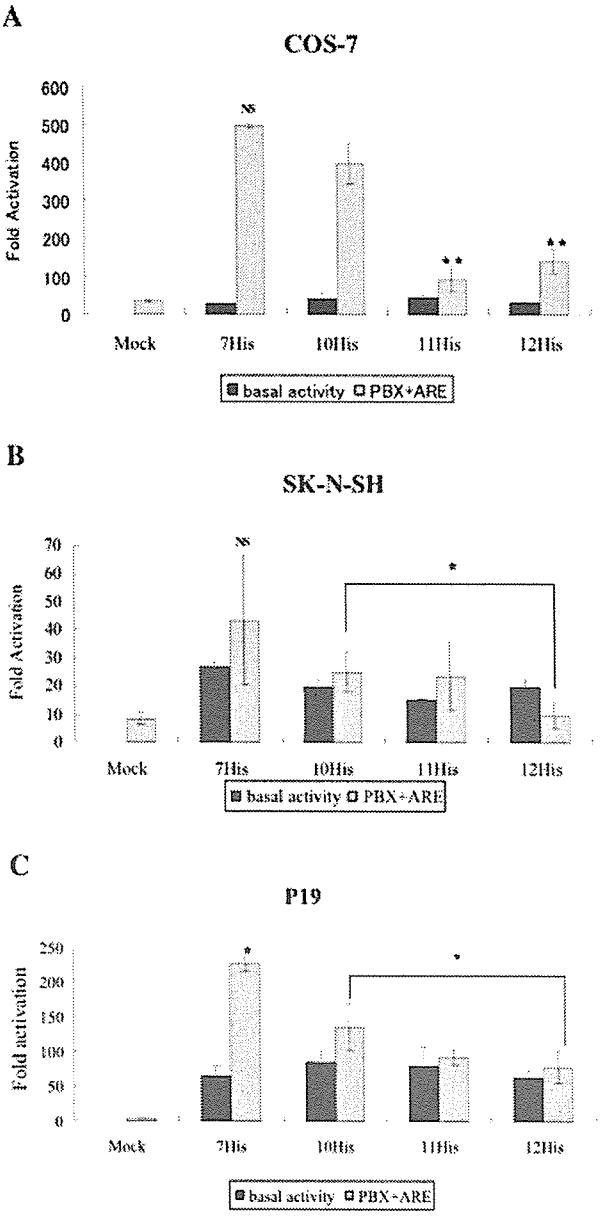

Fig. 3. Expanded HOXA1 reduces PBX1-coupled transcriptional activity. Luciferase assay from the different cell lines. Cells were cotransfected with plasmids containing PBX1, HOXA1, and the enhancer b1-ARE fused to a luciferase reporter gene (Di Rocco et al., 2001). EGFP vector was used as an internal control. **A:** COS-7. **B:** Neuroblastoma cell line SK-N-SH. **C:** Embryonic carcinoma cell line P19 grown in serum-free medium. Bars represent the mean ± SE of at least three independent experiments. $*P < 0.05$, $**P < 0.01$.

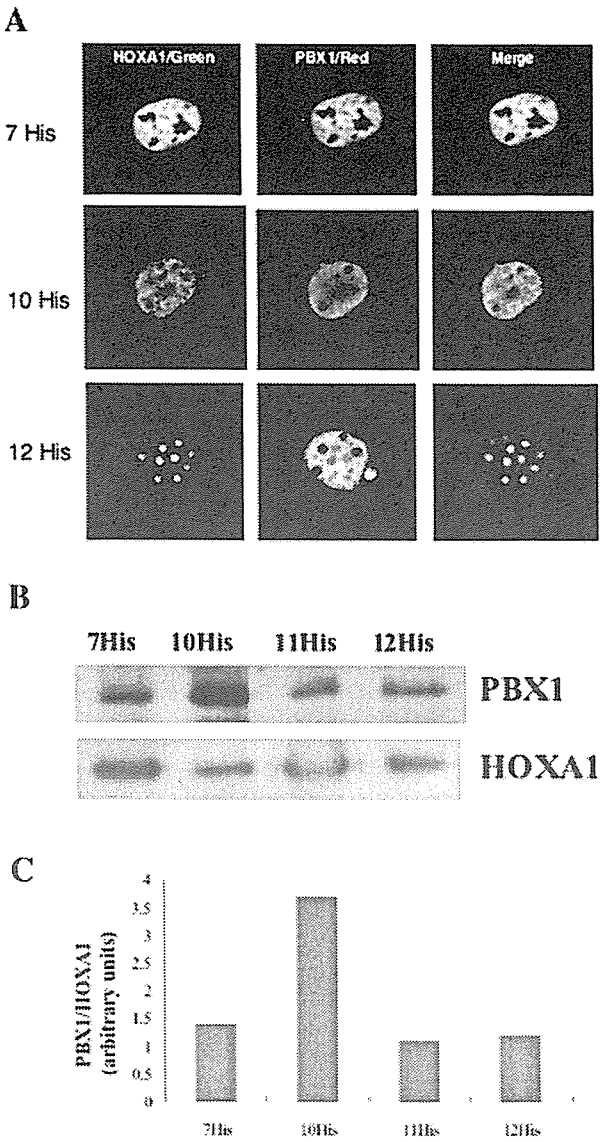

Fig. 4. HOXA1 and PBX1 interaction is impaired in expanded variant of HOXA1. **A:** Immunofluorescence images of COS-7 cotransfected with PBX1, an enhancer bi-ARE, and 7-His, 10-His, or 12-His repeat variants of HOXA1-EGFP. Fluorescence images were taken 24 hr posttransfection. Red signals: anti-PBX1. Note the absence of PBX1 signal in protein aggregates of the 12-His variant. **B:** Immunoprecipitation with anti-GFP and Western blotted with anti-PBX1 and anti-HOXA1. PBX1 protein shows decreased levels in both expanded and deleted variants of HOXA1-EGFP transfected to COS-7 cells. **C:** Quantification of bound PBX1 protein levels per HOXA1 protein from Western blot analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Transcriptional Activities of Polyhistidine Variants of HOXA1 Coupled With PBX1

We next examined the physiological relevance of polyhistidine variants of HOXA1 protein in neuronal cells. PBX1 plays a major role in cooperative transcriptional activation with HOXA1 through an autoregulatory element, the b1-ARE (Di Rocco et al., 2001). To investigate the cooperative transcription of PBX1 with

the different variants of HOXA1 through b1-ARE, we simultaneously expressed HOXA1, PBX1, EGFP (an internal control), and b1-ARE luciferase reporter constructs in COS7, SK-N-SH, and P19 cells. Luciferase assay were performed 24 hr after transfection. The P19 embryonic cell line was grown under serum-free conditions. Growth in serum-free media itself committed EC cells to neural differentiation (Darmon et al., 1981) and addition of retinoic acid (RA) intensified this effect (Tanaka et al., 1992). However, nonneural cell types arise after treatment with RA under serum-containing conditions. Differentiation in serum-free media alone is accompanied by expression of only neuroectodermal/neural mRNAs, but treatment with RA invariably induces the cells to express both neuroectodermal/neural and endodermal mRNAs (Pachernik et al., 2005), so the serum-free medium condition was used in this experiment.

The transcriptional activities of expanded variants were significantly reduced in all cell lines, whereas the activities of deleted variant with 7-polyhistidine were enhanced in P19 cells compared with the activity of 10-polyhistidine variant (Fig. 3A–C). Immunofluorescence images revealed the inability of aggregated forms of expanded HOXA1 protein to bind efficiently with PBX1 protein (Fig. 4A). Inefficient interaction between expanded HOXA1 and PBX1 is clearly indicated by low levels of PBX1 protein in immunoprecipitation assay with anti-GFP (Fig. 4B,C).

Expanded and Deleted Polyhistidine Variants in HOXA1 Inhibited Neuronal Differentiation

Because HOXA1 is one of the neurodevelopmental genes, we examined whether polyhistidine variants had any effect on the process of neuronal differentiation. To accomplish this, RA-induced neuronal differentiation was performed in SK-N-SH cells. Phase microscopy shows the effect of RA treatment on the neurites of these cells (Fig. 5A). Immunocytochemistry with antibody against the neuron-specific protein MAP2 revealed neurite outgrowth in cells expressing the 10-histidine HOXA1-GFP, 42 hr after transfection (Fig. 5B). In contrast, a greater number of cells expressing expanded and deleted forms of HOXA1-GFP failed to initiate neurite outgrowth. Total levels of MAP2 protein in cells overexpressing expanded and deleted variants were also significantly reduced (Fig. 5C,D). The appearance of

