(Caccia et al. 1982; Miller et al. 1992). It has been shown that 1-PP acts as an antagonist at alpha(2A)-adrenoceptors, and that its administration to mice and rats produces hypolocomotion (Newman-Tancredi et al. 1998; Tatarczynska et al. 1989). It is therefore possible that repeated administration of tandospirone inhibits locomotion via a blockade of alpha(2A)-adrenoceptors by its metabolites. There is little information about the effect of 1-PP on PPI; however, yohimbine, an alpha(2A)-adrenoceptor antagonist, disrupts PPI (Powell et al. 2005). These considerations might not be relevant to the results presented here, which indicates that the effect of tandospirone on PPI is mediated by 5-HT1A receptors.

Repeated administration of MK-801-induced behaviours related to schizophrenia, such as deficits in sensorimotor gating, as measured by PPI (Bubenikova-Valesova et al. 2008a). In our study, we used the sub-chronic (4 days) administration of MK-801 and tandospirone. Based on our observations (not shown), we assumed that the 4-day application in our experimental paradigm changed the behaviour and expression of NMDA receptors in a way that mimicked chronic treatment. Antipsychotic agents are expected to reverse the effect of MK-801 while the high-dose tandosprione (5 mg/kg) exacerbated the MK-801-induced PPI deficits, an effect blocked by WAY 100635 (Fig. 2a). Acute administration of MK-801 (0.1 mg/kg) has been shown to enhance locomotion (Bubenikova-Valesova et al. 2008a), and this has been used as a model of the positive symptoms of schizophrenia (van den Buuse 2010); however, repeated administration of the same dose of MK-801 has been shown to induce tolerance and it did not increase locomotor activity (Amitai et al. 2007). Furthermore, tandospirone, at 5 mg/kg, inhibited locomotor activity in MK-801-treated rats, which was not affected by WAY 100635 (Fig. 2c).

Tandospirone decreased locomotor activity in MK-801-treated rats, but exacerbated MK-801-induced PPI disruption (Fig. 1a, c). Clinical trials reporting a beneficial influence of tandospirone on cognition were based on data from schizophrenia patients treated with typical antipsychotic drugs, such as haloperidol, which are D2 antagonists (Sumiyoshi et al. 2000; Sumiyoshi et al. 2001b; Sumiyoshi et al. 2001a). Therefore, we investigated the effect of co-administration of haloperidol on behavioural changes induced by the repeated administration of tandospirone with and without co-treatment by MK-801. Acute administration of haloperidol did not influence PPI or the effect of tandospirone on PPI in rats without MK-801 treatment (Fig. 3a). In accordance to findings in the literature, we found that haloperidol did not block PPI disruption induced by a 5-HT1A agonist (van den Buuse and Gogos 2007). A low dose (0.1 mg/kg) of haloperidol decreased locomotion by itself or when combined with a low, but not high, dose of tandospirone. It is well documented that 5-HT1A agonists ameliorate haloperidol-induced catalepsy (Ohno et al. 2008; Ohno et al. 2009). This line of evidence is consistent with our observations that a high dose of tandospirone counteracted haloperidol-induced hypolocomotion.

In MK-801-treated rats, haloperidol did not reverse PPI disruption induced by tandospirone (5 mg/kg), similar to the effect of 5-HT1A receptor antagonists. Haloperidol by itself did not influence PPI deficits induced by MK-801, in accordance with the results of our previous study (Bubenikova et al. 2005). On the other hand, haloperidol decreased locomotor activity in MK-801-treated rats, which was blocked by tandospirone (5 m/kg) (Fig. 3c).

Augmentation therapy with 5-HT1A partial agonists, such as tandospirone and buspirone, in patients treated with typical or atypical antipsychotics produces benefits to a range of cognitive functions, e.g. verbal memory, executive function, memory organisation and attention/information processing (Sumiyoshi et al. 2001a; Sumiyoshi et al. 2007a; Sumiyoshi et al. 2007b). Therefore, our initial hypothesis was that tandospirone, with or without co-administration with haloperidol, would show antipsychotic-like effects in MK-801-treated rats.

Our results indicate an interaction between 5-HT1A receptors and NMDA receptors on behavioural levels. Several studies show that activation of 5-HT1A receptors inhibits the function of NMDA receptors on the molecular level, although the mechanisms underlying these interactions are largely unknown (Gu et al. 2007; Yuen et al. 2005). Based on this, 5-HT1A agonists were assumed to inhibit the effect of NMDA antagonists. However, our previous study showed that acute administration of the 5-HT1A full agonist 8-OH-DPAT, at a low-dose, blocked MK-801-induced PPI deficits. In addition, it is possible that 5-HT1A partial agonists, such as tandospirone and buspirone (azapirone derivatives), behave differently from full 5-HT1A agonists. Also, active metabolites of these 5-HT1A partial agonists have an affinity for alpha(2A)-adrenoceptors, as discussed above.

The role of 5-HT1A receptors in the pathophysiology and treatment of schizophrenia is still unresolved. The findings in this study suggest that the mode of action of clinically used azapirone derivatives may not be entirely through 5-HT1A receptors. Investigations of 5-HT1A partial agonism, using agents that do not produce active metabolites which influence other neurotransmitter systems, e.g. the adrenergic system, should be the subject of further studies.

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Research Report

Neonatal exposure to MK-801, an N-methyl-D-aspartate receptor antagonist, enhances methamphetamine-induced locomotion and disrupts sensorimotor gating in pre- and postpubertal rats

Takashi Uehara[a,b,*], *Tomiki Sumiyoshi*[a,b], *Tomonori Seo*[a], *Tadasu Matsuoka*[a], *Hiroko Itoh*[a], *Michio Suzuki*[a,b], *Masayoshi Kurachi*[a,b]

[a]*Department of Neuropsychiatry, University of Toyama Graduate School of Medicine and Pharmaceutical Sciences, Toyama, Japan*
[b]*Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan*

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ABSTRACT

Administration of non-competitive N-methyl-D-aspartate (NMDA) receptor antagonists (e.g. phencyclidine, MK-801) has been shown to elicit behavioral abnormalities related to symptoms of schizophrenia, such as spontaneous locomotor activity and impaired sensorimotor gating, as represented by deficits of prepulse inhibition (PPI). We sought to determine whether transient blockade of NMDA receptors at the neonatal stage would produce dopamine supersensitivity around puberty, as manifested by these behavioral measures. For this purpose, we examined methamphetamine (MAP; 1.0 mg/kg, i.p.)-induced locomotor activity and PPI in pre- (postnatal day; PD 36–38) or post- (PD 64–66) puberty in rats administered MK-801 (0.2 mg/kg/day, s.c.) between PD 7 and PD 10. Neonatal MK-801 treatment augmented MAP-induced hyperlocomotion especially in the early adulthood, whereas spontaneous locomotor activity and rearing were not changed. MK-801 administration also disrupted PPI without affecting startle amplitudes around puberty. These findings suggest that transient exposure to MK-801 in the neonatal stage causes exaggerated dopamine transmission and cognitive deficits, particularly in the post-puberty stage.

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1. Introduction

Patients with schizophrenia manifest positive symptoms (e.g., delusions, hallucinations) and negative symptoms (e.g., apathy, social isolation), as well as cognitive disturbances including sensorimotor gating deficits. Although the pathophysiology of schizophrenia remains unclear, some promising hypotheses have been postulated. For example, dysregulation of dopaminergic neurotransmission in limbic brain regions (Carlsson, 1988) has been viewed as convincing, because all antipsychotic drugs block dopamine D2 receptors (Sumiyoshi, 2008). Specifically, a linear relationship exists between clinical potencies of antipsychotic drugs and their affinity for D2 receptors in the brain (Seeman and Lee, 1975; Seeman et al., 1976; Seeman, 2006).

* *Corresponding author.* Department of Neuropsychiatry, University of Toyama Graduate School of Medicine and Pharmaceutical Sciences, 2630 Sugitani, Toyama 930-0194, Japan. Fax: +81 76 434 5030.
E-mail address: **uehara@med.u-toyama.ac.jp** (T. Uehara).

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The N-methyl-D-aspartate (NMDA) receptor hypofunction hypothesis of schizophrenia has also been postulated (e.g., reviewed in (Bubenikova-Valesova et al., 2008; Coyle et al., 2003). This hypothesis is based on clinical observations that NMDA antagonists (e.g. phencyclidine, ketamine, MK-801) produce negative symptoms and cognitive dysfunctions, in addition to positive symptoms in healthy people (e.g., review of (Jentsch and Roth, 1999). On the other hand, morphological studies have demonstrated a reduction in the volume of the prefrontal cortex and the temporal lobes in schizophrenia (Kawasaki et al., 2004; Suzuki et al., 2005), consistent with the neurodevelopmental hypothesis of schizophrenia (Roberts, 1991; Weinberger, 1995). This hypothesis predicts that insults into temporal lobe structures, e.g. hippocampus and parahippocampal gyrus, in fetus results in abnormal development of the frontal cortex, a condition precipitating overt symptoms of schizophrenia at the time of sexual maturation.

The development of animal models of schizophrenia is important to clarify the pathophysiology of the illness and facilitate the development of novel therapeutics. Rats exposed to non-competitive NMDA receptor antagonists (e.g. phencyclidine, MK-801) at the neonatal stage have been shown to elicit behavioral abnormalities related to clinical symptoms of schizophrenia, such as enhanced locomotor activity (Harris et al., 2003; Wang et al., 2001), deficits of prepulse inhibition (PPI), a measure of sensorimotor gating (Rasmussen et al., 2007; Takahashi et al., 2006; Uehara et al., 2009; Wang et al., 2001), and impaired set-shifting test (Stefani and Moghaddam, 2005) in the adult stage.

