(Addington et al. 2007; Corvin et al. 2004; Fukui et al. 2006; Gardner et al. 2006; Harrison and Weinberger 2005; Li et al. 2004; Tang et al. 2004; Thomson et al. 2007; Williams et al. 2003; Yang et al. 2003), although there are several contradictory reports (Ikeda et al. 2008; Iwata et al. 2004; Munafò et al. 2006; Thiselton et al. 2004). Postmortem studies support a genetic contribution of *NRG1* to schizophrenia (Hashimoto et al. 2004; Law et al. 2006). The expression of mRNAs encoding *NRG1* precursors is altered in schizophrenia patients; in particular, the type-I splice variants of *NRG1* mRNA are upregulated in the prefrontal cortex and hippocampus of affected patients (Hashimoto et al. 2004; Law et al. 2006). Although studies have attempted to correlate abnormalities in NRG1 mRNA expression with patient single nucleotide polymorphism (SNP) haplotype (Law et al. 2006), controversy surrounds the use of postmortem samples, especially for mRNA analysis (Chagnon et al. 2008; Harrison et al. 1995; Morrison-Bogorad et al. 1995; Tomita et al. 2004). Terminal conditions, such as coma and hypoxia, might influence *NRG1* gene expression and RNA quality in the brain (Chagnon et al. 2008; Harrison et al. 1995; Morrison-Bogorad et al. 1995; Tomita et al. 2004).

mRNA and protein measurements in blood are often used to diagnose schizophrenia or investigate the pathological contribution of individual molecules (Chagnon et al. 2008; Petryshen et al. 2005; Zhang et al. 2008). Of the molecules examined, growth factors and cytokines displayed marked abnormalities in both central nervous system and peripheral blood (Bellon 2007; Futamura et al. 2002; Sei et al. 2007; Takahashi et al. 2000; Toyooka et al. 2002, 2003). mRNA expression levels for *NRG1* precursors are determined by evaluation of peripheral lymphocytes from schizophrenia patients (Chagnon et al. 2008; Petryshen et al. 2005; Zhang et al. 2008). However, NRG1 peptide levels in peripheral blood have not yet been studied. The production and release of mature NRG1 peptides require proteolytic processing of precursors (i.e., ectodomain shedding) (Marchionni et al. 1993; Shirakabe et al. 2001; Yokozeki et al. 2007). This process liberates mature NRG1 peptides from the cell-anchored precursor proteins, allowing it to activate ErbB receptors. Thus, the measurement of free mature NRG1 peptides is necessary to estimate the biological activity.

Here, we established an enzyme-linked immunosorbent assay (ELISA) for NRG1 and measured trace NRG1-like immunoreactivities (LI) in the cell-free soluble fraction of human serum. ELISA allowed us to estimate the effects of gender, age and schizophrenia diagnosis, disease duration and antipsychotic treatment on human serum NRG1 peptide levels. We also attempted to estimate the contribution of SNPs in *NRG1* genome to serum NRG1-LI levels.

Materials and methods

Subjects

This clinical study was approved by the Ethics Committee on Genetics of the Niigata University School of Medicine. Written informed consent was obtained from all participants. Patients meeting the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria for schizophrenia were recruited from two hospitals. The diagnosis of schizophrenia was based on all available sources of information, including unstructured interviews, clinical observations and medical records. Control subjects were recruited primarily from the staff of participating hospitals and associated laboratories. We matched the ages and genders of the control healthy volunteers to those of the patients examined. Although these subjects were not assessed by a structured psychiatric interview, all of them demonstrated good social and occupational skills and did not report any history of psychiatric disorders.

Three young adult male cynomolgus monkeys (*Macaca fascicularis*), 4 years of age, weighing 3.12–3.98 kg, were used in this study. Monkeys, reared at the animal house of Shinn Nippon Biomedical Lab. Inc. (Kagoshima, Japan), were housed individually in stainless steel cages of 50 cm (*W*) × 86 cm (*D*) × 82 cm (*H*) under temperature-controlled conditions, at 26 ± 2°C and a humidity of 40–60% under a 12/12-h light/dark cycle. Animals were fed 108 g commercial monkey chow daily. Filtered water was delivered by an automatic supplier ad libitum. Experiments were subjected to review by the Ethical Committee of Shinn Nippon Biomedical Lab. Inc., and were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the Guidelines of the Central Research Laboratory.

Blood sampling

Blood was collected in vacuum collection tubes (Neotube, NP-PS0507, Nipro, Osaka, Japan) between 9 and 12 a.m. in the morning. Within 1 h of collection, blood was coagulated at 37°C for 60 min. Serum was separated by centrifugation at 4°C for 15 min and stored at −80°C until use for analysis.

NRG enzyme immunoassay

We produced anti-NRG1β1 polyclonal antibodies by immunizing rabbits and guinea pigs with a mouse cerebellar isoform of NRG1β1 peptide (Ozaki et al. 2000). This type-I isoform, with a molecular weight of 25,500 Da,

consists of an immunoglobulin-like (Ig) domain and an epidermal growth factor (EGF)-like domain alone. Rabbit and guinea pig antisera were subjected to antigen-affinity chromatography (Affi-Gel 10, Bio-Rad, Hercules, CA, USA). We established ELISA using the affinity-purified antibodies according to the previous procedures (Nawa et al. 1995).

In brief, ELISA titer plates were coated with 100 ng rabbit anti-NRG1β1 antibody per well in 0.1 M Tris buffer (pH 9.0) for 12–18 h and then blocked with ELISA buffer [50 mM Tris, 0.5 M NaCl, 0.1% NaN_3, 0.2% Triton X-100, 1% gelatin (pH 6.8)] at 4°C for more than 12 h. Serum (100 μL; quadruplicate) or standard NRG1β3 (10–1,000 pg; quadruplicate) was loaded into wells. After five washes, each well was incubated with 30 ng of guinea pig anti-NRG1β1 antibody followed by rabbit anti-guinea pig Ig biotinylated antibody (1:5,000; Open Biosystems, Huntsville, AL, USA). Biotinylated antibodies bound to wells were incubated with 100 μL avidin-β-galactosidase (1:10,000; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) followed by the fluorogenic substrate, 200 μM 4-methylumbelliferyl-β-D-galactoside (MUG; Sigma Chemicals, St. Louis, MO, USA). The fluorescent product was quantitated using an MTP-601F microplate reader (Corona, Ibaraki, Japan) with excitation and emission at 365 and 450 nm, respectively. To evaluate the cross-reactivity of ELISA, we obtained the following human recombinant factors that exhibit structural homology or similarity to NRG1: betacellulin, EGF, heparin-binding EGF-like growth factor (HB-EGF), epiregulin and transforming growth factor-α (TGFα) (Peprotech, Rocky Hill, NJ, USA, or Sigma Chemicals, St. Louis, MO, USA). The mature human NRG1β3 peptides, which correspond to the respective extracellular domains of NRG1 precursors (Genbank; NM_013956.3 for type I, NM_013962.2 for type II, and NM_013959.3 for type III), were synthesized by an in vitro translation/transcription system (T_NT Quick Coupled transcription/Translation Systems; Promega, Addison, WI, USA) using synthetic cDNAs encoding the corresponding domains (GenScript, Piscataway, NJ, USA). The amount of each type of NRG1β3 peptides was determined by immunoblotting for their histidine tag (R. Wang, unpublished data).

Genotyping

DNA was extracted from blood clots using Puregene core kit A (Qiagen, Germantown, MD, USA). We selected six SNPs from the human *NRG1* genome, which have been intensively characterized previously, to investigate rs35753505 (SNP8NRG221533), SNP8NRG241930, rs6994992 (SNP8NRG243177), rs1081062, rs3924999 and rs2954041 (Ikeda et al. 2008). These six SNPs were genotyped using the TaqMan assay as described previously (Fukui et al. 2006).

Chronic treatment of cynomolgus monkeys with haloperidol

Young adult monkeys (all male) were given oral haloperidol for 2 months. Haloperidol (Wako Chemical Ltd, Tokyo, Japan), suspended in 0.5% methylcellulose solution, was administered at concentrations of 0.125–0.25 mg/mL to monkeys daily with the aid of a gastric tube for 8 weeks. The initial dose of 0.25 mg/kg for 2 weeks was increased to 0.5 mg/kg for the following 6 weeks. We confirmed normal food consumption daily and monitored body weight gain weekly to avoid adverse effects of haloperidol treatment. Whole blood (5 mL/animal) was collected from the femoral vein before treatment and after 4 and 8 weeks of haloperidol treatment. Serum samples were prepared to measure haloperidol concentrations as well as Ig-NRG-LI levels.

Statistical analysis

To analyze multifactorial interactions and ascertain statistical power, we applied parametric analyses to data. Genotype deviation from Hardy–Weinberg equilibrium (HWE) and allele frequency difference between patients and controls were evaluated by using the χ^2 test. ELISA data were initially analyzed with the analysis of variance (ANOVA) using gender, disease, DSM-IV type and/or genotype as between-subject factors. The correlations between each potential confounding factor and Ig-NRG1-LI levels were examined by Pearson correlation analysis. The putative confounding factors included age, gender, duration of illness, age at onset and dose of antipsychotic medication. As we identified a significant and strong correlation of ELISA data with gender in the initial analysis, data were all re-analyzed using analysis of covariance (ANCOVA), adopting gender as a covariate. We used Fisher least significant difference (LSD) test for post hoc analysis. A probability level of $P < 0.05$ was considered to be statistically significant. All data represent the mean ± SE. Statistical analysis was performed using SPSS software (version 11.5).