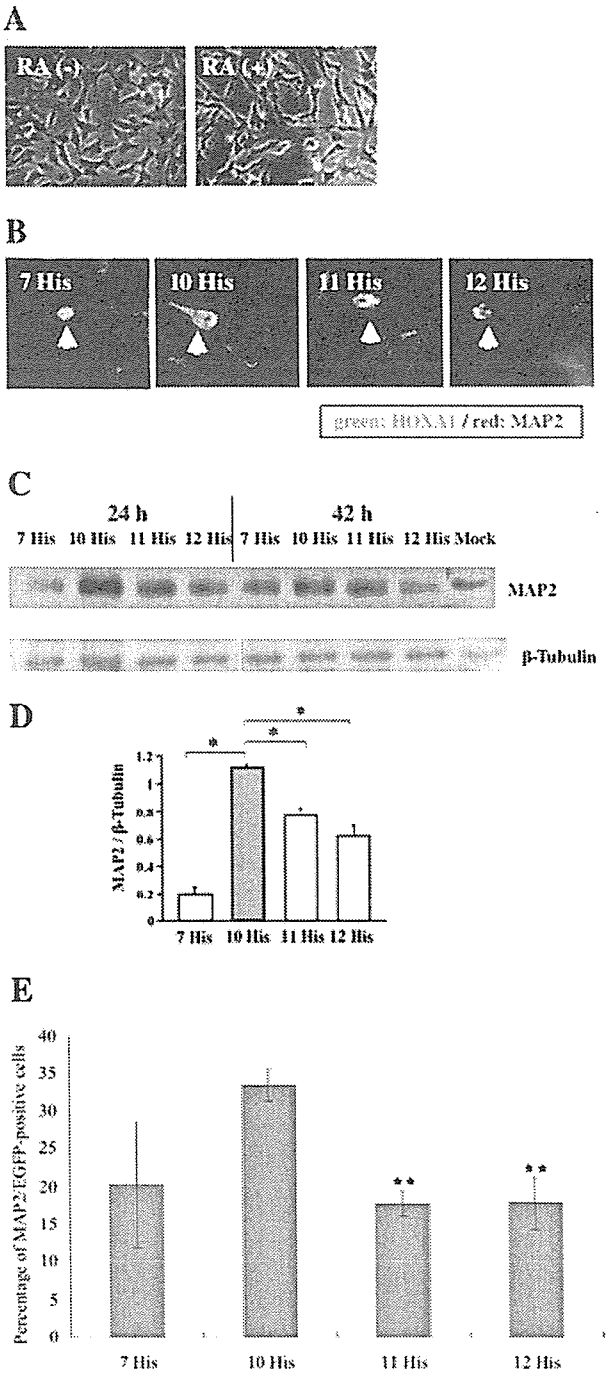

Fig. 5. Expanded and deleted polyhistidine repeats in HOXA1 inhibit neuronal differentiation. **A:** Retinoic acid (RA)-induced neuronal differentiation in SK-N-SH cells Phase images of SK-N-SH cells treated with (right) or without (left) 10 μM RA for 42 hr. **B:** HOXA1-EGFP-transfected SK-N-SH treated with 10 μM RA for 42 hr. Immunofluorescence images where taken 42 hr after transfection. Expanded 11- and 12-His repeat variants as well as deleted 7-His repeat variants inhibit neuronal differentiation. Note the neurite outgrowth immunostained with MAP2 (red signal). **C:** Western blot analysis shows decreased MAP2 expression levels in the expanded and deleted forms. **D:** Quantification of MAP2 expression levels normalized with β-tubulin. **E:** Quantification of the percentage of EGFP-MAP2-double-positive cells. Error bars represent SEM; n = 3. $*P < 0.05$, $**P < 0.01$. *P* values from a paired *t*-test in all experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

differentiated cells was also examined by scoring MAP2-positive transformants expressing the HOXA1-EGFP variants. EGFP-MAP2 double-positive cells quantification also confirmed that there is a significant decrease in the percentage of MAP2 levels particularly in the expanded variants.

DISCUSSION

In this current study, we have shown that polyhistidine repeat tract variants compromised HOXA1 function in transcription and neuronal differentiation. Moreover, we have presented evidence that cell death resulting from polyhistidine repeat expansion is mediated primarily by authophagy and not by a caspase-dependent mechanism. Authophagy is a type of cell death involving bulk degradation of cytoplasmic proteins or organelles in the lytic compartment. Inhibition of autophagy enhances protein aggregation and cell death (Ravikumar et al., 2002). Our data showed that 3-MA accelerates protein accumulations and enhances cell death, particularly in the expanded variants (Fig. 2E,F). Conversely, Rapamycin, an inducer of the autophagic process, decreases protein aggregates and reduces cell death (Fig. 2G). Taken together, our findings indicate that autophagy is involved in the degradation and clearance of aggregations of expanded HOXA1 variants.

HOXA1 splice variant 1 is reported to be active in E7–E8.5 and functions in the presumptive rhombomere 3 and 4 regions of the developing hindbrain (Zhang et al., 1994). However, the nonhomeodomain-containing variant is expressed in the endodermal derivative after E8.5 to the adult stage (Godwin et al., 1998). Therefore, we speculate that splice variant 1 may be active only in early stages of embryonic neurodevelopment. As one of the DNA-binding proteins and transcription factors in the HOX gene family, HOXA1 also relies on the activity of transcriptional cofactors aside from its DNA-binding properties (Pearson et al., 2005). A previous study has reported that HOXB1 and PBX1 cooperatively activate transcription under the control of b1-ARE (autoregulatory enhancer). Moreover, HOXA1 and HOXA2 are also able to activate transcription by b1-ARE in cooperation with PBX1 (Di Rocco et al., 2001). We examined the binding ability of our HOXA1 variants to PBX1 and b1-ARE by evaluating the cooperative transcriptional activation using a luciferase assay. Expanded variants transfected into COS-7 as well as SK-N-SH neuroblastoma and P19 embryonic carcinoma cells (EC) that were induced to undergo neuronal differentiation indeed showed significantly reduced activation compared with the wild type, whereas the deleted variant expressed in P19 cell line showed an enhanced activation (Fig. 3). However, the reason for increased transcriptional activity observed in the deleted variant in this cell line is as yet unknown. Immunofluorescence images and immunoprecipitation assay clearly confirmed the inability of aggregated forms of expanded HOXA1 protein to bind efficiently with PBX1 protein. The cooperative interaction between Hox and Pbx is mediated by a conserved hexapeptide sequence located toward the N-terminal region from Hox homeodomain (Phelan and Fetherstone, 1997; Remacle et al., 2004). Our results suggest that polyhistidine variants in HOXA1 might also affect the binding efficiency of its homeodomain to certain cofactors and/or other target genes or proteins.

Human neuroblastoma SK-N-SH cells, from a malignant pediatric tumor derived from the neural crest, retains its ability to differentiate into the neuronal lineage when exposed to RA (Wainwright et al., 2001). HOXA1 is the first target gene activated by RA, followed by a sequential activation of other HOX genes (Simeone et al., 1990; Martinez-Ceballos et al., 2005). We showed an inhibition of neuronal differentiation by not only the extended but also the deleted variants (Fig. 5). Our study provides novel insights on the pathological implications of the polyhistidine tract in HOXA1 and leaves us with the intriguing possibility that polyhistidine repeat expansions and deletions may cause aberrations in neuronal morphogenesis or differentiation in general.

EC cells differentiate into various lineages depending on the presence of activators in the culture medium (Pachernik et al., 2005). Growth under serum-free conditions committed EC cells to neural differentiation. Differentiation of EC cells into endodermal-like cells is induced by serum. Interestingly, we found that, in EC cells committed to neural differentiation, transcriptional activity inversely coincided with polyhistidine repeat length. These results further strengthen our theory that polyhistidine length affects neurodevelopment. In agreement with our results, another study has proposed that HOXA1 may function as a stimulator of neuroectodermal and mesodermal differentiation and a repressor of embryonic endoderm formation (Martinez-Ceballos et al., 2005) and that aberration in HOXA1 could lead to increased expression of endodermal genes by RA and would lead to repression of neuroectodermal and mesodermal markers. Premature death of HOXA1-expressing cells may impair transcription and neuronal differentiation.

Recently, Tischfield and colleagues (2005) reported that patients with Bosley-Salih-Alohrainy syndrome were homozygous for HOXA1 truncating mutations, whereas heterozygotes had normal phenotypes. This mutation resulted in abnormal development of the central nervous system in the brainstem. Distinguishing phenotypes include horizontal gaze abnormalities, mental retardation, and autism spectrum disorder. Even low levels of Hoxa1 expression in $Hoxa1^{+/-}$ cells are sufficient for normal activation of the Hoxa1 pathway and may explain why mice heterozygous for HOXA1 mutations appear normal (Pasqualetti et al., 2001; Martinez-Ceballos et al., 2005). In our previous report, we identified heterozygous polyhistidine repeat variants in HOXA1 gene from a Japanese population comprising normal and autistic individuals. No individuals homozygous for these mutations have been found. We speculate that there is a possibility

that phenotypic aberrations may exist in homozygous individuals. Future in vivo studies are essential to examine the physiological functions of the polyhistidine variants of HOXA1.