In this study, we sought to determine: (1) whether transient neonatal blockade of NMDA receptors would produce hyperdopaminergic activity in limbic area which is related to positive symptoms of schizophrenia, as well as sensorimotor deficits, in the young adult stage; and (2) if these abnormalities were identified, at which developmental stage they would become evident. For this purpose, we examined methamphetamine (MAP) -induced locomotor activity and rearing in pre- (postnatal day; PD 36–38) and post- (PD 64–66) puberty in rats administered MK-801 between PD 7 and PD 10. Locomotor activity and exploratory (rearing) behavior are governed by the mesolimbic dopaminergic pathway (Fink and Smith, 1980; Kelly et al., 1975; Pijnenburg et al., 1976; Thiel et al., 1999). We chose these two developmental points because positive psychiatric symptoms become overt at the time of sexual maturation in patients with schizophrenia (Weinberger, 1995). So far, this animal model has been shown to elicit impairment of set-shifting test, a measure of the function of prefrontal cortex, in early adulthood (Stefani and Moghaddam, 2005).

2. Results

2.1. *Body weight*

Body weight (g, mean±SEM) for each group was: vehicle 101.4±4.5 (n=10) and MK-801 103.0±2.8 (n=10) on PD 35; vehicle 265.6±3.2 (n=11) and MK-801 237.2±10.2 (n=10) on PD63. ANOVA revealed a significant main effect of treatment [$F(1,37)=5.26$, $P=0.028$] and treatment×age interaction [$F(1,37)=6.592$, $P=0.014$]. Subsequent analysis was conducted to examine treatment effects on PD 35 and PD 63 separately. One-way ANOVA revealed a significant effect of treatment [$F(1,19)=7.72$, $P=0.012$] on PD 63 but not PD 35 [$F(1,18)=0.09$, $P=0.77$].

2.2. *PPI*

One-way ANOVA revealed no significant main effect of treatment status on PPI at PD 35 [$F(1,18)=0.46$, $P=0.44$] and PD63 [$F(1,20)=2.46$, $P=0.13$], respectively.

Repeated ANOVA was conducted to examine treatment effects on PPI at PD 35 and PD 63 separately, because our major purpose was to clear when the behavioral abnormalities become evident. On PD 35, repeated measures ANOVA revealed no significant difference between treatment status (MK-801, vehicle). Treatment×prepulse intensity interaction [$F(4,72)=0.89$, $P=0.47$] and a main effect of treatment status [$F(1,18)=1.41$, $P=0.25$] were not significant. However, there was a significant main effect of treatment status [$F(1,19)=1.98$, $P=0.003$] and marginal treatment×time interaction [$F(4,76)=0.96$, $P=0.06$] on PD 63 (Figs. 1-A and B). PPI deficits were noted with 70 dB, 74 dB, 78 dB and 86 dB prepulse intensity ($Ps<0.01$, one-way ANOVA followed by Dunnet). These findings overall indicate that neonatal MK-801 treatment disrupted PPI on PD63 but not PD 35 without affecting SAs.

2.3. *Locomotor activity*

The effects of neonatal MK-801 treatment on spontaneous and MAP-induced locomotor activity were examined separately at PD 35 and 63. There were no significant treatment×time interaction [$F(1,18)=1.38$, $P=0.26$] and main effect of treatment status [$F(1,18)=0.003$, $P=0.96$] on spontaneous locomotor activity at PD35. However, main effect of treatment status on PD 63 was significant [$F(1,19)=5.26$, $P=0.03$], whereas treatment×time interaction was not [$F(1,19)=0.05$, $P=0.83$]. Post hoc test did not reveal significant differences both 0–15 and 16–30 min intervals.

Repeated ANOVA was conducted to examine treatment effects on MAP-induced locomotor activity at PD 35 and PD 63 separately. On PD 35, treatment status (MK-801 vs. vehicle) produced no significant difference. Treatment×time interaction [$F(7,126)=0.86$, $P=0.86$] and a main effect of treatment status [$F(1,18)=0.33$, $P=0.58$] were not significant, either. On PD 63, there was a significant main effect of treatment status [$F(1,19)=12.22$, $P=0.002$] with no significant treatment×time interaction [$F(7,133)=0.96$, $P=0.46$] (Figs. 2-A and B). The augmentation of an increase in MAP-induced locomotor activity in MK-801 treated animals was significant from 16 to 90 min after MAP injection ($Ps<0.03$, one-way ANOVA followed by Dunnet). These results indicate that spontaneous and MAP-induced locomotor activity was increased by neonatal treatment with MK-801 on PD63 but not PD 35.

Repeated measures ANOVA did not demonstrate significant differences in spontaneous rearing between vehicle- and MK-801 treated groups [treatment×time interaction; $F(1,18)=3.29$, $P=0.09$, main effect of treatment status; $F(1,18)=0.38$, $P=0.55$] and MAP-induced rearing [treatment×time interaction; $F(7,126)=0.51$, $P=0.83$, main effect of treatment status; $F(1,18)=1.22$, $P=0.28$] on PD 35 (Fig. 3-A). On PD 63, there were no significant differences in spontaneous rearing [treatment×time interaction; $F(1,19)=0.09$,

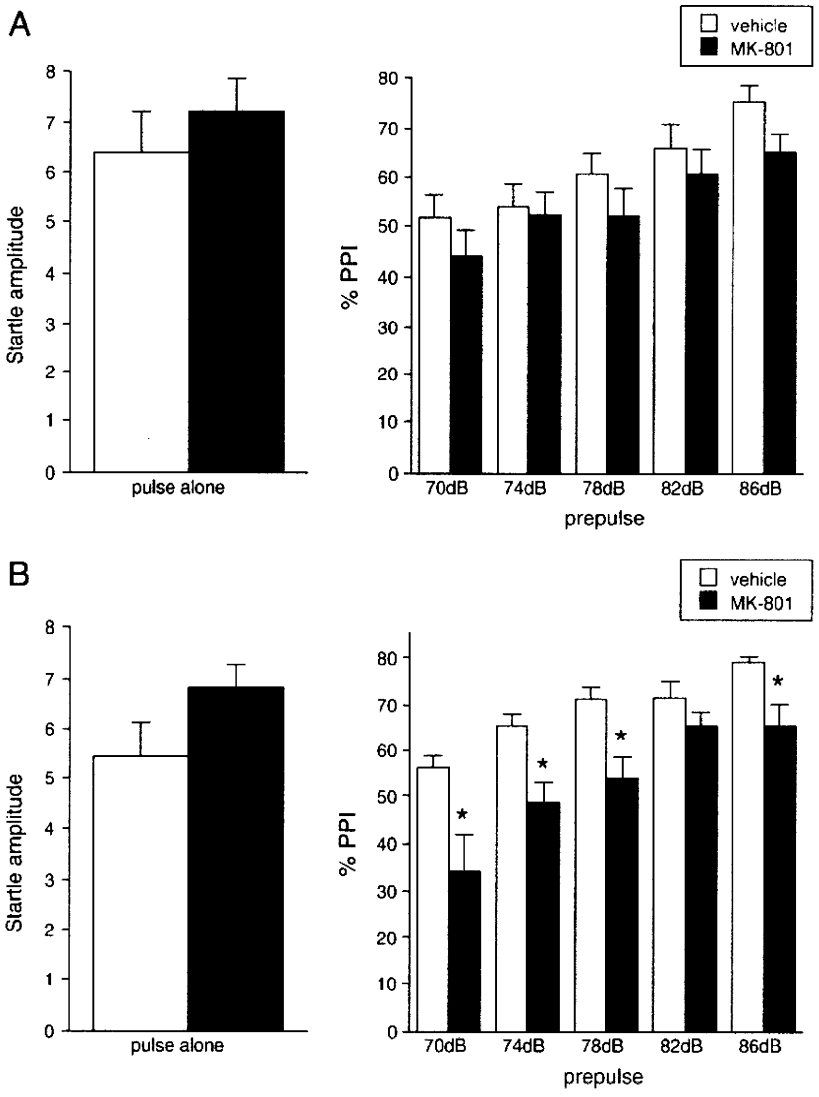

Fig. 1 – Effect of neonatal MK-801 treatment on startle amplitudes (SAs) and prepulse inhibition (PPI) tested on PD 34–35 (A) and PD 62–63 (B). Data of vehicle-treated rats and MK-801-treated rats are shown by open bars and shaded bars, respectively. SAs were obtained from single noise (120 dB) trials during PPI sessions. Values are expressed as mean ± SEM. Asterisks indicate a significant difference from the vehicle group.

P=0.77, main effect of treatment status; F(1,19)=0.05, P=0.82] and MAP-induced rearing [treatment × time interaction; F(7,133)=0.50, P=0.84, main effect of treatment status; F(1,19)=2.99, P=0.10] (Fig. 3-B). Overall, treatment-dependent differences in rearing were not significant on either occasion.

3. Discussion

The results of this study indicate that transient exposure to MK-801 at the neonatal stage augmented spontaneous and MAP-induced hyperlocomotion after the time of puberty, whereas rearing was not affected either pre- or post puberty. MK-801 treatment also disrupted PPI after puberty. These findings suggest that neonatal exposure to MK-801 causes delayed enhancement of dopamine responsivity and sensorimotor gating deficits, especially in the early adulthood stage.

Neonatal MK-801 treatment (PD 7 through PD 10) decreased body weight after puberty, which is in line with previous studies (Stefani and Moghaddam, 2005; Uehara et al., 2009). On the other hand, it is reported that neonatal PCP treatment (10 mg/kg, PD 7, 9 and 11) decreases body weight by 17–21% and whole brain weight by 10% (Boctor and Ferguson, 2009). These findings suggest that neonatal blockade of NMDA receptors exerts long-term toxic consequences. In this study, all pups from the same litter received the same treatment to avoid the influence of cross-fostering and minimize maternal care effects. In fact, there were no statistical litter effects on all parameters (data not shown).