Results

Establishment of a sandwich ELISA for NRG1

We raised antisera directed against recombinant mouse NRG1β1 (type I, soluble mature form) in rabbits and guinea pigs and established a sandwich ELISA for NRG1

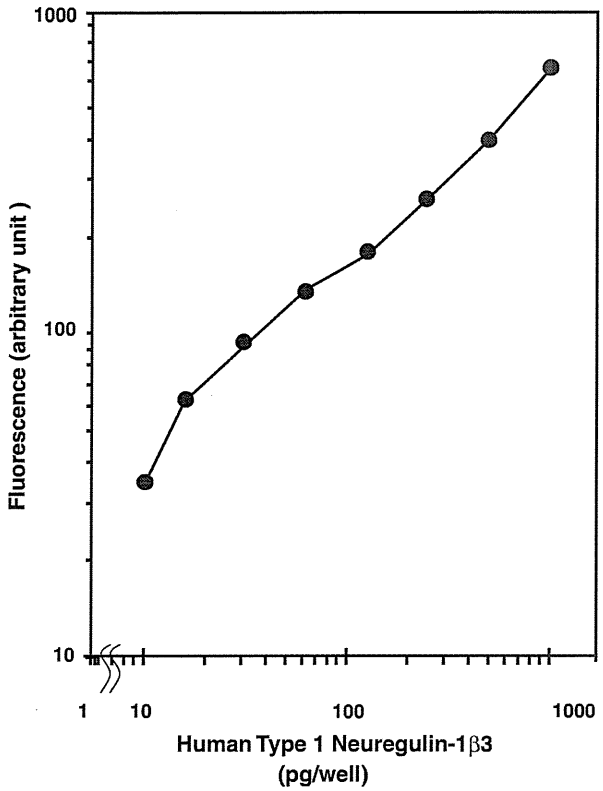

Fig. 1 A standard curve of ELISA with control type-1 NRG-1 peptide. Various amounts of human recombinant NRG1β3 (10–1,000 pg/well/100 μL) were applied to ELISA as a standard. Total immunoreactivity for NRG1 trapped by the primary antibody was determined using the guinea pig secondary anti-NRG1β1 antibody followed by detection with a β-galactosidase-conjugated antibody against guinea pig immunoglobulin. Enzymatic activity in each well was measured using the fluorogenic substrate MUG. The enzymatic product derived from MUG exhibits fluorescence at 450 nm with 365 nm excitation

(Nawa et al. 1995). The combination of these antibodies in ELISA generated a linear standard curve for concentrations of 10–1,000 pg/well for a soluble form of human NRG1β3 (Fig. 1). As NRG1 structurally belongs to the EGF family, we tested the cross-reactivity of this ELISA system to other members in this family, including EGF, HB-EGF, TGFα, betacellulin and epiregulin (Table 1). The cross-reactivity to these factors was <0.1% of the signal for type-I human NRG1β3. We also tested the reactivity of ELISA to the EGF domain of human NRG1 as well as other splice variants of human NRG1. Reactivity to the EGFβ1 domain was negligible (<0.01%), and that to the type-II and type-III variants was 61.0 and 7.2% of the levels seen for type-I NRG1 used as a standard, respectively. These results suggest that this ELISA primarily recognizes not the common EGF domain, but the Ig-like domain of NRG1, which is present in type-I and type-III NRG1, but not type-II NRG1. Using this novel ELISA, we measured

Table 1 Cross-reactivity to types I–III NRG-1 and other neurotrophic factors

Factor	Reactivity (%)
Human NRG1β3 (type I)	100
Human NRG1β3 (type II)	61.0
Human NRG1β3 (type III)	7.2
Human NRG1 core EGFβ1 domain	<0.01
Human betacellulin	<0.1
Human epidermal growth factor (EGF)	<0.01
Human heparin binding-EGF-like growth factor (HB-EGF)	<0.01
Human epiregulin	<0.01
Human transforming growth factor-α (TGF-α)	<0.01

Purified cytokines, growth factors and neurotrophic factors (10 ng) were applied to ELISA. The intensities of their signals were compared to those of human NRG1β3 (type I) in a standard curve

Table 2 Profiles of patients with schizophrenia and control volunteers

	Patients	Controls
Age (mean ± SD) (years)	32.7 ± 6.7	33.3 ± 6.9
Gender		
Men	20	28
Women	20	31
Duration of illness (mean ± SD) (years)	9.4 ± 5.7	
Age at onset (years)	23.2 ± 4.8	
Dose of antipsychotic medication[a]	716 ± 338	
Medication[b]		
Olanzapine (5–30 mg/day)	18	
Quetiapine (200–600 mg/day)	10	
Risperidone (2–9 mg/day)	6	
Perospirone (36–48 mg/day)	4	
Aripiprazole (12–18 mg/day)	4	
Haloperidol (4–12 mg/day)	2	
Others (2)[c]	2	

[a] Chlorpromazine-equivalent dose (mg/day)

[b] Some patients took multiple antipsychotics

[c] Other patients took clozapine (500 mg/day) or levomepromazine (25 mg/day)

According to DSM-IV diagnostic criteria, patients consisted of 13 disorganized types, 4 paranoid types and 23 undifferentiated types

Ig-like domain-containing NRG1-like immunoreactivity (Ig-NRG-LI) in human serum.

NRG1 immunoreactivity in sera of schizophrenia patients and control subjects

We obtained sera from schizophrenia patients ($n = 40$) and age- and gender-matched healthy volunteers ($n = 59$) (Table 2). The time of blood sampling, type of sampling

tube and coagulation procedures were consistent between patients to minimize the influence of differences in sampling conditions (see "Materials and methods"). Human serum samples from healthy volunteers contained Ig-NRG-LI with concentrations ranging from 0.4 to 14.0 ng/mL (Fig. 2). The concentrations in healthy women samples (mean 8.02 ± 1.33 ng/mL) were significantly higher than those in men (mean 5.61 ± 0.91 ng/mL, $P = 0.048$). Two-way ANOVA using subject factors of diagnosis (patient vs. control) and gender (men vs. women) revealed main effects for both diagnosis ($F_{(1,95)} = 11.50$; $P = 0.001$) and gender ($F_{(1,95)} = 9.23$; $P = 0.003$) without an interaction between these two factors ($F_{(1,95)} = 0.24$; $P = 0.627$). Mean Ig-NRG1 immunoreactivity in the schizophrenia group was 63.2% of the mean level observed in the control group. Mean Ig-NRG1 immunoreactivity in the women's group was 147.1% of that detected in the men's group.

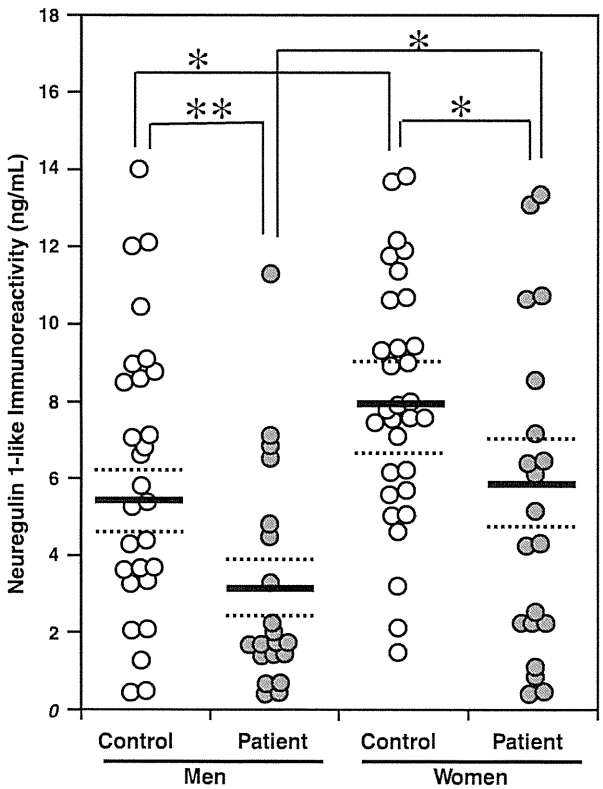

Fig. 2 Serum Ig-NRG1-LI levels in patients with schizophrenia and age-matched controls. Serum samples were collected from 40 patients with schizophrenia ($n = 20$ for men and $n = 20$ women) and 59 healthy volunteers ($n = 28$ for men and $n = 31$ women) and subjected to ELISA to detect Ig-NRG-LI. Mean levels are marked with *thick horizontal lines*, while SE levels are denoted by *horizontal broken lines*. Post hoc analysis detected a significant difference between groups; $^{**}P < 0.01$ and $^{*}P < 0.05$

Table 3 Correlations between levels of neuregulin and clinical parameters in patients with schizophrenia

Pearson correlation	r
Age[a]	0.340*
Age at onset	0.333*
Duration of illness	(0.114)
Duration of medication	(−0.006)
Dose of antipsychotic medication[b]	(−0.235)

* $P < 0.05$, parentheses indicates insignificance

[a] In control subjects; $R = 0.432$, $P = 0.006$

[b] Chlorpromazine-equivalent mg per day

To estimate the influence of patient clinical features on Ig-NRG-LI levels, we performed Pearson correlation analysis with compensation for gender effect (Table 3). There were modest correlations of both age ($R = 0.340$; $P = 0.032$) and disease onset ($R = 0.333$; $P = 0.036$) with Ig-NRG-LI. Neither disease duration nor medication length, however, correlated significantly with Ig-NRG-LI. As the prescribed antipsychotics differed between patients, we calculated chlorpromazine-equivalent doses based on an antipsychotic potency table of commercial drugs (Inada 2006) (Table 2). We did not find a significant correlation between chlorpromazine-equivalent dose and Ig-NRG-LI level ($R = -0.235$; $P = 0.144$). Although we also attempted to assess an interaction between DSM-IV category and Ig-NRG-LI, one-way ANOVA failed to detect a significant difference in Ig-NRG-LI levels among DSM-IV types ($F_{(2,39)} = 0.15$; $P = 0.865$).

Association between NRG1 immunoreactivity and SNP types

We examined the six SNP sites within the *NRG1* gene that are reported to be associated with schizophrenia risk: SNP8NRG221533, SNP8NRG241930, SNP8NRG243177, rs1081062, rs3924999 and rs2954041 (Bakker et al. 2004; Fukui et al. 2006; Hall et al. 2006; Ikeda et al. 2008; Iwata et al. 2004; Law et al. 2006; Li et al. 2004, 2006; McIntosh et al. 2008; Munafò et al. 2006; Nicodemus et al. 2009; Stefansson et al. 2002; Williams et al. 2003; Yang et al. 2003). The genotype distributions of the SNPs examined did not differ significantly from HME in either group (patient: $P = 0.203$–0.747, control: $P = 0.096$–0.678), with the exception of SNP8NRG221533 (patient: $P = 0.016$, control: $P = 0.052$; χ^2 test). With the given sample sizes, we failed to detect any significant difference in genotype distributions at all SNP loci between patient and control groups ($P = 0.073$–0.90; χ^2 test). As there were significant gender differences in Ig-NRG-LI, we adopted ANCOVA with a covariate of gender to estimate SNP effects.