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REVIEW ARTICLE

Review of animal models for autism: implication of thyroid hormone

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ABSTRACT **Autism is a behaviorally defined disorder associated with characteristic impairments in social interactions and communication, as well as restricted and repetitive behaviors and interest. Its prevalence was once thought to be 2/10 000, but recently several large autism prevalence reviews revealed that the rate of occurrence was roughly 30/10 000. While it has been considered a developmental disorder, little is certain about its etiology. Neuroanatomical studies at the histological level in the brains of autistic patients provide many arguments in the etiology of autism. Results from postmortem and imaging studies have implicated many major structures of the brain including the limbic system, cerebellum, corpus callosum, basal ganglia and brainstem. There is no single biological or clinical marker for autism. While several promising candidate genes have been presented, the critical loci are yet unknown. Environmental influences such as rubella virus, valproic acid, and thalidomide exposure during pregnancy are also considered important, as concordance in monozygotic twins is less than 100% and the phenotypic expression of the disorder varies widely. It is thus hypothesized that non-genetic mechanisms contribute to the onset of autistic syndrome. In light of these ambiguities, hope is held that an animal model of autism may help elucidate matters. In this article, we overview most of the currently available animal models for autism, and propose the rat with mild and transient neonatal hypothyroidism as a novel model for autism.**

Key Words: animal model, autism, hypothyroidism, thalidomide, rat

INTRODUCTION

Autism is a severe neurobiological disorder that develops in the first 3 years of life. It is characterized by impairments in social interactions and communication, as well as restricted and repetitive behaviors and interest. Its prevalence was once thought to be 2/10 000, but recently several large autism prevalence reviews revealed that the rate of occurrence is roughly 30/10 000, and its incidence is progressively increasing (Stokstad 2001; Muhle *et al.* 2004; Honda *et al.* 2005). The etiology of autism remains to be clarified. Since the first description by Kanner in 1943, autism has been attributed as the earliest manifestation of schizophrenia and then to a failure of parental nurturing. Currently its etiology is unanimously thought to derive from some developmental disorder of communication with a neurobiological basis.

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The genetic component clearly plays an important role in the pathophysiology of the disorder, as there is a concordance rate of approximately 2–6% in dizygotic twins as opposed to the 60–95% concordance rate in monozygotic twins (Ritvo *et al.* 1985; Bailey *et al.* 1995). The prevalence rate of non-twin siblings of children with autism varies from 1–6% (Hallmayer *et al.* 2002). Nevertheless, the finding of the increasing prevalence rate of autism during the past 10 years may cast some doubt on whether genetics alone can explain the whole picture. A case-control study of a group of Swedish adults with Asperger syndrome (1999) has noted that the rate of autistic children with mild mental retardation remains relatively stable, while the rate is increased in children with severe mental retardation and with normal intelligence (Gillberg & Wing 1999). Some other epidemiological studies (Ehlers & Gillberg 1993; Kadesjo *et al.* 1999) indicate that the recent increase of autistic children is mainly attributable to the increase of so-called atypical autism characterized by a lesser degree of mental retardation or normal intelligence. This higher prevalence rate of high function autism or Asperger syndrome encouraged us to accept the concept of autism spectrum disorders (ASD).

Environmental factors can cause developmental disabilities. Case reports of autism associated with environmental factors, such as rubella virus, valproic acid, and thalidomide exposure during pregnancy, lead to the hypothesis that non-genetic mechanisms may also produce an autistic syndrome (Chess 1977). Although there clearly exists a genetic component in the pathophysiology of autism, ASD appears to be a syndrome of complex genetic traits, rather than the outcome of any single mutation. Furthermore, a varied burden of environmental factors may contribute to the broad spectrum disorders of autistic syndrome with a higher prevalence rate.

Since the 1970s, researchers have known that autism is a complex genetic disorder. Thus far a number of research groups including an international consortium have tried to identify the responsible gene(s) in autism. However, although several promising candidates have been presented, the critical loci are still not known. Therefore, the animal models for autism currently available are mainly derived from empiric data such as viral infection, thalidomide exposure and valproate exposure in human subjects. The rationale for some models arises from the similarities between clinical manifestations in autism and behavioral abnormalities exhibited by treated animals. In this review, we briefly introduce currently available animal models of autism, and then present our hypothesis, the pivotal role of mild neonatal hypothyroidism, as a putative animal model for studying the underlying mechanisms of autism and/or related neurodevelopmental disorders.

NEUROANATOMICAL AND NEUROIMAGING ASPECTS OF AUTISM

Neuroanatomical studies at the histological level in the brains of autistic patients provide many arguments in the etiology of autism.

Results from postmortem and imaging studies have implicated many major structures of the brain including the limbic system, cerebellum, corpus callosum, basal ganglia and brainstem.

Areas of the forebrain that have been found to be histologically abnormal include the hippocampus, subiculum, entorhinal cortex, amygdala, mammillary body, anterior cingulate gyrus and septum, structures which comprise a major portion of the limbic system. In comparison with controls, these areas showed reduced neuronal cell size and increased cell packing density (increased numbers of neurons per unit volume) bilaterally (Bauman & Kemper 1994). Golgi analysis of CA1 and CA4 pyramidal neurons has shown decreased complexity and extent of dendritic arbors in these cells (Raymond *et al.* 1996). In the amygdala, the most significant increase in cell packing density was noted in the most medially placed nuclei. With the exception of a single child of normal intelligence, the lateral nucleus has appeared to be uninvolved.

Outside of the limbic system, the most apparent and consistent abnormalities have been confined to the cerebellum and related inferior olive. Regardless of age, sex, or cognitive abilities, all the autistic brains reported to date have shown a significant decrease in the number of Purkinje cells (Bailey *et al.* 1998). With few exceptions, there has been an absence of glial hyperplasia (Bauman & Kemper 1996; Bailey *et al.* 1998) suggesting the cerebellar lesions are acquired early in development. A similar pattern of change in cell size has also been observed in the inferior olive of the brainstem but the number of neurons has been found to be preserved. Bailey *et al.* (Bailey *et al.* 1998) have noted neocortical malformations to be a prominent feature in their autopsy material. They found evidence of thickened cortices, areas of increased neuronal density, irregular laminar patterns, increased number of neurons in layer I, and abnormally oriented pyramidal cells.

The observation of postnatal brain enlargement is intriguing and a number of hypotheses have been posed to explain its origins. Clinically, the head circumference of the autistic child has been said to be either normal or slightly small at birth but later increases in size during early to mid-childhood (Courchesne *et al.* 2003; Lainhart 2003). More recently, imaging studies have indicated increased brain volume in autism (Aylward *et al.* 1999; Sparks *et al.* 2002; Herbert *et al.* 2003; Courchesne & Pierce 2005), most prominent between 2–4.5 years of age, and later appear to plateau during adolescence (Courchesne *et al.* 2001). Schumann *et al.* (Schumann *et al.* 2004) reported that children with autism (7.5–12.5 years of age) had larger right and left amygdala volumes as well as a right hippocampal volume larger than typically developing controls, even after controlling for total cerebral volume. Brambilla *et al.* (Brambilla *et al.* 2003) reviewed original MRI research papers published from 1966 to 2003 and concluded that increased total volumes of the brain, parieto-temporal lobe, and cerebellar hemisphere were the most replicated abnormalities in autism. Interestingly, recent papers suggest the size of amygdala, hippocampus, and corpus callosum may also be abnormal, although the results are controversial (Abell *et al.* 1999; Aylward *et al.* 1999; Sparks *et al.* 2002).

CURRENT ANIMAL MODELS FOR AUTISM

Neonatally Borna disease virus infected rat

Borna disease virus (BDV) is a negative strand, non-segmented RNA virus that is the prototypic member of Bornaviridae, a new class of virus in the Mononegavirales order, and is a human pathogen (De La Torre 1994). Host factors including the age at the time of inoculation, the genetic background and the immune status, as well as viral factors, influence the course of infection. In adult rats, BDV usually causes an immune-mediated biphasic behavioral disease. After a varied incubation period, the onset of a hyperactive phase is observed, which can lead to rapid death in some animals. Excitability and hyperactivity, together with movement and posture disorders, are consistent clinical features in both natural and experimental infections. Some animals may have stereotyped behaviors. A chronic hypoactive phase with somnolence follows in conjunction with a decrease in the inflammatory reaction and high levels of virus in the Central Nervous System (CNS). During this chronic phase, symptoms resembling those of the initial phase may reemerge in the form of recurrent episodes (Taieb *et al.* 2001). Heightened viral gene expression in limbic system structures, together with astrocytosis and neuronal structural alterations within the hippocampal formation are the main histopathologic hallmarks of BDV infection in adult rats (De La Torre 2002).

When BDV is inoculated into a newborn Lewis rat, it causes a life-long persistent infection that is characterized by the lack of any significant inflammatory cell infiltration within the central nervous system (CNS) and the absence of clinical signs of BDV (Pletnikov *et al.* 1999a).

Intracranial injection of the BDV in a newborn rat pup within the first 24–48 h after birth is the most common way of inducing neonatal BDV infection in rats (Pletnikov *et al.* 2003). Infected rats have normal body shape and proportion but are overall smaller than uninfected control pups. Injury to the cerebellum is one of the most salient morphological features of neonatal infection. BDV infection induces a prominent loss of Purkinje cells (PC), with up to 75% of PCs dropping out by seven months. A loss of PCs and their dendrites in the molecular layer has been suggested to play a major role in markedly reducing cerebellum size (Hornig *et al.* 1999).