Neonatal MK-801 treatment affects spontaneous locomotor activity on PD 63, but not PD35, while rearing was unaffected on either occasion. During the first (0–15 min) time interval immediately after the placement in the test chamber, rats were exposed to novelty environment

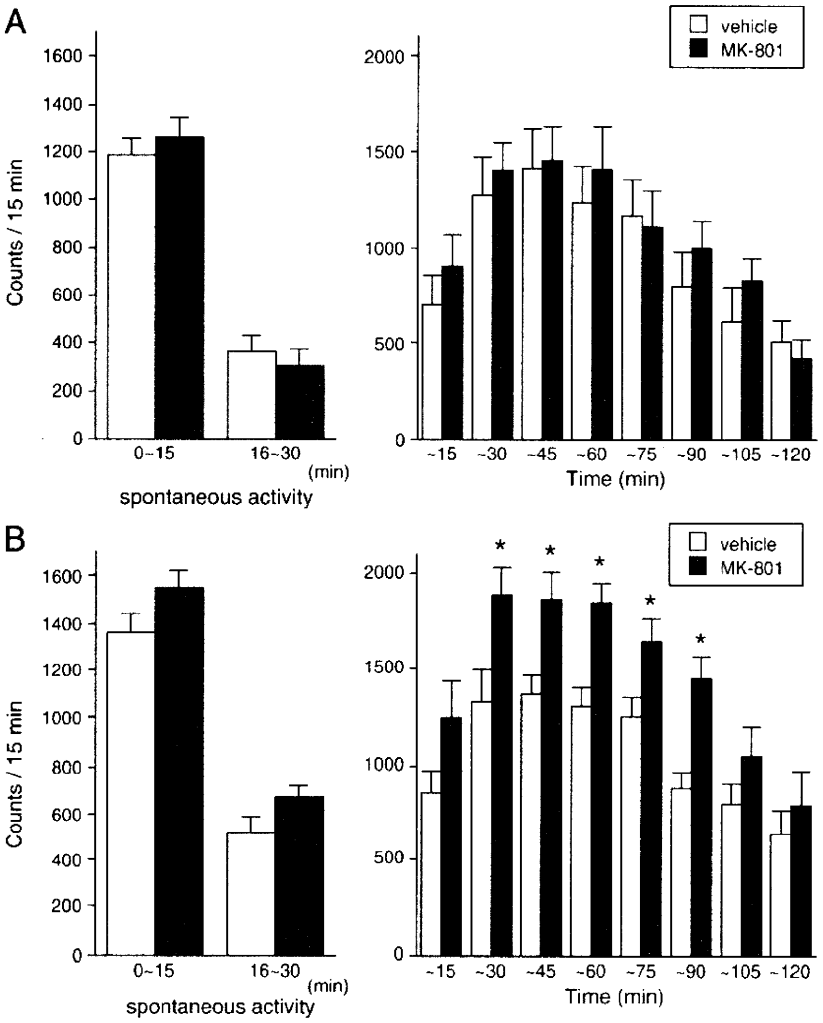

Fig. 2 – Time course of spontaneous (left) and methamphetamine (1.0 mg/kg, i.p.)-induced (right) locomotor activity on PD 36–38 (A) and PD 64–66 (B). Open bars shows activity counts/15 min for vehicle-treated rats and shaded bars for MK-801-treated rats. Values are expressed as mean ± SEM. Asterisks indicate a significant difference from the vehicle group.

(Bubenikova-Valesova et al., 2007). Previous studies demonstrated that NMDA receptor blockade at the neonatal stage did not affect novelty-induced and spontaneous locomotor activity both in the pre- and post-pubertal periods (Beninger et al., 2002; Harris et al., 2003; Stefani and Moghaddam, 2005). These findings may not be consistent with the results in this study. Previous studies used Sprague-Dawley rats (Beninger et al., 2002; Harris et al., 2003; Stefani and Moghaddam, 2005), while we used Wistar rats, suggesting the effect of NMDA receptor blockade on spontaneous locomotor is strain-dependent. Another factor may be the timing of examinations; novelty-induced and spontaneous locomotor activity was not affected on PD35 or 56, which is in line with our previous report (Uehara et al., 2009).

To our knowledge, this study provides the first evidence that neonatal exposure to MK-801 augmented MAP-induced locomotor activity after puberty (PD 63). On the other hand, spontaneous- or MAP-induced locomotor activity and rearing were not affected pre-puberty (PD 35). These results indicate that transient neonatal blockade of NMDA receptor causes delayed emergence of hyper-dopaminergic activity, which is reminiscent of the manifestation of positive symptoms of schizophrenia at the time of sexual maturation (Weinberger, 1995). It has been reported that rats treated with PCP (10 mg/kg, s.c.) on PD 7, 9 and 11 showed enhancement of PCP-induced locomotor activity on PD 42 compared with vehicle-treated animals (Wang et al., 2001). MK-801 (0.5 mg/kg i.p.) treatment on PD 3 was reported to increase amphetamine-induced locomotion on PD 35, but not PD56, whereas treatment with a larger dose (1.0 mg/kg) on PD 35 did not affect locomotion on either occasion (Beninger et al., 2002). These results suggest behavioral supersensitivity related to dopaminergic function as a result of blockade of NMDA receptors depends on the timing of manipulation.

The precise mechanisms underlying how neonatal blockade of NMDA receptors causes delayed sequence of behavioral supersensitivity is currently unknown, but several candidate hypotheses are worth considering. One possibility is a role of testosterone in potentiating the response to dopamine agonists,

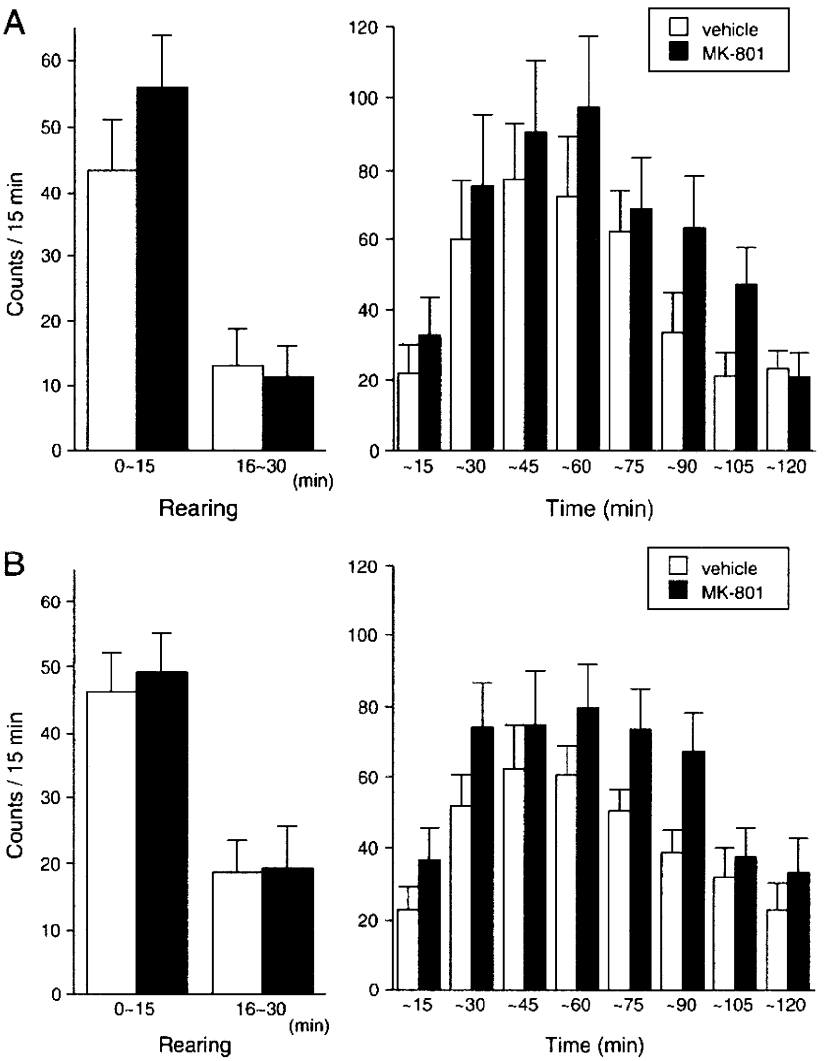

Fig. 3 – Time course of spontaneous (left) and methamphetamine (1.0 mg/kg, i.p.)-induced (right) rearing on PD 36–38 (A) and PD 64–66 (B). Open bars shows activity counts / 15 min for vehicle-treated rats and shaded bars for MK-801-treated rats. Values are expressed as mean ± SEM.

such as MAP. Serum testosterone is reported to be less than 1.0 ng/ml until postnatal 5 weeks, and begins to increase at postnatal 6 weeks (1.5–2.0 ng/ml), which continues until postnatal 10 weeks (about 4.0 ng/ml) in male Wistar rats (Vilamaior et al., 2006). Testosterone increases dopamine release in the nucleus accumbens in rats (de Souza Silva et al., 2009), and enhances Fos expression in ventral tegmental area (VTA) in hamsters (Dimeo and Wood, 2006). These findings indicate testosterone secretion at the time of sexual maturation may mediate exaggerated dopamine transmissions around puberty in the MK 801-treated rats, as indicated here.

It is noteworthy that MAP-induced hyperlocomotion has also been observed in animals with neonatal excitotoxic lesions of hippocampus (Lipska et al., 1993a; Lipska et al., 1993b; Lipska et al., 1995). Perinatal transient blockade of NMDA receptors has been shown to accelerate apoptotic cell death in the hippocampus depending on the timing of treatment (Ikonomidou et al., 1999; Pohl et al., 1999). For example, neonatal treatment with PCP or MK-801 enhanced apoptotic cell death (Beninger et al., 2002; Harris et al., 2003). Specifically, MK-801 dose-dependently produced apoptotic neurodegeneration, which progressed in the 12- to 24-hour interval (Ikonomidou et al., 1999). These results suggest that apoptosis induced by neonatal treatment with NMDA antagonists depends on dose and frequency of treatments. Rats treated neonatally with MK-801 elicited decreased volume of the hippocampus and neuronal number on PD 60 (Harris et al., 2003). Therefore, it is speculated that neuropathological insults are present in specific brain regions, e.g., hippocampus, in rats exposed to NMDA receptor blockade at the neonatal stage. Further studies are warranted to determine if histological changes in the hippocampus, nucleus accumbens, and other brain areas are present in our animal model.