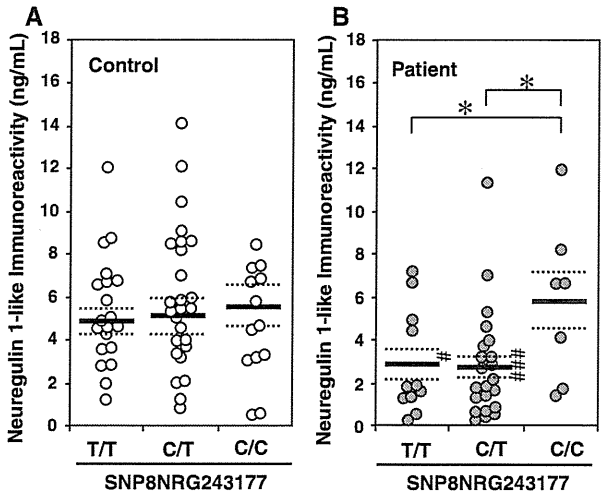

Fig. 3 Association between SNP8NRG243177 and serum Ig-NRG1-LI. Serum Ig-NRG-LI levels detected in individuals with each SNP genotype (SNP8NRG243177; T/T, C/T, C/C) were plotted for the control and patient groups. Mean levels are marked with *thick horizontal lines*, while SE levels are denoted by *horizontal broken lines*. $^{*}P < 0.05$, compared between genotypes of patient group, and $^{\#\#\#}P < 0.001$ and $^{\#}P < 0.05$, compared between patient and control groups of the same genotype

At the site of SNP8NRG243177, two-way ANCOVA revealed a main effect of diagnosis ($F_{(1,93)} = 4.78$; $P = 0.031$), but not that of genotype ($F_{(2,93)} = 2.00$; $P = 0.141$), with an significant interaction between the two subject factors ($F_{(2,93)} = 4.43$; $P = 0.015$), suggesting differential contribution of genotype to the disease-associated decreases in Ig-NRG1-LI levels (Fig. 3). Post hoc analysis revealed that patients with the SNP T allele (T/T or C/T) exhibited lower levels of Ig-NRG-LI than schizophrenia patients bearing the homozygous C allele ($P = 0.038$ for T/T vs. C/C, $P = 0.016$ for T/C vs. C/C) or controls with the same allele ($P = 0.025$ for T/T, $P = 0.0003$ for T/C). The allelic difference in serum Ig-NRG1-LI levels was not observed in the control group. Thus, these results suggest that the T allele at SNP8NRG243177 is one of the biological contributors to the disease-associated decreases in serum Ig-NRG-LI levels. We also analyzed the influences of the other SNP genotypes on serum Ig-NRG-LI. However, there were no significant differences detected at these SNP loci ($F_{(2,93)} = 0.43$–2.28; $P = 0.108$–0.645) without interaction ($F_{(2,93)} = 0.28$–4.70; $P = 0.098$–0.753).

Influence of chronic haloperidol administration to monkeys

To estimate the potential influence of antipsychotic medications given to patients with schizophrenia, we administered haloperidol (0.25–0.50 mg/kg) to three cynomolgus monkeys for 2 months and monitored Ig-NRG1-LI levels in serum before and during treatment. Serum concentrations of haloperidol were 4.00 ± 0.62 ng/mL after 4 weeks of treatment and 4.03 ± 0.52 ng/mL after 8 weeks of treatment. Mean levels of IG-NRG1-LI were 7.0 ± 2.1, 7.5 ± 2.6 and 8.4 ± 2.9 ng/mL before and 4 and 8 weeks after beginning treatment, respectively. One-way ANOVA failed to detect any significant difference during drug treatment ($F_{(2,5)} = 4.07$; $P = 0.865$).

Discussion

To evaluate the pathological influences of schizophrenia on NRG1 protein, we established ELISA for NRG1 and measured NRG1 immunoreactivity in serum. We found that the human sera contains high concentrations of Ig-NRG1-LI and that the concentrations are significantly lower in men as well as in patients with schizophrenia. In addition, the schizophrenia-associated decrease in serum Ig-NRG1-LI is apparent only in patients carrying the schizophrenia risk allele at the SNP8NRG243177 site.

Neuregulin-1 variants possess a common EGF-like core domain that is responsible for its biological activity, thus sharing significant structural similarity with other EGF-like peptides. It was thus important to evaluate the cross-reactivity of the ELISA system with other EGF-like peptides. Despite careful examination of six distinct peptides in the EGF family, none exhibited significant cross-reactivity. As multiple gene promoters and alternative splicing produce distinct structural variants of *NRG1* precursors (Falls 2003; Harrison and Law 2006), it was essential to determine which NRG1 subtypes were recognized by this ELISA system. The antigen used to raise antibodies was a soluble mature form of type-1 NRG1β1 derived from mouse cerebellum, which contains only an Ig-like domain and EGF-like domain (Ozaki et al. 2004). Resulting antibodies derived from rabbits and guinea pigs failed to react with the EGF-like domain, leading to the assumption that these antibodies primarily recognize the Ig-like domain of NRG1. In support of this assumption, ELISA detected type-II NRG1, which contains the Ig domain with high efficiency. Reactivity to type-III NRG1, which lacks the Ig-like domain, was <10% of that to type-I NRG1.

Several clinical features of schizophrenia patients exhibited modest, but significant, correlations with Ig-NRG-LI levels using our novel ELISA. Serum Ig-NRG-LI levels weakly correlated with age ($R = 0.340$) and disease onset ($R = 0.333$) following gender compensation. To confirm the statistical results of ANOVA, we also performed ANCOVA, adopting each of these factors individually or together as covariate(s), and yet obtained the same statistical conclusion (data not shown). It is

noteworthy that our statistical analysis did not detect a significant correlation between antipsychotic medication and serum Ig-NRG-LI levels, although calculating chlorpromazine equivalents of second-generation antipsychotics is controversial among researchers (Woods 2003; Inada 2006). This statistical result of antipsychotic effects appeared to be supported by the present monkey experiment: the chronic treatment of monkeys with haloperidol had no significant effect on serum Ig-NRG1-LI. Pharmaceutical effectiveness of haloperidol in monkeys was controlled by measuring its blood concentrations. Concentrations of haloperidol in monkey serum corresponded to the human therapeutic range of the antipsychotic medication (3–17 ng/mL) (Guthrie et al. 1987). As the given species of antipsychotics to monkeys and patients were not fully identical, however, the interpretation of the present monkey results is limited and needs to be re-evaluated in drug-naïve schizophrenia patients.

In the present assay, we found that human serum contains markedly high concentrations of Ig-NRG1-LI (mean 5.97 ± 0.40 ng/mL, ~213 ± 14 pM) in comparison to the concentrations of other growth factors and cytokines. Concentrations of EGF, HB-EGF and TGFα are 100–400 pg/mL in human serum (Futamura et al. 2002), and those of inflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor alpha are also below 1 ng/mL (Akiyama 1999; Schmitt et al. 2005). Our preliminary study indicates that the concentrations of Ig-NRG1-LI in human plasma were as high as in serum (M. Shibuya, unpublished data). As NRG-1 concentrations in human blood are above the reported dissociation constant of the NRG-1 receptor (k_d; ~60 pM for ErbB3) (Carraway et al. 1994), human NRG-1 peptides in circulation presumably exert a biological activity or influences on tissue targets.

There was a significant gender difference in serum Ig-NRG-LI levels between men and women. Ig-NRG-LI quantities in females were significantly higher than those observed in males, irrespective of the presence of schizophrenia. This trend contrasts the fact that another neurotrophic factor, nerve growth factor, is enriched in the serum of human males (Martocchia et al. 2002). LaCroix-Fralish et al. (2006, 2008) report that protein and mRNA expression levels of NRG1 are higher in female rats. As there were no significant gender differences in *NRG1* mRNA levels observed in postmortem brains (Hashimoto et al. 2004), this gender difference may be limited to NRG1 expression in the peripheral blood.

Although there are several studies examining the expression of *NRG1* mRNA in postmortem brains of patients with schizophrenia, most have reported an increase in mRNA levels. The hippocampus and prefrontal cortex of schizophrenia patients contain higher levels of type-I mRNA than samples from control subjects (Hashimoto et al. 2004; Law et al. 2006). Petryshen et al. (2005) report an increase in mRNA encoding type-III NRG1 precursor (SMDF), while Zhang et al. (2008) detect decreases in type-I and type-II mRNAs encoding NRG1 precursors (HRG-β3 and GGF2) in patient lymphocytes. The latter report is consistent with our present findings on NRG1 expression in blood, assuming that our novel ELISA primarily detected the type-I and type-II NRG1 peptides.

The schizophrenia-associated SNP sites within the *NRG1* gene are located in the 5′-flanking region of the gene promoter (Harrison and Law 2006; Lawrie et al. 2008; Mei and Xiong 2008). Of the six SNPs examined, patients with the T allele at the SNP8NRG243177 site displayed a reduction in serum Ig-NRG-LI levels. Brain imaging studies reveal that the T allele is associated with the reduction of white matter density in the anterior limb of the internal capsule (McIntosh et al. 2008). This finding is in agreement with the fact that NRG1 is a positive regulator for myelination and oligodendrocyte survival (Fernandez et al. 2000; Corfas et al. 2004). In this context, it is possible that the serum reduction in Ig-NRG1-LI might contribute to the white matter deficits of schizophrenia patients.

This SNP site is located at the 5′ region of transcription initiation sites of type-II and type-IV mRNAs, which both encode Ig-NRG1 (Li et al. 2006). The T allele-specific reduction in Ig-NRG-LI was only found in the patient group and is therefore consistent with the finding that this allele is associated with schizophrenia susceptibility (Law et al. 2006; Steinthorsdottir et al. 2004). It is unknown, however, whether the allele-dependent Ig-NRG-LI reduction is ascribed to the difference in basal or regulated transcription rates of *NRG1* gene (Tan et al. 2007). A postmortem brain study combining RT-PCR for *NRG* mRNAs and SNP typing comes to a conclusion in conflict with our findings. The hippocampus of human subjects bearing the risk allele expresses higher levels of type-IV variants of *NRG1* mRNA, irrespective of the diagnosis (Law et al. 2006). Although it remains to be determined whether *NRG1* gene transcription is under the same regulation in both periphery and brain, the answer to this discrepancy awaits future experiments with a larger number and the combination of peripheral and brain samples. We hope that the ELISA system for NRG1 peptide will help to explore its biological role in schizophrenia pathogenesis or diagnosis.

Acknowledgments The authors thank the patients and healthy volunteers for their participation. This work was supported by Health and Labor Sciences Research Grants, a grant from the Promotion of Niigata University Research Projects, Core Research for Evolutional Science and Technology from the JST Corporation, and a grant-in-aid from the Ministry of Health, Labor and Welfare, Japan.

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Phenotypic Characterization of Transgenic Mice Overexpressing Neuregulin-1

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Abstract

Background: Neuregulin-1 (NRG1) is one of the susceptibility genes for schizophrenia and implicated in the neurotrophic regulation of GABAergic and dopaminergic neurons, myelination, and NMDA receptor function. Postmortem studies often indicate a pathologic association of increased NRG1 expression or signaling with this illness. However, the psychobehavioral implication of NRG1 signaling has mainly been investigated using hypomorphic mutant mice for individual NRG1 splice variants.

Methodology/Principal Findings: To assess the behavioral impact of hyper NRG1 signaling, we generated and analyzed two independent mouse transgenic (Tg) lines carrying the transgene of green fluorescent protein (GFP)-tagged type-1 NRG1 cDNA. The promoter of elongation-factor 1α gene drove ubiquitous expression of GFP-tagged NRG1 in the whole brain. As compared to control littermates, both heterozygous NRG1-Tg lines showed increased locomotor activity, a nonsignificant trend toward decreasing prepulse inhibition, and decreased context-dependent fear learning but exhibited normal levels of tone-dependent learning. In addition, social interaction scores in both Tg lines were reduced in an isolation-induced resident-intruder test. There were also phenotypic increases in a GABAergic marker (parvalbumin) as well as in myelination markers (myelin basic protein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase) in their frontal cortex, indicating the authenticity of NRG1 hyper-signaling, although there were marked decreases in tyrosine hydroxylase levels and dopamine content in the hippocampus.

