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

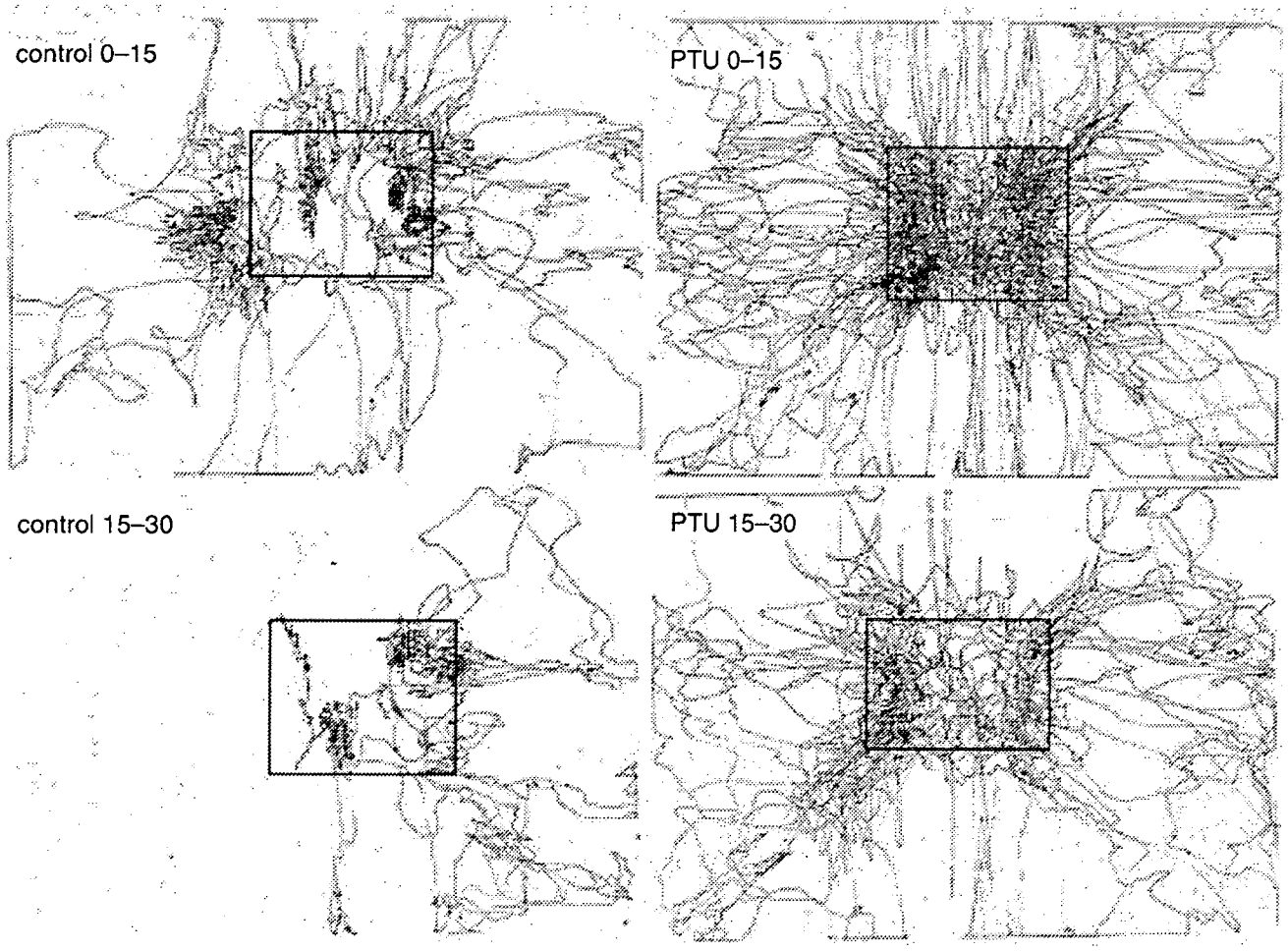

Fig. 1 Comparison of spontaneous movement in a PTU rat (right) and its littermate control (left) as detected by a multidimensional behavioral analyzer (Animex) at the age of 10 weeks. The device recorded the linear locomotion of the animal for two consecutive 15-min periods. The rectangle in the center 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).

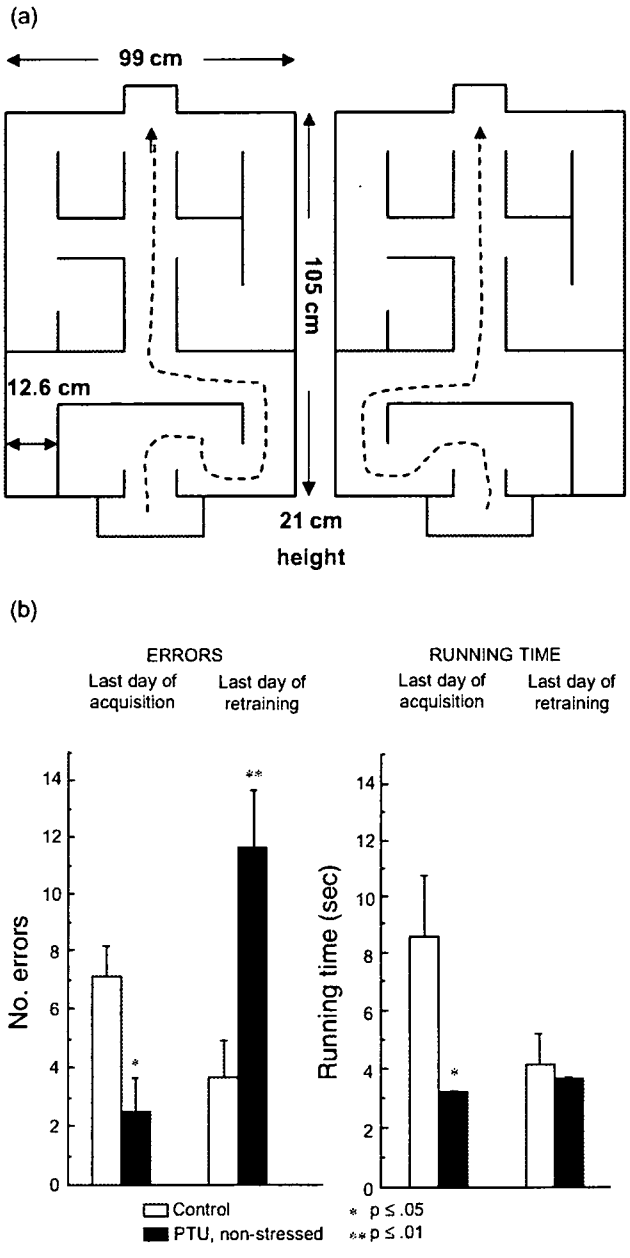

Fig. 2 (a) Diagram of T-maze for study of learning ability in rats. Rats were placed on a 23 h food deprivation schedule and trained to run the maze for food reinforcement once daily in 10 trial sessions. Route 'a' was employed for the learning phase of the study and route 'b' (the mirror image of 'a') 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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ARTICLE

Maternal separation stress drastically decreases expression of transthyretin in the brains of adult rat offspring

Kazuhisa Kohda[1], Seiichiro Jinde[1], Kazuya Iwamoto[2], Miki Bundo[2], Nobumasa Kato[1] and Tadafumi Kato[2]

[1] *Department of Neuropsychiatry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[2] *Laboratory of Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Wako City, Saitama 351-0198, Japan*

Abstract

Adversity in early life has been recognized as a risk factor for psychiatric disorders. In experimental animals, maternal separation (MS) during the neonatal period has been shown to be critical for susceptibility to stress in adult offspring. In this study, we used DNA microarray analysis of rat hippocampal samples to investigate differential gene expression caused by 8-hour MS (MS-8h) every other day during the neonatal period. We found 15 up-regulated and 9 down-regulated genes. We added samples from a daily 15-minute MS (MS-15m) group and performed quantitative real-time PCR to validate the results. Expression of transthyretin (TTR), which is specifically expressed in the choroid plexus (CP), was drastically reduced in the MS-8h group. Two other CP-enriched genes, angiotensin I converting enzyme I and insulin-like growth factor II (IGF-II), were also significantly down-regulated in the MS-8h rats, while significant reduction of IGF-II expression was also found in the MS-15m group. These MS-induced differential gene expressions could be involved in the molecular mechanisms of stress susceptibility. Our findings indicate that the CP, in addition to the neuronal and glial system, might play an important role in determining stress susceptibility.

