAP (4.8 mg/kg, s.c.) to augment L-Arg (Fig. 1) and L-Asn (Fig. 2) levels (Table 7 and 8). The MAP-induced increase in the neocortical, but not striatal, L-Asn/



**Fig. 1** Effects of intraperitoneal injection of HAL or CLZ on the ability of MAP to increase levels of L-Arg in the (a) neocortex and (b) striatum of the rat. MAP (4.8 mg/kg, s.c.) or saline (SAL) was administered 60 min, and HAL (1 mg/kg, i.p.), CLZ (10 mg/kg, i.p.) or vehicle (VEH) was injected 90 min, before the animal was killed. Results are mean  $\pm$  SEM values from 6–16 animals and are expressed as a percentage of control values, which were: neocortex,  $0.107 \pm 0.002$   $\mu\text{mol}$  per g tissue; striatum,  $0.136 \pm 0.003$   $\mu\text{mol}$  per g. \* $p < 0.05$ , \*\* $p < 0.01$  versus vehicle-treated saline-injected controls. + $p < 0.05$ , ++ $p < 0.01$  (ANOVA followed by Scheffé post-hoc test).



**Fig. 2** Effects of intraperitoneal injection of HAL or CLZ on the ability of MAP to increase levels of L-Asn in (a) neocortex and (b) striatum of the rat. MAP (4.8 mg/kg, s.c.) or saline (SAL) was administered 60 min, and HAL (1 mg/kg, i.p.), CLZ (10 mg/kg, i.p.) or vehicle (VEH) was injected 90 min, before the animal was killed. Results are the mean  $\pm$  SEM of values obtained from 6–16 animals and are expressed as a percentage of control values, which were: neocortex,  $0.128 \pm 0.003$   $\mu\text{mol}$  per g tissue; striatum,  $0.107 \pm 0.001$   $\mu\text{mol}$  per g. \* $p < 0.05$ , \*\* $p < 0.01$  versus vehicle-treated saline-injected controls. ++ $p < 0.01$  (ANOVA followed by Scheffé post-hoc test).

L-Asp ratio was inhibited by HAL and CLZ (Table 9). CLZ tended to produce a greater antagonism of the effects of MAP than HAL (Figs 1 and 2; Tables 7 and 8). Indeed, the levels of neocortical L-Arg after MAP in combination with CLZ were significantly lower than those after MAP with HAL (Fig. 1a).

The MAP-induced increase in neocortical L-Thr content was also reversed by CLZ pretreatment, whereas neither HAL nor CLZ significantly inhibited the reduction in L-Asp content (Table 7) and in the L-Gln/L-Glu ratios (Table 9), or the increase in L-Glu levels (Table 7). In the striatum, HAL or CLZ given systemically 30 min before MAP injection blocked the up-regulation by MAP of the concentrations of L-Glu, L-Ser, L-Thr, Gly and L-Ala (Table 8). CLZ, but not

HAL, attenuated the MAP-induced decrease in the L-Gln/L-Glu ratio (Table 9).

## Discussion

The basal values obtained here for tissue concentrations of neocortical and striatal amino acids are compatible with those in our previous studies (Hashimoto *et al.* 1992b; Takahashi *et al.* 1997) and other reports (Ramirez de Guglielmo and Gomez 1966; Tossman *et al.* 1986). These results support the reliability of our assay method for amino acids.

The present study is the first to demonstrate that acute systemic administration of MAP, a schizophrenomimetic, markedly augments the net concentrations of L-Arg and L-Asn in the neocortex and striatum in an antipsychotic-reversible fashion. Moreover, MAP induction of the striking increase in neocortical, but not striatal, L-Asn/L-Asp ratios is attenuated by HAL and CLZ treatment. MAP also produces a slight-to-moderate and HAL- and/or CLZ-sensitive increase in levels of neocortical L-Thr and of striatal L-Glu, L-Ser, L-Thr, Gly and L-Ala. A MAP-induced small reduction in L-Gln/L-Glu ratio in the neocortex is insensitive to the two antipsychotics, whereas that in the striatum is sensitive to CLZ, but not HAL.