In addition to injury of the cerebellum, neonatal BDV infection affects the postnatal maturation of the hippocampus. BDV infection of dentate gyrus neurons is associated with their continuing loss and eventual complete disappearance by 45–55 postnatal days (PNDs) and replaced by reactive glial cells (Hornig *et al.* 1999; Gonzalez-Dunia *et al.* 2000).

Neonatal BDV infection also induces cortical shrinkage. It has been shown that up to 30% of cortical neurons are lost in BDV-infected rats by PND 45. Similar to the hippocampus, diminished immunoreactivity for GAP-43 and synaptophysin is observed in the neocortex of neonatally BDV-infected rats (Gonzalez-Dunia *et al.* 2000).

Neonatally BDV-infected rats have very robust astrocytosis. Astrocytes, oligodendrocytes, ependymal cells and Schwann cells in the peripheral nervous system all express BDV markers (Bautista *et al.* 1995; Pletnikov *et al.* 2002). In the late stages of neonatal infection, BDV spreads centrifugally by anterograde axonal transport and infects most inner organs innervated by peripheral or autonomic nerves.

BDV-induced neuroanatomical damage is likely to underlie the behavioral abnormalities observed in BDV-infected rats. BDV-associated behavioral deficits are as follows; (i) selectively deficient social behaviors; (ii) changes in emotional behavior; (iii) selectively reduced cognitive abilities in spatial memory and learning/contextual fear conditioning; and (iv) spontaneous locomotor hyperactivity, hyper-reactivity and stereotypy along with mild gait ataxia (Dittrich *et al.* 1989; Hornig *et al.* 1999; Bauer *et al.* 2002). Neonatally BDV-infected rats show no evidence of gross ataxia and have normal swimming speeds despite the significant cerebellar lesions (Bautista *et al.* 1995).

Pletnikov *et al.* (Pletnikov *et al.* 1999a) showed that BDV-infected adult Lewis rats exhibited locomotor hyperactivity and elevated defecation in a highly aversive and brightly lit open field,

whereas uninfected control rats showed slightly decreased ambulation when compared in a less aversive, dimly lit open field. Moreover, compared to controls, neonatally BDV-infected rats exhibited attenuated habituation of the acoustic startle at PND 23 and decreased startle responsiveness at PND 30. Prepulse inhibition of the acoustic startle reflex remained unaltered in BDV-infected rats (Pletnikov *et al.* 2001).

Another behavioral task requiring the integrity of the limbic system, particularly the hippocampus, is contextual fear conditioning. Freezing behavior and defecation response can be used in rats for assessing the amount of contextual fear conditioning. BDV-infected rats demonstrated attenuated conditional freezing in the context previously paired with either sudden loud noise or foot shock compared to control rats (Pletnikov *et al.* 1999a).

Both the hippocampus and cerebellum play a major role in the acquisition phase of the spatial navigation task and lesioning of these areas impairs acquisition of a hidden platform location in the Morris water maze (MWM). In the MWM, neonatally BDV-infected rats exhibited a performance deficit. At PND 72, BDV-infected rats had difficulties in learning the location of the platform over a series of swim trials (Rubin *et al.* 1999).

Neonatal BDV infection induced abnormal social interaction and communication in Lewis rats when tested as old as 30–35 days of age. Studies were conducted using the resident/intruder paradigm. A resident rat was isolated for one week in order to increase social motivation. An unfamiliar rat (intruder) was placed in the resident's cage. This scenario is conducive to social interactions between the rats, often resulting in play behavior (measured as number of pins, similar to a pin observed in a wrestling match) (Pletnikov *et al.* 1999b). As the result, control rat pairs exhibited significantly more pins than the pairs where either one or both rats were BDV-infected rats. Similarly, play soliciting behaviors (e.g. pounce, crawl over/under and darting) were reduced in BDV-infected rats. Nonsocial exploratory activity (e.g. ambulation and rearing) was similar in BDV-infected and non-infected residents. Duration of non-play social investigation (e.g. sniffing, approach, and follow) was higher in BDV-infected rats as compared to non-infected controls.

However, there is little serological evidence that suggests BDV infects humans (Chalmers *et al.* 2005), and its role in psychiatric disorders remains controversial.

Chemical teratogenic model of autism

Thalidomide exposure: rats

Thalidomide (THAL) was used worldwide at the end of the 1950s and beginning of the 1960s for the treatment of anxiety and insomnia. Lenz carried out analysis of hypoplastic malformations of the limbs and reported a correlation between the intake of THAL during pregnancy and the observed birth defects (Lenz *et al.* 1962). In addition to limb defects, THAL may give rise to a wide spectrum of malformations of various organ systems. Anomalies noted are heart defects, laryngeal and tracheal abnormalities, anotia, microtia, and hearing impairment, choanal atresia, microphthalmia, cloboma, intestinal atresia, aplastic or hypoplastic gallbladder, renal anomalies, criptorchism, vaginal and anal atresia, as well as dysfunction of cranial nerves, notably the 6th and 7th nerve (Miller & Stromland 1999).

Recent epidemiological studies have revealed that THAL exposure during the first trimester in humans causes higher incidence of autism in the offspring. Exposure between the 20th and 24th day of gestation led to an incidence of autism of 5 out of 15 cases (Stromland *et al.* 1994; Miller *et al.* 2005). This critical period for exposure corresponds to the time of early development of the Central Nervous System (CNS), when the neural tube begins to form. On the basis of somite numbers in early embryos of rats and humans, E9-E11 in rats is considered to be from early somite stage corresponding to approximately E20-E24 in human embryos (Rice & Barone 2000). Models exposed to THAL showed a reduction of cell numbers in the cranial nerve motor nuclei, reductions in Purkinje cell number and cerebellar volume, and retarded migration of 5-HT neuron (Rodier *et al.* 1997; Narita *et al.* 2002). Narita *et al.* (Narita *et al.* 2002) reported that a significant increase of hippocampal serotonin concentration was observed in the group exposed to THAL on E9. E9 THAL exposure resulted in an increase of hippocampal serotonin and frontal cortex dopamine, as well as hyperserotonemia. These observations all parallel the reported human autistic pathologic findings (Rodier *et al.* 1997).

Although neurobehavioral investigations have been scarce, Vorhees *et al.* (Vorhees *et al.* 2001) have reported that male THAL exposed rat pups show significant increases in errors and latency in the multiple-T Cincinnati water maze. They also indicated that THAL exposure induced increased preweaning mortality and male specific, late onset reduction in growth in rat pups (Vorhees *et al.* 2001).

Valproic acid exposed rat model

While THAL may have a teratogenic effect in rodents that differs from that in primates (Schumacher *et al.* 1972), valproic acid (VPA) has a similar effect in rodents and humans (Kemper & Bauman 1993).The effect of VPA are observed if the rat brainstem is exposed to VPA *in utero* and the somatic effects are similar to those of THAL (Kemper & Bauman 1993). Offspring of female rats injected with VPA at the time of neural tube closure show brain abnormalities resembling those found in autistic patients (Christianson *et al.* 1994). There are several brainstem abnormalities found so far in rats exposed to VPA *in utero*; (i) diminished number of motor neurons in the oculomotor, trigeminal, abducens, and hypoglossus nuclei of cranial nerves; (ii) shortening of the region caudal to the facial nucleus and lengthening of the region rostral to the facial nucleus; (iii) smaller cerebellum with reduction of a number of Purkinje cells both in the hemispheres and vermis; and (iv) reduced cerebellar nucleus interpositus (Rodier *et al.* 1997; Ingram *et al.* 2000a)

Schneider *et al.* (Schneider *et al.* 2001) have suggested that rats exposed to VPA during gestation may resemble the abnormalities seen in autism both neurophysiologically and behaviorally. They have demonstrated that VPA exposed rat offspring exhibit (i) lower sensitivity to pain and higher sensitivity to non-painful stimuli; (ii) diminished acoustic prepulse inhibition; (iii) locomotor and repetitive/stereotypic-like hyperactivity combined with lower levels of exploratory activity; and (iv) decreased number of social behaviors and increased latency to social behaviors.

Neonatal amygdala lesioned rat

Results from neuroanatomical studies indicate that medial temporal lobe structures, especially amygdala, may be implicated in the pathogenesis of autism (Bachevailier 1996; Baron-Cohen *et al.* 2000). Some authors have noted similarities between autism and the Kluver–Bucy syndrome, a syndrome caused by bilateral lesions to the anterior temporal lobes in monkeys (Baron-Cohen *et al.* 2000). Monkeys with the Kluver–Bucy syndrome display features often seen in autistic subjects such as absence of social chattering, lack of facial expression and absence of emotional reactions. Other such similarities include repetitive abnormal movement patterns, increased aggression, and the tendency to examine objects by mouth or smell. Several post-mortem studies in autistic subjects

have demonstrated amygdale abnormalities with small neuronal size and increased cell-packing density (Bauman & Kemper 1985; Kemper & Bauman 1993; Bailey *et al.* 1998).