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Key words: Choroid plexus, DNA microarray, hippocampus, maternal separation, transthyretin.

Introduction

Adversity in early life has been recognized as a critical factor determining susceptibility to psychiatric disorders as well as physical illnesses in humans. Maltreatment, such as abuse and neglect, increases the risk for depression and anxiety disorders (Bifulco et al., 1991; Brown et al., 1999; Holmes and Robins, 1987, 1988). In experimental animals, consequences of stress exposure in the early postnatal period have been extensively investigated, commonly using methods of maternal separation (MS) or neonatal handling (Meaney, 2001). MS is thought to increase an animal's susceptibility to stress. In previous studies, MS rats have shown enhanced hypothalamo–pituitary–adrenal (HPA) responses to stress in adulthood, probably due to increased expression of corticotropin-releasing factor (CRF) in the hypothalamus and decreased expression of glucocorticoid receptors (GR) in the hippocampus (Francis et al., 2002; Ladd et al., 2004; Liu et al., 2000; Patchev et al., 1997; Plotsky and Meaney, 1993), both of which should result in attenuated feedback in the HPA system. These rats reportedly showed increased fearfulness and cognitive deficits (Caldji et al., 2000; Patchev et al., 1997). On the other hand, postnatal handling, which involves brief mother–pup separation, induced precisely the opposite effects: enhanced inhibition of the HPA response, increased GR in the hippocampus, and reduced CRF in the hypothalamus (Meaney, 2001). These findings suggest that aversive rearing environments in the early neonatal period, especially maternal maltreatment, contribute to stress susceptibility through plastic changes of the pup brain that persists throughout the lifespan (Francis et al., 1999; Liu et al., 1997).

The hippocampus is known to control the feedback loop of the HPA axis and to be vulnerable to stress (McEwen, 1999). Therefore, in this study, our

Address for correspondence: Dr K. Kohda, Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
Tel.: +81-3-5363-3749 *Fax*: +81-3-3359-0437
E-mail: kkohda@sc.itc.keio.ac.jp

aim was to comprehensively search for genes differentially expressed in the hippocampus that might generate stress susceptibility, utilizing DNA microarray in MS rats. Among several differentially expressed genes, we found a drastic decrease in expression of transthyretin (TTR) in the 8-hour MS (MS-8h) rats; this is a gene that is specifically expressed in the choroid plexus (CP) in the brain. Angiotensin I converting enzyme I (ACE) and insulin-like growth factor II (IGF-II), also enriched in the CP, were significantly down-regulated. These findings indicate that the CP, in addition to the neuronal and glial system, might play a role in determining stress susceptibility.

Materials and methods

Animals and maternal separation

Fisher 344 rats were purchased from SLC (Hamamatsu, Japan) and bred in our facility under controlled illumination (12 h/12 h, lights on at 08:00 hours) and ambient temperature (22–23 °C). Each pregnant female rat was housed individually with free access to food and water. New-born litters were culled to eight pups on postnatal day 1 (PD1). Between PD2 and PD10, all the MS-8h pups were separated from their dams for 8 h (11:00 to 19:00 hours) every other day (Patchev et al., 1997). As for the 15-minute MS (MS-15m) group, these pups were removed daily from their mother for 15 min between PD1 and PD14. The pups were placed individually in a plastic container during separation. The control pups remained in their home cage without any manipulation. All rats were weaned in postnatal week (PW) 3 and housed individually in PW7. Body weight (BW) of the pups was measured on PD8, 14, 28, 42, 56 and 70. Although both male and female pups were involved in the MS protocol, only male rats were subjected to the following experimental procedures.

DNA microarray analysis

In PW13, the male rats were sacrificed. Hippocampal slices of 500 μm thickness were made in a choline solution containing (in mM) 124 choline-Cl, 3 KCl, 2 $CaCl_2$, 4 $MgSO_4$, 1.25 NaH_2PO_4, and 10 D-glucose at 4 °C using a rotary slicer (Dosaka, Kyoto, Japan). After overnight incubation in RNAlater (Ambion, Austin, TX, USA) at 4 °C, slices were frozen and stored at −80 °C.

The sample preparation procedures for DNA microarray analysis have been described previously (Iwamoto et al., 2004). Briefly, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and purified with a RNeasy column (Qiagen, Valencia, CA, USA). Purity and integrity of total RNA were checked by OD measurement.

DNA microarray assay was performed using RNA derived from individual rats according to the protocols of the manufacturer (Affymetrix, Santa Clara, CA, USA). We used 10 μg of total RNA to synthesize cDNA. Biotinylated cRNA was generated from the cDNA. The cRNA was fragmented and applied to a Test2Chip (Affymetrix) to assess sample quality. For DNA microarray assay, rat U34A (Affymetrix) was used. The hybridization signal was scanned with a HP GeneArray scanner (Hewlett-Packard, Palo Alto, CA, USA) and processed using GeneSpring software (Silicon Genetics, Redwood City, CA, USA).

For normalization, the expression values of the genes were divided by the median value. We defined the differentially expressed genes based on the following criteria: (i) 1.5-fold or greater change in the mean expression level and (ii) $p < 0.05$ in the two-tailed Welch test.

Quantitative real-time PCR (qPCR)

We carried out qPCR to quantify mRNA obtained from individual rats with an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) following the protocols of the manufacturer. Measurement was done in duplicate. Normalization was performed by calculating the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or prostaglandin D synthase (PGDS). Primers were obtained from Applied Biosystems ('Assays-on-Demand').

Statistical analysis

The BW and qPCR data were analysed using one-way ANOVA with Tukey post-hoc tests. Differences were considered significant when $p < 0.05$.

Results

Hippocampal samples derived from the MS-8h and control rats were subjected to DNA microarray analysis to study differential gene expression caused by MS in the early neonatal period. Filtering the microarray data based on the criteria described in the Materials and methods section, we obtained 15 up-regulated and 9 down-regulated genes (Tables 1 and 2 respectively). The most notable finding was a 4.85-fold decrease in TTR expression in the MS-8h group, whereas the fold changes of other genes were <2 (Table 2, Figure 1a).

