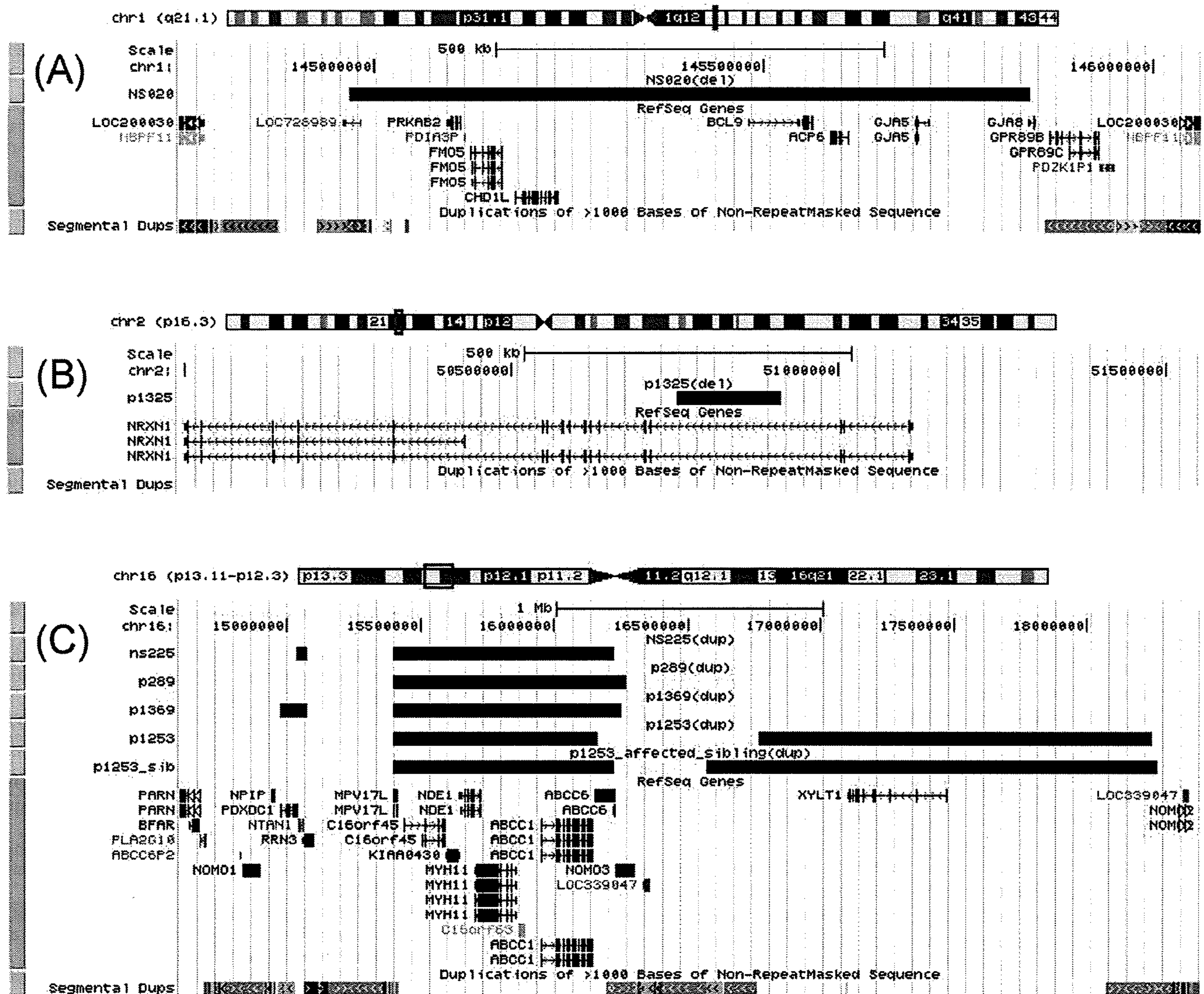

Figure 1. Positions of CNVs according to the validation experiments. CNV validation was undertaken using Illumina HumanHap 660W quad bead arrays (for CNVs at 1q21.1 and *NRXN1*) or 610-quad bead arrays (for CNVs at 16p13.1). Figures are produced on the UCSC Genome Browser according to NCBI Build 36.1, March 2006, hg18 (http://www.genome.ucsc.edu/) and indicate the positions of the CNVs: **(A)** 1q21.1; **(B)** *NRXN1*; and **(C)** 16p13.1: the last trace is that of the affected sibling of "p1253." CNV, copy number variation; NCBI, National Center for Biotechnology Information; UCSC, University of California, Santa Cruz.

implicated CNV risk factors for schizophrenia (4,8). Among large duplications, the most notable is that on 16p13.1, which was found in four cases and one control subject, while one more control subject had the reciprocal deletion (Fisher exact test $p = .19$, one-tailed). These CNVs in cases were confirmed using Illumina arrays (Figure S1 and Tables S1 and S2 in Supplement 2). One of the patients with 16p13.1 duplication had an affected sibling and unaffected mother who had also provided DNA. The duplication was found in the affected sibling but not the unaffected mother (DNA from the father was not available and there is no indication that he suffers with mental illness). The duplication in this family extends further on the centromeric side compared with the region usually included in CNVs of this region (Figure 1).

Of the remaining susceptibility loci reported in the recent studies (4,7,8), we found no deletions at 22q11.2 or 15q13.3. We also find no support for the 15q11.2 locus, where three deletions were found in control subjects and only one in a case (Fisher exact test $p = .37$, two-tailed, a trend in the opposite direction).

We also searched for CNVs that intersected genes and were present only in cases, reasoning as have others (2,3) that such CNVs are good candidates (Tables S3 and S4 in Supplement 2). One of the singleton deletions was in *NRXN1*, a gene implicated in previous studies (2,6,7,9,10) (Figure S1 and Table S2 in Supplement 2). Several more contain intriguing candidate genes (e.g., deletions in *PARK2*, *GRIK2*, *MAGEL2*, and *ATXN2L* and duplications in *CHRNA7* and *NRG4*), which have been implicated in neurodegenerative disorders or have possible functional relevance for neurodevelopment.

Discussion

In this study, we do not find a significant increase in the burden of CNVs in schizophrenia, either overall or for any

specific size range of CNVs, as proposed in previous studies (2–4,7). We did, however, find several trends in the same direction and of a similar magnitude as the largest global CNV survey of schizophrenia (4). Not all research has found such an increased burden, e.g., no evidence was obtained from a study in the Chinese population (5). It is possible that genuine population differences might drive this discrepancy between Caucasian and Asian samples, as might our exclusion of subjects with mental retardation or epilepsy. Sample size could also have played a role. Our sample had a modest power of ~.65 to detect a single CNV in a case for the following very strong candidate loci: 1q21.1, 15q13.3, and 22q11.2 and *NRXN1*, where approximately .2% of affected persons have deletions. In fact, we did find one deletion each in two of these loci (1q21.1 and *NRXN1*).

We found stronger support for association with duplications at 16p13.1, which contain the candidate gene *NDE1*. It is within the interval duplicated in all patients (Figure 1). Deletions and duplications of this region were implicated in autism (13) and schizophrenia (7), while deletions have been implicated in mental retardation (14). The most recent study surveying children with unexplained intellectual disability also reported significant association for both deletions and duplications at this locus ($p = 4.7 \times 10^{-5}$) (15), suggesting that this duplication is also pathogenic for a broad range of neuropsychiatric disorders. Our result for an excess of duplications in schizophrenic probands does not reach statistical significance; however, the frequency of the duplication is fourfold higher in cases than in control subjects (.8% vs. .2%), which is very similar to the rate found in our previous study from the United Kingdom (.6% vs. .2%) (7) and in the ISC study (.4% vs. .2%) (4). We found an identical duplication in an affected sibling. Larger CNVs in this locus, as in one of our probands, were also found in three cases and two control subjects in the ISC (4). The four probands in our study who carry 16p13.1 duplications do not appear to share any specific clinical features (Table S2 in Supplement 2).

We also found one deletion in a case at 1q21.1 and *NRXN1* and none in control subjects, which is close to the reported frequency of .2% in cases. Unlike those deletions of *NRXN1* that were associated with schizophrenia in a previous study (10), the CNV reported here does not intersect exons (10). However, it is large compared with most exon-sparing deletions reported in control subjects (10), and a new reanalysis of all *NRXN1* deletions shows that large (>100 kb) deletions in this gene might be almost as relevant as those affecting exons (16). The relevance to schizophrenia of the other CNVs found only in cases can only be assessed in future meta-analyses of such studies, but we note here that the three deletions we found in *PARK2* are of particular interest, as they have been implicated as a susceptibility factor for autism (17).

In summary, we provide support for the role of CNVs at 16p13.1, 1q21.1, and *NRXN1* in the etiology of schizophrenia. Although we find similar, but not significant, trends for an increased overall burden of CNVs, as well as for the involvement of duplications in the 100 kb to 200 kb range as proposed in the ICS study (4), in this population we could not find an increased burden of very large CNVs (>500 kb) in schizophrenia, which has been the main finding in recent studies (4,7). The discrepancy with previous studies could be due to our exclusion of patients with neurodevelopmental disorders, epilepsy, or known mental retardation, as such features are found in many of the carriers of large CNVs, e.g., 15q13.3 (15). Given the rarity of the CNVs that have been implicated so far in schizophrenia, there is a need for more large studies, studies in non-European populations, and meta-analyses.

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ORIGINAL INVESTIGATION

Effect of aripiprazole, risperidone, and olanzapine on the acoustic startle response in Japanese chronic schizophrenia

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Abstract

Background Studies have also shown that differences in the kind of the antipsychotics influenced disruption of the sensorimotor gating system, including prepulse inhibition (PPI), acoustic startle reflex (ASR), and habituation (HAB). We investigated the influence on startle response in chronic schizophrenia in 20 patients with schizophrenia taking risperidone, 21 patients with schizophrenia taking olanzapine, and 20 patients with schizophrenia taking aripiprazole.

Method The patients who participated in this study were on maintenance therapy with only one antipsychotic drug for 4 months. We performed the test for the association between all PPI measures (ASR, HAB, and PPI at prepulse sound pressure intensities of 82, 86, and 90 dB) and each the risperidene, olanzapine, and aripiprazole groups, with analysis of covariance (ANCOVA; using age, duration of illness, and daily dose of the antipsychotic as covariates). Also, when significant difference was detected in ANCOVA, the differences of PPI measures between every pairs of two drug groups were tested as a post hoc analysis with the use of *t* test and Bonferroni's correction of multiple tests.

Result We found that PPI90 showed significant differences with ANCOVA among patients with schizophrenia taking each of the antipsychotics. When we performed a post hoc analysis for PPI90, the value was higher in the aripiprazole group than in the olanzapine group and higher in the risperidone group than in the olanzapine group.

Conclusion Aripiprazole and risperidone may improve PPI90. ASR, HAB, PPI82, and PPI86 were no different among the Japanese schizophrenic patient groups with different antipsychotics.