Spontaneous or MAP-induced rearing was not affected by neonatal treatment with MK-801. Rearing behavior in general may reflect stereotypy, whereas rearing in a novel environment

has been suggested to be a measure of exploratory behavior (Bubenikova-Valesova et al., 2009). Dopamine transmission in the striatum plays a key role in psychostimulant-induced stereotypy (Kelly et al., 1975). Therefore, our findings suggest that transient neonatal blockade of NMDA receptor did not affect dopamine transmission in the nigrostriatal area.

PPI deficits are one of the most widely used neurophysiological markers in the study of schizophrenia, and have been suggested to represent an aspect of cognitive deficits (Geyer, 2006), and be correlated with disability associated with schizophrenia (Karper et al., 1996). The findings presented here suggest transient blockade of NMDA receptors in neonatal periods disrupted PPI on PD 63, consistent with our previous report (Uehara et al., 2009). PPI has been suggested to be mediated by activation of the limbic and cortico–pallido–striato–thalamic circuitry (Koch and Schnitzler, 1997; Swerdlow et al., 2001). Specifically, dopamine projections to the accumbens nucleus are considered to play an important role in the generation of PPI (Swerdlow et al., 1990a; Swerdlow et al., 1990b; Zhang et al., 2000). These neural substrates are likely to also explain enhanced MAP-induced locomotion observed in our animal model, suggesting increased dopaminergic transmissions in response to external stimuli.

PPI has been suggested to be affected by magnitudes of baseline SAs (Csomor et al., 2008). Although not statistically significant, there appears to be a greater startle response in MK-801 exposed rats compared to vehicle-treated animals at PD63 (Figs. 1-A and B). Therefore, we calculated data to compare between treatment groups with equivalent baseline startle levels, by excluding two animals showing the highest SA in the MK-801-treated rats. This additional analysis confirmed that neonatal MK-801 treatment disrupted PPI on PD 63 even when SA magnitudes are leveled between MK-801-treated rats and control animals.

In conclusion, transient exposure to MK-801 at the neonatal stage augmented MAP-induced locomotion and disrupted PPI in the adolescence or early adulthood stage in rats. These findings suggest that neonatal blockade of NMDA receptors provide an animal model of psychotic symptoms and cognitive disturbances of psychiatric disorders, such as schizophrenia.

4. Experimental procedures

4.1. Animals

Female Wistar rats obtained at 14 days of pregnancy (Japan SLC, Japan) were housed individually at 24±2 °C under a 12-h light (0700–1900 h)–12-dark cycle with free access to food and water. At the time of weaning (PD 21), animals were grouped into four to six per treatment in a cage with free access to food and water. The procedures complied with the National Institutes of Health guide for the care and use of Laboratory animals. All experiments were reviewed and approved by the Committee of Animal Research, University of Toyama.

4.2. Drug treatment

At 7 days postnatal (PD), male pups (7–15 g) were divided into four groups: vehicle group (n=10) and MK-801 group (n=10) which were tested on PD34-38; vehicle group (n=11) and MK-801 group (n=10) tested on PD 62–66. They received MK-801 (dizocilpine, 0.20 mg/kg, Sigma-Aldrich, St. Louis, MO; MK-801 group), or an equal volume of saline (control; vehicle group) once daily for 4 days. Pups received injection between 8:00–10:00. All treatments were given subcutaneously (s.c.). All pups from the same litter received the same treatment to avoid the influence of cross-fostering and minimize maternal care effects. The number of litter for each group was at least 2 groups. There were no statistically significant litter effects on all behavioral parameters evaluated in this study.

4.3. PPI testing

Rats were tested on PD 34–35 or PD 62–63. PPI was measured according to our previous studies (Seo et al., 2008; Uehara et al., 2007). All testing occurred within startle chambers (Ohara & Co., LTD, Tokyo), which was housed in a sound-attenuated room with a 60 dB ambient noise level. Each startle chamber consisted of a Plexiglas cylinder 9.4 cm in internal diameter resting on an 11 cm×22 cm Plexiglas stand. Acoustic stimuli and background noise were given via speakers mounted 12.2 cm above the Plexiglas cylinders, controlled with a computer box (Ohara & Co., LTD, Tokyo). A piezoelectric device mounted below the Plexiglas stand detected and transduced motion within the cylinder. Rats were placed in a startle chamber, and 5 min after the acclimation period, they were exposed to six blocks of six different stimulus types, i.e. pulse-alone; 40 ms 120 dB white noise bursts: prepulse-pulse; 20 ms, white noise pulse of 70, 74, 78, 82 and 86 dB followed by 40 ms 120 dB white noise pulse at a fixed inter-stimulus interval (ISI) of 100 ms. Trials were presented in randomized order, with 20, 25 and 30 s randomized interval.

4.4. Locomotor activity testing

After the PPI examination, locomotor activity was tested on PD 36–38 or PD 64–66. Locomotion was measured in an ambulation observation chamber (blackened vinyl chloride cages, 40 cm× 40 cm×40 cm; AMB-3001, Ohara & Co., Ltd., Tokyo, Japan) equipped with 6×6 photoelectric light sources spaced at 7-cm intervals and 2.5 cm (for horizontal locomotion) above the floor (AMB-2020, Ohara & Co., Ltd.) (Sumiyoshi et al., 2004; Uehara et al., 2007). Rats were brought to the testing room in their home cages, and were immediately placed in the test chamber. Spontaneous locomotor activity was measured thereafter for 30 min., and then each rat was administered intraperitoneally with 1.0 mg/kg MAP (3.0 mg/ml; Dainippon Sumitomo Pharmaceuticals, Tokyo, Japan). Upper activity (rearing) was measured by photoelectric light sources spaced at 12 cm (on PD 36–38) or 19 cm (on PD 64–66) above the floor. Interruptions of light beams were registered as activity counts, and were summarized every 5 min by the Logger Interface control system (IF-10-LOG, Ohara & Co., Ltd.). For convenience, the test days are referred to as PD 35 and PD 63 throughout the manuscript.

4.5. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using SPSS software (version 16.0J for Mac, SPSS Inc.). For comparison of

body weight, two-way ANOVA was performed with treatment status (MK-801, vehicle) and age (PD35, PD63) as between-subject factor, followed by one-way ANOVA on PD 35 and PD63 separately. PPI data were presented as percentage of PPI (%PPI), which was calculated from startle amplitudes (SAs) using the following formula: %PPI=100−[(SA for prepulse–pulse trials)/(SA for pulse-alone trials)×100]. Between-group comparisons were performed by repeated measures ANOVA with treatment status as between-subject factor, whereas prepulse intensity was treated as repeated measures variable. Since our a priori hypothesis predicted a difference in PPI between the two treatment status (MK-801, Vehicle), we performed analysis with treatment status as a between subject factor at each age (PD 35, PD 63) separately. When appropriate, this was followed by one-way ANOVA and post hoc Dunnet test to evaluate between-group difference. For comparisons of SAs, one-way ANOVA was performed with treatment status as between-subject factor. For comparison of spontaneous locomotor activity and rearing, data were divided into two periods (0–15 min and 16–30 min). For comparisons of MAP-induced locomotor activity and rearing, activity counts were obtained for every 15 min. Repeated measures ANOVA with treatment status as between-subject factor, and time as within-subject factor was treated as repeated measures variable. When appropriate, this was followed by one-way ANOVA and post hoc Dunnet test to evaluate between-group difference. A probability (P) of less than 0.05 was considered to be significant.

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BASIC NEUROSCIENCES, GENETICS AND IMMUNOLOGY - ORIGINAL ARTICLE

Change in the expression of myelination/oligodendrocyte-related genes during puberty in the rat brain

Tadasu Matsuoka · Tomiki Sumiyoshi · Masahiko Tsunoda · Ichiro Takasaki · Yoshiaki Tabuchi · Takashi Uehara · Hiroko Itoh · Michio Suzuki · Masayoshi Kurachi

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Abstract Age-dependent changes of gene expression in the prefrontal cortex (PFC) of rats around the time of puberty were investigated by means of microarray and quantitative polymerase chain reaction (qPCR). About 127 and 138 genes were increased and decreased, respectively, in the PFC of rats at post-puberty (PD56) compared with those at pre-puberty (PD35). Functional analysis showed significant associations of these genes with aging, cellular development, and neuropsychological disorders. qPCR analysis confirmed down-regulation of seven genes related to myelination. As these genes have been reported to be diminished in the brain of patients with schizophrenia, the results of this study suggest an exaggerated maturation process may contribute to the pathogenesis of psychotic disorders.

Keywords Development · Age · Brain · Myelination · Microarray · Quantitative PCR · Rat · Schizophrenia

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T. Matsuoka · T. Sumiyoshi (✉) · M. Tsunoda · T. Uehara · H. Itoh · M. Suzuki · M. Kurachi
Department of Neuropsychiatry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
e-mail: tomikisumiyoshi840@hotmail.com

I. Takasaki · Y. Tabuchi
Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Toyama, Japan

T. Sumiyoshi · M. Suzuki · M. Kurachi
Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan

Introduction

The incidence of several psychiatric disorders is increased in the adolescent period (Paus et al. 2008). Specifically, the prodromal phase and onset of schizophrenia are frequently observed at late adolescence. The biological basis for schizophrenia has been suggested to include a variety of abnormalities in frontal and temporal lobes (Suzuki et al. 2002), along with decreased expression of genes related to myelination and oligodendroglial cells in postmortem prefrontal cortex (PFC) of patients with schizophrenia (Hakak et al. 2001). Expression levels of some of the myelination-related genes have been reported to be altered in the adolescence period (Harris et al. 2009).

White matter expansion has been shown to progress linearly from ages 4 to 20 years due to increased axonal growth and myelination (Giedd et al. 1999). The time course of myelination process varies depending on brain regions. Among them, neurons in frontal and temporal lobes are myelinated last (Terry et al. 1987), and the change in white matter volume of these brain portions occurs predominantly in adolescence (Suzuki et al. 2005).

In rats, the adolescent stage corresponds to the period delineated by postnatal day (PD) 35 (pre-puberty) and PD56 (post-puberty) (Lipska et al. 1993; Andersen 2003).