These phenomena do not seem to be due to an artifact because PCP, another schizophrenomimetic with a pharmacological profile distinct from that of MAP, did not alter the net content of the amino acids quantified, apart from a decrease in L-Asp levels in the neocortex and striatum. Reduced L-Asp levels in the striatum were also noted by Yonezawa *et al.* (1992). These differences could, at least in part, underlie differences in behavioral or psychotomimetic effects between MAP and PCP.

Only slight alterations in the concentrations of a few amino acids in the neocortex and striatum of MAP-treated rats were observed 360 min after injection, suggesting that the effects of MAP on cerebral amino acids are relatively short lasting. Because HAL is a D2-preferring dopamine receptor antagonist (Miyamoto *et al.* 2002), the HAL-sensitive nature of MAP-induced changes in cortical L-Arg and L-Asn and in striatal L-Arg, L-Asn, L-Glu, Gly, L-Ser, L-Thr and L-Ala supports the idea that these changes are likely to be associated with increased dopaminergic neurotransmission owing to the facilitation of release and inhibition of reuptake of dopamine by MAP (Seiden *et al.* 1993).

This theory seems to be consistent with the observation using an *in vivo* dialysis technique that application of exogenous L-Arg via the dialysis tubing augments extracellular dopamine concentrations in the striatum (Strasser *et al.* 1994), indicating an interaction between L-Arg and the dopaminergic system. The fact that MAP- and L-Arg-induced striatal dopamine release is eliminated by a NO synthase inhibitor *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) and that some behavioral effects of MAP are blocked by L-NAME (Ohno and

Table 7 Effects of acute MAP injection on the net concentrations of neocortical various amino acids in the rats pretreated with HAL or CLZ

		Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)				
		NMDA receptor-related amino acids				
		Glutamate site				Nitric oxide
Pretreatment (mg/kg i.p.)	Injection (mg/kg, s.c.)	L-Glu	L-Gln	L-Asp	L-Asn	L-Arg
Vehicle	Saline	15.5 $\pm$ 0.2	5.48 $\pm$ 0.06	3.80 $\pm$ 0.05	0.138 $\pm$ 0.003	0.107 $\pm$ 0.002
Vehicle	MAP (4.8)	17.7 $\pm$ 0.2**	5.41 $\pm$ 0.06	3.49 $\pm$ 0.06*	0.180 $\pm$ 0.007**	0.186 $\pm$ 0.014**
HAL (1.0)	MAP (4.8)	17.2 $\pm$ 0.2**	5.32 $\pm$ 0.07	3.51 $\pm$ 0.04*	0.145 $\pm$ 0.006++	0.134 $\pm$ 0.011**+#
CLZ (10.0)	MAP (4.8)	16.6 $\pm$ 0.1**	5.51 $\pm$ 0.11	3.31 $\pm$ 0.03**	0.130 $\pm$ 0.002++	0.097 $\pm$ 0.006**+#
HAL (1.0)	Saline	15.0 $\pm$ 0.1	5.58 $\pm$ 0.06	3.88 $\pm$ 0.07	0.136 $\pm$ 0.002	0.113 $\pm$ 0.003
CLZ (10.0)	Saline	14.9 $\pm$ 0.2	5.79 $\pm$ 0.13	3.84 $\pm$ 0.13	0.130 $\pm$ 0.002	0.096 $\pm$ 0.002

		Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)					
		NMDA receptor-related amino acids					
		Glycine site			Other amino acids		
Pretreatment (mg/kg i.p.)	Injection (mg/kg, s.c.)	Gly	D-Ser	L-Ser	Tau	L-Thr	L-Ala
Vehicle	Saline	1.05 $\pm$ 0.02	0.266 $\pm$ 0.003	1.09 $\pm$ 0.02	6.15 $\pm$ 0.06	0.666 $\pm$ 0.009	0.748 $\pm$ 0.011
Vehicle	MAP (4.8)	1.11 $\pm$ 0.03	0.275 $\pm$ 0.006	1.16 $\pm$ 0.03	6.17 $\pm$ 0.12	0.770 $\pm$ 0.018**	0.816 $\pm$ 0.011**
HAL (1.0)	MAP (4.8)	1.06 $\pm$ 0.02	0.276 $\pm$ 0.003	1.09 $\pm$ 0.02	6.15 $\pm$ 0.07	0.705 $\pm$ 0.012	0.789 $\pm$ 0.010
CLZ (10.0)	MAP (4.8)	1.00 $\pm$ 0.03	0.272 $\pm$ 0.004	1.03 $\pm$ 0.03**	6.13 $\pm$ 0.10	0.683 $\pm$ 0.018++	0.766 $\pm$ 0.011
HAL (1.0)	Saline	1.02 $\pm$ 0.01	0.268 $\pm$ 0.003	1.09 $\pm$ 0.01	6.21 $\pm$ 0.10	0.661 $\pm$ 0.012	0.726 $\pm$ 0.013
CLZ (10.0)	Saline	1.05 $\pm$ 0.01	0.264 $\pm$ 0.003	1.08 $\pm$ 0.02	6.14 $\pm$ 0.04	0.651 $\pm$ 0.014	0.755 $\pm$ 0.010