Keywords Acoustic startle response · Risperidone · Aripiprazole · Olanzapine · Schizophrenia · Prepulse inhibition · Antipsychotic

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Abbreviations

PPI	Prepulse inhibition
ASR	Acoustic startle reflex
HAB	Habituation
OLZ	Olazapine
RIS	Risperidone
HPD	Haloperidol
ARP	Aripiprazole
SD	Standard deviation
SCID-1	Structured Clinical Interview for DSM-IV disorders
LI	Lead interval
PA trial	Pulse alone trials
PP trials	Trials of pulse with prepulse
ANOVA	Analysis of variance

ANCOVA	Analysis of covariance
5-HT1A receptor	Serotonin 1A receptor
5-HT1B receptor	Serotonin 1B receptor
α2c receptor	Adrenergic alpha2c receptor
MDMA	Methylenedioxy-substituted phenalkylamines 3,4-methylenedioxy-*N*-methylamphetamine
M5 receptors	Muscarinic acetylcholine 5 receptors

Introduction

Disruption of the sensorimotor gating system, including prepulse inhibition (PPI) deficit, the acoustic startle reflex (ASR), and habituation (HAB), is suggested to be one of involved in the pathophysiology of schizophrenia (Braff et al. 1992; Kunugi et al. 2007; Takahashi et al. 2008; Walters and Owen 2007). These abnormalities are also considered to be endophenotypes for schizophrenia (Braff et al. 1992; Kunugi et al. 2007; Takahashi et al. 2008; Walters and Owen 2007). Recently, we detected significant differences in ASR, HAB, and each PPI (82, 86, and 90 dB) between patients with schizophrenia and controls (Moriwaki et al. 2009). Several investigations have reported that the ASR and PPI are influenced by factors such as gender and smoking state (Kumari et al. 1996, 1997, 2004; Swerdlow et al. 1999). However, we found no correlation between gender or current smoking state and ASR, HAB, or any PPI in a multiple regression analysis (Moriwaki et al. 2009).

It has also been reported that several measures of the startle response were influenced by the kind of antipsychotic. Wynn and colleagues also reported that olazapine (OLZ) improved the startle acoustic response significantly compared with risperidone (RIS) and haloperidol (HPD) in people with schizophrenia (Wynn et al. 2007). The antipsychotic aripiprazole (ARP) is known to be a dopamine system stabilizer, the pharmacological mechanism of which is a unique partial agonistic action on dopamine 2 receptors (Burris et al. 2002; Jordan et al. 2002). To our knowledge, there is no investigation on the influence ARP in the startle response in people with schizophrenia. Therefore, we investigated whether some antipsychotics, including ARP, RIS, and OLZ, influenced the startle response in chronic schizophrenia.

Materials and methods

Subjects

One hundred forty-one patients with schizophrenia (91 males and 50 females: mean age standard deviation (SD) 49.8±15.6 years) were recruited. This study increased 26 patients compared with our previous study (Moriwaki et al. 2009). However, because 17 patients were nonresponders, these patients excluded the further analysis. Detailed information about exclusion criteria can be seen in the paper of Takahashi et al. (2008). Among 124 patients, 71 patients were taken monotherapy antipsychotics. Also, 53 patients were taken polytherapy antipsychotics. All subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of a structured interview using the Structured Clinical Interview for DSM-IV disorders (SCID-1) and a review of medical records. All met the following inclusion criteria: (1) age 25 to 70 years, (2) no systemic or neurologic disease, (3) no electroconvulsive therapy, (4) no history of head trauma, (5) no lifetime history of substance dependence or history of substance abuse within 3 months, and (6) use of only one antipsychotic drug therapy for 4 months. All patients were hospitalized at the time of measurement. The study was described to subjects, and written informed consent was obtained from each. This study was approved by the ethics committees at Fujita Health University School of Medicine and Okehazama Hospital.

Startle response measurement

Apparatus and stimuli

We measured startle response using a commercial computerized human startle response monitoring system (Startle Eyeblink Reflex Analysis System Map1155SYS, Nihonsanteku Co., Osaka, Japan). Startle eyeblink electromyographic responses were recorded from the left orbicularis oculi muscle with two Ag/AgCl disposable electrodes (sensor area 15 mm^2), filled with wet gel. The first electrode (Blue Sensor N-00-S, Ambu, Ballerup, Denmark) was positioned approximately 1 cm directly below the pupil of the left eye and low enough to not touch the lower eyelid, while the second electrode (Blue Sensor M-00-S, Ambu, Ballerup, Denmark) was placed laterally and slightly superior to the first one, with the centers of the electrodes separated by approximately 2 cm. The impedance between the two electrodes was measured and deemed acceptable if below 5 kΩ. The impedance was measured with an electrode impedance meter (MaP811, Nihonsanteku Co., Osaka, Japan) at a measurement frequency of 30 Hz. The ground electrode (Blue Sensor M-00-S) was placed on the left angle of the mandible. We used a method same as in the study of Takahashi and colleagues (Takahashi et al. 2008). Detailed information can be seen in their paper.

The stimulus sequence, procedure, and response scoring and data reduction

Measurements were made with startle paradigm constructed of three blocks with continuous background white noise of 70 dB SPL. Pulse stimuli consisted of broadband white noises with an instantaneous rise/fall time lasting for 40 ms and presented at 115 dB SPL. Prepulse stimuli were also broadband white noises with an instantaneous rise/fall time lasting for 20 ms, presented at three different intensities (82, 86, and 90 dB SPL). The LI (from prepulse onset to pulse onset) in our study was set at 120 ms. In block 1, the startle response for pulse alone trials (PA trial) was recorded six times. Block 2 consisted of PA trials or trials of pulse with prepulse at three intensities (PP trials), performed eight times for each condition. Block 3 was the same as block 1, to measure habituation. All trials were presented in a fixed pseudorandom order, separated by inter-trial intervals of 15–25 s (20 s on average). The startle paradigm consisted of a total of 44 trials. Together with 5 min acclimation to the background noise, the session lasted approximately 20 min. We used the same method as Takahashi and colleagues (2008). Detailed information can be seen in their paper.

Statistical analysis

The numbers of patients, who took monotherapy antipsychotics, were 20 in the RIS group, 21 in the OLZ group, 20 in the ARP group, four in the quetiapine group, one in the perospirone group, two in the blonanserin group, two in the HPD group, and one in the timiperone group. Because our samples of quetiapine, perospirone, blonanserin, HPD, and timiperone group were small, we included only the RIS, OLZ, and ARP groups in this study. All demographic data were analyzed by one-way analysis of variance (ANOVA). As described in the "Results" section, the mean age of ARP group was youngest among three drug groups; meanwhile, the duration of illness was shortest, and the daily dose of the antipsychotic was the largest in ARP group. So we performed analyses of covariance (ANCOVA) for comparing the PPI measures among the three drug groups, using the above three parameters as covariates to adjust possible confounding. When significant difference was detected in ANCOVA, the differences of PPI measures between every pairs of two drug groups were tested as a post hoc analysis with the use of t test and Bonferroni's correction of multiple tests. The significance level for all statistical tests was 0.05.

All statistical analyses were performed using SPSS (SPSS 12.0, SPSS Japan Inc., Tokyo, Japan).

Results

The mean age of ARP group was youngest among three drug groups; meanwhile, the duration of illness was shortest, and the daily dose of the antipsychotic was the largest in ARP group. Although one-way ANOVA did not show significant difference as to these three parameters among the three drug groups (Table 1), we conducted ANCOVA for comparing the PPI measures among three drug groups, using the above three parameters as covariates to adjust possible confounding. We found that PPI90 were significantly different among the ARP, RIS, and OLZ groups with the use of ANCOVA (P=0.0320; Table 2). We then performed a post hoc analysis of PPI90 and found that the ARP group had higher PPI90 than the OLZ group (P= 0.0178; Fig. 1). Also, PPI90 was higher in the RIS group than in the OLZ groups (P=0.0368; Fig. 1).

Discussion

We first investigated whether ARP influenced the startle response. Also, because the participating patients took only one antipsychotic, we considered the effect of each antipsychotic on acoustic startle response in people with

Table 1 Schizophrenic patients' demographics and disposition

	Aripiprazole	Risperidone	Olanzapine	P value
N	20	20	21	
Sex (males/females)	14/6	10/10	13/8	0.231
Age, years (mean±SD)	46.7±17.1	56.9±16.8	51.0±13.9	0.140
Current smoker/non-smoker, n (%)	8 (40.0%)	7 (47.6%)	10 (35.0%)	0.709
Clinical diagnosis, n (Dis/Res/Par)	2/14/4	1/15/4	0/17/4	0.551
Duration of illness (day, mean ± SD)	8,070±547	10,500±435	9,790±391	0.235
PANSS total score	84.7±20.6	76.3±19.5	76.3±19.5	0.220
Antipsychotics (mg/day)[a]	626±119	505±188	599±245	0.156
Anxiolytics/hypnoticse, n (%) (mg/day)[b]	10.3±12.2	12.1±10.5	7.56±6.46	0.337

Dis disorganized type, *Res* residual type, *Par* paranoid type

[a] Chlorpromazine-equivalent

[b] Diazepam-equivalent

Table 2 ANCOVA of startle measure with three antipsychotics groups

Startle measure	Aripiprazole	Risperidone	Olanzapine	*P* value[a]
ASR (μV, mean ± SD)	162±160	74.9±72.3	70.3±30.1	0.108
HAB (%, mean ± SD)	23.3±27.1	24.0±28.3	29.6±25.6	0.103
PPI82 (%, mean ± SD)	32.0±25.7	18.2±16.9	22.6±17.5	0.171
PPI86 (%, mean ± SD)	41.6±25.7	29.7±24.0	23.7±16.0	0.0685
PPI90 (%, mean ± SD)	39.9±31.1	34.9±22.9	21.1±15.9	**0.0320**

Bold represents significant *P* value

ASR acoustic startle reflex, *HAB* habituation, *PPI* prepulse inhibition

[a] Calculated by ANCOVA. *P* values were obtained by ANCOVA using age, duration of illness, and daily antipsychotic dose

schizophrenia directly. In this study, the ARP group had higher scores than the OLZ group in PPI90. Also, the RIS group showed significant difference than the OLZ group in PPI90. We found that PPI86 showed marginal difference among RIS, OLZ, and ARP groups with the use of ANCOVA (P=0.0685). When we performed a post hoc analysis for PPI86, the value was higher in the ARP group than in the OLZ groups (P=0.0634). From this result, we considered that ARP and RIS may improve abnormalities in PPI. We found large differences in the pharmacological profiles of ARP, RIS, and OLZ as follows: first, ARP has stronger affinity with the serotonin 1A (5-HT1A) receptor than RIS and OLZ (Burris et al. 2002; Jordan et al. 2002; Roth et al. 2004). Gogos and colleagues reported that the activation of 5-HT1A receptors increased PPI (Gogos et al. 2008). Also, RIS has stronger affinity with the serotonin 1B (5-HT1B) receptor, serotonin 7 receptor, adrenergic alpha2c (α2c) receptor, adrenergic alpha1a receptor, and adrenergic alpha1b receptors than ARP and OLZ (Roth et al. 2004). Dulawa and colleagues reported that a5-HT1B receptors have relation with ASR, HAB, and PPI in mice (Dulawa et al. 1997, 1998). RU24969, which is 5-HT1A/1B agonist, reduced PPI in WT mice (Shanahan et al. 2009). Dulawa and colleagues reported that the methylenedioxy-substituted phenalkylamines 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) increase PPI in 5-HT1B knockout mice, but not WT mice (Dulawa et al. 2000). These authors suggested that the activation of 5-HT1B receptors by 5-HT decreases PPI (Dulawa et al. 2000). Also, the adrenergic α2c receptor knockout mice showed increased ASR reduced PPI (Sallinen et al. 1998). On the other hand, OLZ has stronger affinity with serotonin 6 receptor, hisitamine 1 receptor, and muscarinic acetylcholine 5 (M5) receptors than RIS and ARP (Roth et al. 2004). Several animal studies using the M5 receptor knockout mice have shown significantly decreased PPI compared to wild-type (Thomsen et al. 2007); however, results have been rather inconsistent (Wang et al. 2004). Second, the antipsychotic aripiprazole is known to be a dopamine system stabilizer, the pharmacological mechanism of which is a unique partial agonistic action on dopamine 2 receptors. Several animal studies using mice reported that ARP restored the abnormalities in the PPI induced by apomorphine (Auclair et al. 2006; Nakai et al. 2008; Nordquist et al. 2008). These mechanisms may be involved in the different effects seen among the antipsychotic groups. However, this has, in seeming contrast to findings by Wynn and colleagues (2007), who reported that OLZ improved the startle acoustic response significantly, compared with RIS in people with schizophrenia. We consider that a replication study using larger samples or samples of other populations will be required for conclusive results.