We herein compared gene expression levels in medial prefrontal cortex (mPFC) of rats between pre- and post-puberty comprehensively using microarray. After obtaining the results of microarray assay, we performed quantitative polymerase chain reactions (qPCR) to test the hypothesis that expression of genes responsible for the myelination process is down-regulated from pre-puberty to adulthood (PD77), i.e., a developmental period associated with the onset of schizophrenia.

Materials and methods

Animals

Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were housed in groups of four in a plastic cage at 24 ± 2°C under a 12 h:12 h light/dark cycle (light on from 05:00 to 17:00). Food and water were available ad libitum. All procedure was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was reviewed and approved by the Committee of Animal Research, University of Toyama.

RNA isolation

The brains were rapidly removed at PD35, PD56, or PD77. Bilateral mPFC was carefully dissected using the following coordinates: anterior 2.7–3.7 mm, lateral 0–1 mm, and ventral 4.5–5.5 mm with respect to the bregma (Paxinos and Watson 1998). They contained predominantly gray matter. Mean weight of tissue was 17.7 mg. Total RNA was extracted from the brain tissues using an RNeasy Protect Mini Kit (Qiagen), and was treated with DNase I (RNase-free DNase kit, Qiagen) for 15 min at room temperature to remove residual genomic DNA. Total RNA were purified from each animal separately. There were no significant differences in concentrations of total RNA among PD35, PD56 and PD77. We measured the OD260/280 ratio. The ratios for our samples fell into the range from 1.9 to 2.0

Microarray analyses

Based on the previously described methods (Wong et al. 2005; Matsuoka et al. 2008), total RNA from six animals within each group (PD35, PD56) was pooled.

Gene expression was analyzed using a GeneChip® system with Rat Expression Array 230A which is spotted with 15,923 probe sets (Affymetrix, Santa Clara, CA). Sample preparation for array hybridization was carried out following the manufacturer's instructions. Scanned chips were analyzed using GeneChip Analysis Suite software (Affymetrix). Hybridization intensity data were converted into a presence/absence call for each gene, and changes in gene expression between ages (PD35 vs. PD56) were detected by comparison analysis. Data were further analyzed using GeneSpring software (Silion Genetics, Redwood City, CA) to extract significant genes.

To examine gene ontology, including biological processes, cellular components, and molecular functions, the data were analyzed using Ingenuity Pathway Analysis tools (Ingenuity System, Mountain View, CA), a web-delivered application. The gene lists identified by the GeneSpring containing Affymetrix gene ID and natural legalism were uploaded into Ingenuity Pathway Analysis. Fischer's exact test was used to calculate a *P* value deterring the possibility that each biological function and/or disease assigned to that data set is due to chance alone.

Real-time quantitative PCR assay

We selected seven myelination/oligodendrocyte-related genes based on the relevant literature: myelin-associate glycoprotein (Mag), myelin oligodendrocyte glycoprotein (Mog), claudin 11 (Cldn11), SRY-box containing gene 10 (Sox10), myelin and lymphocyte protein (Mal), myelin-associated/oligodendrocytic basic proteon-99 (Mobp), and cyclic nucleotide phosphodiesterase 1(Cnp1). These genes have been reported to be down-regulated in the PFC of patients with schizophrenia (Hakak et al. 2001; Tkachev et al. 2003; Iwamoto et al. 2005; Dracheva et al. 2006).

Real-time qPCR was performed on a Real-Time PCR system (Mx3000P, Stratagene Japan K.K., Tokyo, Japan) using SYBR® Premix Ex TaqTM (TAKARA BIO INC., Japan) according to the manufacturer's protocol. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen K.K.) was carried out with DNase-treated total RNA by using an oligo (dT)15 primer. Real-time qPCR was performed using the following specific primers: glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) forward primer, tgaggaccaggttgtctcct; reverse primer, atgtaggccatga ggtccac; *Mag* forward primer, ccttcaacctgtctgtgg; reverse primer, actccctctctgtctcgttc; *Mog* forward primer, ggacag aagaacccacaac; reverse primer, gccaaagcaagatggtg; *Cldn11* forward primer, ctgttcgctcctttcctc; reverse primer, tccatt gtcgctggtaac; *Sox10* forward primer, ggaaagaatcagaggtgtcc; reverse primer, gtgcgaggcaaaggtag; *Mal* forward primer, caaagtgggagattgagacc; reverse primer, ccaggagaagagtgctt acc; *Mobp* forward primer, ataggagcacacagtagccc; reverse primer, agacaagcaagcactcagg; *Cnp1* forward primer, agcca gcaagagtaagcc; and reverse primer, acagggtgagggtaggaac. Temperature cycling conditions for each primer consisted of 10 min at 95°C followed by 40 cycles for 10 s at 95°C, and 40 s at 60°C. Dissociation analysis was carried out over the range from 60 to 95°C by monitoring SYBR Green fluorescence. PCR-specific products were determined as a single peak at the melting curves of more than 80°C. In addition, the specificity of primers was confirmed as a single band with the correctly amplified fragment size through an agarose gel electrophoresis of the real-time quantitative PCR products. Each expression level was normalized to the expression level of glyceraldehydes 3-phosphate dehydrogenase (*Gapdh*) (*cf.* there is a suggestion that the use of more than one reference genes is desirable for normalizing; Taylor et al. 2010).

Statistical analysis

Data are presented as means with SD. Comparisons of expression levels among groups were made by analysis of variance (ANOVA) using Stat View-J 4.5 software. Values were considered statistically significant when P was <0.05.

Results

Microarray assay

Of the 15,923 probe sets analyzed, we identified 10,231 (percentage present: 64.2%) and 10,202 (64.0%) probe sets in PD35 samples, and 9,800 (61.5%) and 9,470 (59.4%) probe sets in PD56 samples.

Supplementary Table shows the genes that were up- or down-regulated by >1.5-fold in the mPFC from rats at PD56 compared with those from animals at PD35. The ratio was calculated based on scanned data from four arrays (two arrays per condition) using GeneSpring software. All presented results represent raw data showing >1.5-fold changes. We found 127 up-regulated genes and 138 down-regulated genes (Supplementary Table).

Functional analyses

Functional analysis of up- or down-regulated probe sets was conducted using the Ingenuity Pathways Analysis Knowledge Base (Takasaki et al. 2007). Based on the order of significance level of genes, the top five subcategories affected by age (PD35 vs. PD56) are represented for "Diseases and Disorders" category and "Molecular and Cellular Functions" category. Within the Diseases and Disorders category, the top five subcategories were developmental disorders (significance 3.34E-06 to 3.34E-06, associated gene number 35), cancer (6.55E-06 to 6.66E-03, 102), genetic disorders (4.19E-05 to 4.63E-03, 164), neurological diseases (4.47E-05 to 6.47E-03, 105), and psychological disorders (4.47E-05 to 2.51E-04, 19). Within the Molecular and Cellular Functions category, the top five were cellular development (2.25E-09 to 6.55E-03, 82), cell morphology (1.16E-06 to 6.59E-03, 52), cellular growth and proliferation (1.38E-06 to 6.52E-03, 90), gene expression (1.83E-06 to 2.74E-03, 48), and cellular assembly and organization (2.26E-06 to 6.59E-03, 43).

RT-qPCR assay

Based on the findings in microarray assay, genes related to oligodendrocyte/myelination were further investigated.

To confirm the results of the microarray analysis, expression levels of myelination/oligodendrocyte-related genes were analyzed by real-time quantitative PCR (RT-qPCR). Total RNA from eight animals within each group (PD35, PD56 and PD77) was analyzed individually.

Figure 1 demonstrates gene expression levels, calculated as the ratio to Gapdh mRNA, in the mPFC of rats at PD35, PD56, and PD77. Relative expressions of Mag ($F = 9.49$, $P < 0.05$), Mog ($F = 13.45$, $P < 0.05$), Cldn11 ($F = 5.64$, $P < 0.05$), Sox10 ($F = 9.26$, $P < 0.05$), Mal ($F = 6.88$, $P < 0.05$), Mobp ($F = 8.22$, $P < 0.05$), and Cnp1 ($F = 7.51$, $P < 0.05$) were significantly reduced at PD56 and PD77 compared with those at PD35. There were no significant differences in the expression levels between PD56 and PD77 (post hoc analysis by Scheffe's test Fig. 1).

Discussion

To our knowledge, this study was the first to examine the effect of development around the time of puberty on gene expression in the brain of the rat. Functional analysis of microarray data indicates the effect of age on expression of genes associated with developmental disorder, cancer, genetic disorder, and neurological/psychological disorder. Expression levels of molecule-encoding genes were altered for cellular development, morphology, growth, and proliferation.

Findings from the RT-qPCR analysis confirmed the results of the microarray assay and are considered to reflect a normal age-dependent myelination process in the PFC of rats during adolescence. It is noteworthy that expression levels of some of the genes related to myelination, e.g.,

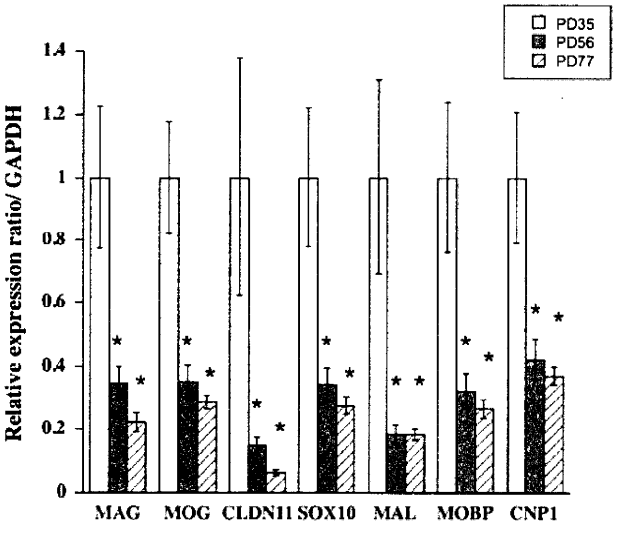

Fig. 1 Age-dependent changes in the expression of myelination/oligodendrocyte-related genes in the medial prefrontal cortex of rats. *$P < 0.05$ compared with PD35

Mag, Mog, Cldn11, Sox10, Mal, Mobp, and Cnp1, have been reported to be decreased in the PFC of patients with schizophrenia, (Hakak et al. 2001; Tkachev et al. 2003; Iwamoto et al. 2005; Dracheva et al. 2006). In our results of microarray assay, Plp1 (proteolipid protein 1) and Pllp (plasma membrane proteolipid) were included in myelination-related genes. These genes have been reported to be down-regulated in the brain of schizophrenia. Because of the difficulty in appropriately preparing their specific primers, RT-qPCR data of Plp1 and Pllp were not available. It would be worthwhile to determine temporal and/or causative interactions among these myelination/oligodendrocyte-related genes in future studies.