Conclusions: These findings suggest that aberrant hyper-signals of NRG1 also disrupt various cognitive and behavioral processes. Thus, neuropathological implication of hyper NRG1 signaling in psychiatric diseases should be evaluated with further experimentation.

Citation: Kato T, Kasai A, Mizuno M, Fengyi L, Shintani N, et al. (2010) Phenotypic Characterization of Transgenic Mice Overexpressing Neuregulin-1. PLoS ONE 5(12): e14185. doi:10.1371/journal.pone.0014185

Editor: Thomas Burne, University of Queensland, Australia

Received January 22, 2010; **Accepted** November 10, 2010; **Published** December 9, 2010

Funding: This work was supported by Health and Labor Sciences Research Grants, a grant for Promotion of Nigata University Research Projects, Core Research for Evolutional Science and Technology from the Japan Science and Technology Agency, and a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan. Hiroyuki Nawa was a recipient of research grants from Astrazeneca Pharmaceutical. No funders had a role in either the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: HN was a recipient of the research grants from Astrazeneca Pharmaceutical, Inc. This does not alter the authors' adherence to the PLoS ONE policies on sharing data and materials.

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Introduction

A genetic association between the neuregulin-1 (NRG1) gene and schizophrenia has been documented in various human populations. However, the exact biological relationship is still unclear [1–3]. Many model studies have used NRG1 hypomorphic mutant mice to study the phenotypic consequences of decreased NRG1 signaling as well as its pathologic contribution to schizophrenia [2], [4–13]. Differential promoter usage and alternative splicing produce a large variety of structural variants of NRG1 precursor proteins. For example, the type-1, -2, and -4 subgroups of NRG1 contain a immunoglobulin-like domain and a transmembrane domain while the type-3 variant carries two transmembrane domains and a cystein-rich domain [14], [15]. Mutant mice deficient in NRG1 have been found to exhibit schizophrenia-associated behavioral abnormalities in sensorimotor gating [2], [4], social interactions [5], [9–11], latent inhibition [12], and locomotor activity [8], [13], although neurobehavioral features of the individual mutants significantly differ depending upon the targeted isoforms of NRG1 [2], [6], [7–13]. NRG1 has neurotrophic activities to promote NMDA receptor expression, GABA synthesis, and myelination, all of which are diminished in postmortem brain of schizophrenia patients [14], [15]. The animal and patient studies suggest that

decreased NRG1 signals are responsible for the pathophysiology of schizophrenia [14], [15]. However, this argument is not supported by all types of studies. For example, postmortem studies report that higher levels of type-1 and type-4 NRG1 mRNA are present in the hippocampus and prefrontal cortex of schizophrenic patients as well as in patients' lymphocytes, compared to control subjects [16–19]. Similarly, the up-regulation of the NRG1 protein or its signaling is detected in the brains of schizophrenic patients [20], [21]. Thus, these patient studies rather suggest a biological link between increased NRG1 signaling and the pathophysiology of schizophrenia. As the type-1 NRG1 variant display marked mRNA increase in patients' postmortem brain and single nucleotide polymorphisms (SNPs) of its corresponding genome locus are implicated in genetic vulnerability to schizophrenia [18], [19], we have established mouse transgenic lines carrying the transgene of mouse type-1 NRG1 cDNA and examined whether NRG1 hypermorphic mice, as opposed to NRG1 hypomorphic mice, are an appropriate animal model for schizophrenia. In the present investigation, we analyzed two independent transgenic (Tg) mouse lines to minimize the effects of the transgene insertion in their host genome as well as those of the genetic inhomogeneity of mouse genetic background. Further, we examined neurochemical consequences of NRG1 overexpression in several neuronal and glial markers in one of the Tg lines. Behavioral similarity and difference between the present Tg mice and reported NRG1-knockout mice are also discussed.

Results

Generation of transgenic mice overexpressing NRG1

We constructed the Tg vector that contained the promoter of a house keeping gene, elongation-factor 1α (EF1α) and GFP-tagged NRG1β1 cDNA. The GFP-tag facilitated transgene expression and detection in mice. The Tg vector was injected to fertilized eggs to generate transgenic mice (**Fig. 1A**). The modification of GFP tagging is known not to affect NRG1 function [22]. We selected two independent NRG1-Tg lines, Tg5 and Tg7, which were viable and healthy with normal body weights and reproduction (data not shown). The number of transgene copies integrated in genome was estimated by polymerase chain reaction (PCR). Densitometric measurement revealed that PCR products from Tg5 DNA first appeared at ~22 cycle and that from Tg7 at ~24 cycle, 3–4 and 1–2 cycle earlier than the emergence of wild type (WT) mice product (2 copies of wild allele) respectively (**Fig. 1B**). Based on the present efficiency of PCR amplification (1.6±0.1 fold/cycle), we estimated that Tg5 mouse contained 5~6 copies of the transgene and Tg7 mouse carried ~2 copies. To confirm the expression of the transgene in the brain, we performed an immunoblotting analysis with anti-NRG1 and anti-GFP antibodies. There were NRG1-like and GFP-like immunoreactivities at the same size (55 kDa) in whole brain lysates of Tg5 and Tg7 (**Fig. 1C**). The size approximately matches the sum of molecular weights of GFP (25 kDa) and a shedded mature form of NRG1β1 (30 kDa). The Tg5 line contained higher levels of

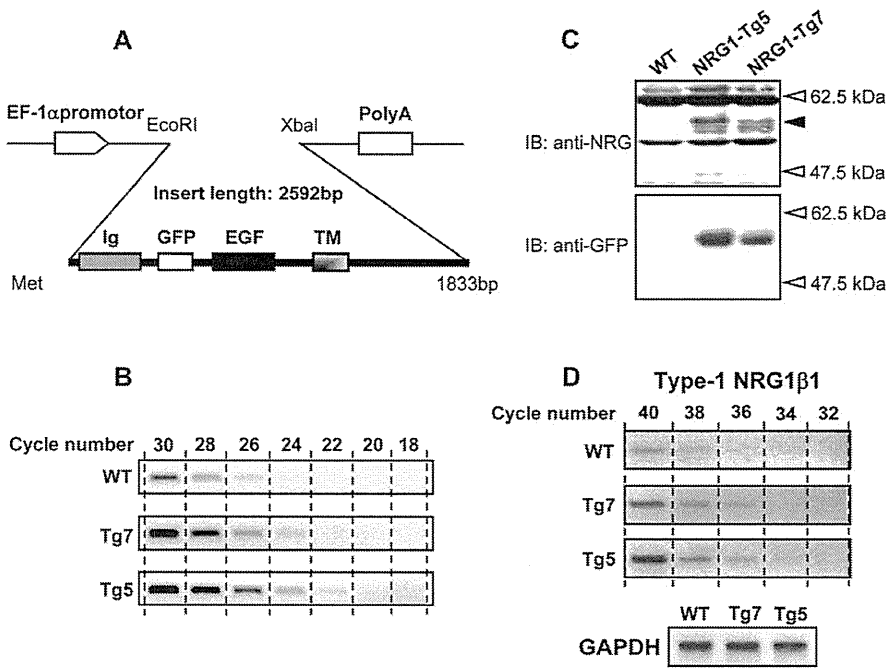

Figure 1. Establishment of GFP-tagged NRG1 transgenic mice with EF1α-promoter. (**A**) A schematic illustration of a transgene construct carrying EF-1α genomic promoter, NRG1β1 cDNA, GFP-tag insertion, and poly A signal. (**B**) Estimation of the copy number of the transgene by PCR. The exon 3 fragment of NRG1 genome was amplified with 18–30 cycles using tail DNA from Tg5 and Tg7 and separated in an agarose-gel. (**C**) Protein lysate was prepared from whole brain of adult male NRG1-Tg mice (Tg5 and Tg7) and WT littermate and subjected to immunoblotting with anti-NRG1 and anti-GFP antibodies. A closed arrowhead marks the transgene products. (**D**) Quantification of mRNA levels for type-1 NRG1 by RT-PCR. cDNA fragments specific for type-1 NRG1 and GAPDH mRNAs were amplified in the presence of SYBR Green I. PCR amplification curves and difference in Ct were analyzed by a real-time temperature cycler (LightCycler, Roche Molecular Biochemicals). For figure display, RT-PCR products were also separated by agarose-electrophoresis and visualized with ethidium bromide staining.
doi:10.1371/journal.pone.0014185.g001

the transgene product than the Tg7 line. To confirm the overexpression of type-1 NRG1 mRNA in the transgenic mice, we carried out real-time quantitative reverse transcription (RT)-PCR for type-1 NRG1 mRNA in the presence of SYBR green I. Calculation of PCR amplification curves and the threshold cycle (Ct) suggested that the transgenic mice expressed approximately 4.3-fold (Tg5) and 2.2-fold (Tg7) higher levels of type-1 NRG1 mRNA than wild type littermates. The mRNA increases were also apparent in agarose gel electrophoresis (**Fig. 1D**).

The expression pattern of the GFP-NRG1 protein within the Tg5 line was examined by GFP-fluorescence. Under the control of the EF1α promoter, GFP signals were ubiquitously and homogeneously distributed throughout the brain including the cerebral cortex, striatum, and hippocampus (**Fig. 2A–F**). There was a similar distribution pattern of GFP signals in the Tg7 line (data not shown).

Gross physical conditions of NRG1-Tg mice

The physical abilities of mice, such as sensory ability, motor reflex and coordination, directly and indirectly influence performance scores in behavioral tests. To estimate health and physical conditions of NRG1-Tg mice, we investigated various physical and behavioral parameters and compared between genotypes (transgenic vs wild), between lines (Tg5 vs Tg7) and between genders. MANOVA revealed that there was no significant main effect of genotype [$F(16, 30) = 1.249$, $P = 0.290$] or any significant interactions of genotype with line [$F(16, 39) = 0.974$, $P = 0.506$] or gender [$F(16, 39) = 118.50$, $P = 0.226$]. This suggests that the NRG1 transgene did not significantly influence gross health and physical conditions in NRG1-Tg mice. As MANOVA also detected significant main effects and/or interactions of gender and Tg line (see details in **Table S1 and Table S2**), we analyzed two Tg lines independently and tested a gender x genotype interaction in the following individual behavioral tests.