Our previous study reported significant differences in ASR, HAB, and each PPI (82, 86, and 90 dB) between 115 patients with schizophrenia and 111 controls in multiple regression analysis (there were 15 nonresponders in the patient group and four in the control group; therefore, we performed the analysis of startle measure with 100 patients and 107 controls) (Moriwaki et al. 2009). When all PPI measures (except HAB) in the ARP group were compared with our 107 healthy control subjects with the use of ANCOVA (using age and sex as covariates), there was no statistical difference between the ARP group and controls

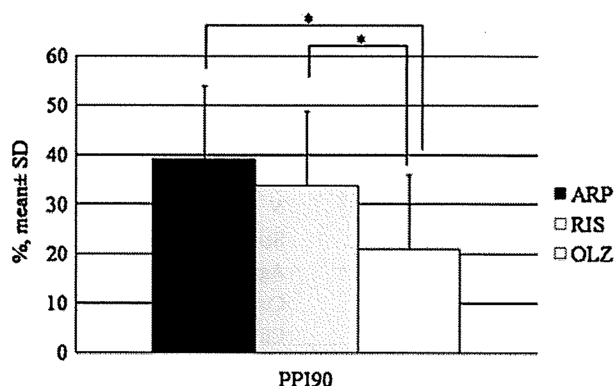

Fig. 1 Bonferroni's *post hoc* analysis of PPI90 in three antipsychotic groups *P<0.05, (μV, mean ± SD). ARP group=39.9±31.1, RIS group=33.7±22.9, and OLZ group=21.0±17.9

(detailed information about startle response on controls can be seen in our previous paper and Supplementary Table 1; P_{ASR}=0.686, P_{PPI82}=0.903, P_{PPI86}=0.703, and P_{PPI90}= 0.141) (Moriwaki et al. 2009). In addition, when all PPI measures (except HAB) in the RIS group were compared with our 107 healthy control subjects with the use of ANCOVA (using age and sex as covariates), there was no statistical difference between the RIS group and controls (Supplementary Table 1; P_{ASR}=0.485, P_{PPI82}=0.267, P_{PPI86}=0.900, and P_{PPI90}=0.331) (Moriwaki et al. 2009). On the other hand, when HAB in the ARP or RIS group were compared with our healthy control subjects with the use of ANCOVA (using age and sex as covariates), ARP or RIS group data were significantly different than that of control subjects (Supplementary Table 1; P_{ARP}= 0.00000109 and P_{RIS}=0.00840). Also, we performed ANCOVA for comparing the PPI measures among three groups (ARP, RIS, and healthy control groups), using above two parameters (age and sex) as covariates to adjust possible confounding. We found that HAB were significantly different among the ARP, RIS, and healthy control groups with the use of ANCOVA (P=0.00000736). However, we did not detect that other PPI measures were significantly different among the ARP, RIS, and healthy control groups with the use of ANCOVA (P_{ASR}=0.514, P_{PPI82}=0.327, P_{PPI86}=0.705, and P_{PPI90}=0.288). HAB was not statistically different between the antipsychotic groups among Japanese patients with schizophrenia (Table 2). This lack of difference in HAB among the Japanese patients with schizophrenia using different antipsychotics suggested that HAB was a common endophenotype of schizophrenia. Although ASR, PPI82, and PPI86 were not different in each of the antipsychotic groups, there was no statistical difference between the ARP or RIS groups and controls when ASR, PPI82, PPI86, and PPI90 in the ARP or RIS group were compared with our 107 healthy control subjects (Supplementary Table 1). Also, PPI90 was significantly different among the antipsychotic groups. Because it was possible that ARP and RIS led to the improvement of disruption of PPI90 to the level of healthy controls, we considered that ASR, PPI82, PPI86, and PPI90 might not be an endophenotype of schizophrenia.

There are a few limitations to this study. First, there is no control in this study. We did not measure the acoustic startle response when patients participating in this study did not take antipsychotics. Because each of the startle responses measured in the ARP group in a drug-naïve state might have higher scores than those of other antipsychotic groups in a drug-naïve state, our results must be interpreted careful. However, measuring the startle response of people with schizophrenia in the drug-naïve state is very difficult. Second, the positive association may be due to a small sample size. Third, because our samples were small, the statistical errors are possible in the results of these statistical association analyses. To overcome this limitation, a replication study using larger samples or samples of other populations will be required for conclusive results.

In conclusion, ARP and RIS may improve PPI90. ASR, HAB, PPI82, and PPI86 were no different among the Japanese schizophrenic patient groups with different antipsychotics. Since HAB showed no difference between the antipsychotic groups of Japanese patients with schizophrenia, we suggest that HAB may not be influenced by several clinical factors. However, since our samples are small, it will be necessary to conduct a replication study using larger samples.

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ORIGINAL ARTICLE

Transient exposure of neonatal mice to neuregulin-1 results in hyperdopaminergic states in adulthood: implication in neurodevelopmental hypothesis for schizophrenia

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Neuregulin-1 (NRG1) is implicated in the etiology or pathology of schizophrenia, although its biological roles in this illness are not fully understood. Human midbrain dopaminergic neurons highly express NRG1 receptors (ErbB4). To test its neuropathological role in the neurodevelopmental hypothesis of schizophrenia, we administered type-1 NRG1 protein to neonatal mice and evaluated the immediate and subsequent effects on dopaminergic neurons and their associated behaviors. Peripheral NRG1 administration activated midbrain ErbB4 and elevated the expression, phosphorylation and enzyme activity of tyrosine hydroxylase (TH), which ultimately increased dopamine levels. The hyperdopaminergic state was sustained in the medial prefrontal cortex after puberty. There were marked increases in dopaminergic terminals and TH levels. In agreement, higher amounts of dopamine were released from this brain region of NRG1-treated mice following high potassium stimulation. Furthermore, NRG1-treated mice exhibited behavioral impairments in prepulse inhibition, latent inhibition, social behaviors and hypersensitivity to methamphetamine. However, there were no gross abnormalities in brain structures or other phenotypic features of neurons and glial cells. Collectively, our findings provide novel insights into neurotrophic contribution of NRG1 to dopaminergic maldevelopment and schizophrenia pathogenesis.
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Keywords: NRG1; dopamine; schizophrenia; ErbB4; TH; neurotrophic factor

Introduction

Neurodevelopmental deficits are considered to be the key features of schizophrenia, which is a multifactorial disease involving environmental factors/insults and genetic predispositions, such as a genetic polymorphism of the neurotrophic factor *neuregulin-1* (*NRG1*). *NRG1* and its receptor (*ErbB4*) were identified as susceptibility genes for schizophrenia.[1,2] Changes in the expression levels of NRG1 splicing isoforms and ErbB4 protein are also found in postmortem brains and peripheral blood cells of schizophrenia patients,[3–6] although the pathophysiological contribution of abnormal NRG1/ErbB4 signaling to schizophrenia is largely unresolved. In this neurodevelopmental hypothesis of schizophrenia, the environmental factors include maternal infection, abnormal delivery and neonatal hypoxia, which presumably interact with the risk genes of schizophrenia.[7,8] For example, schizophrenia-related single-nucleotide polymorphisms (SNPs) of the *NRG1* gene are often located in its promoter regions and positively regulate gene transcription.[1,5] NRG1 expression is induced by adult ischemic and traumatic brain injury, as well as by neonatal hypoxia.[9–11] Thus, it is possible that the more abnormal expression of NRG1 is induced in human embryos or neonates carrying these SNPs by these environmental insults, the more severely this factor might impair brain development to increase the risk of schizophrenia.

Neuregulin-1 is one of the neurodevelopmental regulators that are involved in neuronal migration, axon pathway finding, myelination and synaptogenesis.[12–15] Thus, the abnormality in its expression can be implicated in the neurodevelopmental hypothesis mentioned above. Accordingly, various exons of the

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NRG1 genome have been disrupted by homologous recombination in mice and their neurobehavioral traits have been investigated.[1,16–19] In adults, the mutant mice of NRG1 variants often exhibit schizophrenia-associated behavioral abnormalities. As hypomorphic or hypermorphic NRG1 signals persists throughout life in these genetic mutants (that is, is not temporally controlled), the evaluation of these models is challenging in regard to the neurodevelopmental hypothesis.

Recently, we described the localization of ErbB4 mRNA in the midbrain dopaminergic neurons in mice and primates including humans.[20,21] Our *in situ* hybridization detected high levels of ErbB4 mRNA signals in almost all midbrain dopaminergic neurons. In particular, the expression is higher from the late embryonic stage to neonatal stage when these neurons are vigorously developing.[20,22] However, the nature of NRG1 activity on dopaminergic development or function is still poorly understood.[23]

In this study, we have designed an experimental protocol based on the neurodevelopmental hypothesis of schizophrenia[24–26] and assessed the pathological roles of excess NRG1 signals on dopaminergic neurons and their functions during and after development.[27] As upregulation of type-1 NRG1 expression is reported in schizophrenia brain pathology,[4,5] we selected this isoform of NRG1 protein and administered it to the periphery of mouse pups. The immediate and delayed impact of NRG1 treatment on developing dopaminergic neurons was analyzed using neurochemical and anatomical approaches. Furthermore, we discuss the use of NRG1-treated mice as a model for schizophrenia and compare it with other genetic mutant mice of this gene.

Materials and methods

Generation of recombinant NRG1β1 protein

A cDNA for the ectodomain (46–634 nucleotides) of mouse NRG1β1[28] was subcloned into the pET-22b (+) vector (Novagen, Madison, WI, USA) and expressed as histidine-tagged recombinant protein in *Escherichia coli* (BL21 DE3, Novagen). Inclusion bodies were denatured and solubilized by 6 M guanidine–HCl, and then NRG1β1 was purified with HiTrap Chelating HP column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Denatured NRG1β1 protein was refolded by gradual removal of guanidine–HCl by means of stepwise dialysis in the presence of L-arginine and oxidized glutathione.[29] We purified a biologically active form of NRG1β1 protein with cation exchange chromatography (HiTrap-CM-FF, GE Healthcare). The peak fractions that induced ErbB4 phosphorylation in cultured neocortical neurons were used in this study (Supplementary Figure S1). The full mature form of recombinant NRG1β1 protein carries the tag sequence of six histidine residues at its carboxyl terminal end and has calculated molecular weight of 25 400 Da. Alternatively, we obtained the core epidermal growth factor domain of human NRG1β1 (eNRG1; molecular weight 7500 Da, PeproTech EC, London, UK), which is an artificial product common to all NRG1 splice variants.