Taken together, the results of this study are consistent with the suggestion that an exaggerated maturation process may contribute to the pathogenesis of psychotic disorders, including schizophrenia. Alternatively, it is possible that down-regulation of these genes in the postmortem brain from patients with schizophrenia may represent a failure to maintain myelin-related gene expression in adulthood. Also, some mechanisms causing white matter abnormalities in schizophrenia (Kanaan et al. 2005) may be associated with normal maturational process. Since a recent human postmortem study also reports a decrease in myelin-related genes in the PFC from adolescence to adults (Harris et al. 2009), some common processes may exist in the brain maturation in this period between the two species. It would be worthwhile to see the overall between species difference in gene expression in the PFC.

In our study, some interesting genes were found to be down-regulated during the puberty (Supplementary Table). For example, Drd1a (dopamine receptor D1a) is worth mentioning, as dopamine D1 receptors have been reported to be reduced in the PFC in patients with schizophrenia (Okubo et al. 1997). Likewise, Sema6d (semaphorin 6D) and Dcx (doublecortin) have been associated with migration process, while Tnc (Tenacin C) have been linked to cellular organization process (Supplementary Table from the use of Ingenuity Pathway Analysis, http://www.ingenuity.com). Further analysis of these genes would be worthwhile in future investigations.

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Impaired ability to organize information in individuals with autism spectrum disorders and their siblings

Chika Sumiyoshi[a,*], Yuki Kawakubo[b,c,d], Motomu Suga[b], Tomiki Sumiyoshi[e], Kiyoto Kasai[b]

[a] Faculty of Human Development and Culture, Fukushima University[1], Fukushima, Japan
[b] Department of Neuropsychiatry, Graduate School of Medicine, University of Tokyo[2], Japan
[c] Japan Science and Technology Agency (JST)[3], Japan
[d] Core Research of Evolutional Science & Technology (CREST)[4], Japan
[e] Department of Neuropsychiatry, University of Toyama Graduate School of Medicine and Pharmaceutical Science, Toyama, Japan

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ABSTRACT

Despite rigorous research on disturbances of executive function and social cognition in autism spectrum disorders (ASD), little information has been available concerning higher cognitive functions, such as the ability to focus and associate relevant features to form categories, or 'organizing of information'. The purpose of this study was to investigate this issue by using the Wisconsin Card Sorting Test (WCST) and the Verbal Learning Task (VLT). Cognitive assessments were conducted in 22 individuals with ASD, 14 non-affected siblings, and 15 age-matched control subjects. Overall, individuals with ASD performed significantly worse on the WCST and VLT compared to their siblings and normal control subjects. Although siblings performed generally well on both tasks, they exhibited similar degree of perseverative responses in the WCST compared to the probands. A linear increase of the memory organization score in the VLT was also absent in siblings as well as the ASD group. These results suggest an impaired ability to organize information is one of the cognitive endophenotypes for ASD.

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1. Introduction

High-functioning autism (HFA) and Asperger syndrome (AS) are defined as a part of autism spectrum disorders (ASD), characterized by difficulties in establishing social relationships, poor communication skills, lack of imaginative behavior, and repetitive stereotypic behaviors (American Psychiatric Association, 1994; World Health Organization, 1992). Although subjects with HFA or AS do not show a significant delay in intelligence, they have been reported to elicit disturbances of some domains of cognitive function, e.g. social cognition (Baron-Cohen et al., 1985) and executive function (Hill, 2004). For example, they perform poorly on various types of Theory of Mind tasks, ranging from perceptual (e.g. The Eyes Task), verbal (e.g. The Strange Stories) (Kaland et al., 2008a) to emotional ones (identification of emotional states of others) (Shamay-Tsoory, 2008). These results suggest the inability to recognize thoughts and feelings to understand how others act. Also, subjects with ASD have been reported to show impaired executive function, specifically, cognitive flexibility (Geurts et al., 2004) and inhibition (Happe et al., 2006).

Although previous studies have identified some aspects of cognitive disturbance associated with ASD, more specific assessments of higher cognitive functions would help further understand the psychopathology of the disorder. Specifically, 'organizing infor-

Abbreviations: ASD, autistic spectrum disorders; WCST, Wisconsin Card Sorting Test; VLT, verbal learning task; SCR, stimulus category repetition.

* Corresponding author at: Faculty of Human Development and Culture, Fukushima University, 1 Kanayagawa Fukushima, Fukushima 960-1296, Japan. Tel.: +81 24 548 8161; fax: +81 24 548 8161.
E-mail addresses: sumiyoshi@educ.fukushima-u.ac.jp (C. Sumiyoshi), yukik-tky@umin.ac.jp (Y. Kawakubo), mosuga-tky@umin.net (M. Suga), sumiyo@med.u-toyama.ac.jp (T. Sumiyoshi), kasaik-tky@umin.net (K. Kasai).

[1] The AQ is a self-report questionnaire consisting of five domains of questions regarding the psychopathology of ASD: social skills, attention switching, attention to detail, communication, and imagination.

[2] The CARS is a behavior rating scale completed by clinician or parents based on subjective observation. The scale contains 15 items (e.g. relationship to people, imitation, and so on) and each item is rated with 1 (normal for child's age) to 4 (severely abnormal).

[3] This BAP scale covers three domains of autism, i.e. 'communication impairment', 'social dysfunction', and 'stereotyped and repetitive behavior'. Each domain includes items coded as either 'presence' or 'absence' of autistic symptoms. A subject would be classified as BAP if his/her total score of each domain exceeds the designated cut-off point.

[4] There were two outliers in the %PEM, deviating 2SD from the average of the ASD group. We re-analyzed the data excluding these deviations but the main results have remained the same.

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mation', i.e. the process of focusing and associating relevant information to form categories, appears to be worth investigating, as they are assumed to be pertinent to some cardinal traits, such as inflexible and perseverated behavior or restricted interests (Kenworthy et al., 2005).

The Wisconsin Card Sorting Test (WCST; Heaton et al., 1993) has been used to provide a good measure to evaluate the focusing process. This test consists of four stimulus types, and requires subjects to detect sorting principles from stimulus cards and categorize a response card. The task proceeds through the shifts of sorting principles, i.e. color, form and number. Successful performance on the WCST depends on the ability to detect the correct sorting principle on the basis of feedback, and maintain the principle until it is replaced by a new one.

From 1980s onward, more than 30 studies have been conducted to examine the WCST performance in individual with ASD. The majority of them have reported the degradation in performance on some measures of the task (generally, the number of categories achieved and perseverative errors. For review, Hill (2004), Pennington and Ozonoff (1996), for recent studies, Ambery et al., 2006; Geurts et al., 2004; Hill, 2004; Hill and Bird, 2006; Kaland et al., 2008b; Lopez et al., 2005; Pennington and Ozonoff, 1996; Sergeant et al., 2002; Voelbel et al., 2006; Winsler et al., 2007). Studies targeting adults with ASD, however, have been relatively limited (Ambery et al., 2006; Ciesielski and Harris, 1997; Lopez et al., 2005; Rumsey, 1985; Rumsey and Hamburger, 1988, 1990). Most of these investigations have reported poor achievement of the task in adults with ASD, as has been observed in the studies for younger samples. Ambery et al. (2006), for example, has reported that adults with ASD produced substantially greater perseverative errors, indicating that the ability to find and utilize relevant features of in-coming information may not have been well developed in people with ASD.

There is also paucity of information as to the ability to associate information for category formation in individuals with ASD, besides the findings from free-recall task (Tager-Flusberg, 1991) and the California Verbal Learning Task (CVLT; Delis et al., 1987). Both tasks are similar in that they require subjects to learn orally presented word lists and let them recognize the association of presented stimulus; the association process is evaluated to calculate the difference in the number of recalled words between related (members of a particular category) and unrelated word lists in the free-recall task, or by the number of voluntarily re-organized category-wise responses in the CVLT. Subjects with ASD have been reported to fail to achieve better in the related lists compared to the unrelated lists in the free-recall paradigm, unlike IQ-matched normal controls (Bowler et al., 1997). On the other hand, a study with the CVLT (Minshew and Goldstein, 1993) has not detected clear differences in the semantic cluster ratio, a measure of category formation, between subjects with ASD and normal controls.

Given the mixed results from these verbal learning tasks, more sensitive methods to evaluate the ability to associate information for categorization is needed. The Verbal Learning Task (VLT; Gold et al., 1992; Yamashita et al., 2000), which has been used in the studies of schizophrenia, would be appropriate for this purpose. The VLT consists of three types of word lists: the Random, Blocked, and Semi-blocked lists. The Random list consists of semantically unrelated nouns while the Blocked and Semi-blocked lists contain category exemplars. In the Blocked list, the words are presented on a category-basis, while the words of the same category are never presented consecutively in the Semi-blocked list. One of the strength of the VLT is the inclusion of the SCR (Stimulus Category Repetition; Bousfield and Bousfield, 1966), which quantifies the category-wised responses in the Semi-blocked list. With this measure, the process of category-formation is directly evaluated.