Table 1. Fifteen up-regulated genes due to the 8-hr MS stress

Affymetrix ID	Public ID	Symbol	Gene title	Category/function	Fold change
rc_AA894210_at	AA894210	–	EST198013	Unknown	1.90
X04070_at	X04070	Gjb1	Gap junction membrane channel protein beta 1	Cell contact	1.78
rc_AA875577_at	AA875577	–	Similar to dapper2 (LOC308212), mRNA	Unknown	1.72
rc_AA800551_at	AA800551	Hsj2	DnaJ-like protein	Stress response	1.71
rc_AA866369_at	AA866369	–	Transcribed sequences	Unknown	1.68
rc_AI228407_s_at	AI228407	Adcyap1	Adenylate cyclase activating polypeptide 1	Signal transduction	1.63
rc_AA892637_at	AA892637	Grp58	Glucose regulated protein, 58 kDa	Stress response	1.61
rc_H33461_at	H33461	Oxr1	Oxidation resistance 1	Stress response	1.61
rc_AI639001_at	AI639001	Ptprm	Protein tyrosine phosphatase, receptor-type, M	Signal transduction	1.60
rc_AA894168_at	AA894168	–	Similar to PHD finger protein 3 (LOC363210), mRNA	Unknown	1.60
AA850219_at	AA850219	Anx3	Annexin III (Lipocortin III)	Phospholipid binding protein	1.59
U35371_at	U35371	Cntn4	Contactin 4	Cell contact	1.53
rc_AA875023_at	AA875023	–	Similar to RIKEN cDNA 2410005K17 (LOC362578), mRNA	Unknown	1.53
rc_AA866299_g_at	AA866299	–	Transcribed sequences	Unknown	1.52
AF003835_at	AF003835	Idi1	Isopentenyl-diphosphate delta isomerase	Lipid metabolism	1.50

Differential expression was defined as follows: (i) 1.5-fold or greater change in the mean expression level; (ii) $p<0.05$ by two-tailed Welch test.

Table 2. Nine down-regulated genes due to the 8-hr MS stress

Affymetrix ID	Public ID	Symbol	Gene title	Category/function	Fold change
rc_AA945169_at	AA945169	Ttr	Transthyretin	Carrier protein	4.85
D28560_at	D28560	Enpp2	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	Myelin formation	1.97
U03734_at	U03734	Ace	Angiotensin 1 converting enzyme 1	Renin-angiotensin system	1.92
rc_AA684641_at	AA684641	–	Transcribed sequences	Unknown	1.84
X17012mRNA_s_at	X17012	Igf2	Insulin-like growth factor 2	Signal transduction	1.71
D49847_at	D49847	Grb2	Growth factor receptor bound protein 2	Signal transduction	1.60
U04835_at	U04835	Crem	cAMP responsive element modulator	Transcription	1.58
D85035_g_at	D85035	Dpyd	Dihydropyrimidine dehydrogenase	Pyrimidine metabolism	1.58
X56596_at	X56596	RT1-Bb	RT1 class II, locus Bb	Rat MHC class II	1.51

Differential expression was defined as follows: (i) 1.5-fold or greater change in the mean expression level; (ii) $p<0.05$ by two-tailed Welch test.

Brief daily neonatal handling of rats has the opposite effect of hours-long separation (for review, see Meaney, 2001). We therefore generated the MS-15m group for comparison with the MS-8h rats. We used qPCR to validate the microarray analysis data. Normalized by GAPDH expression level, one-way ANOVA indicated significant difference in TTR expression ($F_{2,12}=8.08$, $p<0.01$). TTR expression in

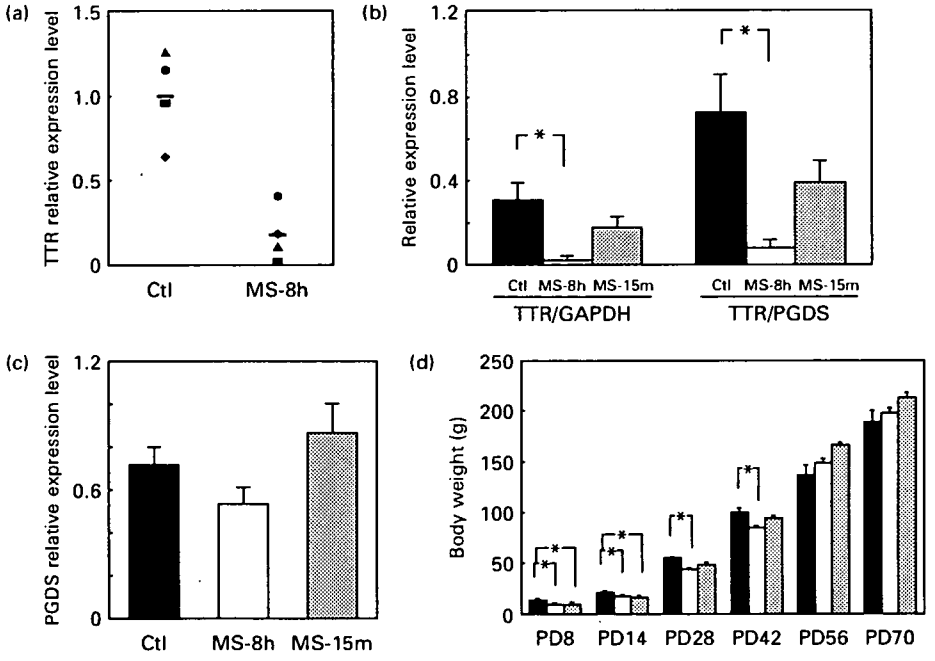

Figure 1. Drastic reduction of TTR expression in MS rats. (a) Distribution of relative TTR expression level of control and MS-8h rats in DNA microarray analysis. In the diagram, the mean value of the control group TTR expression was set as 1. The bars indicate the mean value ($n=4$ for both groups). (b) The difference in TTR expression was validated by qPCR. TTR expression was normalized by GAPDH or PGDS. Drastic decrease of TTR in the MS-8h group was validated in either case ($n=4$–5 per group). (c) Comparison of PGDS expression level in the control, MS-8h, and MS-15m groups ($n=4$–5 per group) by qPCR. There was no statistical difference between the three groups. (d) The effects of MS on body weight (BW) of the pups. On PD8 and PD14, the MS-8h and MS-15m pups weighed significantly less than the control rats. Although the BW of the MS-15 group caught up with the control group by PD28, a significant difference between the MS-8h and control groups remained until PD42. The mean and S.E.M. are shown in (b), (c), and (d). Ctl, control (■); MS-8h, maternal separation for 8 h (□); MS-15m, maternal separation for 15 min (▨). * $p<0.05$.

the MS-8h rats was, indeed, significantly decreased in comparison with controls (post-hoc test, $p=0.008$; Figure 1b). The MS-15m group indicated reduced expression of TTR, although there was not significant difference compared with either controls or MS-8h rats (vs. controls, $p=0.22$; vs. MS-8h, $p=0.12$; Figure 1b) It was previously reported that TTR is expressed almost exclusively in the CP in the brain, but not in neurons (Herbert et al., 1986; Schreiber et al., 1993). Since we did not control the amount of CP included during hippocampal slice preparation, it was possible that this result might have simply reflected variance in the amount of CP contained in the samples. Thus, we normalized the qPCR results by measuring PGDS mRNA, which is also expressed predominantly in the CP (Hayaishi, 1999). To our knowledge, PGDS has never been suggested to correlate with stress reaction or depression. Again, the TTR expression level was significantly lower in the MS-8h group compared with controls ($F_{2,12}=9.44$, $p<0.01$; post-hoc test, $p=0.005$; Figure 1b). In addition, one-way ANOVA revealed that PGDS expression levels were not different among the three groups ($F_{2,12}=0.50$, $p=0.62$; Figure 1c). These data indicate that differential TTR expression resulted from MS, rather than from accidental differences in the amount of CP included in the samples.