Animals and drug treatment

Pregnant C57BL/6NCrj mice were purchased from Nihon Charles River (Kanagawa, Japan), and their newborn pups were used in the following experiments. NRG1β1 or eNRG1 (both 1.0 $\mu g\,g^{-1}$ body weight) was administered subcutaneously daily to half of the littermates during postnatal days (PNDs) 2–10.[30] Control littermates received an injection of phosphate-buffered saline (vehicle) of the same volume. The given dose of NRG1β1 was the highest one that did not produce growth retardation in neonatal mice. A dose of NRG1β1 (3.0 $\mu g\,g^{-1}$ body weight) significantly attenuated body weight gain in the postnatal period (88.0 ± 1.3%, compared with control mice).

Risperidon (Risperdal, Janssen Pharmaceutical KK, Tokyo, Japan) was daily administered (1.0 $\mu g\,g^{-1}$) intraperitoneally on PNDs 42–70 to induce the chronic medication state of patients.[31–33] The same volume of physiological saline was administered as a control. Behavioral testing was conducted 24 h after the final antipsychotic administration to minimize sedative effects of risperidon. All the animal procedures were approved by the Animal Use and Care Committee of Niigata University and performed in accordance with the National Institute of Health (NIH) guideline (USA).

In situ *hybridization and immunohistochemistry*

Mice were anesthetized with halothane (Takeda Pharmaceutical, Osaka, Japan) and transcardially perfused with 4% paraformaldehyde. Brains were immersed in 30% sucrose and embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). Coronal sections (20- to 40-μm thick) were prepared for *in situ* hybridization and immunohistochemistry. Alternatively, histopathological examination was performed by Klüver–Barrera stain (see Supplementary Materials and Methods).

For *in situ* hybridization, sections were hybridized with digoxigenin (DIG)-labeled antisense cRNA probe to ErbB4 mRNA (GenBank: NM_010154. 429–1042 nucleotides) and then with alkaline phosphatase-conjugated anti-DIG antibody as fully described previously.[20] For immunostaining, alternatively, sections were incubated with anti-c-fos (1:20000, Calbiochem, La Jolla, CA, USA) or anti-TH (1:1000, Millipore, Bedford, MA, USA) antibodies, followed by the biotinylated anti-rabbit immunoglobulin antibody (1:200, Vector Laboratories, Burlingame, CA, USA). The detection of primary antibodies or injected biotinylated NRG1β1[34] was performed with the conventional peroxidase-conjugated avidin complexes. To confirm the specificity of the avidin/biotin reaction, some of the sections were pretreated with the avidin/biotin blocking agent (Vector Laboratories). Immunoreactivity was observed using an all-in-one

microscope (BZ-9000, Keyence, Osaka, Japan) and a BZ-Analyzer (Keyence).

Immunoprecipitation and immunoblotting
For immunoprecipitation, whole brain or midbrain of mice (PND 2) was homogenized in RIPA buffer (50 mM Tris–HCl buffer pH 7.4 plus 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). Protein lysate (2 mg protein) was then incubated with the anti-ErbB4 antibody (2 μg) overnight. The antigen–antibody complex was recovered with Protein G Sepharose beads (GE Healthcare) and subjected to immunoblotting as described below.

Brain tissues were homogenized in the sample lysis buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 0.5% NP-40, 5 mM EDTA) plus the protease inhibitor cocktail. Protein samples (5–50 μg per lane) were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with antibodies directed against phosphotyrosine (1:1000, Millipore), ErbB4 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), tyrosine hydroxylase (TH) (1:1000, Millipore), dopamine-β-hydroxylase (DBH) (1:500, Millipore), dopamine transporter (DAT) (1:1000, Millipore), vesicular monoamine transporter 2 (vMAT2) (1:1000, Millipore), D1 dopamine receptor (D1DR) (1:500, Santa Cruz Biotechnology), D2 dopamine receptor (D2DR) (1:250, Millipore), catechol-*O*-methyltransferase (COMT) (1:8000, BD Transduction Laboratories, Lexington, KY, USA), norepinephrine/noradrenaline transporter (NET) (1:500, Millipore) and β-actin (1:4000, Millipore). Alternatively, immunoblots were probed with antibodies directed against glutamatergic, GABAergic, glial markers and phospho/nonphospho-TrkB proteins (see Supplementary Materials and Methods). Immunoreactivity on membranes was detected by peroxidase-conjugated anti-immunoglobulin antibodies followed by chemiluminescence reaction combined with X-ray film exposure (ECL kit, GE Healthcare).

Behavioral testing
All behavioral tests were performed at PNDs 56–84. Spontaneous locomotor activity, acoustic startle response and prepulse inhibition (PPI) were measured as fully described previously.[35] The test paradigm of context- and tone-dependent fear learning was performed in a conditioning chamber and different test chamber (both; $10\,L \times 10\,W \times 10\,H$ cm box; Obaraika, Tokyo, Japan).[35,36] Freezing behavior was automatically monitored by a video camera during all sessions and analyzed by imaging software (Obaraika). The latent inhibition test was performed with the same conditioning and test chambers.[37] For auditory brainstem-evoked response testing, social interaction and further details of individual behavioral tests, see Supplementary Materials and Methods.

Methamphetamine challenge
The effects of methamphetamine (MAP) were monitored with an automated locomotor activity monitor (Med Associates, St Albans, VT, USA). Mice were placed in an activity chamber, and their horizontal activities were recorded at 5-min intervals. Mice were first habituated to the apparatus for 60 min and then challenged by MAP (Dainippon-Sumitomo Pharmaceuticals, Osaka, Japan, 1.0 or 2.0 μg g^{-1}, intraperitoneally) or saline. For quantification of c-fos-positive cells, mice were fixed as described above 2 h after a single injection of MAP. We quantified the number of c-fos-positive cells in digital microscopic images using the NIH Image cell-counting system (ver. 1.61).[38]

Surgery and microdialysis
Mice were anesthetized with pentobarbital (50 mg kg^{-1}, Somnopentyl, Schering Plough Animal Health, Kenilworth, NJ, USA) and mounted on a stereotaxic frame. Stainless guide cannula (AG-4, Eicom, Kyoto, Japan) with a dummy probe (AD-4, Eicom) was placed in the medial prefrontal cortex (mpFC, equivalent to prelimbic cortex) (coordinates: anterior +2.0 mm, lateral +0.5 mm, ventral −6.0 mm relative to the bregma). A probe was perfused with artificial cerebrospinal fluid (ACSF: 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$) or ACSF containing high potassium (69.7 mM NaCl, 80 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$) at the flow rate of 0.7 μl min^{-1}. Dialysate samples (20 μl) were collected every 30 min.

Quantification of monoamines, their metabolites and L-DOPA
The enzymatic activity of TH was assessed by monitoring the production of 3,4-dihydroxy-L-phenylalanine (L-DOPA) from tyrosine using high-performance liquid chromatography (HPLC) equipped with an electrochemical detector as fully described previously.[39] Contents of dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and L-DOPA were determined by HPLC using a C18 column (model CA-5ODS, 4.6 × 150 mm, Eicom).[39] Dialysis samples were directly applied onto the HPLC system equipped with BDS Hypersil C18 column (1.0 × 100 mm, Keystone Scientific, Bellefonte, PA, USA). For details, see Supplementary Materials and Methods.

Statistical analysis
Statistical analyses were performed using SPSS 11.0 (SPSS Co., Tokyo, Japan). As the initial analyses of variance of behavioral data yielded no significant outcomes involving the gender variable, the data of the two genders were combined for final analyses. Fisher's least significant difference *post hoc* analysis was used to detect differences of absolute behavioral values. Alternatively, univariate data in two groups were subjected to unpaired two-tailed *t*-test. A *P*-value <0.05 was regarded as statistically significant.

Results

Peripheral NRG1β1 crosses BBB and reaches the midbrain of mouse neonates

As the blood–brain barrier (BBB) of neonatal mice is not fully developed and may allow cytokines to penetrate into the brain,[30,40] we tested the BBB permeability of the full-length mature type-1 NRG1β1 (hereafter, referred to as NRG1β1) in mouse pups. We administered NRG1β1 (1.0 μg g^{-1}, subcutaneously) to PND 2 mice and examined the phosphorylation of NRG1 receptors (ErbB4). NRG1 administration increased the immunoreactivity for phospho-ErbB4 in the whole brain as well as in the midbrain (Figures 1a and b). The maximal activation of