Experimental lesion studies in non-human primates provide further evidence for medial temporal lobe involvement in autism. Bilateral lesions to the medial temporal lobe in infant rhesus monkeys have resulted in long-term deficits in social behavior, an effect that is absent in monkeys receiving similar lesions in adulthood (Bachevailier 1996). Monkeys subjected to bilateral removal of the amygdala, hippocampus, and adjacent cortical areas were uninterested in and avoided social contacts. Those monkeys also developed autistic-like characteristics, such as unexpressive faces, very little eye contact, locomotor stereotypies, and self-directed activity (Prather *et al.* 2001; Bauman *et al.* 2004).

Neonatal ibotenic acid lesion of the amygdala in the rat has also been proposed as an animal model of autism. Excitotoxic lesions of the amygdala at PND 7, but not PND 21 in rat, produce multiple behavioral abnormalities persisting into adulthood, indicating neurodevelopmental deficits of structures connected to the amygdala (Daenen *et al.* 2002a). Lesioning the amygdala on PND 7 resulted in an adult animal with stereotypic-like increased ambulatory behaviors and decreased investigatory behaviors. Moreover, those animals exhibited increased locomotor reactivity to challenge with a low dose of apomorphine, reminiscent of supersensitivity of postsynaptic dopamine systems in the nucleus accumbens (Wolterink *et al.* 2001; Daenen *et al.* 2002a).

Other lesioned animals

There have been several reports suggesting that neonatally ventral hippocampus (VH) lesioned rats show many aspects of abnormalities in behavior and cellular formation reminiscent of schizophrenia. When tested as juveniles (PND 35), rats with the neonatal VH lesions are less social than controls (Sams-Dodd *et al.* 1997), but otherwise behave normally in motor tests involving exposure to stress and dopamine agonists. In adolescence and adulthood (PND 56 and older), lesioned animals display markedly changed behaviors such as motor hyperresponsiveness to stress and stimulants, and enhanced stereotypies. They also show deficits in PPI and latent inhibition, impaired social behaviors and working memory problems (Lipska & Weinberger 1993; Lipska & Weinberger 1994; Lipska *et al.* 1995).

However, other reports found that rats lesioned in the VH on PND 7 or PND 21, showed no differences in social behavior related or unrelated to social play behavior early in life or in adulthood (Wolterink *et al.* 2001; Daenen *et al.* 2002b). In monkeys, emotional behavior was not disturbed with damage in the hippocampal area only (Bauman *et al.* 2004). Wood *et al.* (Wood *et al.* 1997) suggested that the pattern of impairments associated with the excitotoxic VH lesion varies depending on the age at which lesioning occurs. Consequently, VH lesioned rats are still considered to be controversial as a model of autism.

Early prefrontocortical damage in humans has been shown to impair cooperative and reciprocal behavior, social interactions, and social cognition (Eslinger *et al.* 2004). It is suggested that dysfunctions and morphological abnormalities of the prefrontal cortex (PFC) are implicated in the pathophysiology of autism (Baron-Cohen *et al.* 1999). Neonatal PFC lesions have also been proposed as an adequate model to investigate early developmental aberrations (Schneider & Koch 2004). The total amount of self-grooming and social behaviors was reduced in PFC lesioned animals compared to controls. Neonatal PFC lesions reduced pinning in juvenile rats and lesioned rats showed an increase in the total number of so called 'partial rotations'. Partial rotation is an adult-like pattern of defense, so investigators suggested that neonatal lesions of PFC lead to a behavioral shift of social play in juvenile rats to an adult-like pattern of defense (Schneider & Koch 2004).

There is growing evidence that the cerebellum is implicated in autism. Recently, many studies have demonstrated that the cerebellum is involved not only in the regulation of motor skills, but also in more complex integrated functions, such as classical conditioning, learning of motor skills, spatial learning, habituation of exploratory behavior and the acoustic startle response (McCormick & Thompson 1984; Leaton & Supple 1986; Leaton & Supple 1991; Dahhaoui *et al.* 1992a,b; Molinari *et al.* 1997). The cerebellum is further implicated in motivations and emotional behavior as well (Heath *et al.* 1980; Caston *et al.* 1998). Adult rats with midline lesions of the cerebellum performed at PND 10 exhibited the hyperactivity in the open field test as well as overt disinhibition tendencies in the anxiety and social discrimination tests (Bobee *et al.* 2000). These results indicate the involvement of the cerebellar vermis in the pathology of autism, considering a number of autistic subjects have a hypoplasia of cerebellar vermal lobules.

Genetic model

Recently, overwhelming evidence of genetic underpinnings of autism has generated much research. As this field is rapidly developing, many candidate loci for autism have been published in recent years. Spontaneous mutants or transgenic animal models can greatly help to delineate the role of these candidate genes.

The nonapeptide oxytocin (OT) is synthesized in the hypothalamus and released into the blood stream via axon terminals in the posterior pituitary or neurophypophysis. OT receptors are concentrated in several brain regions involved in social behavior in the mouse, including the olfactory bulbs, piriform cortex, amygdala and lateral septum. OT facilitates the formation of the mother-infant bond in sheep and stimulates nurturing behaviors in rodent females toward pups. In male rats, chronic OT treatment doubles the time spent in social contact. OT knockout mice (OTKO) fail to remember recently encountered individuals despite apparently normal olfactory and general cognitive abilities (Young 2001; Winslow & Insel 2002). Central injections of OT prior to the first encounter, but not after, completely rescue this very specific deficit and infusions of an OT antagonist inhibit social recognition in normal wild-type (WT) mice (Ferguson *et al.* 2000). Both WT and OTKO mice showed a similar neuronal activation in the initial encounter, as evidenced by the comparable c-Fos immunoreactivity in olfactory bulbs, piriform cortex, cortical amygdala, and the lateral septum. However, WT mice, but not OTKO mice, exhibited an induction of c-Fos in the medial amygdala, whereas OTKO, but not WT mice, showed dramatic increases in c-Fos in the somatosensory cortex and the hippocampus (Ferguson *et al.* 2000). These findings have an interesting parallel with recent neuroimaging studies in autistic human patients, suggesting that people with autism utilize alternative cortical areas to process social cues, areas that are typically activated by non-social cues in normal subjects (Schultz *et al.* 2000).

Recent genetic reports implicate a number of genes in the causation of autism and the Reelin gene (*RELN*) is one such gene (Fatemi *et al.* 2001). Persico *et al.* (Persico *et al.* 2001; Zhang *et al.* 2002) reported that individuals inheriting alleles of the Reelin gene that contain 11 CGG repeats in the 5′-UTR of the *RELN* mRNA have an increased risk of autism. Another group has reported that autistic patients and their first-degree relatives show significantly reduced plasma levels of full-length Reelin and its low molecular weight isoforms (Fatemi *et al.* 2001). The reeler mutation is a spontaneous recessive mutation in mice that leads, in the homozygous

state, to the absence of Reelin and to severe disorganization of cortical, hippocampal, and cerebellar development. In comparison to WT mice, heterozygous reeler mice (rl/+) displaying Reelin levels reduced by 50% do not show gross developmental abnormalities of the CNS, but do show a progressive loss of Purkinje cells in the cerebellum during the first postnatal weeks (Tueting *et al.* 1999). The loss of Purkinje cells is seen only in male rl/+, and not in female rl/+mice (Hadj-Sahraoui *et al.* 1996).

Another interesting example of genetically altered mouse models presenting autistic-like features is mice deficient for Dishevelled-1 (Dvl1) proteins. Dvl1 is one of three mouse homologs of the Drosophilla segment polarity gene Dishevelled. Mice deficient in Dvl1 were reported to exhibit abnormal social interaction as well as deficits in sensorimotor gating, as measured by impaired prepulse inhibition (PPI) (Lijam *et al.* 1997). These mice have been noted as a potent model for autism or schizophrenia, but the deficits in social memory task and PPI were not replicated in Dvl1-null mice in a later study (Long *et al.* 2004).

NEONATAL HYPOTHYROIDISM RATS

Thyroid hormone is essential for brain development and maintenance of basal metabolic rates. The manipulation of thyroid hormone in laboratory animals typically increases activity levels and decreases performance during motivated learning tasks. It is well-known that hypothyroidism during the critical period of brain development induces irreversible dysfunction of the central nervous system. The timing of thyroid hormone manipulation plays a critical role in the degree to which developmental sequelae are expressed. The anatomical bases of behavioral and intellectual deficits may result from global reductions in brain size, premature termination of neuronal proliferation, non-migrated granule cells in the cerebellar cortex and caudate nucleus, decreased synaptic junctions in cerebellar cortex and malformed dendrites on Purkinje cells (Lewis *et al.* 1976). Humans with primary or secondary congenital hypothyroidism demonstrate deficits in academic skills as children, and as adults, decreased performance on neuropsychological tests and prolongation of latencies for visual- and auditory-evoked potentials (Murphy & Nagy 1976; Osterweil *et al.* 1992).