MAP or saline was administered 60min, and HAL, CLZ or vehicle was injected 90 min before sacrifice. The amino acids quantified in this study are divided into the following four groups that are defined as indicated in the subheading of Table 1. Results represent the mean with SEM of the data obtained from 7-16 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle-pretreated saline-injected controls. ++ $p < 0.01$  compared to vehicle-pretreated MAP-injected animals. # $p < 0.05$ , compared to haloperidol-pretreated MAP-injected animals.

Watanabe 1995; Inoue *et al.* 1996) raises the possibility that the stimulatory effect of MAP on striatal L-Arg levels may lead to augmented dopamine release owing to overproduction of NO from the increased L-Arg by neural NO synthase. Moreover, previous observations of an MAP-induced increase in extracellular L-Glu levels in the frontal cortex (Shoblock *et al.* 2003) and striatum (Zhang *et al.* 2001) and of NMDA receptor-mediated activation of the NO-generating system (Southam *et al.* 1991) allow us to postulate that the increased levels of neocortical and striatal L-Glu after MAP administration in this study might stimulate NO synthesis through an increased extracellular release of L-Glu and NMDA receptor stimulation. The plausible metabolic links among L-Arg, NO, L-Glu NMDA receptor and dopamine might represent an additional mechanism underlying MAP-induced hyperdopaminergic activity. These data suggest that an increase in neocortical and striatal L-Glu as well L-Arg following acute MAP administration would also be connected to the interaction between dopamine and the NO system.

Our results concerning L-Asn levels and L-Asn/L-Asp ratios indicate that L-Asn metabolism might be under dopaminergic regulation in the neocortex and striatum. This regulation appears to be related to the cerebral dopamine-excitatory amino acid interaction, because (i) an excitatory amino acid L-Asp is shown to be formed by deamination of L-Asn which is catalysed by the enzyme asparaginase in rat brain (Dieterich *et al.* 2003), (ii) [ $^{14}\text{C}$ ]Asn has been reported to be converted to a great extent to [ $^{14}\text{C}$ ]Asp which is decreased by lesioning of cortico-striatal pathways (Reubi *et al.* 1980), and (iii) evidence has accumulated indicating that the excitatory amino acid system including L-Asp, L-Glu and NMDA co-agonists Gly and D-Ser have mutual interactions with dopaminergic systems in the cortical and subcortical brain areas through the NMDA or non-NMDA ionotropic or metabotropic Glu receptors (Nishijima *et al.* 1994; 1996; Umino *et al.* 1998; Sesack *et al.* 2003). Interruption of excitatory amino acid transmission via the NMDA receptor has been observed to inhibit the development of behavioral sensitization induced by amphetamines

**Table 8** Effects of acute MAP injection on the net concentrations of striatal various amino acids in the rats pretreated with HAL or CLZ

		Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)				
		NMDA receptor-related amino acids				
		Glutamate site				Nitric oxide
Pretreatment (mg/kg i.p.)	Injection (mg/kg, s.c.)	L-Glu	L-Gln	L-Asp	L-Asn	L-Arg
Vehicle	Saline	11.5 $\pm$ 0.1	5.76 $\pm$ 0.05	2.42 $\pm$ 0.04	0.107 $\pm$ 0.001	0.136 $\pm$ 0.002
Vehicle	MAP (4.8)	12.8 $\pm$ 0.1**	5.68 $\pm$ 0.04	2.22 $\pm$ 0.04	0.166 $\pm$ 0.009**	0.217 $\pm$ 0.016**
HAL (1.0)	MAP (4.8)	12.1 $\pm$ 0.1**,**	5.76 $\pm$ 0.06	2.09 $\pm$ 0.02	0.129 $\pm$ 0.006**,**	0.146 $\pm$ 0.009**
CLZ (10.0)	MAP (4.8)	11.8 $\pm$ 0.1**	5.83 $\pm$ 0.13	2.13 $\pm$ 0.03	0.111 $\pm$ 0.002**	0.125 $\pm$ 0.004**
HAL (1.0)	Saline	11.2 $\pm$ 0.1	5.96 $\pm$ 0.07	2.24 $\pm$ 0.29	0.111 $\pm$ 0.003	0.145 $\pm$ 0.004
CLZ (10.0)	Saline	11.0 $\pm$ 0.1	6.04 $\pm$ 0.09	2.25 $\pm$ 0.25	0.097 $\pm$ 0.002	0.122 $\pm$ 0.003

		Amino acid concentration ( $\mu\text{mol/g}$ of wet weight)					
		NMDA receptor-related amino acids					
		Glycine site			Other amino acids		
Pretreatment (mg/kg i.p.)	Injection (mg/kg, s.c.)	Gly	D-Ser	L-Ser	Tau	L-Thr	L-Ala
Vehicle	Saline	0.808 $\pm$ 0.010	0.223 $\pm$ 0.003	0.696 $\pm$ 0.006	8.84 $\pm$ 0.09	0.630 $\pm$ 0.009	0.67 $\pm$ 0.01
Vehicle	MAP (4.8)	0.959 $\pm$ 0.043**	0.236 $\pm$ 0.006	0.891 $\pm$ 0.048**	8.86 $\pm$ 0.15	0.774 $\pm$ 0.028**	1.03 $\pm$ 0.04**
HAL (1.0)	MAP (4.8)	0.852 $\pm$ 0.023*	0.237 $\pm$ 0.004	0.768 $\pm$ 0.019**,**	8.77 $\pm$ 0.05	0.684 $\pm$ 0.017*	0.90 $\pm$ 0.02**,**
CLZ (10.0)	MAP (4.8)	0.787 $\pm$ 0.016**	0.232 $\pm$ 0.004	0.690 $\pm$ 0.009**	8.60 $\pm$ 0.08	0.655 $\pm$ 0.014**	0.84 $\pm$ 0.01**,**
HAL (1.0)	Saline	0.809 $\pm$ 0.024	0.222 $\pm$ 0.004	0.712 $\pm$ 0.019	8.82 $\pm$ 0.06	0.646 $\pm$ 0.018	0.75 $\pm$ 0.03
CLZ (10.0)	Saline	0.775 $\pm$ 0.011	0.220 $\pm$ 0.003	0.668 $\pm$ 0.009	8.82 $\pm$ 0.10	0.601 $\pm$ 0.010	0.68 $\pm$ 0.01

MAP or saline was administered 60min, and HAL, CLZ or vehicle was injected 90 min before sacrifice. The amino acids quantified in this study are divided into the following four groups that are defined as indicated in the subheading of Table 1. Results represent the mean with SEM of the data obtained from 7-14 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle-pretreated saline-injected controls. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle-pretreated MAP-injected animals.

**Table 9** Effects of acute MAP injection on the ratios of L-Asn/L-Asp and L-Gln/L-Glu in the rat brain pretreated with HAL or CLZ