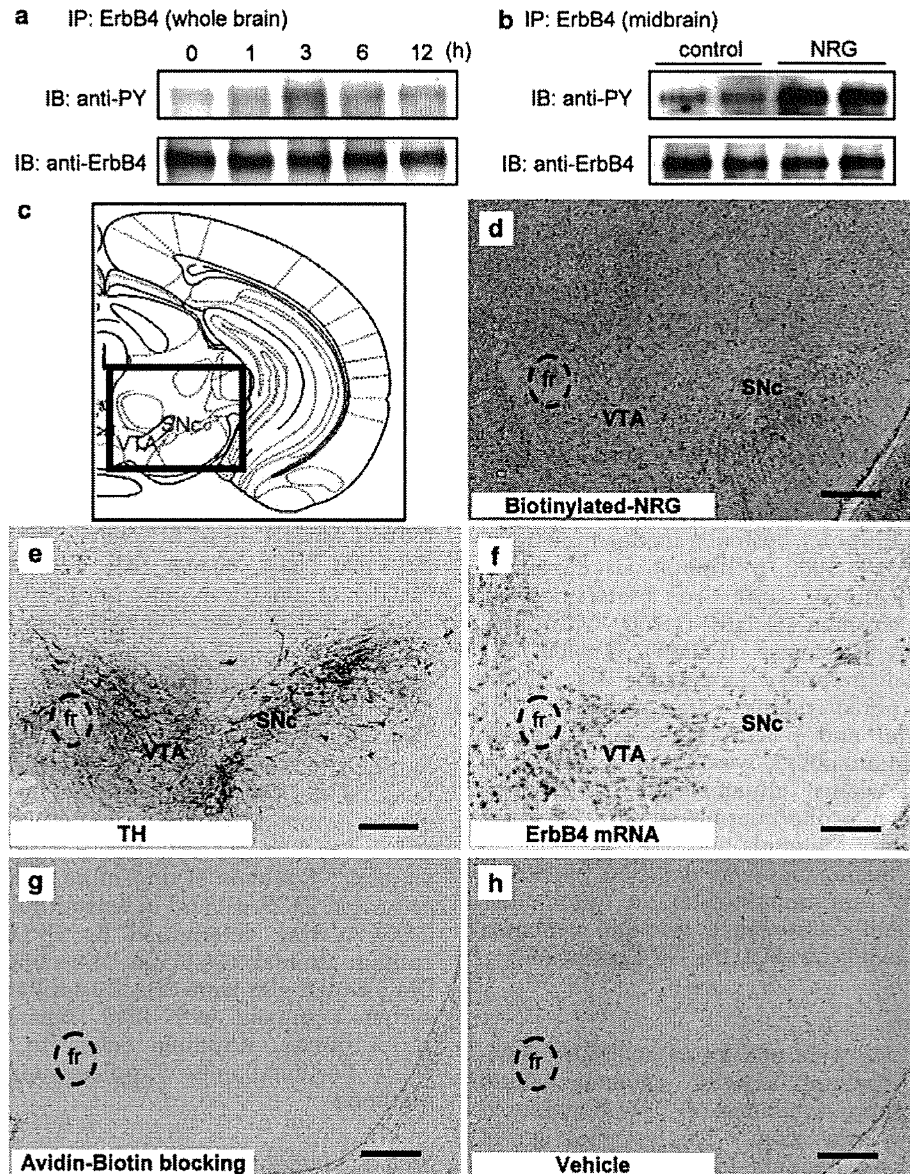


Figure 1 Penetration of administered neuregulin-1 (NRG1)β1 through the blood–brain barrier. (**a**) The whole brain lysates were prepared at 0, 1, 3, 6 and 12 h after subcutaneous injection of NRG1β1 to neonatal mice (postnatal day (PND) 2), immunoprecipitated (IP) with the anti-ErbB4 antibody and subjected to immunoblotting (IB) with anti-phosphotyrosine (anti-PY) or anti-ErbB4 antibodies. (**b**) ErbB4 phosphorylation in the midbrain was similarly examined 3 h after NRG1β1 treatment (N = 4 mice per group). Distributions of biotinylated NRG1β1 in the midbrain region of the enclosed area in (**c**) were examined with the avidin/biotinylated horseradish peroxidase complex. (**d**) Brain section was prepared from biotinylated-NRG1β1-injected mice. Adjoining serial sections were stained with (**e**) the anti-tyrosine hydroxylase (TH) antibody or (**f**) an *in situ* hybridization probe to ErbB4 mRNA. (**g**) Section prepared from biotinylated-NRG1β1-injected mice was pretreated with the avidin/biotin blocking reagent. (**h**) Brain section was prepared from vehicle-injected mice. fr, fasciculus retroglexus; a landmark for the midbrain; VTA, ventral tegmental area; SNc, substantia nigra pars compacta. Scale bar: 200 μm.

ErbB4 was obtained ~3 h after injection presumably because of slow penetration rates across BBB as seen with other cytokines.[30] By injecting the biotinylated form of NRG1β1, we confirmed its penetration across the neonatal BBB. Significant levels of biotin signal were detected in the intracellular space as well as on cell surfaces in the midbrain (Figure 1d), potentially representing endocytosis of the ligand-bound ErbB4 receptors.[41] This region contained TH-positive cells and ErbB4 mRNA (Figures 1e and f). Biotin signals were also detected in other brain regions (data not shown) but not when sections were pretreated with an avidin/biotin blocking agent (Figure 1g). The control sections prepared from vehicle-treated animals failed to exhibit a biotin signal (Figure1h). These results suggest that, during neonatal and possibly during perinatal stages, NRG1 circulating in the periphery can reach the midbrain and activate ErbB4 receptors of dopaminergic neurons.

Effects of neonatal NRG1β1 treatment on developing dopaminergic system

ErbB4, the receptor for NRG1, is expressed exclusively by the midbrain dopamine neurons.[20] To study the developmental effects of exogenous NRG1 on these neurons, we repeatedly administered NRG1β1 to mouse neonates (subcutaneously, PNDs 2–10). We then determined the protein expression, phosphorylation and enzyme activity of TH, a rate-limiting enzyme of dopamine and noradrenaline synthesis. We found a significant increase in TH protein levels in the whole frontal cortex (FC) ($P<0.05$) but not in the striatum of NRG1β1-treated mice at PND 11 (Figures 2a and b). As FC receives both dopaminergic and noradrenergic innervations, we also examined the protein levels of DBH, the enzyme that converts dopamine to noradrenaline. There was no significant change in DBH levels in FC, suggesting limited effects of NRG1β1 on noradrenergic neurons. Although NRG1β1 treatment did not affect TH levels in the striatum, there was a significant increase in ser-40 phosphorylation of this enzyme ($P<0.05$) (Figure 2b), a process known to elevate the enzyme activity of TH.[42] We also tested the core epidermal growth factor domain peptide of NRG1β1 (eNRG1, common for all splice variants) as a positive control and detected similar increases in TH and its phosphorylation (Supplementary Figure 2).

We found that NRG1β1 treatment in neonates significantly increased the enzyme activity of TH in the striatum and FC (both $P<0.05$) (Figure 2c). In parallel, dopamine contents in FC and the striatum were elevated in NRG1β1-treated mice at PND 11 (FC: $P<0.05$, striatum: $P<0.01$) (Figure 2d). However, there were no differences in the dopamine metabolites, DOPAC and HVA, or in noradrenaline (DOPAC: 95.7 ± 3.0%; HVA: 107.2 ± 1.9%; noradrenaline: 94.1 ± 7.1% of control). These results suggest that neonatal treatment with NRG1β1 promotes aberrant phenotypic development of midbrain dopamine neurons during neonatal and postnatal stages.

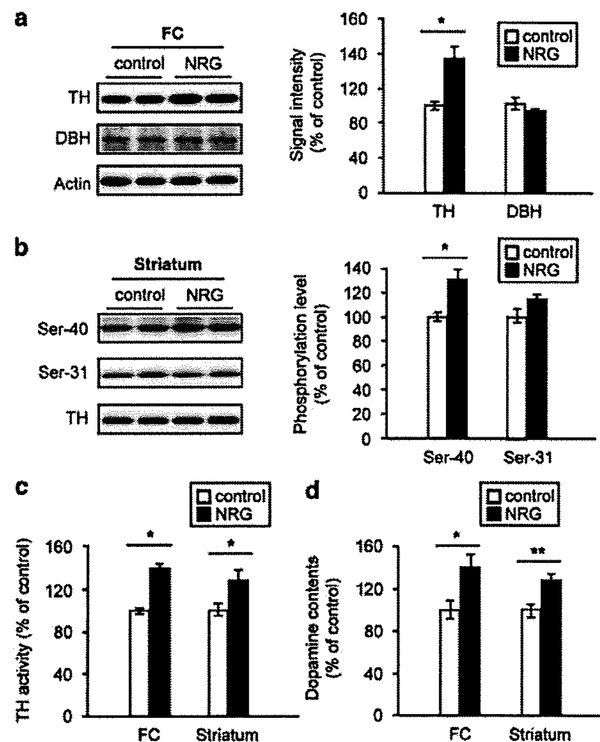


Figure 2 Effects of neonatal neuregulin-1 (NRG1)β1 treatment on developing dopaminergic neurons. NRG1β1 (1.0 μg g^{-1}) or vehicle (control) was administered (subcutaneously) daily to mouse pups during postnatal days (PNDs) 2–10, and effects on dopaminergic systems were evaluated. (**a**) Protein levels of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) in the frontal cortex (FC) were analyzed by immunoblotting and quantified by densitometric analysis, and normalized to β-actin levels ($N=6$ mice per group). (**b**) The phosphorylation (ser-40 and ser-31) levels of TH in the striatum were analyzed by immunoblotting and are presented as the ratio of phospho-TH immunoreactivity to total TH levels ($N=6$–7 mice per group). (**c**) Enzyme activity of TH was analyzed in FC and the striatum ($N=7$–11 mice per group). (**d**) The dopamine contents were measured in FC and the striatum on PND 11. Data are expressed as mean ± s.e.m ($N=7$–8 mice per group). *$P<0.05$, **$P<0.01$, by unpaired two-tailed t-test.

We also examined NRG1β1 effects on mouse physical development. There were no effects on body weight at PNDs 11 and 56, but there was a slight acceleration of eyelid opening and tooth eruption in NRG1β1-treated mice (Supplementary Tables S1 and S2). Thus, the transient exogenous supply of NRG1β1 seems to produce limited influences on physical indices in mouse development.

Neonatal NRG1β1 treatment induces dopaminergic hyperinnervation in adult FC

To estimate the long-term effect of neonatal NRG1β1 treatment on the dopaminergic system, we measured the levels of dopamine and its metabolites in FC, the nucleus accumbens (NAc) and striatum at the adult

stage as well (PNDs 56–70). We found significant increases in dopamine metabolites, DOPAC and HVA, in FC (DOPAC: 130.0 ± 14.0%, $P<0.05$; HVA: 131.0 ± 13.5% of control, $P<0.01$, $N=10–12$ mice per group). However, there was no significant effect on dopamine in FC (105.6 ± 6.9% of control) or on dopamine and its metabolites in the other regions (dopamine, 104.0 ± 7.7%; DOPAC, 101.1 ± 8.0; HVA, 100.1 ± 8.0% of control in NAc, $N=9$ mice per group; and dopamine, 110.8 ± 6.6%; DOPAC, 106.9 ± 8.7%; HVA, 105.5 ± 4.2% of control in the striatum, $N=10–12$ mice per group). To evaluate morphological influences, we marked the dopaminergic somata and fibers with TH immunostaining and examined them in serial coronal sections of FC and the midbrain at the adult stage. Denser or thicker axon terminals appeared to be distributed in the deep cortical layers of mpFC (i.e. prelimbic cortex) of NRG1β1-treated mice (Figures 3a–f). In contrast, there were no apparent differences in the frequency and arborization of these axons in other subregions of FC or in the midbrain (Supplementary Figure S3).