Lactating rats receiving 0.02% propylthiouracil (PTU) in their drinking water transfer the goitrogenic effect to the offspring through their milk. This treatment induces a temporary mild hypothyroid condition of the pups (Van Middlesworth & Norris 1980). We conducted experiments to investigate the effects of temporary neonatal PTU-induced hypothyroidism on the behavior of rats. Rat pups were treated with 0.02% PTU in drinking water to dams from day 0–19 post partum (Kato *et al.* 1982). The serum T4 level was depressed below the limit of detection at 2 weeks of age, but recovered to the normal level at 4 weeks of age (Akaike *et al.* 1991). The open field test was conducted at 3, 6, and 9 weeks of age. At 3 weeks of age, the number of ambulations did not differ between PTU rats and controls. At 6 and 9 weeks of age, the number of ambulations of the PTU rats was significantly greater than that of the control rats. Kato *et al.* reported extensive hyperactivity (Akaike *et al.* 1991; Akaike & Kato 1997) and attenuated habituation in the open field test in PTU rats after maturation, as shown in Figure 1 (Kato *et al.* 1992).

Spatial learning ability was further investigated in the PTU rats. Biel water maze tests at the age of 6 weeks showed an increase in errors with prolonged swimming time in the PTU rats. The radial arm maze test was performed to evaluate spatial maze learning. The test started at 13 weeks and revealed that the PTU animals required more trials until they showed the first well-performed trial. The PTU rats showed more active moving from arm to arm compared to controls. However, while the number of total choices of PTU rats was increased the number of correct choices was smaller than the control values (Akaike *et al.* 1991; Akaike & Kato 1997). The performance of PTU rats was further assessed by the modified T-maze test and then the mirror image of the first trial (Fig. 2a). The performance of PTU rats was superior to that of the controls in the initial maze test, but it was clearly inferior to that of the controls in terms of a higher error frequency and a longer running time upon reversal of the route to the mirror image of the original (Fig. 2b). This was interpreted as inability to adapt to changes in the environment and a reference for the highly repetitive and routine response pattern initially acquired.

As stated earlier, the most apparent and consistent neuropathology in autistic patients lies in the cerebellum. In this regard, it might be of significance that PTU rats have retarded granular cell migration in the external granular layer (Sadamatu & Watanabe 2005). Furthermore, PTU rats exhibited a marked susceptibility to audiogenic seizures, starting from the age 7 weeks and persisting into adulthood (Yasuda *et al.* 2000).

These results suggest that mild hypothyroidism around the critical period causes permanent impairment of brain function, as manifested by hyperactivity, lack of habituation, spatial learning impairment and auditory hypersensitivity. It is thus expected that PTU-treated rats may serve a useful model for autism.

DISCUSSION

The extremely high concordance rate of autism in monozygotic twins, as compared in dizygotic twins, clearly indicates the important role of genetic factors. The published genome screens have found convergent evidence for linkage in several genomic regions, with regions on chromosome 2, 7, 15, 16 (IMGSAC 2001a). In particular a region on chromosome 7q showed increased allele sharing in all screens (Risch *et al.* 1999; IMGSAC 2001a,b; Bartlett *et al.* 2005). *RELN* (Persico *et al.* 2001) and *HOXA1* (Ingram *et al.* 2000b), both on chromosome 7q22, are the most prospective candidate genes. In human subjects, one report showed that blood levels of Reelin were reduced (Fatemi *et al.* 2002). Although the case-control and affected sib-pair findings fail to support a role for *RELN* in susceptibility to Autism Spectrum Disorder (ASD), the more powerful family–based association study demonstrates that *RELN* alleles with larger numbers of CGG repeats may play a role in the etiology of some cases of ASD, especially in children without delayed phrase speech (Zhang *et al.* 2002; Bonora *et al.* 2003). Recent studies have reported conflicting findings of an association between a variant of the *HOXA1* gene and autism (Ingram *et al.* 2000b; Conciatori *et al.* 2004; Gallagher *et al.* 2004). Thus, so far a single gene responsible for the pathogenesis of autism has not been found and, it seems unlikely that any single gene can explain the whole picture of autism.

Genes have two broad roles, the first being the template function and the second the transcriptional function. Although the template function is largely independent of outside forces, the transcriptional function is highly regulated and responsive to environmental factors.

The question of whether or not the actual number of autistic patients has increased is also a matter of debate. Honda *et al.* (Honda *et al.* 2005) first reported that childhood autism was more frequent in Japan than previously estimated. Cumulative incidence of childhood autism up to 5 years in the birth cohort in the Yokohama increased up to 27.2 per 10 000 in 1991 in the strictest sense, whereas it was 16.2 in 1988. If indeed, the prevalence of autism is

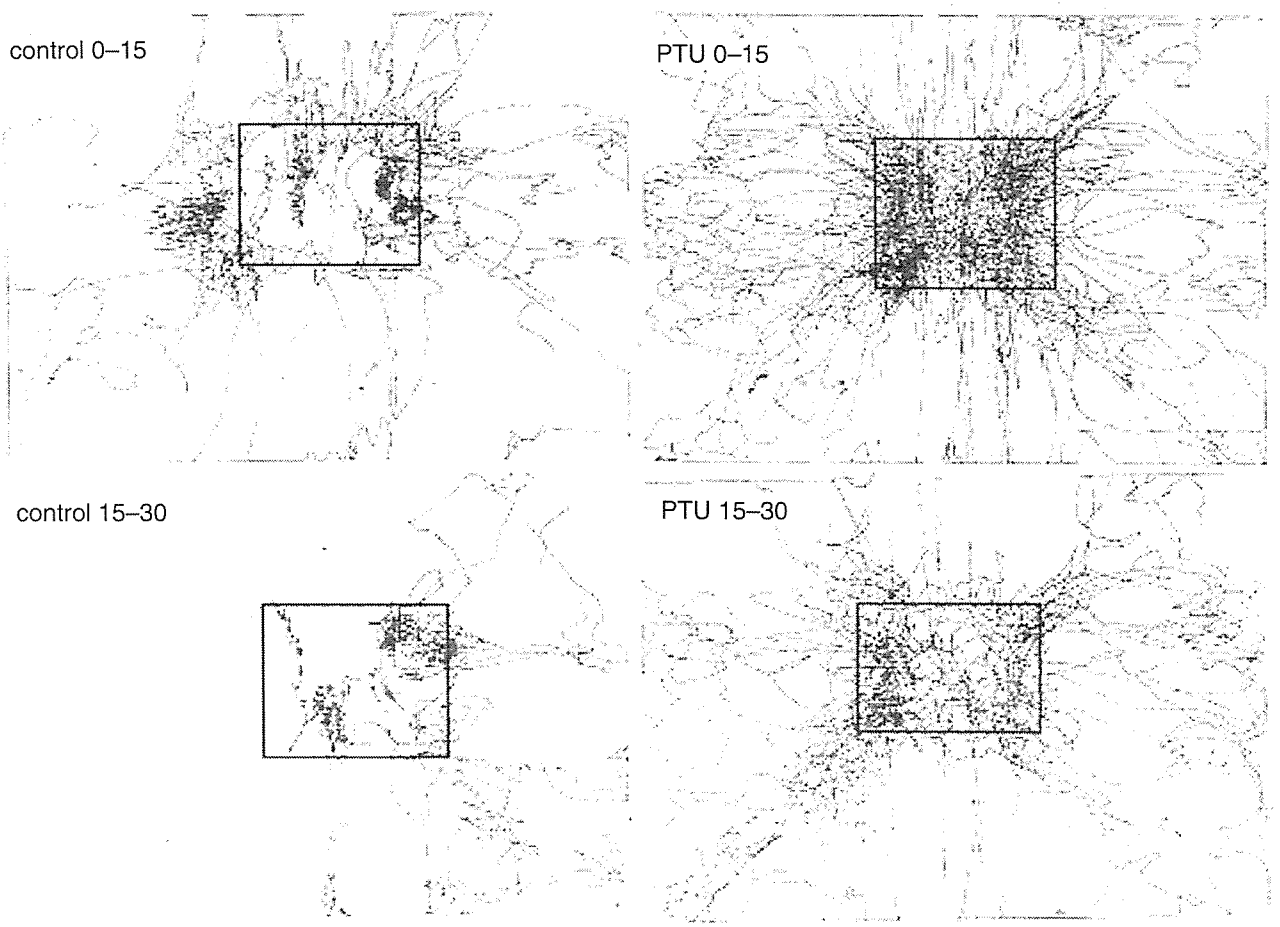

Fig. 1 Comparison of spontaneous movement in a PTU rat (right) and its littermate control (left) as detected by a multidimensional behavioral analyzer (Animex) at the age of 10 weeks. The device recorded the linear locomotion of the animal for two consecutive 15-min periods. The rectangle in the center of each figure indicates the base of the cage and traces outside the rectangle indicate rearing (Kato *et al.* 1992).

growing recently in some urban areas like Yokohama regardless of the rate, it seems plausible that environmental factors might contribute to the incidence of autism. In view of this, autism–like syndrome(s) due to environmental factors may not necessarily be the same as classical autism with mental retardation.