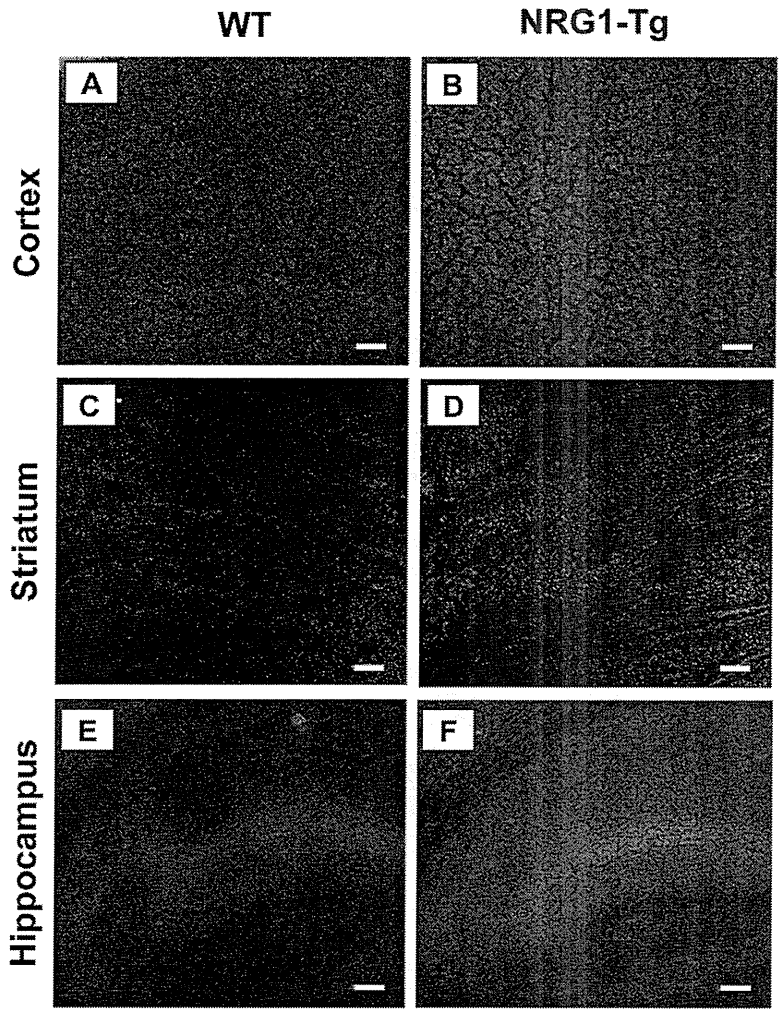

Figure 2. Detection of the transgene expression by GFP fluorescence in the brain. The transgenic line (NRG1-Tg5) was fixed and slices were prepared from their brain. The green fluorescence by GFP protein was examined in the cortex (**A, B**), striatum (**C, D**), and hippocampus (**E, F**), and compared between male NRG1-Tg and WT mice. Scale bars = 50 μm in A, B, and 100 μm in C–F. Note: Fixed brain of WT mice also exhibits autofluorescence but its intensity is lower than that of NRG1-Tg mice.
doi:10.1371/journal.pone.0014185.g002

Hyperlocomotor activity of NRG1-Tg mice in a novel environment.

We assessed behavioral pathology of adult NRG1-Tg mice by measuring locomotor activity, prepulse inhibition (PPI), fear learning, and social interaction, which are often implicated in schizophrenia animal models. First, we used an open field task to examine the locomotor behavior of the NRG1 Tg lines, Tg5 and Tg7. A two-way repeated ANOVA using a between-subjects factor of genotype and a within-subjects factor of time revealed a significant main effect of genotype in both lines [Tg5: $F(1, 40) = 10.79$, $P<0.05$; Tg7: $F(1, 27) = 5.19$, $P<0.05$]. These results indicated that increases in NRG1 expression led to hyperactivity in a novel environment (**Fig. 3A, B**). The significant effect of time [Tg5: $F(11, 440) = 118.50$, $P<0.001$; Tg7: $F(11, 297) = 25.03$; $P<0.001$] and the lack of interaction between genotype and time [Tg5: $F(11, 440) = 0.60$, $P=0.83$; Tg7: $F(11, 297) = 0.72$, $P=0.72$] suggested that mice exhibited a decrease in locomotor activity over time. Furthermore, the rate of habituation was not significantly different between NRG1-Tg and WT mice. The novelty-induced rearing behavior of NRG1-Tg mice was simultaneously scored and compared with that of wild littermates. There were no significant differences in rearing behavior [genotype, Tg5: $F(1, 40) = 3.47$, $P=0.07$; Tg7: $F(1, 27) = 2.17$, $P=0.15$] (**Fig. 3C, D**).

NRG1-Tg mice exhibit normal prepulse inhibition and startle responses

Using different prepulse intensities, we examined and compared PPI levels of adult NRG1-Tg mice and WT littermates from the two Tg lines. Both NRG1-Tg mice revealed a non-significant trend toward decreasing PPI levels compared to WT mice [genotype, Tg5: $F(1, 39) = 3.39$, $P=0.073$; Tg7: $F(1, 21) = 3.36$, $P=0.081$]. PPI levels were dependent on prepulse intensity [Tg5: $F(3, 117) = 64.54$, $P<0.001$; Tg7: $F(3, 63) = 76.9$, $P<0.001$]. There was no significant interaction between genotype and prepulse intensity [Tg5: $F(3, 117) = 0.02$, $P=0.99$; Tg7: $F(3, 63) = 0.05$, $P=0.99$] (**Fig. 3E, F**). In pulse-alone startle responses, the amplitude of startle responses was dependent on pulse intensity [Tg5: $F(7, 140) = 90.14$, $P<0.001$; Tg7: $F(7, 119) = 73.61$, $P<0.001$]. There was no difference between the genotypes in both lines [genotype, Tg5: $F(1, 20) = 0.21$, $P=0.65$; Tg7: $F(1, 17) = 0.06$, $P=0.82$] and no significant interaction between genotype and tone intensity [Tg5: $F(7, 140) = 0.76$, $P=0.62$; Tg7: $F(7, 119) = 1.15$, $P=0.19$] (**Fig. 3G, H**).

Impaired context-dependent fear learning in NRG1-Tg mice

The effect of NRG1 overexpression on learning performance was examined in adulthood by measuring freezing behavior following fear conditioning. In this task, electric shock was coupled with a context plus a tone. In both mouse Tg lines, there was no significant difference in freezing rates during conditioning between NRG1-Tg mice and their WT littermates [genotype, Tg5: $F(1, 37) = 0.68$, $P=0.42$; Tg7: $F(1, 21) = 0.05$, $P=0.83$] (**Fig. 4A, B**). This finding suggests that there was no significant influence of the NRG1 transgene on shock sensitivities. A two-way repeated ANOVA using a between subjects factor of genotype and a within-subjects factor of time detected a significant effect of genotype on freezing rates when the test was coupled with the context [Tg5: $F(1, 37) = 8.45$, $P<0.01$; Tg7: $F(1, 21) = 15.86$, $P<0.01$]. However, there was no interaction between genotype and time [Tg5: $F(5, 185) = 0.66$, $P=0.65$; Tg7: $F(5, 105) = 0.66$, $P=0.65$] (**Fig. 4C, D**). In contrast, there was no significant difference in tone-dependent learning between NRG1-Tg mice and their WT littermates [genotype: Tg5: $F(1, 37) = 1.84$, $P=0.18$; Tg7: $F(1, 21) = 0.70$, $P=0.41$] (**Fig. 4E, F**). These results indicate that the overexpression of NRG1 in the Tg mice specifically impairs context-dependent learning ability.

Social behavior of NRG1-Tg mice in an isolation-induced resident-intruder test

The effect of the NRG1 transgene on social behavior was examined using an isolation-induced resident-intruder test. In this assay, a resident male, who was previously housed alone, was exposed to an unfamiliar intruder male. The social (**Fig. 5A–D**) and aggressive behaviors (**Fig. 5E, F**) of the resident male in response to the intruder were monitored and scored. The NRG1-Tg residents displayed a significant decrease in the duration of social behaviors compared to those of WT male residents (anogenital sniffing, Tg5: $P<0.05$; Tg7: $P<0.05$; non-anogenital sniffing, Tg7: $P<0.05$, non-agonistic social behavior, Tg5: $P<0.01$; Tg7: $P<0.01$, unpaired two tailed t-test) (**Fig. 5A, B**). There was no significant difference in non-anogenital sniffing between Tg5 and wild mice, however (non-anogenital sniffing, Tg5: $P=0.13$, unpaired two tailed t-test). This trend was also supported by a decrease in the frequency of these behaviors (anogenital sniffing, Tg5: $P<0.001$; Tg7: $P<0.001$; non-anogenital sniffing, Tg5: $P=0.42$; Tg7: $P<0.05$; non-agonistic social behavior, Tg5: $P<0.01$; Tg7: $P<0.01$, unpaired two tailed t-test) (**Fig. 5C, D**). In aggressive behaviors, Tg5 male residents displayed an increase in frequency of aggressive following behavior ($P<0.05$), but the frequency of attack and threat behaviors of Tg5 residents was indistinguishable from that of WT resident males (attack: $P=0.39$; threat: $P=0.90$) (**Fig. 5E**). In contrast, Tg7 resident males showed an increase in all indices of aggressive behaviors (aggressive following: $P<0.05$ attack: $P<0.01$; threat: $P<0.05$) (**Fig. 5F**). Although the Tg line-specific behavioral changes require further investigation, our results from the resident-intruder test indicate that NRG1 overexpression has significant influences on social behavior.

Analysis of neurochemical markers for excitatory and inhibitory neurons and glial cells

NRG1 is involved in the regulation of GABAergic development, myelin formation, and NMDA receptor expression and function [23–26]. To explore whether the NRG1 transgene influenced these processes, we determined protein levels of molecular markers for GABAergic neurons, oligodendrocytes, and excitatory synapses and compared those between Tg5 mice and their WT littermates. Immunoblotting revealed that the immunoreactivity for parvalbumin, one of the phenotypic markers for cortical GABAergic neurons, was elevated in the frontal cortex of Tg5 mice ($P<0.05$) (**Fig. 6A**). Furthermore, we also found significant increases in myelin-basic protein (MBP, $P<0.01$) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, $P<0.05$) in the same region (**Fig. 6A**). This result at least verified the hyper-signaling of NRG1 expressed from the transgene. There were no significant alterations in protein levels of glutamate decarboxylase (GAD) 65/67, NMDA receptor1 (NR1), and NMDA receptor 2A/2B (NR2A/2B) in the fontal cortex (**Fig. 6A**) as well as in all markers examined in other brain regions (**Fig. 6B, C**).