To ascertain that neonatal NRG1β1 treatment resulted in the dopaminergic hyperinnervation of

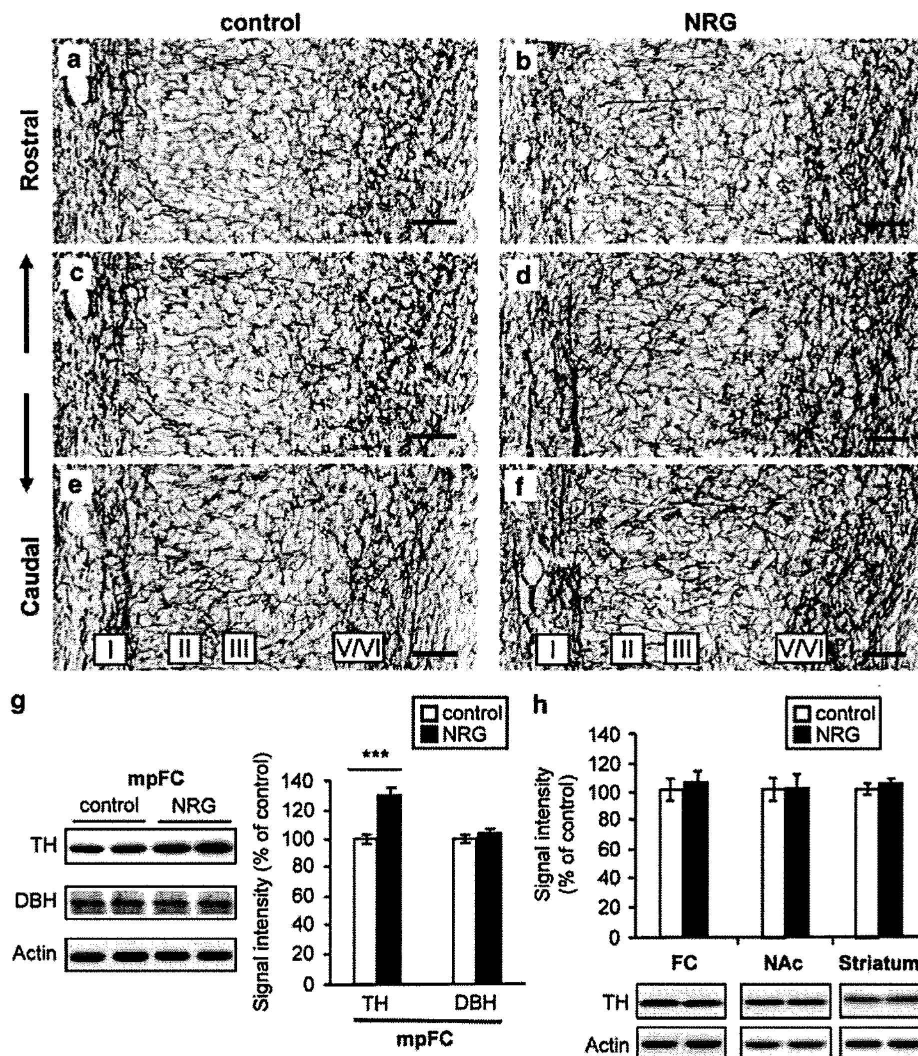


Figure 3 Neonatal exposure to neuregulin-1 (NRG1)β1 results in persistent increases in tyrosine hydroxylase (TH)-positive terminals and TH protein levels in adult medial prefrontal cortex (mpFC). Serial coronal sections of FC were prepared from vehicle-treated control and NRG1β1-treated mice and immunostained with the anti-TH antibody. TH-immunoreactive fibers in mpFC of vehicle-treated control (**a**, **c** and **e**) and NRG1β1-treated mice (**b**, **d** and **f**) are shown at the positions (**a**, **b**) +2.22 mm, (**c**, **d**) +1.98 mm and (**e**, **f**) +1.70 mm, all from the bregma. ($N=3$ mice per group). Scale bar: 100 μm. TH protein levels in mpFC, whole FC (FC), the nucleus accumbens (NAc) and striatum were measured by immunoblotting at the adult stage. (**g**) Protein levels of TH and dopamine-β-hydroxylase (DBH) in the mpFC were analyzed by immunoblotting and quantified by densitometric analysis and normalized to β-actin levels ($N=6$ mice per group). (**h**) Similarly, protein levels of TH in whole FC (FC), NAc and the striatum were analyzed ($N=5–6$ mice per group). $^{***}P<0.001$, by unpaired two-tailed t-test.

mpFC at the adult stage, we examined TH protein levels with immunoblotting. In agreement, there was a significant increase in TH protein levels in the mpFC (131.0 ± 4.3% of control, $P<0.001$) (Figure 3g). There was no change in DBH levels in the mpFC of NRG1β1-treated mice. This TH increase was not manifested by immunoblotting for the whole FC, NAc or striatum (Figure 3h).

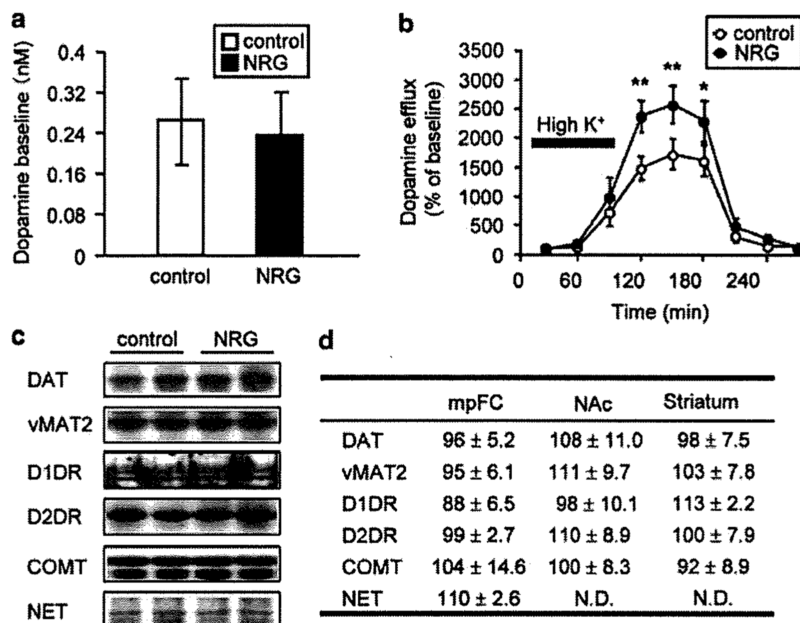
Higher levels of dopamine release in the NRG1β1-treated mice verify their hyperdopaminergic states

The increases in dopamine metabolites and TH protein levels may lead to enhanced dopamine transmission at the adult stage. To test this hypothesis, we carried out *in vivo* microdialysis in mpFC. Although neonatal NRG1β1 treatment had no effect on the basal levels of extracellular dopamine (Figure 4a), potassium depolarization evoked higher amounts of dopamine release in mpFC of NRG1β1-treated mice (NRG1 treatment, $F_{(1,12)}=4.00$, $P<0.05$) (Figure 4b). The reason underlying failure in detecting the difference in basal dopamine release awaits further investigation. We also examined molecular markers related to dopamine transmission; DAT, vMAT2, D1DR, D2DR, COMT and NET. We did not detect significant changes in these proteins in mpFC, NAc or the striatum of NRG1β1-treated mice (Figures 4c and d).

In addition to the effects on the dopaminergic system, neonatal exposure to NRG1β1 might also influence the development of gamma-aminobutyric acid (GABA) neurons, glutamatergic synapses, glial cells or other neurotrophic signaling.[12,13,43–45] Using the conventional pathological staining as well as immunoblotting, we examined brain structures and neuronal/glial markers of NRG1β1-treated mice in the adult stage (PND 56). Klüver–Barrera staining revealed that there were no apparent structural abnormalities in adult NRG1β1-treated mice (Supplementary Figure S4). In addition, there were no significant differences in the protein levels of the glutamatergic, GABAergic and glial markers (Supplementary Figure S5), and TrkB phosphorylation (Supplementary Figure S6) between NRG1β1-treated and vehicle-treated control mice.

Neonatal NRG1β1 treatment enhances behavioral and neurochemical sensitivity to MAP

We tested whether neonatal NRG1β1 treatment alters acute responsiveness to MAP in this study. A systemic challenge of MAP ($1.0\,\mu g\,g^{-1}$, intraperitoneally)



	mpFC	NAc	Striatum
DAT	96 ± 5.2	108 ± 11.0	98 ± 7.5
vMAT2	95 ± 6.1	111 ± 9.7	103 ± 7.8
D1DR	88 ± 6.5	98 ± 10.1	113 ± 2.2
D2DR	99 ± 2.7	110 ± 8.9	100 ± 7.9
COMT	104 ± 14.6	100 ± 8.3	92 ± 8.9
NET	110 ± 2.6	N.D.	N.D.

Figure 4 Analysis of neonatal neuregulin-1 (NRG1)β1 treatment effects on adult dopamine release and on neurochemical markers of dopaminergic neurons. Neonatal mice were treated with NRG1β1 or vehicle (control) as described in Figure 2. At the adult stage (postnatal days (PNDs) 63–70), microdialysis study was carried out in the medial prefrontal cortex (mpFC). (**a**) Basal extracellular levels of dopamine were monitored for 150 min ($N=7$ mice per group). (**b**) Dopamine release was evoked by perfusion of 80 mM KCl over 90 min (solid bar) and monitored for 270 min. Data represent relative dopamine levels in 30-min fractions (% of baseline, mean ± s.e.m, $N=7$ mice per group). $^{*}P<0.05$, $^{**}P<0.001$, by Fisher's least significant difference. Protein extracts were prepared at the adult stage (PNDs 56–84) from mpFC, the nucleus accumbens (NAc) and striatum of mice that were neonatally treated with NRG1β1 or vehicle (control) and subjected to immunoblotting with antibodies directed against the indicated dopaminergic markers. (**c**) Typical immunoreactive signals of two mpFC samples were displayed. (**d**) Immunoreactivities were measured by densitometric analysis and normalized to β-actin levels ($N=5$–7 mice per group). Relative levels of the protein markers in NRG1β1-treated mice are presented (% of control; mean ± s.e.m). ND, not determined. Note: There were no significant differences in all pairs.

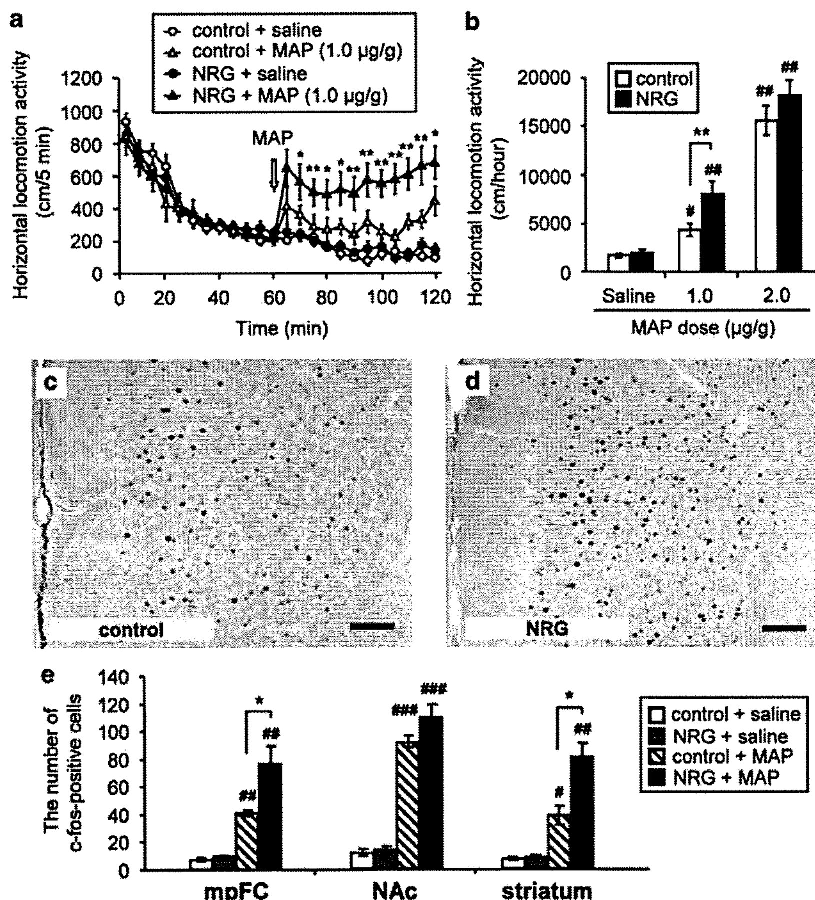


Figure 5 Neonatal exposure to neuregulin-1 (NRG1)β1 enhances locomotor activity and c-fos expression following methamphetamine (MAP) challenge. (**a**) Horizontal locomotor activity was monitored before and after MAP (1.0 μg g^{-1}) or saline challenge at the adult stage. (**b**) Total locomotor activity was calculated and presented for the 60-min period after saline or MAP (1.0 or 2.0 μg g^{-1}) challenge (N = 9–10 mice per group). (**c**) Vehicle-treated control and (**d**) NRG1β1-treated mice were subjected to c-fos immunohistochemistry 2 h after MAP (1.0 μg g^{-1}) challenge. Typical pictures of the medial prefrontal cortex (mpFC) are shown for mice challenged with MAP. Scale bar, 100 μm. (**e**) The number of c-fos-positive cells in the above microscopic field (725 × 965 μm) was counted bilaterally using the NIH Image cell-counting system (ver. 1.61) using five to seven sections of FC (+1.70 to +1.98 mm from the bregma), the nucleus accumbens (NAc) and striatum (+1.18 to +1.54 mm from the bregma), averaged for each mouse and subjected to statistical analysis (N = 4 mice per group). Data are expressed as mean ± s.e.m. *P < 0.05, **P < 0.01, compared between marked groups. #P < 0.05, ##P < 0.01, ###P < 0.001, compared between MAP-challenged and -unchallenged groups by Fisher's least significant difference.