Results from postmortem and imaging studies have implicated many major structures of the brain including the limbic system, cerebellum, corpus callosum, basal ganglia and brainstem. However, is any single brain region able to explain such a broad spectrum dysfunction of ASD? Baron-Cohen *et al.* (Baron-Cohen *et al.* 2000) proposed the amygdala as an area responsible for the impairment of social behavior in autism, but recent data on the effects of amygdala lesions in macaque monkeys did not support their hypothesis (Amaral *et al.* 2003). Alternatively, a different hypothesis as to the brain region responsible for ASD may be derived from recent neuroimaging studies with human patients with the disorder. When viewing images of faces, autistic subjects, compared with unaffected subjects, exhibit a decreased activation of both the amygdala and cortical 'face' areas, and interestingly, also show an increase in other cortical regions typically activated while viewing non-social objects (Critchley *et al.* 2000). Autistic patients may have genetic and/or environmental impairments in some specific brain areas, which, in turn, activate a different set of brain structures during social recognition (Ferguson *et al.* 2001).

Most of the neuroanatomical features highlighted by recent studies of autistic subjects indicate the aberration of very early fetal development, such as shortening and elongation of the brainstem, increased cell packing in the cerebral cortex and preceding enlargement of brain volume. This may imply the significance of a critical period when some genetic and/or environmental factors work in the fetus. The period determines the extent of organs involved, and each organ has its own period for maturation. Some environmental factors such as VPA or THAL disrupt specific points of cell proliferation and differentiation, and some factors such as thyroxine affect the maturation of some sets of organs in the CNS.

Neonatal mild hypothyroidism may provide a useful model for autism. The importance of thyroid hormone in brain development has been extensively documented. Recent studies further demonstrate that relatively subtle changes in circulating levels of thyroid hormone in pregnant women can affect the neurological outcome of their children (Morreale De Escobar *et al.* 2004; Pop & Vulsma 2005). One candidate that affects thyroid function is endocrine disruptors. We currently focus on bisphenol-A, one of the endocrine disruptors known to alter thyroid function (Moriyama *et al.* 2002).

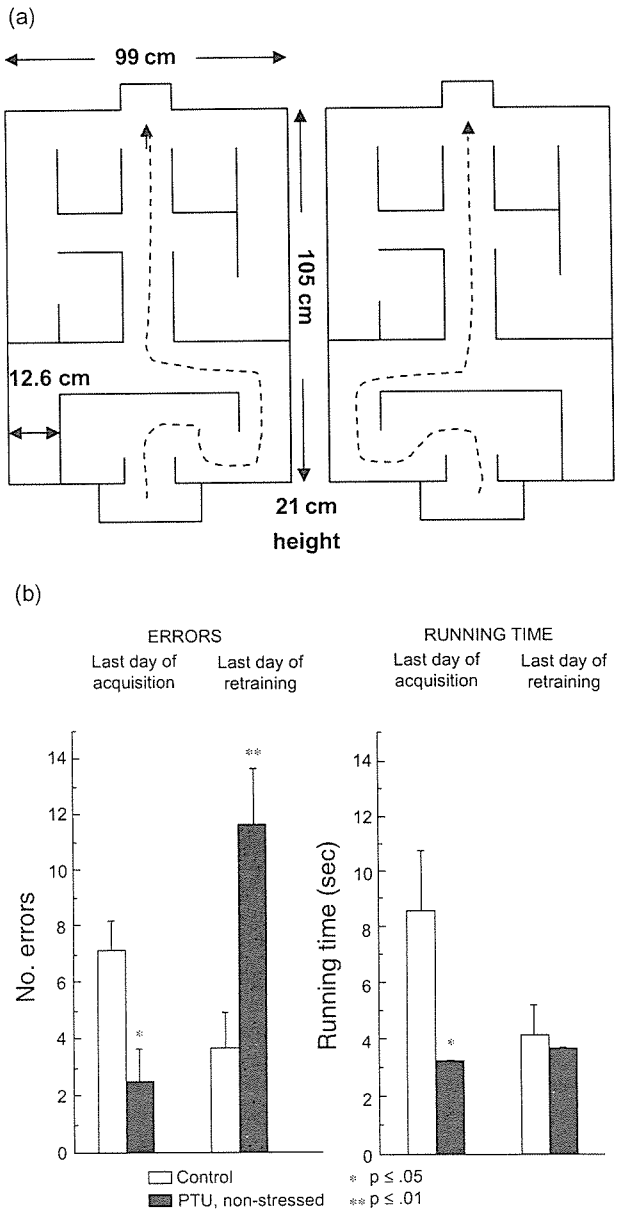

Fig. 2 (a) Diagram of T-maze for study of learning ability in rats. Rats were placed on a 23 h food deprivation schedule and trained to run the maze for food reinforcement once daily in 10 trial sessions. Route 'a' was employed for the learning phase of the study and route 'b' (the mirror image of 'a') for the relearning phase. (b) Maze-learning ability of PTU versus control rats. Average number of errors and running time were evaluated on the last day of task acquisition in the 'a' maze and the last day of retraining in the 'b' maze. Both error frequency and total running time were reduced in the PTU rats on the last day of task acquisition. In contrast, error frequency was significantly increased in the PTU rats on the last day of retraining. (Akaike *et al.* 1991; Akaike & Kato 1997)

Our preliminary data indicates that the administration of bisphenol-A at the environmental dose during the early postnatal period induces hyperactivity and learning impairment in male, but not female, rats after maturation (unpublished data).

Our understanding of the neuropathology of autism has advanced substantially over the past 20 years, but there are still so many questions that remain unsolved. Each of these models mentioned above seems to capture at least one of the pieces of the autism puzzle. It is hoped further studies will elucidate the whole picture of the neuropathology of autism.

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Tachykinin 1 (TAC1) gene SNPs and haplotypes with autism: A case-control study

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Abstract

Autism (MIM 209850) is a severe neurodevelopmental disorder characterized by disturbances in social interaction and communication, by repetitive body movements and restricted interests, and by atypical language development. Several twin and family studies have shown strong evidence for genetic factors in the etiology of autism. Glutamate is a major excitatory neurotransmitter in the human brain. Glutamate systems are involved in the pathophysiology of autism. There are many similarities between the symptoms evoked by glutamate antagonist treatment and symptoms of autism found in several human and animal studies. To elucidate the genetic background of autism, we analyzed the relationship between three single nucleotide polymorphisms (SNPs) of the Tachykinin 1 gene (TAC1) and autism, because TAC1 is located in the candidate region for autism and produces substance P and neurokinins. These products modulate glutamatergic excitatory synaptic transmission and are also involved in inflammation. Many different inflammation-related mechanisms could be involved in the autistic brain. Therefore, TAC1 may have some functions associated with the presumable pathophysiology of autism. We compared the allele and haplotype frequencies between autistic patients ($n = 170$) and normal controls ($n = 214$) in the Japanese population, but no significant difference was observed. Thus, the TAC1 locus is not likely to play a major role in the development of autism.

Keywords: Autistic disorder; Chromosome 7; TAC1; Genetic association; Glutamate system; Haplotype block

1. Introduction

Autism is a neurodevelopmental disorder characterized by early onset of the three cardinal symptoms: disturbance of social interaction, atypical language

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development, and restricted, repetitive, stereotyped patterns of behavior and interests [1]. The genetic involvement of autism has been supported by its higher concordance rates for monozygotic twins than for dizygotic [2,3] and the several chromosomal loci possibly linked to autism, including 7q22-q31 [1].

Glutamate is one of the neurochemicals speculated to contribute to the pathogenesis of autism, because (1) there are many similarities between the symptoms evoked by glutamate antagonist treatment and the symptoms of autism found in several human and animal studies [6]; (2) several neuropathological and brain-imaging studies of autistic patients have shown involvement of the cerebral regions where glutamatergic neurons originate [6]; (3) the mRNA levels of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor increases in autistic subjects [7]; (4) the aspartate/glutamate carrier SLC25A12 gene and metabotropic glutamate receptor 8 gene have been reported to be associated with autism [8,9].

In this study, we focused on the Tachykinin 1 gene (TAC1; MIM162320) because it is located in 7q21-q22 and encodes a precursor containing substance P and other neurokinins (Neurokinin A, Neurokinin K, and Neuropeptide γ) [10], some of which are implicated in the modulation of glutamate-driven neurotransmission and excitotoxicity in the basal forebrain and other CNS regions [11]. Moreover, TAC1 mutant mice are less sensitive to nociceptive stimulation, which reminds us of some autistic patients ignorant of pain [6,13]. An increase in proinflammatory cytokines and the activation of microglia and astrocytes in the brain of autistic patients may be also associated with the inflammatory responses of TAC1 products [14].

Based on TAC1 biological information and its chromosomal location, we considered the gene worth analyzing as one of the autism candidate genes. In the present study, we tested for the presence of an association of three single nucleotide polymorphisms (SNPs) of TAC1, and haplotypes consisting of the SNPs, with autism, using case-control design.

2. Subjects and methods

The patients comprised 170 unrelated Japanese with autism (147 males and 23 females, mean age = 20.8 years within a range of 3–41 years). The patients were recruited from the outpatient clinics of the departments of psychiatry, Tokyo University Hospital and Tokai University Hospital, and seven daycare facilities for subjects with developmental disorders. All the hospitals and facilities were located around Tokyo. All the subjects met the DSM-IV criteria for autistic disorder. The diagnoses were made by one or two experienced child psychiatrists through interviews and reviews of clinical records. Apparent physical anomalies were not observed in the subjects. The controls consisted of 214 unrelated Japanese healthy volunteers (145 males and 69 females, mean age = 34.6 years within a range of 21–65 years). They were mainly recruited from the hospital and facility staff, and all of them resided in the same area (Kanto District or around Tokyo) as the patients. All the patients and controls were ethnically Japanese, with no parents or grandparents of ethnicity other than Japanese.