The plasma TTR level is known as a sensitive indicator of nutritional state, although the concentration of TTR in the cerebrospinal fluid (CSF) is reportedly less sensitive to dietary changes than is plasma TTR (Dickson et al., 1986; Wade et al., 1988). BW changes were measured to determine whether maternal separation affected gross development of the pups. One-way ANOVA revealed significant difference in BW on PD8 ($F_{2,21}=28.92$, $p<0.001$), PD14 ($F_{2,21}=6.94$, $p<0.05$), PD28 ($F_{2,21}=8.77$, $p<0.005$), and PD42 ($F_{2,21}=4.00$, $p<0.05$) among the control, MS-8h, and MS-15m groups (Figure 1d). Post-hoc tests indicated that rats in the MS-8h and MS-15m groups weighed

significantly less, on average, than those in the control group on PD8 ($p<0.001$ for both groups) and PD14 ($p<0.01$ for MS-8h and $p<0.05$ for MS-15m). By PD28, the control and MS-15m rats did not differ from one another in BW, whereas the MS-8h rats still remained smaller in BW than the controls ($p<0.01$). The BW of the MS-8h group caught up with the controls by PD56 (PD56: $F_{2,21}=3.04$, $p=0.07$; PD70: $F_{2,21}=1.60$, $p=0.22$). These findings suggested that there was no significant difference present in gross development at the time of DNA microarray or qPCR analysis (PW13) among the three groups, although BW gain was delayed in the MS-8h pups for a longer period than in the MS-15m pups. Therefore, the results of TTR expression level should not be affected by the nutritional state of the rats.

It should be noted that the MS-15m rats showed significantly smaller BW on PD8 and PD14. This suggested that 15-min separation, a standard manipulation for postnatal handling, worked as weak MS stress in this study (see Discussion). Therefore, the results obtained from the MS-15m rats should be consistent with those from the MS-8h.

Among the genes whose expression was decreased significantly in the MS-8h group in DNA microarray analysis, we found ACE and IGF-II. Interestingly, it was previously reported that their expression is enriched in the CP, although not limited to this region (Chai et al., 1987; Newton et al., 2003). Normalized by either GAPDH or PGDS, qPCR revealed the significantly different expression level of ACE ($F_{2,12}=9.15$, $p<0.01$ for ACE/GAPDH; $F_{2,12}=5.08$, $p<0.05$ for ACE/PGDS) and IGF-II ($F_{2,12}=11.45$, $p<0.005$ for IGF-II/GAPDH; $F_{2,12}=10.24$, $p<0.005$ for IGF-II/PGDS; Figure 2a, b). Post-hoc tests indicated that ACE and IGF-II were expressed less in the MS-8h group than in the control group (ACE/GAPDH, $p=0.004$; ACE/PGDS, $p=0.03$; IGF-II/GAPDH, $p=0.002$; IGF-II/PGDS, $p=0.004$), whereas the MS-15m data indicated significant reduction in expression of IGF-II (IGF-II/GAPDH, $p=0.04$; IGF-II/PGDS, $p=0.02$; Figure 2b), but not in ACE (ACE/GAPDH, $p=0.10$; ACE/PGDS, $p=0.12$).

In the MS model, in-situ hybridization studies have indicated that GR expression was down-regulated in the subregions of hippocampus (Francis et al., 2002; Ladd et al., 2000, 2004). We found no significant difference in GR expression between the MS-8h and control groups in DNA microarray analysis (data not shown). In addition, qPCR revealed no significant difference in the MS-8h, MS-15m and control groups ($F_{2,12}=0.27$, $p=0.76$ for GR/GAPDH; data not shown). This apparent discrepancy between the previous reports and the present study is probably due to the fact that we used the whole hippocampi for microarray analysis.

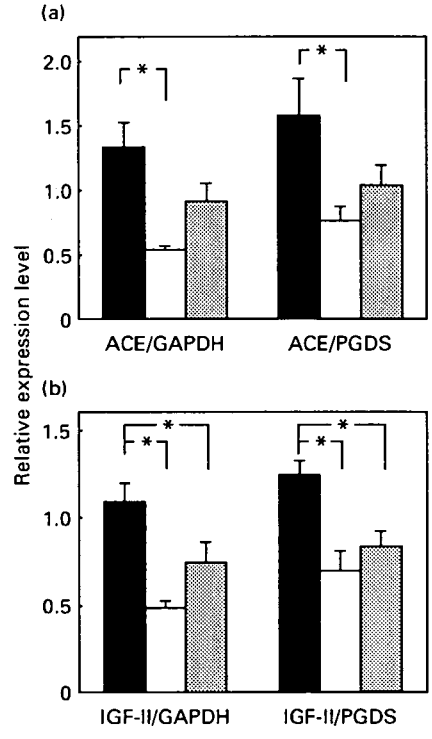

Figure 2. Reduced expression of ACE and IGF-II in the MS rats. The results of qPCR normalized by GAPDH or PGDS are shown. (a) ACE reduction was significant in the MS-8h group, normalized by either GAPDH or PGDS. (b) Expression of IGF-II was significantly decreased in both MS-8h and MS-15m rats. The mean and S.E.M. are shown in the diagrams. Ctl, control (■). MS-8h, maternal separation for 8 h (□). MS-15m, maternal separation for 15 min (▨). * $p<0.05$.

Discussion

Since MS rats show increased fearfulness and attenuated feedback of the HPA axis in adulthood, it is assumed that MS stress produces plastic changes in the brains of pups, leading to stress susceptibility that lasts throughout life. In this study, using hippocampal samples and DNA microarray analysis, we intended to comprehensively search the genes that might be involved in the molecular mechanisms of MS-induced stress susceptibility. We found several differentially expressed genes between the MS-8h and control groups.

Quantitative real-time PCR, indeed, revealed significant decrease of TTR expression in the MS-8h group

in comparison with the control group. Nutritional state should not affect the result, since BW of the MS-8h pups caught up with the other two groups by PD56. In addition, the MS-15m rats also showed reduced, but not significant, TTR expression compared with the control group. This result was rather unexpected, since they reportedly showed the opposite characteristics in behavioural and neuroendocrinological responses to the rats of hours-long MS (Meaney, 2001). In the previous reports, the delay of BW gain was not observed in 15-min separation, whereas hours-long MS resulted in significantly less weight gain (Barna et al., 2003; Huot et al., 2004; Ploj et al., 2003). The significantly smaller BW in the MS-15m rats suggested that 15-min separation did not work as the 'neonatal handling' manipulation, but rather that it worked as MS stress in our study. This might be due to differences of the strains used for the experiments. The parallel changes in expression of TTR, ACE and IGF-II found in the MS-8h and MS-15m groups should be consistent in the view of MS stress in the early postnatal period.

TTR, a carrier protein of thyroxine and retinol, is found in the plasma and CSF (Davis et al., 1970; Hagen and Elliott, 1973; Schreiber, 2002) and its expression is almost specifically restricted in the CP in the brain (Herbert et al., 1986; Schreiber et al., 1993). Since the biologically active compounds triiodothyronine (T3) and retinoic acid are indispensable for brain function and development (Anderson, 2001; Bernal, 2002; Morriss-Kay and Ward, 1999), and TTR is the only thyroid hormone-binding protein found at a substantial level in the CSF (Herbert et al., 1986), it is possible that TTR reduction in MS-8h rats could affect supply of T3 and retinoic acid, which might cause developmental abnormalities and/or dysfunction of the brain.