increased locomotor activity in both NRG1β1-treated and control groups (MAP, $F_{(1,36)}$ = 16.30, P < 0.001). However, the magnitude of MAP-induced locomotor activity was significantly higher in NRG1β1-treated group than the control group (NRG1 treatment, $F_{(1,36)}$ = 5.14, P < 0.05; NRG1 treatment × MAP, $F_{(1,36)}$ = 4.34, P < 0.05) (Figure 5a). The acceleration of MAP sensitivity by neonatal NRG1β1 treatment seemed to depend on the MAP dose (MAP dose, $F_{(1,54)}$ = 162, P < 0.001). At a dose of 2.0 μg g^{-1} of MAP, the effect of NRG1β1 treatment became less apparent (P = 0.052) (Figure 5b). Thus, these results indicate that mice neonatally exposed to NRG1β1 exhibited higher sensitivity to the lower dose of MAP.

We also examined c-fos expression in the brain following MAP challenge (1.0 μg g^{-1}) (Figure 5e). We found that MAP challenge induced a greater number of c-fos-positive cells in mpFC and the striatum in NRG1β1-treated mice than in control mice (both P < 0.01) (Figures 5c–e). In contrast, there were no significant effects of NRG1β1 treatment in NAc (Figure 5e).

Neonatal NRG1β1 treatment impairs sensorimotor gating at the adult stage

We assessed sensorimotor gating of NRG1β1-treated mice by measuring PPI, which is often implicated in schizophrenia pathology or dopaminergic

dysfunction.[46,47] We found a significant reduction of PPI in the NRG1β1-treated group (NRG1 treatment, $F_{(1, 22)}=6.91$, $P<0.05$) (Figure 6a) with no significant effect on pulse-alone startle (Figure 6b). As NRG1 signaling is involved in the survival of cochlear sensory neurons,[48] we also tested hearing ability by measuring auditory brainstem-evoked response thresholds. There were no differences in the auditory stimulus thresholds at any frequency between the two groups (Figure 6c).

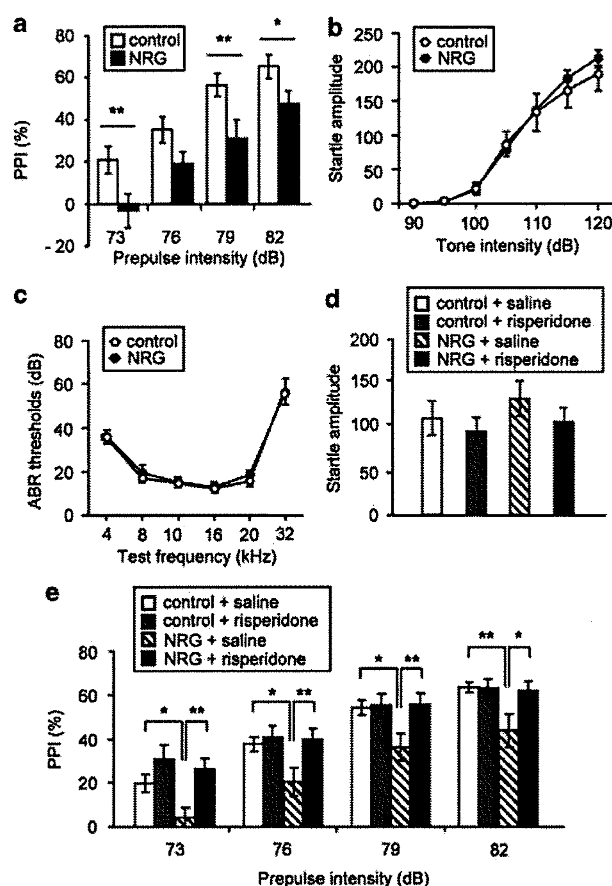


Figure 6 Effects of neonatal exposure to neuregulin-1 (NRG1)β1 on hearing, startle responses and PPI. Neonatal mice were treated with NRG1β1 or vehicle (control) as described in Figure 2. At the adult stage, PPI levels, acoustic startle responses and brainstem-evoked response (ABR) thresholds were determined. (**a**) PPI was measured with 73, 76, 79 and 82 dB prepulse stimuli ($N=12$ mice per group). (**b**) Relative amplitudes of startle responses were monitored with 90, 95, 100, 105, 110, 115 and 120 dB tones ($N=10$ mice per group). (**c**) ABR thresholds were examined with specific auditory stimuli (4, 8, 10, 16, 20 and 32 kHz) by varying the sound pressure levels ($N=9$–10 mice per group). Pharmacological responses of NRG1β1-treated mice to risperidone were examined by PPI measurement. Mice daily received risperidone ($1.0\,\mu g\,g^{-1}$, intraperitoneally) or saline during postnatal days (PNDs) 42–70. On 1 day after final administration of risperidone, PPI test was performed. (**d**) Effects of risperidone on pulse-alone startle (120 dB) were presented ($N=10$ mice per group). (**e**) Amelioration of PPI deficits in NRG1β1-treated mice by risperidone ($N=10$ mice per group). Data are expressed as mean ± s.e.m. $*P<0.05$, $**P<0.01$, by Fisher's least significant difference.

Risperidone, an atypical antipsychotic, has been shown to reduce psychotic symptoms and ameliorate PPI deficits in schizophrenia patients and in the animal models of schizophrenia.[49] Using a chronic administration schedule, we investigated the effect of risperidone ($1.0\,\mu g\,g^{-1}$, daily) on PPI deficits in NRG1β1-treated mice. Risperidone administration significantly improved PPI deficits of NRG1β1-treated mice compared with control mice (drug, $F_{(1, 36)}=7.46$, $P<0.01$) (Figure 6e). However, it did not significantly alter the amplitudes of pulse-alone startle (Figure 6d).

Neonatal NRG1β1 treatment alters latent inhibition and social behaviors

We assessed the basal locomotor activity of adult mice, which influences behavioral evaluation of learning. There was no significant difference in locomotor activity scores between NRG1β1-treated and vehicle-treated control mice (NRG1 treatment, $F_{(1, 23)}=1.17$, $P=0.29$) (Supplementary Figure S7a). Then learning ability of these mice was measured with context- and tone-dependent fear conditioning. We found that NRG1β1 treatment in neonates did not alter learning performance in either paradigm (NRG1 treatment, $F_{(1, 20)}=0.41$, $P=0.53$ for the context-dependent learning; $F_{(1, 20)}=0.21$, $P=0.66$ for the tone-dependent learning) (Supplementary Figure S7b).

As NRG1β1-treated mice exhibited normal locomotion and fear learning, the mice were subjected to latent inhibition test. Latent inhibition of learning is considered to be an ability to ignore irrelevant stimuli and has been shown to involve the dopaminergic system.[50,51] This process is often disrupted in schizophrenia patients.[52] Although basal learning scores were indistinguishable in non-pre-exposure (NPE) groups, the inhibitory effect of pre-exposure (PE) on freezing scores was different between vehicle-treated and NRG1β1-treated groups (NRG1 treatment × pre-exposure, $F_{(1,48)}=4.23$, $P<0.05$) (Figures 7a and b). In contrast to the significant latent inhibition of vehicle-treated mice ($P<0.01$), there was no significant difference in freezing rates between the NPE and PE groups of NRG1β1-treated mice ($P=0.78$) (Figure 7c), indicating their lack of latent inhibition.

To investigate the impact of neonatal NRG1β1 treatment on adult social behaviors in adulthood, we used a resident-intruder behavioral assay. In this assay, a group-housed male mouse (intruder) was placed in another home cage where a resident mouse had been housed alone until the test day. We found that male NRG1β1-treated mice (residents) showed a significant decrease in the duration and frequency of social interactions compared with vehicle-treated control male residents (both $P<0.01$) (Figures 7d and e). On the other hand, the duration of fighting behavior was indistinguishable between NRG1β1- and vehicle-treated control mice (Figure 7f).

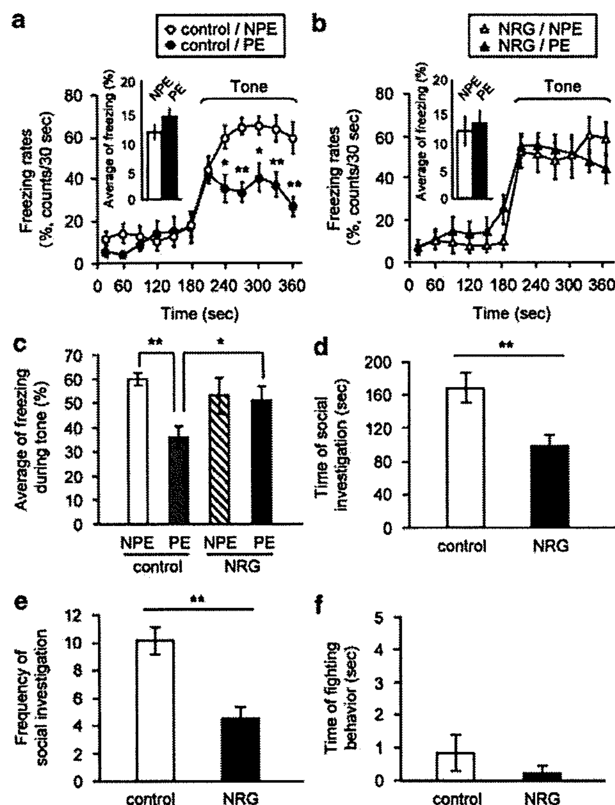


Figure 7 Mice neonatally exposed to neuregulin-1 (NRG1)β1 show impaired latent inhibition and reduced social interaction. NRG1β1-treated and vehicle-treated mice were pre-exposed to tone cue (PE group) or not exposed to the cue (non-pre-exposure (NPE) group) and then subjected to tone-footshock pairs. On 1 day after conditioning, freezing rates (%) of (**a**) vehicle-treated control and (**b**) NRG1β1-treated mice were measured every 30 s in a different chamber before and during tone exposure. Insets reveal mean freezing rates during conditioning (N = 12–14 mice per group). (**c**) Mean freezing rates during tone exposure in test trial. Social interaction was evaluated by the resident-intruder assay. We measured (**d**) time spent by the resident males over 10-min period actively pursing social investigation of the intruder mouse, (**e**) frequency of social behavior and (**f**) total time duration of fighting behavior (N = 7–8 mice per group). Data are expressed as mean ± s.e.m. *P < 0.05, **P < 0.01, by Fisher's least significant difference.