		Neocortex		Striatum	
Pretreatment (mg/kg i.p.)	Injection (mg/kg, s.c. or i.p.)	L-Asn/L-Asp	L-Gln/L-Glu	L-Asn/L-Asp	L-Gln/L-Glu
Vehicle	Saline	0.034 $\pm$ 0.001	0.361 $\pm$ 0.006	0.042 $\pm$ 0.001	0.479 $\pm$ 0.005
Vehicle	MAP (4.8)	0.050 $\pm$ 0.002**	0.313 $\pm$ 0.005**	0.070 $\pm$ 0.003**	0.423 $\pm$ 0.004**
HAL (1.0)	MAP (4.8)	0.038 $\pm$ 0.001**	0.317 $\pm$ 0.003**	0.059 $\pm$ 0.003	0.455 $\pm$ 0.007
CLZ (10.0)	MAP (4.8)	0.037 $\pm$ 0.001**	0.338 $\pm$ 0.006	0.051 $\pm$ 0.001	0.471 $\pm$ 0.009**
HAL (1.0)	Saline	0.033 $\pm$ 0.001	0.377 $\pm$ 0.005	0.058 $\pm$ 0.010	0.501 $\pm$ 0.006
CLZ (10.0)	Saline	0.032 $\pm$ 0.001	0.398 $\pm$ 0.006**	0.047 $\pm$ 0.007	0.523 $\pm$ 0.005

MAP or saline was administered 60min, and HAL, CLZ or vehicle was injected 90 min before sacrifice. Results represent the mean with SEM of the data obtained from 6-16 animals. \*\* $p < 0.01$  compared to vehicle-pretreated saline-injected controls. \*\* $p < 0.01$  compared to vehicle-pretreated MAP-injected animals.

and other psychostimulants (Vanderschuren and Kalivas 2000). Therefore, MAP may modify behavioral expression or mental function partially by influencing L-Asp-L-Asn metabolism.

As for L-Ser, L-Thr and L-Ala, there are currently no data concerning the relationship between dopaminergic transmission and their metabolism. Further investigations are needed to clarify the exact mechanisms underlying dopaminergic



control over neocortical and striatal levels of the neutral amino acids. Based upon no direct conversion of dopamine and its metabolites to any of the amino acids quantified here, the dopaminergic regulation could directly or indirectly influence synthesis, release, uptake or degradation of these amino acids.

The observation that CLZ produced a more complete antagonism of the effects of MAP on L-Arg and L-Thr than HAL seems to conflict with their rank order potency as dopamine receptor antagonists. This discrepancy might be explained by the influence of CLZ on the possible direct action of MAP on L-Arg and L-Thr metabolism or on extra-dopaminergic systems, for example, the serotonin or norepinephrine system. The hypothesized extra-dopaminergic effects of CLZ might partially explain its atypical actions that ameliorate typical antipsychotic-resistant schizophrenic symptoms.

It should be noted that HAL failed to significantly attenuate MAP-induced changes in L-Asp concentrations and L-Gln/L-Glu ratios in the neocortex and striatum. Moreover, even CLZ did not reverse the reduction in neocortical and striatal L-Asp content and the neocortical L-Gln/L-Glu ratio following MAP administration. Our findings that MAP causes alterations in L-Asp, L-Asn, L-Glu and L-Gln concentrations, and L-Gln/L-Glu and L-Asn/L-Asp ratios agree with previous reports showing the substantial modifications by amphetamines of excitatory amino acid metabolism in rat brain (Bustamante *et al.* 2002; Shoblock *et al.* 2003), supporting the involvement of an excitatory amino acid system in the pathophysiology of schizophrenic symptoms. Together with the clinical observations that MAP elicits antipsychotic-resistant and negative symptom-like schizophrenic disturbances in some patients with a history of abuse of these drugs (Tomiya 1990; Sato 1992; Flaum and Schultz 1996; Mikami *et al.* 2003), the present findings indicate that the HAL- and CLZ-insensitive modification of cerebral amino acids by MAP might participate in precipitation and/or lasting of incurable schizophrenic symptoms.

In conclusion, we have revealed prominent and antipsychotic-reversible changes in the net levels of endogenous L-Asn and L-Arg following acute MAP treatment in the neocortex and striatum. We have also found (1) HAL- and CLZ-sensitive, (2) HAL-insensitive and CLZ-sensitive, or (3) HAL- and CLZ-insensitive alterations in various other amino acids in the two tissues. The present findings indicate that certain signal pathways involving these amino acids would be dysfunctional in schizophrenic brains and be suitable targets for the development of novel antipsychotic drugs. Although the schizophrenomimetics and antipsychotics tested in this series of experiments have been expected to affect D-Ser metabolic pathways, including D-Ser levels, because of the anti-MAP (Hashimoto *et al.* 1991) and anti-PCP (Tani *et al.* 1991, 1994; Umino *et al.* 1998) effects of the D-amino acid, there were minimal changes in neocortical and striatal D-Ser concentrations at the doses used in this study.