Analysis of dopaminergic markers, tissue contents of dopamine and its metabolites in NRG1-Tg mice

Recently we found that transient exposure of type-1 NRG1 protein to mouse pups produces persistent hyperdopaminergic

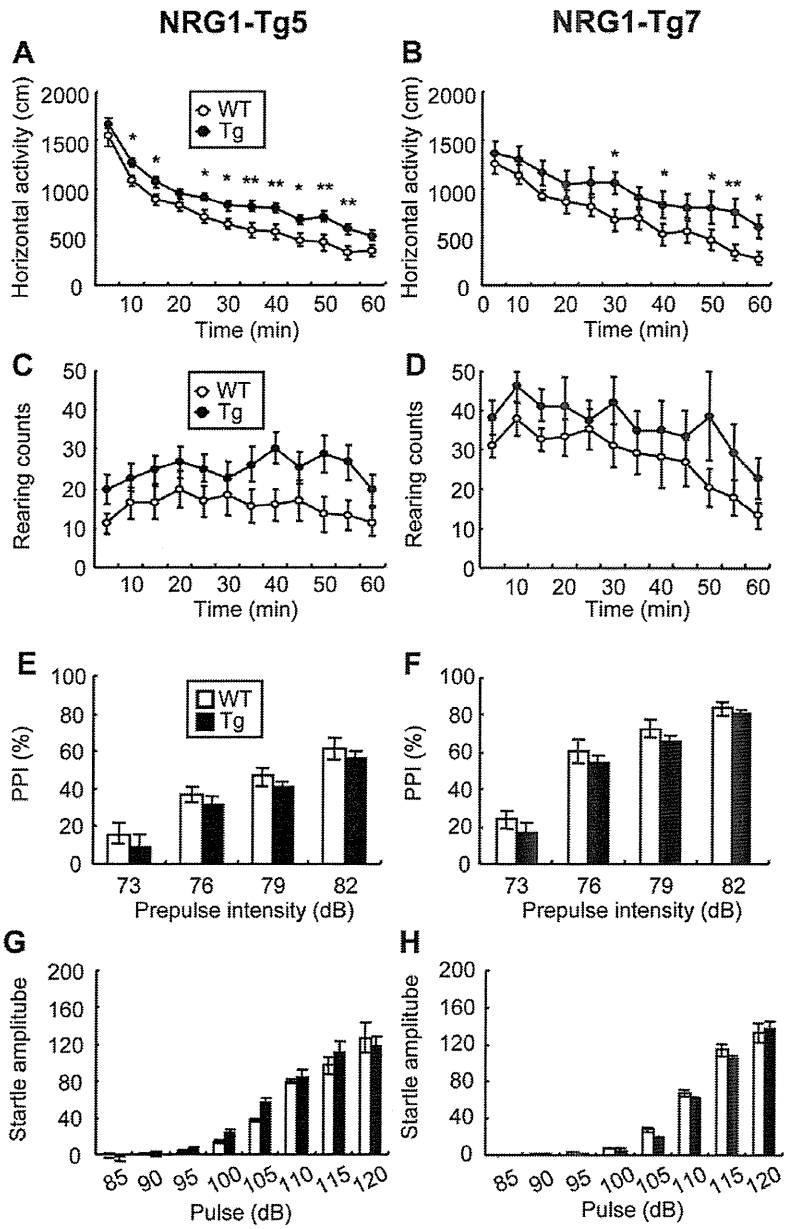

Figure 3. Locomotor activity and sensorimotor gating of NRG1-Tg mice. Behavioral traits were compared between NRG1-Tg5 mice and WT littermates (**A, C, E, G**) and between NRG1-Tg7 mice and WT littermates (**B, D, F, H**) at the adult stage (postnatal day PND 56–84). (**A, B**) Horizontal locomotor activity was scored every 5 min in a novel environment. (**C, D**) Rearing behavior was counted similarly [N = 23 (male: N = 9, female: N = 14) for Tg5, N = 18 (male: N = 7, female: N = 11) for WT; and N = 15 (male: N = 8, female: N = 7) for Tg7, N = 14 (male: N = 6, female: N = 8) for WT]. There was neither significant or marginal tread in a gender x genotype interaction; Tg5: $F(1, 37) = 1.06$ (locomotor activity) and 0.70 (rearing behavior), $P = 0.31$ (locomotor activity) and 0.79 (rearing behavior); Tg7: $F(1, 25) = 0.01$ (locomotor activity) and 0.11 (rearing behavior), $P = 0.94$ (locomotor activity) and 0.69 (rearing behavior)]. (**E, F**) Prepulse inhibition (PPI) percentages are shown with prepulses of 73, 76, 79 and 82 dB [N = 23 (male: N = 9, female: N = 14) for Tg5, N = 18 (male: N = 7, female: N = 11) for WT; and N = 15 (male: N = 8, female: N = 7) for Tg7, N = 14 (male: N = 6, female: N = 8) for WT]. There was neither significant or marginal tread in a gender x genotype interaction; Tg5: $F(1, 37) = 1.90$, $P = 0.18$; Tg7: $F(1, 19) = 0.11$, $P = 0.74$]. (**G, H**) Relative amplitudes of startle responses to white noise at 75, 80, 85, 90, 95, 100, 105, 110, 115 and 120 dB tones are shown [N = 12 (male: N = 6, female: N = 6) for Tg 5, N = 10 [male: N = 5, female: N = 5) for WT; and N = 10 (male: N = 5, female: N = 5) for Tg7, N = 10 (male: N = 5, female: N = 5) for WT]. There was neither significant or marginal tread in a gender x genotype interaction; Tg5: $F(1, 18) = 0.64$, $P = 0.43$; Tg7: $F(1, 15) = 1.59$, $P = 0.23$]. Data are expressed as mean±S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ compared to WT mice by Fisher's LSD.
doi:10.1371/journal.pone.0014185.g003

states in the frontal cortex [27]. To assess the effects of NRG1 transgene on the dopamine system, we measured the levels of dopamine and its metabolites [dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] in various brain regions of adult mice. There were significant decreases in dopamine and DOPAC levels in the hippocampus of NRG1-Tg compared to

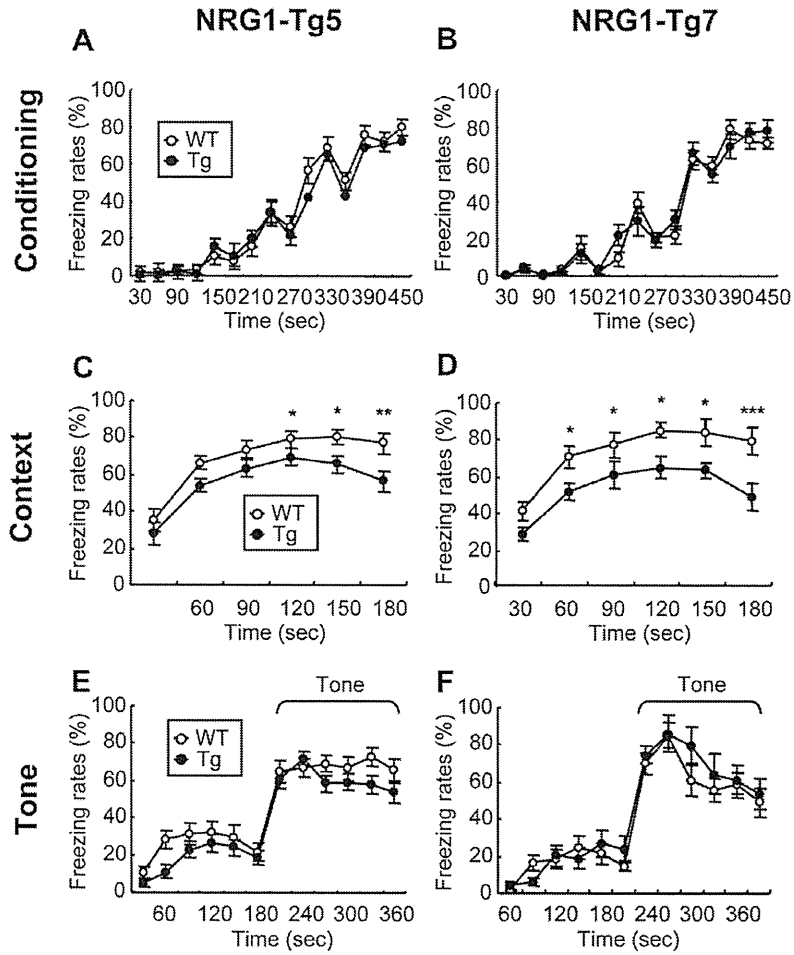

Figure 4. Context-dependent and tone-dependent fear learning in NRG1-Tg mice. Learning ability was compared between NRG1-Tg5 mice and WT littermates (**A, C, E**) and between NRG1-Tg7 mice and WT littermates (**B, D, F**). NRG1-Tg mice and WT littermates were subjected to shock-paired contextual conditioning with a tone cue. One day after conditioning, their learning performance was measured in the presence of a contextual or tone cue. (**A, B**) Freezing rates (time %) were compared between NRG1-Tg mice and WT littermates during conditioning. (**C, D**) Freezing rates during context exposure are shown. (**E, F**) Freezing rates were compared between NRG1-Tg mice and WT littermates during tone exposure [N = 21 (male: N = 11, female: N = 10) for Tg5, N = 18 (male: N = 10, female: N = 8) for WT; and N = 12 (male: N = 6, female: N = 6) for Tg7, N = 11 (male: N = 6, female: N = 5) for WT]. There was neither significant or marginal tread in a gender × genotype interaction; Tg5: F(1, 35) = 0.06 (conditioning), 0.01 (context) and 0.19 (tone), $P = 0.81$ (conditioning), 0.99 (context) and 0.66 (tone); Tg7: F(1, 19) = 0.01 (conditioning), 0.10 (context) and 0.91 (tone), $P = 0.94$ (conditioning), 0.76 (context) and 0.35 (tone). Data are expressed as mean±S.E.M. *$P<0.05$, **$P<0.01$, *** <0.001 compared to WT mice by Fisher's LSD.

doi:10.1371/journal.pone.0014185.g004

WT mice (dopamine: $P<0.01$; DOPAC: $P<0.001$) although there were no differences in the HVA ($P = 0.12$) (**Fig. 7C**). In the frontal cortex, there was a trend toward decreasing dopamine content of NRG1-Tg mice, but not statistically significant (dopamine: $P = 0.065$; DOPAC: $P = 0.19$; HVA: $P = 0.41$) (**Fig. 7A**). In the striatum, there were no differences in the dopamine and its metabolites (dopamine: P = 0.20; DOPAC: P = 0.54; HVA: $P = 0.88$) (**Fig. 7B**).

To explore the molecular mechanism underlying the changes in dopaminergic metabolism, we examined the protein markers related to dopamine synthesis and transmission [tyrosine hydroxylase (TH), dopamine beta hydroxylase (DBH), dopamine transporter (DAT), D1 dopamine receptor (D1DR), D2 dopamine receptor (D2DR), and vesicular monoamine transporter (vMAT2)] in the frontal cortex, hippocampus and striatum. In agreement with the above change in dopamine metabolism, we found a significant protein decrease in TH, a rate-limiting enzyme of dopamine and noradrenaline synthesis, in the hippocampus ($P<0.05$) (**Fig. 7F**). The decrease in TH levels was manifested in the frontal cortex as well. In addition, we found significant increase in D2DR protein levels in the frontal cortex (**Fig. 7D**). To assess the influence on noradrenergic terminals, we also determined protein levels of dopamine-β-hydroxylase (DBH), the enzyme that converts dopamine to noradrenaline. There was no significant change in DBH levels in the frontal cortex and hippocampus (**Fig. 7D, F**). We also failed to detect significant alteration in DAT, D1DR, vMAT2 in all the regions examined (**Fig.7D–F**). These results suggest that the life-long increase in NRG1-expression disrupts dopaminergic synthesis and transmission in the cortico-limbic system. The present phenomenon contrasts our recent finding that neonatal treatment with NRG1 protein enhances dopamine synthesis and release in adulthood [27].