To determine if the deficits of organizing information, discussed above, are cognitive traits specific to ASD, i.e. a cognitive endophenotype, it would be worthwhile to investigate this cognitive ability in biological relatives. A number of studies have reported that several domains of cognitive function are, to some extent, disturbed in first-degree relatives of individuals with ASD (Bailey et al., 1998; Dorris et al., 2004): executive function (Delorme et al., 2007; Hughes et al., 1999; Kawakubo et al., 2009), central coherence (Baron-Cohen and Hammer, 1997; Baron-Cohen et al., 2006; de Jonge et al., 2006; Fombonne et al., 1997), and Theory of Mind (Smalley and Asarnow, 1990; Szatmari et al., 1993). As to the processes of organizing information, however, clear results have not been obtained. For instance, Ozonoff et al. (1993) has reported that sibling of ASD exhibited no distinct impairments in the overall performance on the WCST. On the other hand, studies using the intradimensional/extradimensional (ID/ED) set-shifting task, a categorization task akin to the WCST, have reported that parents (Hughes et al., 1997) and siblings (Hughes et al., 1999) of ASD probands performed poorly compared with those of typically-developed children. Apart from those contradicted findings for the ability to focus on relevant information, little is known about the process for category formation, at least under the verbal leaning task paradigm, in siblings of individuals with ASD.

The purposes of the current study were two-fold: first, the ability to organize information, specifically focusing and associating the relevant features to form categories, were investigated in subjects with ASD using the WCST and the VLT. The two tasks have been typically used as the measure for executive function (flexibility) and verbal learning (or working memory), respectively. The simultaneous implementation of these two tests, however, would be useful to evaluate the two processes of organizing of information; the percentage of perseverative errors of Milner (%PEM) in the WCST provide the index for the focusing process while the SCR in the VLT represents the one for the association process. Second, the possibility that the deficits of the two cognitive processes are ones of the cognitive markers, i.e. endophenotypes, of ASD was examined by administrating these tasks to siblings of subjects with ASD.

2. Method

2.1. Subjects

Twenty-two individuals with ASD (M/F = 19/3), 14 non-affected siblings (M/F = 8/6), and 15 age-matched normal controls (M/F = 11/4) entered the study. Male/female ratio was not significantly different among the groups ($\chi^2 = 4.01$, $df = 2$, *n.s.*). Subjects in the ASD group met DSM-IV criteria for autistic disorder ($N = 8$), Asperger disorder ($N = 12$) or pervasive developmental disorder not otherwise specified (PDD-NOS) ($N = 2$).

Individuals with ASD and their siblings were recruited from outpatient clinics in the following institutions: Departments of Neuropsychiatry and Child Psychiatry, University of Tokyo Hospital and Mie Prefectural Asunaro Hospital for Children and Adolescent Psychiatry. Participants from public symposia on ASD which took place at The University of Tokyo were also included. Healthy controls were mainly recruited from hospital staff members, their acquaintances and children, and college students. Exclusion criteria were neurological illness, traumatic brain injury with any known cognitive consequences and loss of consciousness for more than 5 min, a history of electroconvulsive therapy, and alcohol/substance abuse or addiction. Besides, based on the Structured Interview Schedule of DSM-IV Axis I Disorders Research Version Non-patient Edition (SCID-I/NP), normal controls were excluded if they or their first-degree relatives had a history of DSM-IV axis I disorders. IQs were evaluated with the WISC-III or WAIS-R (IQ range: normal controls; 87–120, siblings; 90–118, ASD; 58–114). The full

Table 1
Demographic and clinical data.

	Controls	Siblings	ASD[a]
Male/female	11/4	8/6	19/3
Age	29.7(6.4)	24.5 (4.0)	26.5(7.4)
Education	15.9(2.1)	15.3(1.6)	12.5(1.8)**
CARS	–	15.6(0.8)	31.2 (6.1)‡
AQ-J	16.3 (5.6)	22.7 (5.52)	30.6 (8.6)†
Medication (mg/day)[b]	–	–	220.8 (347.7)
IQ[c]	99.7(8.6)	101.2(10.4)	94.1(17.8)

Note: ASD, autism spectrum disorders; CARS, Childhood Autism Rating Scale; AQ-J, Autism-Spectrum Quotient Japanese version.

[a] Missing data in education = 7, CARS = 1, AQ-J = 10.
[b] Chlorpromazine equivalent dose.
[c] Estimated IQ, controls and siblings; Full IQ, ASD.
** $p < 0.01$; compared to controls and siblings.
‡ $p < 0.01$; compared to siblings.
† $p < 0.05$; compared to siblings.

version was administered to subjects with ASD and siblings while the abbreviated one (i.e. Information, Similarities, Picture Completion, and Digit Symbol-Coding) was applied to normal controls. Although 4 subjects with ASD had IQs of less than 70, they were included in the study as they had completed at least high school education (i.e. more than 12 years). In fact, the directions of principal results, presented later, did not change even if data from those cases were excluded. The Ethical Committee of The University of Tokyo Hospital approved this study (receipt No. 630-5). The Mie Prefectural Asunaro Hospital for Children and Adolescent Psychiatry delegated the ethical review to the ethical committee of The University of Tokyo Hospital because they did not have an institutional review board. Written informed consent was obtained from all participants.

The Autism-Spectrum Quotient Japanese version (AQ-J; Kurita and Koyama, 2006)[5] was administered to probands, siblings, and normal controls. In addition, subjects with ASD and siblings were assessed by the Childhood Autism Rating Scale-Tokyo Version (CARS-TV; Kurita et al., 1989)[6] by trained child psychiatrists. One-way ANOVA for AQ-J yielded a significant group difference ($F = 9.65$, $df = 2, 25$, $p < 0.01$). Multiple comparisons with the Tukey method revealed that the ASD group elicited a significantly higher score than other two groups ($p < 0.05$). As to CARS, t-test revealed a significantly higher score for the ASD group compared to siblings ($t = 9.23$, $df = 17$, $p < 0.01$). These results indicate that the ASD group and other two groups were clinically independent. In fact, no siblings were found to elicit autistic features as evaluated by the Broader Autism Phenotype (Le Couteur et al., 1996).[7] All but one on the ASD subjects received medication, with eight of them treated with antipsychotics (risperidone = 4; pimoizide = 2; haloperidol = 2). Other medications included mood stabilizers (e.g. valproate = 6, lithium = 3), benzodiazepines (e.g. bromazepam = 3, nitrazepam = 2, triazolam = 2), anti-depressants (e.g. fluvoxamine = 4, paroxetine = 3), and anti-parkinson drugs (e.g. biperiden = 5, trihexyphenidyl = 2). Demographic and clinical profiles of participants are summarized in Table 1.

[5] The AQ is a self-report questionnaire consisting of five domains of questions regarding the psychopathology of ASD: social skills, attention switching, attention to detail, communication, and imagination.

[6] The CARS is a behavior rating scale completed by clinician or parents based on subjective observation. The scale contains 15 items (e.g. relationship to people, imitation, and so on) and each item is rated with 1 (normal for child's age) to 4 (severely abnormal).

[7] This BAP scale covers three domains of autism, i.e. 'communication impairment', 'social dysfunction', and 'stereotyped and repetitive behavior'. Each domain includes items coded as either 'presence' or 'absence' of autistic symptoms. A subject would be classified as BAP if his/her total score of each domain exceeds the designated cut-off point.

2.2. Design and procedure

WCST. A computerized version of the WCST (WCST-64: Computer Version-2 Research Edition, Psychological Assessment Resources, Inc.) was used. Subjects were requested to sort cards according to one of the implicit principles (i.e. color, shape, and number), which is altered after 10 consecutive correct responses. A test session was terminated when 6 shifts had been completed. Three variables, (1) number of categories achieved (CA), (2) the percentage of perseverative errors of Milner (%PEM), and (3) reaction time (RT), were analyzed.

VLT. The Japanese version of the VLT (JVLT; Yamashita et al., 2000) was used. This task consists of three 16-word lists: Random list, Blocked list, and Semi-blocked list. The Random list consists of 16 unrelated nouns. Other two lists contain four exemplars from four taxonomic categories (the Blocked list: stationery, animal, musical instruments, and sports; the Semi-blocked list: vehicles, seasoning, flower, and countries). In the Blocked list, exemplars in the same category were presented consecutively, while they were never given serially in the Semi-blocked list. Thus, in the Semi-blocked list, the list items would be re-organized in a category-wise manner, if a subject voluntary formed categories to be utilized as recalling cues.

Three trials were conducted and each trial included the Random, Blocked, and Semi-blocked lists. The lists were presented in the fixed order of the Random, Blocked, and Semi-blocked lists. Every word in the lists was presented orally in 1 s basis, and subjects were instructed to learn them. The VLT scores were calculated for each type of list as the average of the three trials. In addition, for the Semi-blocked lists, Stimulus Category Repetition (SCR; Bousfield and Bousfield, 1966) was calculated. SCR is defined as the total number of exemplars in the same category consecutively recalled in each trial. Thus, it was considered to be an index of the degree to which a subject associates information based on implicitly given categories (Koh et al., 1976).

2.3. Statistical analyses

Multivariate analysis of variance (MANOVA) was conducted to examine group differences for demographic variables (age, education, IQ) and three measures of WCST (CA, %PEM, RT). The arcsine transformation and logarithmic transformation were applied for %PEM and RT, respectively. The VLT scores were analyzed by three-way ANOVA with Group (normal controls vs. siblings vs. ASD) as between-subject factor, and Block (Random vs. Blocked vs. Semi-blocked) and Trial (1st vs. 2nd vs. 3rd) as within-subjects factor. The SCR scores were examined by two-way ANOVA, with Group (normal controls vs. siblings vs. ASD) as between-subjects factor and Trial (1st vs. 2nd vs. 3rd) as within-subjects factor. The correlation analyses were conducted between the CARS Total scores and the measures of the WCST and VLT to examine the relationship between the severity of symptoms and cognitive performances.

3. Results

3.1. Demographic variables

Demographic and clinical data are shown in Table 1. MANOVA indicated an overall difference among the three groups (*Wilks' lambda* = 0.52, $F = 4.45$, $df = 6, 68$, $p < 0.01$). Subsequent univariate analyses revealed that the ASD group showed a significantly shorter education period than did other two groups ($F = 13.22$, $df = 2, 39$, $p < 0.01$), while Age ($F = 2.26$, $df = 2, 48$, *n.s.*) and IQ ($F = 2.29$, $df = 2, 44$, *n.s.*) did not differ among the groups.