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Original Article

Case-control association study of human netrin G1 gene in Japanese schizophrenia

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The exact etiology of schizophrenia remains undetermined but accumulating evidence suggests that disturbances in neurodevelopment may represent one contributory factor. Netrin G1, a recently cloned gene from the mouse, has been shown to play a potential role in the formation of neural circuitry. To determine whether this gene is involved in the development of psychosis, we performed a genetic association study of human netrin G1 gene in schizophrenia. First, we determined the human genomic structure of netrin G1 by direct comparisons between cDNA and genome sequences, and by database searches. For the subsequent examination of heterozygosity, we selected 10 single nucleotide polymorphisms (SNPs) for an association test in case ( $n = 180$ ) and control ( $n = 180$ ) samples. Among these SNPs, IVS8-1467C>T showed significant allelic association (nominal  $P = 0.020$ ) with disease. This SNP is located in a haplotype block of ~40 kb and haplotypes in this block also displayed significant association (most significant  $P = 0.017$ ). These findings suggest that netrin G1 or a nearby gene may contribute to the overall genetic risk for schizophrenia.

**Key words:** netrin family, laminin 1, axon guidance, haplotype, linkage disequilibrium

Introduction

Schizophrenia is a common and devastating mental disorder of unknown etiology. Multiple factors including risk-conferring genes and undefined environmental variables are thought to contribute to overall susceptibility.<sup>1</sup> One etiological hypothesis is that neurodevelopmental abnormalities are at least partially involved in the manifestation of schizophrenia. This assertion is supported by a range of epidemiological, clinical and neurobiological evidence.<sup>2</sup>

The developing nervous system is dependent on the actions of various secreted factors and membrane proteins that allow neuronal axons to find their correct targets. The proteins that provide these cues include netrins, ephrins, semaphorins and slits.<sup>3</sup> Classical netrins identified as laminin like molecules that direct migration in *Caenorhabditis elegans* are soluble secreted proteins that provide bifunctional axon guidance signals that can mediate either attraction or repulsion.<sup>4</sup> Three classical netrin molecules (1, 2 and 3) have been characterized in vertebrates.<sup>5</sup> The gene family is structurally related to the short arms of the laminin  $\gamma$  chain, comprising a laminin VI domain, three LE repeats, similar to the laminin V domain and a positively charged heparin-binding carboxyl domain.<sup>6</sup>

Recently, netrin G1 (also called laminin 1) has been identified in the mouse.<sup>7</sup> Its predicted domain structure resembles that of the laminin  $\beta$  chain and the protein is

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linked to the plasma membrane by a glycosyl phosphatidyl-inositol (GPI) lipid anchor, an important feature that distinguishes them from classical netrins.<sup>7,8</sup> Additionally, netrin G1 does not bind to receptors for classical netrins, nor does it attract circumferentially growing axons from the cerebellar plate in explant extracts.<sup>7</sup> No orthologues for netrin G1 gene have been found in the *C. elegans* or *D. melanogaster*. These observations suggest that netrin G1 may play an as yet, undetermined role in cell architecture that is unique to vertebrates.

Based on the potential relevance of netrin G1 to neurodevelopment, we performed a genetic analysis of this gene in schizophrenia.

## Material and Methods

### Subjects

Schizophrenic samples were composed of 90 males (mean age,  $40.3 \pm 8.6$  years) and 90 females (mean age,  $47.1 \pm 13.0$  years). All patients were diagnosed according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) for schizophrenia, to give a best-estimate lifetime diagnosis with consensus from at least two exper-

rienced psychiatrists (Yamada K, Toyota T and Yoshikawa T). The interview parameters included those described in the Structured Clinical Interview For DSM-IV Axis I Disorders. All available medical records and family informant reports were also taken into consideration. Controls comprising 90 males (mean age,  $39.3 \pm 11.5$  years) and 90 females (mean age,  $46.9 \pm 11.9$  years), were recruited from hospital staff and company employees documented to be free from psychoses. All of our samples were collected from central Japan.