Using PCR-based subtractive hybridization, increased TTR expression by stress during fear-conditioning training was shown in samples from mouse basolateral amygdala (Stork et al., 2001). Microarray analysis of rat hippocampal samples indicated that single-prolonged stress, a model of post-traumatic stress disorder, also up-regulated TTR expression (Harada et al., unpublished observations). These findings suggest that stress should up-regulate TTR expression. Considering that the MS rats showed greater susceptibility to stress as well as a drastic decrease in TTR, we suggest that TTR might have a protective role against stress in the brain.

One clinical study reported that TTR expression in CSF was significantly reduced in depressed patients (Sullivan et al., 1999), suggesting that TTR reduction might be involved in the pathophysiology of depression. Since attenuated HPA feedback is also thought to be a biological marker of depression (Carroll, 1982), the increased risk of depression by maltreatment during early life might be partly explained by TTR. It was recently reported that TTR-deficient mice showed behavioural alterations, such as decreased immobility in the forced swimming test and increased exploratory activity in the open-field test (Sousa et al., 2004). Although these alterations apparently indicate a resistance to depression and anxiety, TTR seems to be involved in their molecular mechanisms. Notably, norepinephrine (NE) levels were increased in the limbic forebrain in TTR-deficient mice. Since TTR can also bind the oxidation product of NE (Boomsma et al., 1991) and MS rats indicated increased levels of NE in the frontal cortex, hippocampus and hypothalamus after restraint stress (Daniels et al., 2004), it is possible that reduction of TTR affected the function of the NE system in the brain.

ACE and IGF-II expression, which is normally enriched in the CP (Chai et al., 1987; Newton et al., 2003), was significantly decreased in the MS-8h group. Significant reduction of IGF-II was also found in MS-15m rats. Again, these results should reflect MS stress in both groups.

Regarding ACE, its expression is found in various regions in the brain. It is expressed rather strongly in the dentate gyrus in the hippocampus, in addition to the CP (Chai et al., 1987). Other than for blood pressure control, the renin–angiotensin system seems to be involved in depression and/or anxiety. Although ACE inhibitors have been demonstrated to have antidepressant effects in humans and rats (Martin et al., 1990; Vuckovic et al., 1991; Zubenko and Nixon, 1984) and angiotensinogen-deficient mice showed reduced depression-like behaviour (Okuyama et al., 1999b), enhanced anxiety has been observed in mice lacking angiotensin II type-2 receptor (Okuyama et al., 1999a). Reduced expression of ACE in the hippocampus and/or the CP might be involved in enhanced anxiety of the MS rats.

It has been reported that IGF-II is synthesized predominantly in the leptomeninge, the CP, and microvasculatures, while its immunoreactivity has been detected in various areas of the brain (Logan et al., 1994). This finding indicates that IGF-II produced locally in the non-neuronal system probably spreads to and affects the whole brain. Although the physiological roles of IGF-II in the brain are not well understood, it should be noted that electroconvulsive seizure, an established treatment for depression,

induces IGF-II up-regulation in the CP (Newton et al., 2003), suggesting antidepressant-like roles of IGF-II. Persistently lowered levels of IGF-II in MS-8h and MS-15m rats might have a significant role in their stress susceptibility.

In the hippocampal samples used in this study, we found differential expression of several genes induced by MS stress. Surprisingly, TTR, a gene expressed almost specifically in the CP, indicated the most conspicuous decrease. We also found ACE and IGF-II, which are also enriched in the CP, to be significantly down-regulated. Since the CP is thought to support the brain by releasing several trophic polypeptides into the CSF (Chodobski and Szmydynger-Chodobska, 2001), dysfunction of the CP could conceivably play a role in the pathophysiology of MS rats.

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Statement of Interest

None.

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Neuroanatomy in monozygotic twins with Asperger disorder discordant for comorbid depression

H. Yamasue, MD; M. Ishijima, MD, PhD; O. Abe, MD, PhD; T. Sasaki, MD, PhD; H. Yamada, MD, PhD; M. Suga, MD; M. Rogers, PhD; I. Minowa, MA; R. Someya, BA; H. Kurita, MD, PhD; S. Aoki, MD, PhD; N. Kato, MD, PhD; and K. Kasai, MD, PhD

Significant genetic contributions to autism spectrum disorders (ASDs) have been reported.[1] However, specific genetic variants that contribute to ASD have not been conclusively identified. Recently, interest has centered on an approach aimed at identifying potential intermediate phenotypes such as neuroanatomic abnormalities, as such findings may facilitate the endeavor to localize specific genetic variants.[2] Here we report common and distinct neuroanatomic abnormalities of a pair of monozygotic twins concordant for Asperger disorder (ASP) but discordant for psychiatric comorbidity. The current study applied individual whole-brain voxel-based morphometry (VBM) to quantitatively identify neuroanatomic abnormalities.

Participants. A pair of 22-year-old male twins with ASP was recruited from the Department of Neuropsychiatry, Hospital of Tokyo University, Japan (see table E-1 on the *Neurology* Web site at www.neurology.org). Diagnosis of ASP was determined for each patient according to the Diagnostic and Statistical Manual for Mental Disorders-IV (DSM-IV) (reference E-1) and further confirmed according to the International Classification of Diseases-10 (reference E-2) criteria through a consensus of two trained child psychiatrists. DNA fingerprint probes were used to establish zygosity, using an eight-probe single-locus DNA profile. DNA testing was performed to rule out fragile X syndrome. Although both the twins showed normal intelligence, one of them had current major depression as a psychiatric comorbidity (for detailed clinical characteristics of each twin, see table E-1). Eighty-two Japanese men without neuropsychiatric disorder served as a comparison sample (mean [SD] age = 28.9 [4.0] years, range 22 to 39 years). The participants were interviewed by trained psychiatrists and screened for the presence or absence of DSM-IV axis I disorder (reference E-3). All subjects were right-handed based on the Edinburgh Inventory (reference E-4). The Ethical Committee of the Faculty of Medicine, University of Tokyo, approved of this study. After a complete explanation, written informed consent was obtained from all participants.

MRI acquisition and analysis. The methods of MRI acquisition and image processing have been described in detail elsewhere.[3] In brief, the MRI data with 0.9375 × 0.9375 × 1.5–mm voxels were obtained from all subjects using a 1.5 T scanner. Processing of the acquired images was similar to that described in our previous study[3] except that, rather than SPM99, the current study employed SPM2, which includes spatial normalization using study-specific customized template, tissue segmentation with extracting nonbrain voxels and smoothing with 12-mm full width at half-maximum. Furthermore, global gray matter, white matter, and CSF volumes were calculated from the optimized VBM procedure.[4] Statistical comparisons of the processed images between the twin pair (n = 2) and controls (n = 82) and between each twin (n = 1) and controls (n = 82) were performed using an analysis-of-covariance model with age and intracranial volumes as confounding covariates. For individual VBM, a statistical analysis method similar to that of a previous study[5] was employed. Significance levels were set at corrected $p < 0.05$.

Results. Significantly reduced gray matter voxel densities were found in the left superior temporal gyrus including superior temporal sulcus (STS), left fusiform gyrus, right amygdala, and right prefrontal cortex (PFC) in twins with ASP as compared with control subjects. Individual VBM revealed reduced gray matter densities in the left STS, fusiform, and right PFC commonly in both twins. In contrast, the reduced gray matter densities in the right amygdala were evident in the twin with comorbid depression but not in the co-twin without mood disorder. No significant group difference in voxel density was detected for other gray matter regions or any of the white matter regions (figure, page 492).