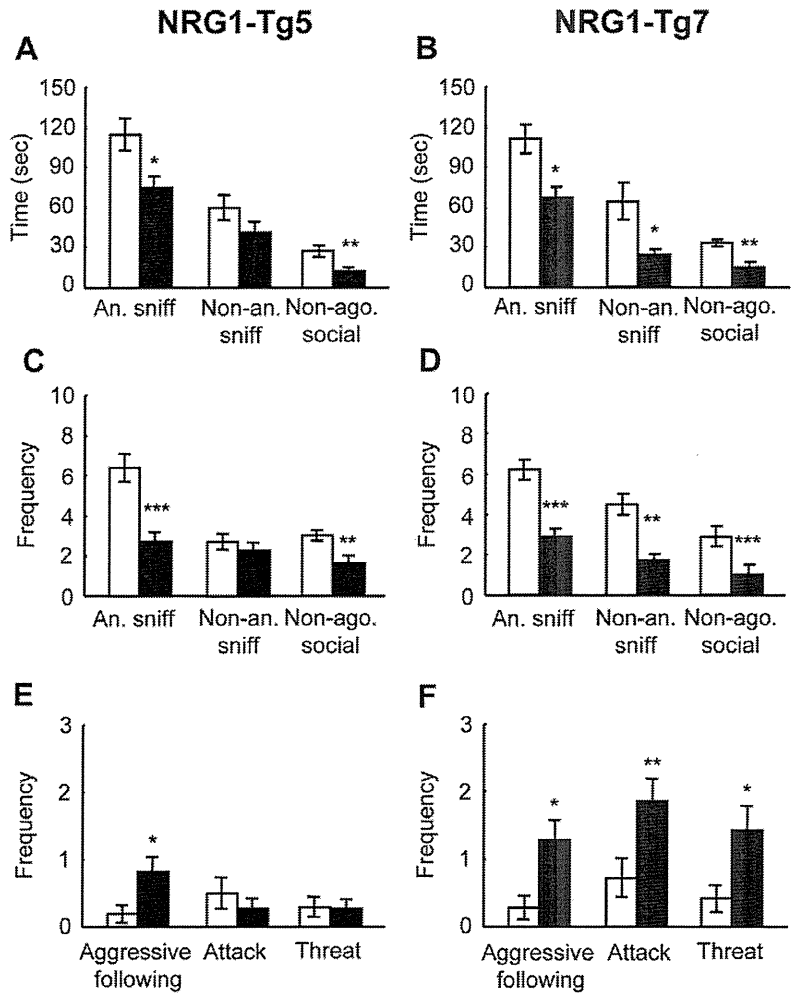

Figure 5. Isolation-induced resident-intruder test. NRG1-Tg5 mice (**A, C, E**) and Tg7 mice (**B, D, F**) and their WT littermates were subjected to a isolation-induced resident-intruder test. (**A–D**) Social scores of anogenital sniffing (An. sniff), non-anogenital sniffing (Non-an. sniff) and non-agonistic social behaviors (Non-ago. social) were measured over a 10-min period. The non-agonistic behaviors represent grooming and lying down next to each other of resident mice. (**E, F**) Aggressive behaviors, which represent aggressive following, attacks and threats, were counted in parallel. (**A, B**) Time spent by the resident males actively pursuing social behaviors. (**C, D**) The frequency of social behaviors in the resident males is shown. (**E, F**) The frequency of aggressive behaviors of the resident males was compared between the NRG1-Tg mice and their WT littermates (N = 11 for Tg5, N = 10 for WT; and N = 7 for Tg7, N = 7 for WT, all males). Data are expressed as mean±S.E.M. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to WT littermates by unpaired two-tailed t-test.
doi:10.1371/journal.pone.0014185.g005

Discussion

To investigate the neurobehavioral consequences of life-long NRG1 hyper-signaling, we established Tg mouse lines carrying the GFP-tagged-NRG1 (type-1) cDNA driven by the ubiquitous transcription promoter. We selected two Tg lines and backcrossed those lines with C57BL/6N mice (more than seven times) to stabilize the transgene in a single genomic integration site. One of the lines carried more copies of the NRG1 transgene than the other. In agreement, the expression of the transgene was higher in the Tg5 line compared to the Tg7 line as shown by immunoblotting as well as by real time RT-PCR. Our results indicated that the expression of the NRG1 transgene was widespread throughout the brain. As indicated the neurotrophic actions of this neurotrophic factor, the Tg mice exhibited the increase in the phenotypic markers of GABAergic neurons and oligodendrocytes [14], [15], [23], [24], [28]. In spite of the reported neurotrophic activity of NRG1 on midbrain dopaminergic neurons [29], [30], the hippocampal decrease in TH and dopamine was observed beyond our expectation.

These two independent Tg mouse lines displayed similar levels of neurobehavioral abnormalities; hyper-locomotor activity in a novel environment, learning deficits in context-fear conditioning, reduced social interactions, and a nonsignificant trend toward decreasing prepulse inhibition. Both lines also exhibited the normal behavioral phenotypes that were indistinguishable from WT littermates in acoustic startle amplitudes, vertical movement, shock sensitivity, and tone-dependent fear learning. These behavioral traits of the Tg mice appear to indicate their normal motor function or sensory abilities in a limited degree. We do not exclude the possibility that unexamined physical functions, such as olfaction, might be altered by NRG1 overexpression and influence social interaction scores of the Tg mice.

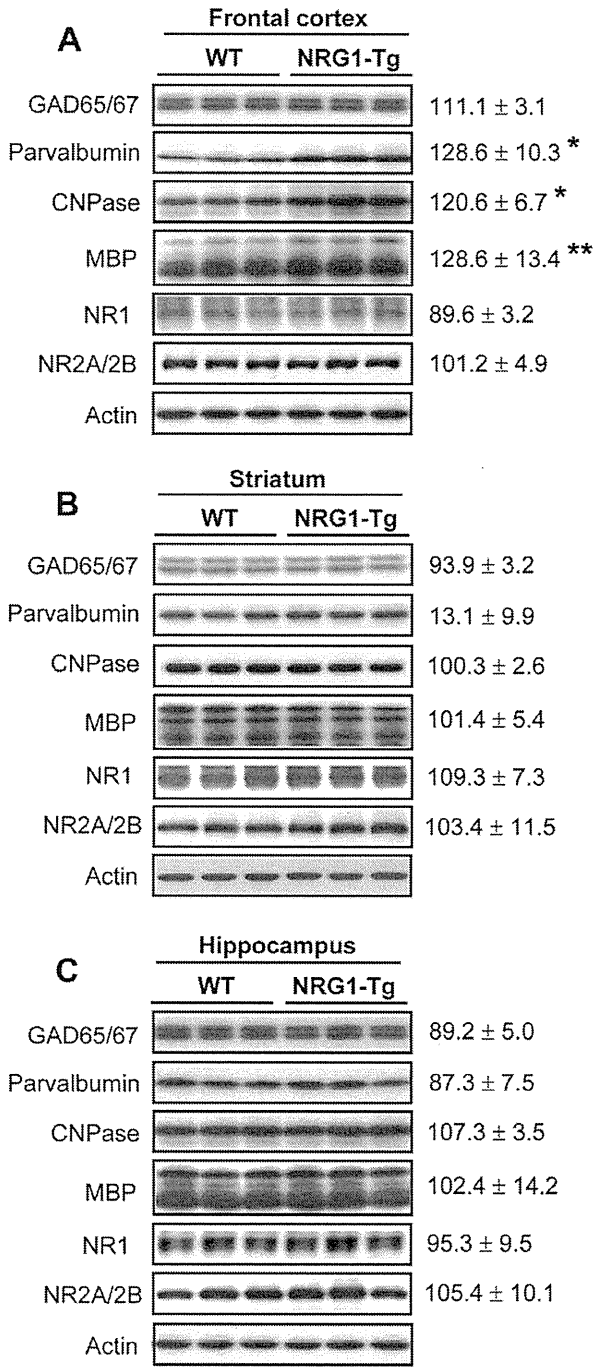

Figure 6. The expression of GABA-, myelin-, and excitatory synapse-associated molecular markers. Protein extract was prepared from (**A**) frontal cortex, (**B**) striatum, and (**C**) hippocampus and subjected to immunoblotting with antibodies directed against the GABAergic markers (GAD65/67 and parvalbumin), oligodendrocyte markers (CNPase and MBP), and excitatory synaptic markers (NR1 and NR2A/2B). Immunoreactivity on immunoblots was measured by densitometric analysis, and normalized to β-actin levels. Percentage ratio to that of WT littermates was calculated (mean±S.E.M, N = 5, all males) and analyzed by unpaired two-tailed t-test.
doi:10.1371/journal.pone.0014185.g006

The behavioral homology between these two independent Tg lines presumably rules out the possibility that genomic disturbance of the transgene integration was involved in these behavioral deficits. In addition, it is also unlikely that the distinct genome background impurities of the two independent Tg lines resulted in the same behavioral traits. In this context, the discordant behavioral trait (social aggression) between the two Tg lines might be illustrated by the distinct genomic disturbance of the transgene integration or different background impurities of the original DBA mouse genome.

The Tg mice in the present study displayed both an increase in horizontal locomotor activity and a decrease in social behavior. Interestingly, hyperlocomotion is typically associated with positive symptoms in a mouse model of schizophrenia whereas reduced social activity is implicated as a negative symptom of this illness [31–33]. The hypo-dopaminergic state is often associated with impairments in social and learning behaviors, and might illustrate some of the behavioral traits of the present transgenic mice [34], [35]. In particular, their hypo-dopaminergic state in the limbic system might impair the hippocampal functions, leading to their context learning deficits [35]. The observed behavioral traits are reported in studies of various NRG1 knockout lines as well [5], [8–11]. Although we failed to detect significant and marginal gender x genotype interactions in individual behavioral tests, the physical examination test detected a significant main effect of gender and an interaction between Tg line and gender, presumably suggesting the dose-dependent NRG1 effects on gender-specific behavioral trends. This agrees with the reports that the down-regulation of NRG1-ErbB signaling in mice exhibit sexually dimorphic changes in several behavioral paradigms such as exploratory and habituation profiles [8], [36], [37]. The biological mechanism underlying the interaction between sex hormone and NRG1 signaling remains to be studied.

Unexpectedly, the present study and previous reports indicate that both hypomorphic and hypermorphic expression of the NRG1 gene may produce several common behavioral phenotypes in mice. This finding is quite surprising but raises a challenging question about the molecular and cellular mechanisms underlying the behavioral deficits common to both the hypermorphic and hypomorphic expression of NRG1.