Table 2
Performance on the Wisconsin Card Sorting Test (WCST) and Verbal Learning Task (VLT).

	Controls	Siblings	ASD
WCST			
CA	6.0(1.7)	5.9(0.5)	4.2 (2.2)
%PEM	9.9 (4.5)	12.1 (9.2)	24.1 (19.5)
RT (min)	1.6(0.3)	1.8(0.8)	2.1(1.1)
JVLT			
Random	9.9(3.6)	10.1 (3.0)	8.6(3.6)
Semi-blocked	11.8(3.3)	12.9 (2.7)	10.0 (4.0)
Blocked	13.8 (2.9)	13.8(2.5)	11.2(4.3)
SCR1	3.0(2.4)	4.6 (2.8)	3.5 (3.2)
SCR2	6.7 (4.3)	7.6(3.8)	5.8 (4.0)
SCR3	8.9(4.3)	8.9(4.5)	5.9 (4.8)

Note: ASD, autism spectrum disorders; WCST, Wisconsin Card Sorting Test; JVLT, Japanese Verbal Learning Task; CA, categories achieved; %PEM, percentages of Milnere-type perseverative errors; RT, reaction time; SCR, stimulus category repetition.

3.2. Performance on the WCST

Mean and SD for variables of the WCST are summarized in Table 2. MANOVA demonstrated significant overall group difference (*Wilks' lambda* = 0.71, $F = 2.70$, $df = 6, 86$, $p < 0.05$). Subsequent univariate analyses revealed main effects of CA ($F = 8.31$, $df = 2, 45$, $p < 0.01$) and %PEM ($F = 5.20$, $df = 2, 45$, $p < 0.01$),[8] but not RT ($F = 0.10$, $df = 2, 45$, *n.s.*). Multiple comparisons showed a significantly lower CA score for the ASD group compared to other two groups ($p < 0.01$). As for the %PEM, the difference was found only between the ASD group and normal controls. Siblings did not differ from either group.

3.3. Performance on the VLT

Mean and SD for variables of the VLT are shown in Table 2. ANOVA showed a significant Block × Group interaction effect ($F = 2.54$, $df = 4, 96$, $p < 0.05$) on the VLT scores. Simple main effects of Group were significant for the Blocked ($F = 4.137$, $df = 2, 144$, $p < 0.05$) and Semi-blocked ($F = 5.87$, $df = 2, 144$, $p < 0.01$), but not the Random ($F = 1.72$, $df = 2, 144$, *n.s.*) lists condition. Multiple comparisons yield significant differences in the Blocked lists condition in the order of controls = siblings > ASD ($p < 0.05$). For the Semi-blocked lists condition, on the other hand, the difference was only found in siblings > ASD ($p < 0.01$).

ANOVA revealed a significant Trial × Group interaction effect ($F = 3.14$, $df = 4, 86$ $p < 0.05$) on the SCR score. Therefore, simple main effect for the Trial condition was examined, which yielded significant results for all groups (normal controls: $F = 49.86$, $df = 2, 129$, $p < 0.01$; siblings; $F = 27.23$, $df = 2, 129$, $p < 0.01$; ASD; $F = 10.22$, $df = 2, 129$, $p < 0.01$). Multiple comparisons revealed a stepwise enhancement on the SCR score (3rd trial > 2nd trial > 1st trial) in normal controls ($p < 0.01$), while the difference was detected only between the first two trials (2nd trial > 1st trial) in siblings ($p < 0.01$) and ASD patients ($p < 0.01$).

3.4. Correlation analyses

No measures in the WCST were significantly correlated with the CARS total score (CA: $r = -0.34$, $df = 19$, *n.s.*; %PEM: $r = -0.12$; RT: $r = 0.15$ $df = 18$, *n.s.*). The same result was obtained in the VLT (Random: $r = -2.66$; Blocked: $r = -0.14$; Semi-blocked: $r = -0.21$, $df = 20$, *n.s.*; SCR1: $r = -0.06$; SCR2: $r = -0.23$; SCR3: $r = -0.16$, $df = 15$, *n.s.*).

[8] There were two outliers in the %PEM, deviating 2SD from the average of the ASD group. We re-analyzed the data excluding these deviations but the main results have remained the same.

4. Discussion

The purpose of the current study was to investigate the ability to organize information in individuals with ASD and their siblings. Specifically, the two cognitive processes in that ability, i.e. focusing on relevant features and associating them to form categories, were examined using the WCST and VLT.

Overall, individuals with ASD performed poorly on the WCST and VLT, as shown by the significantly worse scores on most measures of the tasks (Table 3). Specifically, greater %PEM in the ASD group is in accordance with a previous study (Kenworthy et al., 2005), representing the poor ability to focus on the relevant information in subjects with ASD using other tasks (i.e. the Object Assembly, the Rey-Osterrieth Complex Figure, the Story Sentence Memory, and letter fluency with "F, A, S". Similarly, the lack of an increase in the SCR supports the result from the free-recall task (Bowler et al., 1997), reporting the failure to associate category members in subjects with ASD. Both %PEM and the SCR scores did not correlate with the CARS score in the ASD group. Thus, it is possible that degradation in the cognitive process, such as focusing and associating relevant information, is intrinsic to the symptomatology of ASD irrespective of severity of the illness.

The poor cognitive performance in subjects with ASD cannot be attributed entirely to their shorter education period. First, the IQ level in subjects with ASD on the whole was not significantly different from those in siblings and normal controls, as shown by the analysis of the demographic variables. In addition, the RT in the WCST and performance on the Random List condition in the VLT were equivalent to those for normal controls and siblings. In all, these results suggest that general intelligence, visuo-motor skills, attention, and memory capacity, were relatively uniform among the three groups studied here.

Generally, the sibling group performed almost as equally well as the normal control group; however, there were some noticeable similarities between siblings and subjects with ASD (Table 3, highlighted in bold). First, siblings have failed to show the linear increase in the SCR score in the VLT. Second, the %PEM for siblings was not significantly smaller than that for the ASD group, although it did not differ from that of normal controls, either (Table 3). This result is worth noting as siblings performed better than individuals with ASD on other measures, such as the CA. In all, the results from the two tasks are likely to reflect the limited ability of siblings to focus and conceptualize categories using updated information.

The tendency of 'weak central coherence' may explain the current results for the subjects with ASD. 'Central coherence' is a cognitive trait to extract meaning, gist, and gestalt from given stimuli (Frith and Happe, 1994). Impairment of this ability, or 'weak central coherence', leads to an inclination to focus on individual parts of information without integrating them (Happe and Frith, 2006). This may be related to increased perseverative responses in subjects with ASD; once their attention has been captured by a particular dimension of features, shifting it according to negative feedback would become difficult.

The weak central coherence trait also seems to be related to higher cognitive function such as category formation and organization (Plaisted, 2001); in order to form a category, it is necessary to extract a commonality from each piece of information and ignore idiosyncratic features. This cognitive process may be disturbed in people with ASD, leading to the restriction in forming categories. The lack of an increase in the SCR scores may be accounted for by this cognitive deficit.

Interestingly, the tendency related to weak central coherence has also been reported in first-degree relatives of patients with ASD, especially their fathers (Happe et al., 2001). This seems to be consistent with our observations that siblings of subjects with ASD failed to show a steady increase of the SCR in the VLT, suggesting

Table 3
Summary of performances on the WCST and JVLT.

WCST		JVLT			
		SCORE		SCR	
CA	Controls = Siblings > ASD	**Blocked**	Controls = Siblings > ASD	**Normal controls**	SCR1 > SCR2 > SCR3
%PEM	Controls < ASD	**Semi-**	Siblings > ASD	**ASD**	**SCR1 = SCR2 > SCR3**
	Controls = siblings	**blocked**	Controls = Siblings		
	Siblings = ASD				
RT	Controls = Siblings = ASD	**Random**	Controls = Siblings = ASD	**Siblings**	**SCR1 = SCR2 > SCR3**

Note: ASD, autism spectrum disorders WCST; Wisconsin Card Sorting Test; JVLT, Japanese Verbal Learning Task; SCR, stimulus category repetition.

that degradation in the ability to associate information is one of the cognitive markers, i.e. endophenotypes for people vulnerable to ASD.

It is hypothesized that attenuated neural activities in parahippocampal regions may be responsible for the difficulties in organizing information in subjects with ASD. The left parahippocampal regions, including parahippocampal gyrus, has been suggested to play an important role in sorting, relating, and sending information to hippocampus (Eichenbaum, 1997; Sumiyoshi et al., 2006; Vargha-Khadem et al., 1997). Thus, dysfunction of these regions is assumed to impair the ability to systemize incoming information. In fact, several idiosyncrasies around parahippocampal regions have been reported in individuals with ASD. For example, Boucher et al. (2005) found a smaller parahippocampal volume in patients with ASD compared to normal controls, as well as weaker correlational activities between this region and the hippocampal region.

A key issue for future research is the developmental course of the knowledge structure, that is, semantic association in the long-term memory, in individuals with ASD. Given that the ability to organize information is impaired, knowledge structure would suffer from insufficient maturation in subjects with ASD, as has been reported in children with William's syndrome (Johnson and Carey, 1998). Although several attempts have been already made to address this issue (Dunn et al., 1996), further research is expected to clarify the link between the limited ability to organize information and the development of knowledge structure in individuals with ASD and those who are vulnerable to the disorder.

Finally, several limitations for the present study should be mentioned. First, it would be worthwhile to relate some cognitive measures studied here, e.g. %PEM and SCR, to endophenotypes of ASD in replication studies with a larger sample of ASD probands and their siblings. Second, although clinical variables such as intelligence did not significantly affect current findings, evaluation with more adjusted samples would be desirable in future studies.

5. Conclusion

The current study has identified the limited ability to organize information in individuals with ASD, extending the previous observations that they performed poorly on most of the measures of the WCST and verbal learning compared with normal controls. In addition, their siblings showed similar impairments on the measures of organizing of information derived from these tasks, suggesting that the deficits of this cognitive ability is one of the cognitive endophenotypes of ASD.

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