Discussion. Both of the monozygotic twins concordant for ASP had significantly smaller than normal left STS, left fusiform gyrus, and right PFC, regions important for social cognition and behavior (reference E-5). The current findings are generally consistent with previous structural MRI studies in persons with ASD, although some inconsistencies exist in the literature.[6] These findings further suggest a contribution of shared genetic factors to underlying the structural abnormalities in ASD. Of particular interest, however, reduction of the amygdala was evident only in the twin with comorbid depression. Here the difference in age distribution between the twins and the control group and the medication effect on the depressed twin should be considered. Taking into account the age-associated decrease in brain volume[4] and neurotrophic effects of lithium and antidepressants, (reference E-6) however, the elimination of these effects would likely only strengthen the statistical difference. Taken together with the amygdala volume reduction reported in some forms of depression and anxiety (reference E-7), our results have an important implication for the interpretation of structural abnormality of amygdala, which has been extensively demonstrated in adults with ASD (reference E-8). Our results are also in accordance with recent animal studies[7] suggesting a role of the amygdala in abnormal fear and anxiety rather than abnormal social behavior in ASD.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the August 9 issue to find the title link for this article.

From the Departments of Neuropsychiatry (Drs. Yamasue, Ishijima, Sasaki, Suga, Rogers, Kato, and Kasai, I. Minowa and R. Someya), Radiology (Drs. Abe, Yamada, and Aoki), and Mental Health (Dr. Kurita), Graduate School of Medicine, University of Tokyo, Japan.

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Address correspondence and reprint requests to Dr. H. Yamasue, Department of Neuropsychiatry, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; e-mail: yamasue-tky@umin.ac.jp

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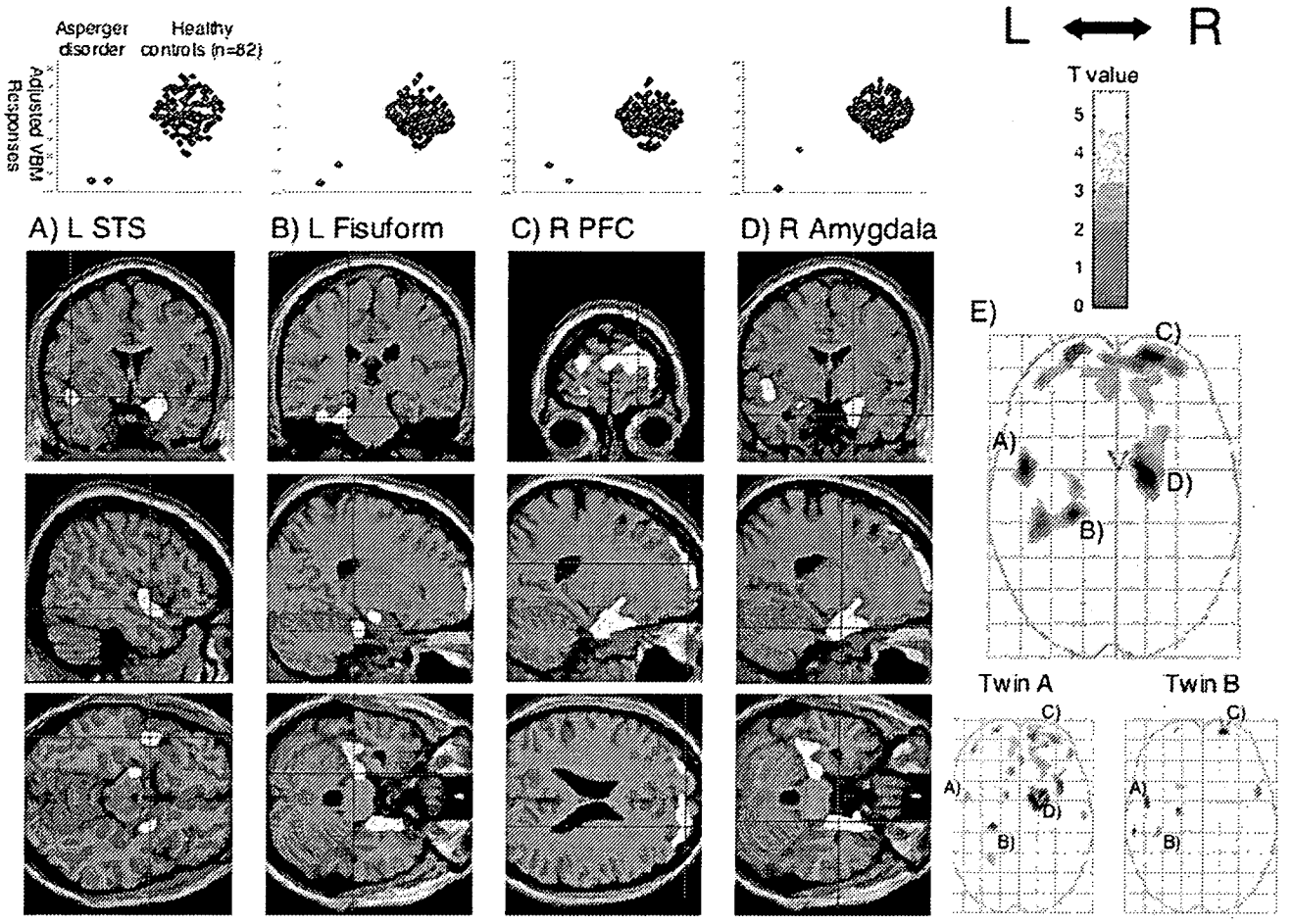

Figure. Morphological abnormalities in twins with Asperger disorder. (Left bottom, A through D) Gray matter voxels with reduced density in the twins with Asperger disorder as compared with normal control subjects (n = 2 vs n = 82) were rendered onto orthogonal slices of the normal template MR images. Voxel threshold: uncorrected p< 0.001; significantly abnormal regions: left superior temporal gyrus including superior temporal sulcus (A): peak coordinate at (x, y, z): (−49, 2, -13), spatial extent k = 1,934, Z(2,81) = 4.94, corrected p = 0.015; left fusiform gyrus (B): (−23, −23, −26), Z = 4.97, k = 3,590, corrected p = 0.013; right prefrontal cortex (C): (20, 61, 23), Z = 5.01, k = 9,617, corrected p = 0.011; right amygdala (D): (18, -3, -26), Z = 5.14, k = 6,514, corrected p = 0.006. (Left top, A through D) Plots of adjusted voxel-based morphometry responses at each brain region. (E) Statistical parametric maps in the axial projection showing gray matter voxels with reduced density in the twins (n = 2; upper map) and each twin (lower maps) as compared with normal controls (n = 82). A significant reduction in the right amygdala (D) found in Twin A was absent in Twin B. Voxel threshold: uncorrected p< 0.001.

A patient with left ventricular thrombus and recurrent stereotypic TIAs

Christine M. Bower, MD; Lola Morgan, MD; and Bruce Ovbiagele, MD

Stereotyped TIAs are presumed to occur secondary to a fixed flow-limiting stenosis of medium/large vessels in the cervicocephalic arterial tree[1] or in situ disease of small deep penetrating arteries in the brain.[2] We report the unusual case of a patient with recurrent stereotypic TIAs associated with the presence of a left ventricular thrombus and with delayed focal ischemia on the T1-weighted MRI sequence.

Case report. A 60-year-old nonsmoking Filipino man, with an unremarkable medical history, reported three distinct episodes of sudden-onset right-sided weakness and numbness and difficulty with expression. These episodes occurred every 2 hours over a 6-hour period. The first two spells lasted 10 minutes, and the third spell lasted 20 minutes.