The present study was approved by the Ethics Committees of RIKEN and Tokyo Medical and Dental University, and all participants provided written informed consent.

### Determination of genomic organization of netrin G1 gene

A mouse netrin G1 cDNA sequence NM\_030699 and a human EST (expressed sequence tag) clone KIAA0976 sequence (NM\_014917) were compared to human BAC clones forming the contig NT\_029860 using BLAST, to determine the intron/exon structure of the human netrin G1 gene. This led to the identification of 10 exons, with translation starting within exon 2 (Fig. 1). The UCSC April 2003 draft assembly of the human

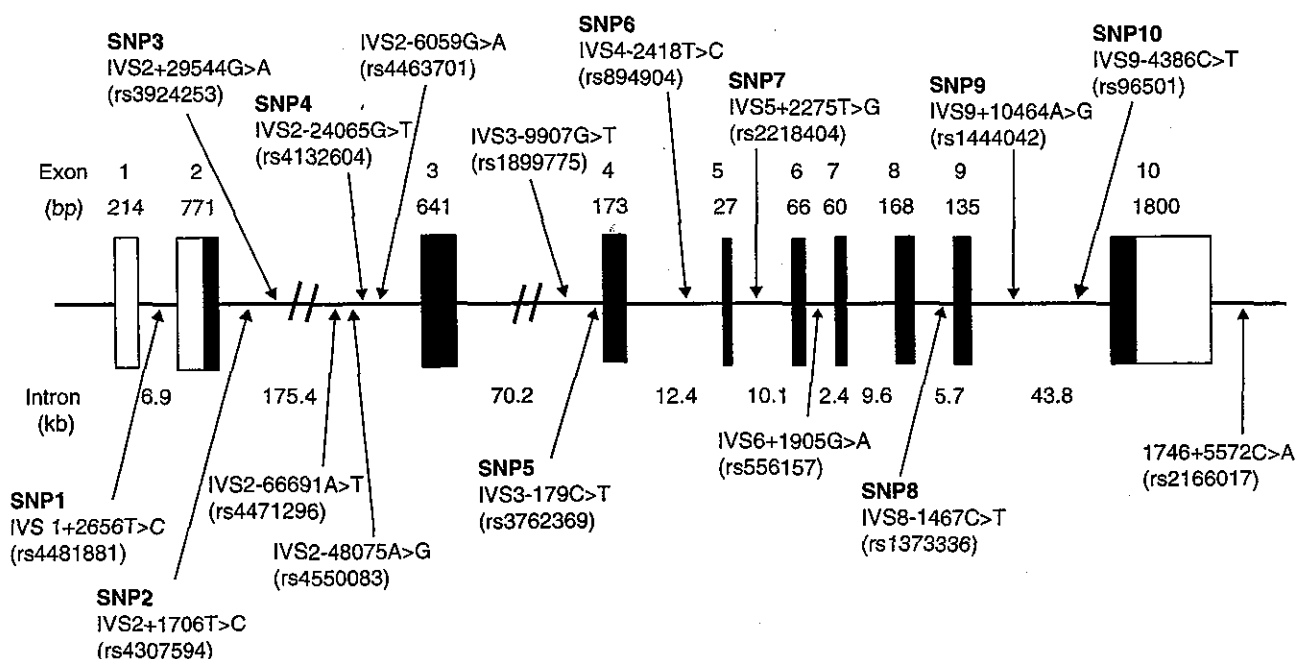


Fig. 1. Genomic structure and locations of polymorphic sites for the human netrin G1 gene. Exons are denoted by boxes, with untranslated regions in open boxes and translated regions in closed boxes. The sizes of exons (bp) and introns (kb) are also shown. The rs number of each SNP is the NCBI SNP cluster ID from the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>).

genome (UCSC Genome Bioinformatics web site, <http://genome.ucsc.edu/>) included only exons 1 to 5 in its gene prediction program. "A" from the ATG initiation codon was considered at +1.