Confirmation of the diagnosis was conducted as follows. Semi-structured behavior-observation of the patients and interviews with them and their parents were conducted for most of the cases by two experienced child psychiatrists independently. At the interview with the parent(s), the Child Behavior Questionnaire Revised (CBQ-R) [12] was used to assist evaluation of autism-specific behavior and symptoms. After the initial observation and interview, we followed up by examining the patients' behavior and symptoms for several months (for at least 6 months in most of the cases) and those who were not considered to meet the DSM-IV criteria during the follow-up were excluded from the sample.

The present study was approved by the Ethical Committee, the Faculty of Medicine of the University of Tokyo and Tokai University. The objective of the present study was clearly expressed and written informed consent was obtained from all subjects and healthy volunteers.

Peripheral blood was obtained and genomic DNA was extracted using the standard phenol–chloroform method. Single nucleotide polymorphisms (SNPs) were

Table 1
Allele frequencies of 3 SNPs of the TAC1 gene in autism patients and controls

Locus	db SNP ID	Allele A/B[a]	Minor allele frequencies				χ^2	*P*-value	Odds ratio	95% confidence intervals		Location
			Patients		Controls					Lower	Upper	
			N	%	*N*	%						
SNP 1	rs2072100	C/T	107	31.8	136	32.2	0.01	0.91	1.02	0.75	1.38	int01
SNP 2	rs1229434	C/T	149	44.3	199	47.2	0.60	0.44	1.12	0.84	1.49	int06
SNP 3	rs1397202	A/G	68	20.1	82	19.2	0.09	0.76	0.95	0.66	1.35	int06

[a] Alleles "B" are minor alleles.

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analyzed using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Three SNPs of the TAC1 gene were selected from the list of Assays-on-Demand™ Products for the ABI 7900HT to cover the full length of the gene, considering minor allele frequencies indicated in the products list. The db SNP IDs of the SNPs are shown in Table 1.

Statistical analyses were performed using the SAS/Genetics 9.1 software (SAS Institute Inc., Cary, North Carolina, USA). The frequencies of alleles and genotypes of each SNP were compared between patients and controls using the χ^2 test. To further analyze the haplotype structure in our sample, we computed the pair-wise D' values that indicated the linkage disequilibrium (LD) between the SNPs. The frequencies of haplotypes consisting of SNPs, which were at a high linkage disequilibrium (haplotype block), were estimated. The exact P-values based on the likelihood ratio test with 10,000 permutations were calculated for comparison of the haplotype frequencies between patients and controls.

3. Results

The allele frequencies of the SNPs of the TAC1 gene are summarized in Table 1. No significant difference was observed in genotypic distributions (not shown in the tables) or allele frequencies of the three markers of the TAC1 gene between patients and controls. The minor allele frequencies of the SNPs were higher than 19% in all three SNPs. For all assayed SNPs, none of the SNPs deviated from Hardy–Weinberg equilibrium.

The pair-wise D' and r^2 values of the SNPs within the TAC1 gene are summarized in Table 2. Considering r^2, these three SNPs within the TAC1 gene were not in complete linkage disequilibrium with each other. However, all the pair-wise D' values of each SNP indicated 1.00, forming a haplotype block. Regarding the pair-wise D' values, therefore, three marker haplotypes of SNP1-3 (rs2072100, rs1229434, rs1397202), were tested.

The estimated haplotype frequencies are shown in Table 3. No significant difference was observed in the estimated haplotype distributions between patients and controls.

Table 2
The strength of LD (denoted as D' and r2) between pairs of SNPs of TAC1 in autism patients (the lower diagonal) and controls (the upper diagonal)

SNP	1	2	3
D'			
1		1.00	1.00
2	1.00		1.00
3	1.00	1.00	
r_2			
1		0.53	0.11
2	0.60		0.22
3	0.12	0.20	

Table 3
Estimated frequencies and permutation P-values for association of major TAC1 haplotypes with rs2072100-rs1229434-rs1397202

Haplotype	Frequencies		χ^2	P-value
	Case	Control		
C-C-A	0.36	0.33	0.42	0.53
T-T-A	0.32	0.32	0.00	0.95
C-C-G	0.20	0.19	0.09	0.78
C-T-A	0.12	0.15	1.33	0.25

Global $P = 0.6958$

Haplotype frequencies were estimated >1%.

4. Discussion

We genotyped three SNPs within the TAC1 gene. No association was found in the allele and haplotype distributions between autistic patients and normal controls.

The 95% confidence intervals of the odds ratios were within 0.66 and 1.49 in all three SNPs of the gene. Considering the sample size and minor allele frequencies in the present study, our results might have adequate statistical power (>0.8) to contradict the effects of the gene with odds ratios of approximately 1.7 or more.

The TAC1 gene is located on 7q21.3 within the candidate region for autism and has 7 exons spanning approximately 8.4 kb of genomic DNA [13]. Neurokinin A, neuropeptide K, and neuropeptide γ, as well as Substance P, are produced from the TAC1 gene as a result of differential splicing and posttranslational processing [14] (Fig. 1). Substance P and other neurokinins are known as a mediators of pain and inflammation. They are produced in nociceptive primary sensory neurons and in many brain regions involved in pain signaling. Pain behavior evoked by thermal, mechanical, and chemical stimulation of somatic and visceral tissues were all reduced in the TAC1 mutant mice [8].

Besides pain and inflammation, the concentration of Substance P was elevated in the cerebrospinal fluid of depressed patients, and in the brain of a rat model of depression. Limbic structures contain a high density of Substance P terminals and Neurokinin 1 receptor sites. The tachykinin system therefore may play an important role in the regulation of emotional states and the development of anxiety disorders and depression [10].

In the Rett syndrome included in pervasive developmental disorders (PDD) with autistic symptoms, reduced expression of the TAC1 gene products was reported [15,16]. This fact may suggest that the TAC1 gene might not be implicated in the whole spectrum of PDD.

Nontheless, several facts, including decreased pain sensitivity, brain inflammation, and association of the

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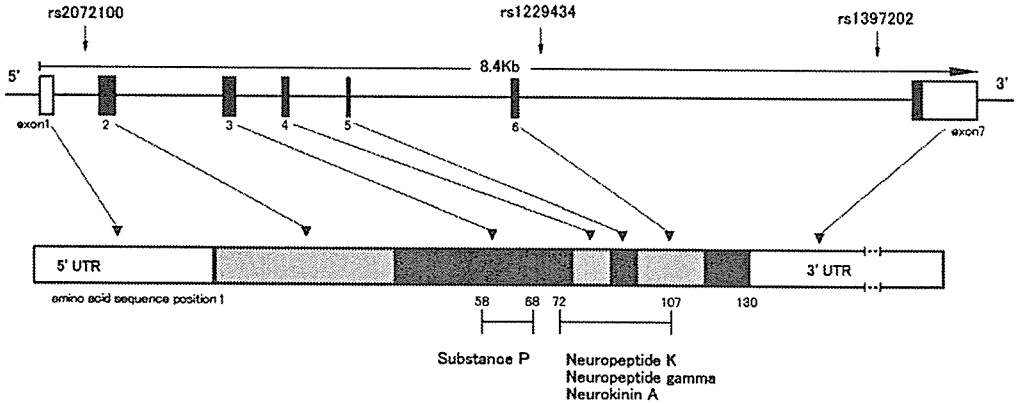

Fig. 1. This figure shows the TAC1 gene structure and positions of the SNPs (presented from 5′ to 3′ of the gene). All SNPs are denoted numerically with reference to Table 1. The vertical black bars indicate exons. Open boxes indicate the 5′ and 3′ untranslated regions. A horizontal line between the exons depicts introns. The TAC1 gene product is also indicated. Neurokinins are produced as a result of differential splicing and posttranslational processing of the product.

TAC1 gene with the glutamate system, suggested that Substance P or other neurokinins, which are products of the TAC1 gene, might contribute to the etiology of a part of autism. Although our data denied the association of the TAC1 gene with autism, these facts possibly support the necessity for this study.

The controls in this study were not age-matched to the patients. However, this is not likely to affect the result, considering the strong effect of genetic factors in autism compared with the rather small effect of environmental factors [2,17].

Another concern may be the population stratification of the sample, which could affect the results of studies in case-control design. This may not, however, significantly affect the present result, because the Japanese population is highly homogeneous, due to no major immigration for more than a thousand years, compared with the European or North American population. No subjects in this study had parents or grandparents of ethnicity other than Japanese.

In conclusion, we analyzed three SNPs in the TAC1 gene to determine whether there exists an association of TAC1 with autism using case-control design. To our knowledge, this is the first study that has examined the role of TAC1 polymorphisms in autism. No association was observed in this study.

5. Uncited references

[4,5].

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No association between the Neuronal Pentraxin II gene polymorphism and autism.

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