On admission, his blood pressure was 155/90 mm Hg and pulse was 61 beats/min. Otherwise, general and neurologic exams were normal. Brain CT showed no evidence of infarct. Because of the temporal and stereotypic nature of his spells, we felt that the patient had a fixed flow-limiting stenosis in his left internal carotid or middle cerebral arteries causing hemodynamic compromise. The patient was admitted to the intensive care unit and placed on a heparin drip. MRI of the brain showed a mild hyperintensity on diffusion-weighted imaging (DWI) with corresponding apparent diffusion coefficient hypointensity in the left head and body of the caudate and a portion of the anterior internal capsule (figure, A and B). There was no corresponding signal change on T1-weighted, T2-weighted, or fluid-attenuated inversion recovery images. MR angiograms of the neck and circle of Willis were within normal limits (see the figure, C and D). Despite his stereo-

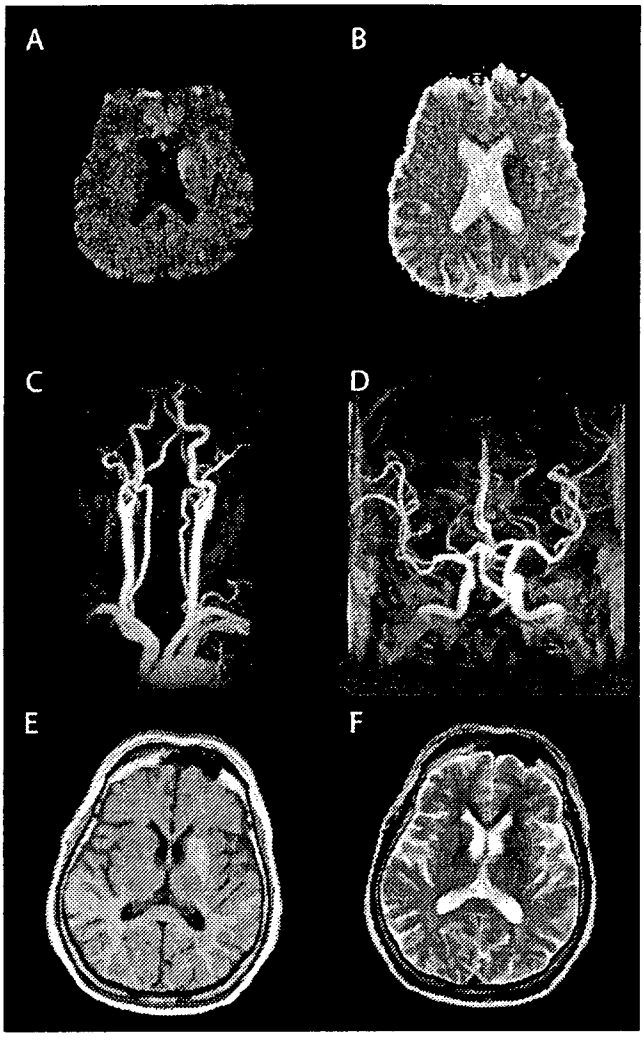

Figure. Initial MRI showing (A) diffusion-weighted imaging hyperintensity in left head and body of caudate and a portion of the anterior internal capsule (B) with corresponding hypointensity on apparent diffusion coefficient. Initial normal MR angiogram of (C) neck and (D) circle of Willis. MRI at 3 months showing (E) delayed T1-weighted hyperintensity in the caudate and putamen (F) with no corresponding change on T2-weighted imaging.

typic clinical presentation and the presence of diffusion MRI abnormality, there was no evidence of any flow-limiting stenosis in his cervicocephalic tree. These observations prompted the search for a cardioembolic source. Transesophageal echocardiogram (TEE) showed normal function and wall motion, but did reveal a 0.3 × 0.4–cm echodensity on the apex of the left ventricle that was interpreted as a blood clot. Laboratory testing showed no evidence of recent cardiac ischemia or hypercoagulable state but did reveal underlying hypercholesterolemia. The patient remained stable and was discharged home on warfarin. Three months after discharge, MRI brain showed T1-weighted hyperintensity in the left caudate and putamen without any corresponding lesion on fluid-attenuated inversion recovery and T2-weighted sequences (see the figure, E and F). TEE 6 months later showed no echodensity, implying resolution of the intracardiac clot.

Discussion. Traditionally, recurrent stereotypic TIAs are attributed to a fixed flow-limiting stenosis[1] or a manifestation of the capsular warning syndrome secondary to intrinsic small-vessel disease.[2] To our knowledge, this is the first reported case of repeated stereotyped TIAs associated with a documented cardioembolic source. How may this presentation be explained? It is known that transient internal carotid–middle cerebral artery occlusion can lead to the syndrome of "spectacular shrinking deficit" (SSD).[3] SSD refers to a sudden major hemispheric stroke syndrome followed by rapid improvement, leaving mild or no deficits.[3] Presumably, SSD is the result of cerebral embolism, usually from a cardiogenic source, with rapid embolic lysis, fragmentation, and migration of residual fragments along the internal carotid artery axis.[3] We contend that our patient had a single transient clot that caused flow-limiting intermittent symptoms lasting long enough to cause incomplete ischemic change. In support of our hypothesis, SSD syndrome has been associated with delayed onset of T1-weighted hyperintensity and T2-weighted hypointensity in the basal ganglia and cortex in humans.[4] Additionally, brief middle cerebral artery occlusion in rats with subsequent reperfusion induces the delayed ischemic changes of T1-weighted hyperintensity and T2-weighted hypointensity in the striatum.[5] The delayed striatal hyperintensity on T1-weighted MRI, which we observed in our patient, is thought to involve tissue manganese accumulation accompanied by manganese superoxide dismutase and glutamine synthetase induction in reactive astrocytes.[6] Of note, however, DWI sequences were not performed in these studies. DWI is a sensitive indicator of acute ischemic deficits even in patients with reversible neurologic deficit. In fact, approximately half of patients with clinical TIAs possess a relevant DWI abnormality.[7]

This case underscores the imprecise relationship between the mode of presentation of a cerebrovascular event and its presumed pathophysiology. Indeed, it has been noted that information on DWI led to a change in suspected anatomic localization, vascular localization, and TIA mechanism in over one-third of TIA patients.[7] Our report illustrates the importance of considering a cardiogenic mechanism in patients presenting with recurrent stereotypic TIAs that occur within a relatively short space of time.

From the Department of Neurology, UCLA Medical Center.

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Address correspondence and reprint requests to Dr. C.M. Bower, Department of Neurology, University of California at Los Angeles, 710 Westwood Plaza, Los Angeles, CA 90095; e-mail: cbower@mednet.ucla.edu

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Delayed automatic detection of change in speech sounds in adults with autism: A magnetoencephalographic study

Kiyoto Kasai[a,*], Ohiko Hashimoto[b], Yuki Kawakubo[a], Masato Yumoto[c], Satoru Kamio[a], Kenji Itoh[d], Ichiro Koshida[e], Akira Iwanami[f], Kazuyuki Nakagome[g], Masato Fukuda[h], Hidenori Yamasue[a], Haruyasu Yamada[i], Osamu Abe[i], Shigeki Aoki[i], Nobumasa Kato[a]

[a]*Department of Neuropsychiatry, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, 113-8655 Tokyo, Japan*
[b]*Child and Adolescent Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[c]*Department of Laboratory Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[d]*Department of Cognitive and Speech Sciences, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[e]*School of Bionics, Tokyo University of Technology, Tokyo, Japan*
[f]*Department of Medical Technology, Aino University, Osaka, Japan*
[g]*Department of Psychiatry, Showa University School of Medicine, Tokyo, Japan*
[h]*Department of Psychiatry and Human Behavior, Gunma University Graduate School of Medicine, Gunma, Japan*
[i]*Department of Radiology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*

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Abstract

Objective: Autism is a form of pervasive developmental disorder in which dysfunction in interpersonal relationships and communication is fundamental. This study evaluated neurophysiological abnormalities at the basic level of language processing, i.e. automatic change detection of speech and non-speech sounds, using magnetoencephalographic recording of mismatch response elicited by change in vowels and tones.