### Single nucleotide polymorphisms (SNPs) search and genotyping

We consulted the JSNP database (<http://snp.ims.u-tokyo.ac.jp/>) and The SNP Consortium Ltd database (<http://snp.cshl.org/>) to find polymorphisms within the netrin G1 gene, and identified a total of 16 SNPs (Fig. 1). We first genotyped these SNPs using 40 randomly chosen schizophrenic samples, and direct sequencing of PCR products (the SNPs located in exons and nearby introns) or the TaqMan method<sup>9</sup> (Applied Biosystems, Foster City, California, US) (the SNPs located in deep introns). The primers used for PCR amplification are shown in Table 1. Sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) and the ABI 3700 Genetic Analyzer (Applied Biosystems). The SNPs that were not poly-

morphic in the 40 samples were excluded from further genetic analyses. These included IVS2-66691A>T (rs4471296, <http://www.ncbi.nlm.nih.gov/SNP/>), IVS2-48075A>G (rs4550083), IVS2-6059G>A (rs4463701), IVS3-9907G>T (rs1899775), IVS6+1905G>A (rs556157) and 1746+5572C>A (rs2166017) (Fig. 1). The remaining 10 variants were genotyped in all samples using the TaqMan method, that utilizes the 5'-exonuclease activity of the Taq polymerase in combination of PCR and competitive hybridization.<sup>9</sup> Probes and primers were designed using the Assays-by-Design File Builder v2.0 software and the Primer Express software (Applied Biosystems) (Table 2). PCR reactions were performed using an ABI 9700 thermocycler, and fluorescence-based genotyping was conducted using an ABI 7900 sequence detection system and SDS v2.0 software (Applied Biosystems). The samples with ambiguous genotypes were not used in statistical analyses.

### Statistical analysis

Deviation from Hardy-Weinberg equilibrium was

**Table 1.** PCR Primers used to examine nucleotide variants in the *NTNG1*

Region	Primers (F, forward; R, reverse)	Product size (bp)	3' end of primer
Exon 2	(F) 5'-TGCTTTATATTGCATCAGACCTC-3'	480	-603 (intron 1)
	(R) 5'-GACCTCAAAGCAGATCCCAAAA-3'		-146 (exon 2)
	(F) 5'-AGTATGTTAGGCTTCCACCAA-3'	566	-218 (exon 2)
	(R) 5'-GTCTTCACACTCACCACATC-3'		+328 (intron 2)
Exon 3	(F) 5'-TAGGGCAAATAAAAATGA-3'	584	+175568 (intron 2)
	(R) 5'-AAAAACGCGAACCTGTC-3'		+176134 (exon 3)
	(F) 5'-ACGAACATGGCAGCCCTATCAG-3'	529	+175966 (exon 3)
	(R) 5'-AATGCCTTCAGAACCTACT-3'		+176473 (intron 3)
Exon 4	(F) 5'-GGCCTGCAAACTATCTCTTACTA-3'	511	+246439 (intron 3)
	(R) 5'-GATGACTGAATTTTACTGAT-3'		+246877 (intron 4)
Exon 5	(F) 5'-TGCACCTGTATTTGTGTGTGTC-3'	283	+258962 (intron 4)
	(R) 5'-CCTATTACATCAGAAATGGACACT-3'		+259221 (intron 5)
Exon 6	(F) 5'-AATTGCTTGCTCTTGTT-3'	347	+269832 (intron 5)
	(R) 5'-TTTCAAAGACATAGCATTTCAT-3'		+270162 (intron 6)
Exon 7	(F) 5'-CTTAATTTAGGGCTACTTTTCA-3'	254	+272404 (intron 6)
	(R) 5'-TACACTTCACAGATATCCAGATT-3'		+272636 (intron 7)
Exon 8	(F) 5'-ATGCCATTCCACCGTCTTT-3'	406	+282063 (intron 7)
	(R) 5'-AGGATATTTTCTACATTGAG-3'		+282450 (intron 8)
Exon 9	(F) 5'-TCATTAATGGACATCTTT-3'	352	+287966 (intron 8)
	(R) 5'-GGATCTTTTCTGCTCTGA-3'		+288300 (intron 9)
Exon 10	(F) 5'-GGCTGAAAACATGATGTACCAGATG-3'	453	+331947 (intron 9)
	(R) 5'-AGGCCCTTCTAGTTTGTACTGTC-3'		+332375 (exon 10)

Nucleotide positions are counted from A of the start codons on the genomic stretches of *NTNG1* (GenBank accession No. NM\_014917).