Discussion

In support of the neurodevelopmental hypothesis for the etiology of schizophrenia, here, we found the neuropathological implication of abnormal signals of the risk gene, *NRG1*. Specifically, we showed the profound influences of transient hyper-NRG1 signals on developing midbrain dopaminergic systems, evaluating the subacute and delayed neurochemical and behavioral consequences. Exogenous administration of NRG1β1 protein to mouse neonates produced the activation of midbrain ErbB4 receptors and caused marked changes in dopaminergic neurons and their associated behaviors: (1) Neonatal treatment of NRG1β1 increased the protein levels, phosphorylation, enzyme activity of TH and elevated dopamine levels in FC and/or the striatum; (2) In adulthood, NRG1β1-treated mice exhibited sustained increases in dopamine metabolism and depolarization-triggered dopamine release in FC; (3) NRG1β1-treated mice were more sensitive to MAP in regard to locomotor activity and c-fos induction; (4) NRG1β1-treated mice showed behavioral abnormalities in PPI, social interactions and latent inhibition. These results reveal a novel neurotrophic activity of NRG1 on developing midbrain dopaminergic neurons *in vivo* and indicate a biological link between prenatal or perinatal NRG1 and the dopaminergic pathology of schizophrenia. In contrast to the abnormalities in the dopaminergic systems described above, our examinations failed to uncover any gross structural deficits and neuronal or glial abnormalities with conventional dye staining and immunostatining. In addition, learning ability and basal locomotor activity were preserved in this animal model.

Influences of NRG1β1 on brain structure and function
Neuregulin-1 has a variety of neurotrophic activities on neuronal migration, axon guidance, myelination and synaptogenesis.[12–15] We assessed the influences of NRG1β1 on these neurotrophic processes in this model by quantifying the following neuronal and glial phenotypic markers in various brain regions: neuron-specific enolase, glial fibrillary acidic protein, myelin basic protein, 2′,3′-cyclic nucleotide 3′-phosphodiesterase, glutamate and GABA receptors (GluR1, GluR2/3, NMDAR1, NMDAR2A/B, GABAARα1), glutamate decarboxylase 65/67 and parvalbumin. However, there were no apparent neurochemical alterations in any of these molecular markers.

In contrast to these results, previous studies on knockout and transgenic mice of *NRG1* or *ErbB* genes show that abnormal NRG1 signals result in phenotypic alterations such as impairments in myelin formation, migration of GABAergic interneurons and glial development.[12,13,44,45] We assume that the absence of phenotypic influences in our model could be due to (1) saturation of neurotrophic supports for these cells by other factors, (2) the distinct developmental time window of individual cell types or (3) difference in biological activities of individual NRG1 splicing variants.

Gene targeting in mice shows that the development of neuronal and glial cells are supported by multiple neurotrophic factors and cytokines.[53] Thus, signal redundancy by multiple neurotrophic factors can help explain the phenotypic differences seen between this NRG1β1 model and the genetic mutant models described earlier. Exogenously supplied NRG1 may not have pronounced effects on GABAergic neurons and glial cells if the same or similar factors endogenously provide a saturated level of neurotrophic support for these cells. Conversely,

midbrain dopaminergic neurons might not receive enough neurotrophic support from endogenous NRG1 and be therefore competent to fully react to exogeneous NRG1 in this model. In this context, it is noteworthy that ErbB4 knockout mice did not exhibit any significant structural or neurochemical alterations in midbrain dopaminergic neurons.[54]

In this study, we limited the exposure period of NRG1β1 to PNDs 2–10, which matches the developmental period of midbrain dopaminergic neurons.[55,56] In this context, it is possible that this cell population is relatively sensitive to NRG1β1 and dynamically reacts with this factor during the used experimental period. Thus, if we changed the timing of the NRG1β1 administration, the effect or affected cell population would differ.

Another possible explanation is based on the difference in biological activities of NRG1 splicing variants (types 1–5).[57–59] Type-1 and type-2 NRG1 proteins comprise the extracellular Ig-like domain that interacts with heparan sulfate proteoglycans and modifies receptor binding and signaling.[60] In contrast, type-3 NRG1 has an activity to regulate oligodendrocyte development and myelination.[13] The specific use of type-1 NRG1β1 in the present experiments might therefore limit its biological effects.

Neurotrophic activity of NRG1 on midbrain dopaminergic neurons

In this study, we found subacute effects of neonatal NRG1β1 treatment on developing dopaminergic systems. Interestingly, the influence of neonatal treatment with NRG1β1 was persistent in mpFC until adulthood. In adult mice treated with NRG1β1 as neonates, there were elevated dopaminergic innervation and metabolism. These results indicate that neonatal exposure to NRG1β1 can lead to lifelong impairment of dopamine synthesis and release in this brain region.

Dopaminergic innervation in the brain is classified into the three fiber routes: mesostriatal, mesolimbic and mesofrontocortical pathways. As ErbB4 mRNA is distributed in almost all classes of midbrain dopaminergic neurons in mice and primates,[20,21] these results raised a question regarding pathway specificity of the long-term effects of NRG1β1 treatment: Why was a dopaminergic abnormality persistent and apparent in mpFC? We would like to elaborate on the differences in developmental schedules among three dopaminergic pathways as follows.

In regard to the temporal schedule of development, there is significant time lag in the development of mesostriatal, mesolimbic and mesofrontocortical dopamine pathways. In rodents, the dopaminergic projections to the striatum and NAc are more extensive during midgestation.[55] In contrast, dopaminergic projections to the neocortical and limbic regions is vigorous at postnatal stages.[56,61] Thus, our hypothesis is that, at the postnatal stage when NRG1β1 was administered, corticolimbic fibers were still growing and capable of responding to this neurotrophic factor.

Behavioral similarity to genetic mutant mice

By targeting individual exons in the *NRG1* gene, various types of knockout mice for *NRG1* gene were developed, and their behavioral deficits were extensively investigated and compared.[1,16,17,19] There are some similarities in behavioral traits between NRG1β1-administered mice in this study and NRG1 knockout mice in published reports, even though these models presumably use an opposite strategy of NRG1 to reduce NRG1 signals. For example CRD-NRG1$^{+/-}$ mice exhibited PPI deficits and Ig-NRG1$^{+/-}$ mice have abnormalities in latent inhibition as seen in the present NRG1-injection model.[16,19] In this context, there is an interesting report that transgenic mice overexpressing type-1 NRG1 exhibited similar behavioral deficits in PPI.[62]

Different types of NRG1 precursors, which are produced from a single gene, carry the distinct transmembrane domain(s) and are subjected to different modes of proteolytic regulation (for example, ectodomain shedding).[57] We established this animal model by stimulating ErbB receptors with processed type-1 NRG1 in a paracrine manner. This counterintuitive similarity between this model and these knockout mice might be explained by determining how individual exon disruption influences juxtacrine and paracrine/autocrine signaling of the remaining NRG1 variants and how it affects the molecular interaction of individual NRG1 variants with distinct ErbB receptors.[12,13,15,16,63] Future studies should elucidate the biological characteristics of individual NRG1 variants and their precursors as well as their compensation or interference.[57–59]

NRG1β1-treated mice as an animal model for schizophrenia

Schizophrenia patients show increased sensitivity to various dopamine agonists or releasers, such as amphetamine and cocaine.[64] Local or systemic administration of dopamine agonists to animals elicits the behavioral deficits in PPI as well as latent inhibition and social interactions that are implicated in schizophrenia,[46,51,65] suggesting pathological contribution of hyperdopaminergic states to this disease.[66] In this study, neonatal NRG1β1 treatment resulted in similar behavioral deficits as well as persistent dopaminergic impairments in adulthood. *In vivo* microdialysis and monoamine measurement verified the hyperdopaminergic state of NRG1β1-treated mice. This argument is also supported by the hypersensitivity of this model to MAP. Furthermore, chronic treatment of risperidone ameliorated the deficits in PPI in NRG1β1-treated mice. Chronic antipsychotic treatment selectively decreases dopamine transmission in FC,[67] although different brain regions are also involved in PPI abnormality.[68] Although a plenty of controversies and discrepancies against the roles of dopamine in schizophrenia remain, our findings with the NRG1β1-treated mice provide evidence in favor of a hyperdopaminergic state in schizophrenia, at least within FC.[68–70]

In contrast, the pathological link between the dopaminergic abnormality and the social deficits of this mouse model is controversial, as the reduction in social interaction is rather ascribed to the hypodopaminegic or serotonergic deficits.[27,71] In this context, we do not exclude the possibility that uncovered neurochemical deficits of NRG1β1-treated mice still remains to be explored.

Pathological implication of NRG1β1 in neurodevelopmental hypothesis of schizophrenia

The SNPs of the *NRG1* gene that associate with the risk for schizophrenia are often located in the promoter region of *NRG1* gene[1] and presumably involved in positive regulation of *NRG1* gene transcription.[5] In agreement, post-mortem studies support the association of increased NRG1 mRNA or protein with schizophrenia.[3–5] Depending on the SNP type of *NRG1*, ischemic and traumatic brain injury may induce higher levels of NRG1 expression.[10,11] In this context, NRG1 is one of the candidate molecules that might be involved in both genetic and environmental vulnerabilities to schizophrenia.[72]

Maternal infections and fetal/neonatal hypoxia are potential environmental risk factors for schizophrenia and are used in its animal modeling.[7,8,73] Interestingly, the animal models established by the immune inflammatory insults also exhibit impaired dopaminergic innervations or metabolism.[74–76] In this context, it is noteworthy that NRG1 is highly inducible in fetuses and neonates in response to these environmental insults.[9,10] Therefore, it is possible that NRG1 and potentially other ErbB4 ligands might contribute to the dopaminergic impairment of these animal models as well as that of schizophrenia.[25,26]

On the basis of the neurodevelopmental hypothesis,[24–26] we have tested various inflammatory cytokines and neurotrophic factors to address the question of whether they can mediate the environmental insults for schizophrenia risk.[77]Among many factors examined, neonatal treatment only with epidermal growth factor, interleukin-1 and NRG1 produce the long-lasting behavioral impairments that are implicated in schizophrenia models.[40,66,78] Interestingly, these factors have a common neurotrophic activity on midbrain dopaminergic neurons.[30,40] However, we did not detect any neurobehavioral influences of control proteins (cytochrome c and albumin) in the *in vivo* experimental paradigm.[40,77,78]

In conclusion, NRG1 is one of the key neurotrophic factors that have crucial impact on dopaminergic development and its neuropathology. We hope that this model established with NRG1 will facilitate the validation of both neurodevelopmental and dopaminergic hypotheses of schizophrenia.

Conflict of interest

HN was a recipient of the research grants from Astrazeneca Pharmaceutical Inc.

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Note added in proof: We also tested the *in vivo* activity of the misfolded NRG1β1 in the peak 1 of the cation-exchange chromatography (Supplementary Figure S1) and did not detect its effects on TH and dopamine content (Supplementary Figure S8).

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