Methods: The auditory magnetic mismatch field (MMF) was evaluated in 9 adults with autism and 19 control subjects using whole-head magnetoencephalography. The MMF in response to the duration change of a pure tone or vowel /a/ and that in response to across-phoneme change between vowels /a/ and /o/, were recorded.

Results: The groups were not significantly different in MMF power under any conditions. However, the autism group showed a left-biased latency prolongation of the MMF particularly under the across-phoneme change condition, and this latency delay was significantly associated with greater symptom severity.

Conclusions: These results suggest that adults with autism are associated with delayed processing for automatic change detection of speech sounds. These electrophysiological abnormalities at the earliest level of information processing may contribute to the basis for language deficits observed in autism.

Significance: These results provide the first evidence for delayed latency of phonetic MMF in adults with autism.

Keywords: Autism; Magnetoencephalography (MEG); Mismatch negativity (MMN); Phoneme; Speech sound; Tone; Vowel

1. Introduction

Autism is a pervasive developmental disorder associated with aberrant social skills, deficient language, abnormal attention, and stereotyped repetitive behaviors (American Psychiatric Association, 1994). Fundamental cognitive deficits in autism are characterized by a lack of normal attentional preference to socially relevant stimuli (Rapin, 1997). For example, individuals with autism spent more time looking at objects and less time looking at people (Swettenham et al., 1998). Moreover, children with autism

* Corresponding author. Tel.: +81 3 5800 9263; fax: +81 3 5800 6894.
E-mail address: kasaik-tky@umin.ac.jp (K. Kasai).

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oriented more poorly to social (both speech and non-speech) than to non-social stimuli (Dawson et al., 1998). However, brain functional basis for deficits in socially relevant auditory stimuli such as speech sounds in autism has been poorly understood. At the earliest stage, i.e. the level of auditory sensory processing, speech sound processing requires the discrimination of phonemes; a process that requires the categorization of the simplest unit of speech sounds according to their acoustic features. Such a process can be indexed by the auditory mismatch negativity (MMN) elicited by speech sounds (Näätänen et al., 2001).

The MMN or its magnetic counterpart (magnetic mismatch field; MMF) is an event-related potential (ERP) or magnetic field peaked at approximately 100–200 ms after the onset of a physically deviant auditory stimulus in identical and repeated sequence (Hari et al., 1984; Näätänen et al., 1978). Näätänen (1992) noted that MMN (MMF) reflects the detection of mismatches between the deviant stimuli and the neural trace encoding the physical features of the standard stimuli and that MMN (MMF) can be elicited even under passive conditions when subjects ignore the stimuli entirely. Thus, MMN (MMF) can be considered an index of the process of automatic detection of acoustic change in humans. A number of researchers have recently extended their investigations into MMN (MMF) in response to speech sound discrimination (reviewed in Näätänen, 2001). Magnetoencephalography (MEG) (Alho et al., 1998a; Koyama et al., 2000; Näätänen et al., 1997; Rinne et al., 1999) and positron emission tomography (PET) (Tervaniemi et al., 2000) studies have demonstrated that the left auditory cortex is predominantly activated during the automatic processing of speech sounds (vowel or consonant–vowel syllables) in normal subjects. Moreover, Kraus (1998) suggested that phonetic MMN showed an increase as a result of cognitive discrimination training; thus it may be an index of language-related plasticity in the central nervous system.

A review of the previous literature on MMN or MMF in individuals with autism, identified 5 studies that employed ERPs (MMN) (Čeponienė et al., 2003; Ferri et al., 2003; Gomot et al., 2002; Kemner et al., 1995; Seri et al., 1999) and one that employed MEG (MMF) (Tecchio et al., 2003); the results of these studies are mixed (reviewed and discussed in detail in the Discussion section). The subjects in all 5 ERP studies were children with autism, and the MEG study by Tecchio et al. employed autism individuals with a broader range of ages (8–32 years). Moreover, only two ERP studies (Čeponienė et al., 2003; Kemner et al., 1995) used speech sounds to elicit MMN: Kemner et al. (1995) reported preserved MMN amplitude in response to change between /ay/ and /oy/ sounds in children with autism; however, no analysis of latency data was reported; Čeponienė et al. (2003) reported intact MMN amplitude and latency elicited by change in vowels as well as simple and complex tones in children with autism. Thus, to date, no studies have evaluated MMN/MMF specifically in adults with autism; no studies have used MEG to record mismatch response to speech sounds in autism. Importantly, no studies have explored the relationship between MMN/MMF indices and clinical symptoms in autism. Additionally, all 5 studies that evaluated tonal MMN/MMF in autism (Čeponienė et al., 2003; Ferri et al., 2003; Gomot et al., 2002; Seri et al., 1999; Tecchio et al., 2003) measured mismatch response to frequency change (frequency MMN/MMF), with none of them assessing MMN/MMF in response to duration change of tones (duration MMN/MMF).

Accordingly, the goal of this study was to investigate, using a whole-head MEG, whether or not a reduction and/or latency prolongation in magnetic mismatch field elicited by across-category change of speech sounds is present in adults with autism. Additionally, we also measured duration MMF using tonal and vowel stimuli. The use of a whole-head MEG instead of a scalp EEG has two advantages. First, the use of a whole-head MEG enables independent investigation of left and right hemispheric functions, because, in contrast to electrical fields, magnetic fields are not influenced by intervening tissues of different conductivities. Second, MEG selectively detects electrical currents tangential to the scalp, whereas EEG is more sensitive to radially oriented currents. Thus, MMF generated in the superior temporal plane constituting the auditory cortex could be selectively detected by MEG recording, while MMF from other generators such as the frontal component (Alain et al., 1998; Alho et al., 1994; Giard et al., 1990; Kasai et al., 1999; Liasis et al., 2001; Umbricht et al., 2000), having preferentially radially oriented currents (Giard et al., 1990; Kasai et al., 1999), is largely filtered out.

2. Methods

2.1. Subjects

Nine right-handed (Edinburgh Inventory [Oldfield, 1971] with laterality index ≥ 0.8 as the cut-off for right-handedness) adults with autism were recruited from the Outpatient Clinic, Department of Neuropsychiatry, University Hospital of Tokyo, Japan. Six were male and 3 were female, and the mean age was 27.2 (SD 7.7). Nineteen age-, gender-, and handedness-matched healthy subjects (mean age 27.3; SD 7.0; 13 males and 6 females) participated in the study. Diagnosis of autism was made according to the DSM-IV (American Psychiatric Association, 1994) criteria for autistic disorder and confirmed using the Childhood Autistic Rating Scale—Tokyo Version (CARS-TV) (Kurita et al., 1989) administered by an experienced child psychiatrist (O.H.). Scores for all subjects in the patient group (mean 33.7 [SD 1.2]) were above the cut-off point of >27 for adult criteria of autism (Mesibov et al., 1989). All subjects with autism were able to construct 3 word sentences. Patient's IQs (mean 57.2 [SD 15.0]) were evaluated using the Wechsler Adult Intelligence Scale-Revised (WAIS-R)