PPI is also implicated in the neuropathology of schizophrenia and its animal models. In contrast to the abnormality in social behavior or locomotor activity, the PPI deficits of these Tg mice appear to be moderate. The NRG1 knockout line (transmembrane-domain of $NRG1^{+/-}$) similarly displayed moderate or non-significant abnormality in PPI levels [2], [13]. Since there are variations in the magnitude of PPI deficits depending upon the targeted exon of NRG1 gene [4], the behavioral phenotype of the present NRG1-Tg mice is not discordant with that of NRG1 knockout mice in this context. The recent report is noteworthy that the specific overexpression of type-1 NRG1 driven by a Thy-1 promoter in brain projection neurons markedly impairs PPI [38]. The use of distinct gene promoters of EF1α and Thy-1 genes differentially regulates timing and cell types of the transgene expression and presumably results in the difference in mouse behavior. Controversy of the behavioral difference between these NRG1 Tg lines awaits further investigations, however.

NRG1 is one of the neurotrophic factors that positively regulate neuronal migration, synaptogenesis, GABAergic and dopaminergic neuronal development, and myelination [14], [15], [27–30], [39–41]. In agreement with the given biological activities of NRG1, we found the increases in parvalbumin, MBP and CNPase. These molecular phenotypes of the NRG1-Tg mice well contrast those of NRG1 knockout mouse lines. ErbB4, the

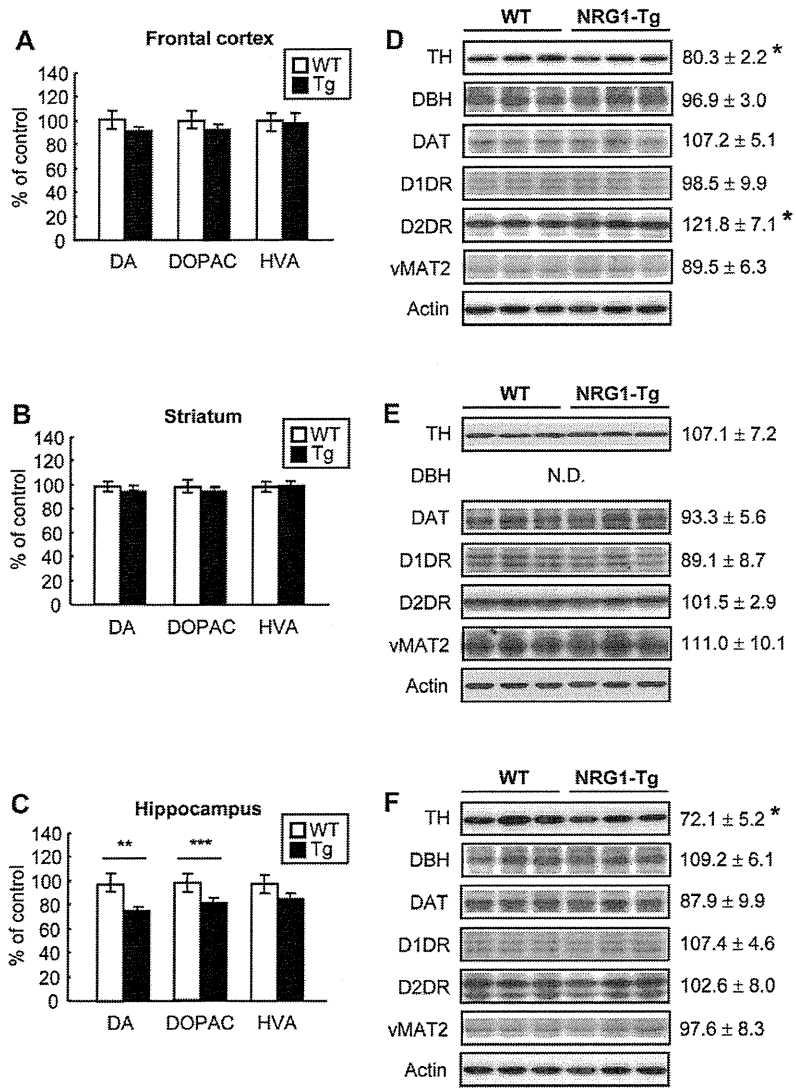

Figure 7. Analysis of dopamine metabolism and neurochemical markers for dopaminergic neurons of NRG1 transgenic mice. Levels of dopamine and its metabolites (DOPAC and HVA) (**A, B, C**) as well as those of dopamine-related molecular markers (**D, E, F**) were measured in (**A, D**) frontal cortex, (**B, E**) striatum and (**C, F**) hippocampus of NRG1-Tg5 and WT mice at the adult stage. Typical immunoblots for TH, DBH, DAT, D1DR, D2DR, and vMAT2 were displayed. Each immunoreactivity was measured by densitometric analysis, normalized to β-actin levels and its ratio to that of WT littermates was displayed (N = 4–5 each, all males). Data are expressed as mean±S.E.M. (% of WT). *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared to WT littermates by unpaired two-tailed t-test.
doi:10.1371/journal.pone.0014185.g007

receptor for NRG1 knockout-mutants exhibit reduced parvalbumin positive cells in the hippocampus [28] and loss of ErbB4 signaling by its dominant-negative form reduced oligodendrocyte number and myelination [41]. These phenotypic abnormalities of NRG1 knockout mice are in agreement with the neuropathological findings on postmortem brains of schizophrenia patients [42–44]. Conversely, the present NRG1-Tg mice, which display the increases in these pathological markers, may be irrelevant to an animal model for schizophrenia in spite of their schizophrenia-like behavioral deficits. In this context, it is a challenging question how the hyper-NRG1 signals reported in patients' postmortem brains is associated with the above neuropathologic deficits.

NRG1 is reported to promote cell survival of midbrain dopaminergic neurons and trigger dopamine release [29], [30], [45–47]. Accordingly, we had expected positive influences of the NRG1 transgene expression on the dopaminergic system in the present experiment. However, the direction of the dopaminergic changes in NRG1-Tg mice was opposite to our expectation. NRG1-Tg mice rather displayed reduction in TH protein levels and dopamine content in the hippocampus and/or frontal cortex. It is a challenging question how the hyper-signaling of NRG1 produced the TH decrease. This discrepancy might be illustrated by the NRG1 action on dopaminergic neurons [46]. NRG1 evokes an almost immediate overflow of striatal dopamine when injected into a region just dorsal to the substantia nigra. Therefore, it is possible that the life-long hyper signals of NRG1 might result in constant dopamine over-flow and produce cytotoxic influences on dopaminergic terminals [48], [49]. As there are many alternative

explanations for this controversy, the exact mechanism underlying this phenomenon remains to be explored.

In summary, our behavioral results from NRG1-Tg mice and previous findings on NRG1 knockout mice highlight the complex dose dependency of NRG1 functioning in brain development or behavioral regulation.

Materials and Methods

Ethics statement

All of the animal experiments described were approved by the Animal Use and Care Committee guidelines of Niigata University and performed in accordance with the guidelines of NIH (USA).

Generation of NRG1-Tg mice

The GFP gene was inserted into the NspV-SacI site between the immunoglobulin (Ig)-like and epidermal growth factor (EGF)-like domains of NRG1β1 cDNA [50]. The 2.6 kb cDNA fragment encoding mouse NRG1β1 and GFP tag was then excised by EcoRI-XbaI digestion, subcloned into the EcoRI-XbaI site of a mammalian vector pT113 (gifted from Dr. Shigekazu Nagata, Osaka University) and ligated to an EF-1α gene promoter. The DNA construct of the transgene was confirmed by DNA sequencing (data not shown). Transgenic mice were generated by pronuclear injection of the fragment (shown in **Fig. 1A**) into fertilized mouse eggs (DBA/2×C57BL/6 F1). The lines of the NRG1-Tg mice (Tg5 and Tg7, chosen for its low copy number of the transgene) were backcrossed with C57BL6NCr mice (purchased from Nihon Charles River, Yokohama, Japan) for 7–9 generations, and their offspring of heterozygous mice was used in this study. Mice were genotyped by PCR using primers corresponding to the Ig-like domain of NRG1 (forward: 5′-TGCCTCCCAGATTGAAAGAG) and the EGF domain of NRG1 (reverse: 5′-TTCTCCTTCTCCGCACACTT), giving a product with 1112 bp. All Tg mice were bred and housed under a 12 h light-dark cycle with free access to food and water. The mice were subjected to behavioral testing during the light phase between postnatal day (PND) 56–84.

Immunoblotting

Whole brain tissues were homogenized in lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.5% NP-40, 5 mM EDTA) with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After centrifugation, the supernatant was collected and protein concentrations were determined. Equal amounts of protein (30 μg/lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with anti-extracellular-NRG1 (7D5, 1:1000, NeoMarkers, Fremont, CA, USA) or anti-GFP (1:2000, Clontech, Palo Alto, CA, USA) monoclonal antibodies. Alternatively, immunoblots were probed with anti-GAD 65/67 (1:5000, Sigma-Aldrich, St Louis, MO, USA), anti-parvalbumin (1:10000, Abcam, Cambridge, UK), anti-MBP (1:1000, Millipore, Bedford, MA, USA), anti-CNPase (1:1000, Millipore), anti-NR1 (1:250, Millipore), anti-NR2A/B (1:150, Millipore), and anti-β-actin (1:4000, Millipore) antibodies. Immunoblots were alternatively probed with antibodies directed against the following dopamine-related molecules; TH (1:1000, Millipore), DBH (1:1000, Millipore), DAT (1:1000, Millipore), D1DR (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), D2DR (1:250, Millipore), and vMAT2 (1:1000, Millipore). Immunoreactivity was detected by peroxidase-conjugated anti-rabbit or peroxidase-conjugated anti-mouse Ig antibody followed by a chemiluminescence reaction combined with X-ray film exposure (ECL kit; GE Healthcare, Little Chalfont, UK).

Analysis of NRG1 mRNA expression

Real-time RT- PCR was performed in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction. Total RNA was isolated from whole brain tissue with the guanidinium-phenol solution (Isogen, Nippon Gene, Osaka, Japan) and treated with DNase I (20 U/ml) to remove contaminating genomic DNA. NRG1 mRNA was detected by recombinant *Thermus thermophilus* DNA polymerase (High-Plus, Toyobo, Osaka, Japan) using the forward primer (5′-GCAAAGAAGGCAGAGGCAAG) and the reverse primer (5′-GCTACGGTTCAGCTCATTCC), which correspond to exon 2 and exon 3 sequences of mouse NRG1 genome, respectively. The primer set was designed to amplify mRNA transcripts specific for type-1 NRG1. RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was similarly carried out with the forward primner (5′-TGCACCACCAACTGCTTAGC) and the reverse primer (5′-GATGCAGGGATGATGTTCTG). These primer sets was designed to span intron(s) to distinguish PCR products of mRNA from those of genomic DNA. The lengths of the expected products were 230 bp for NRG1β1 mRNA and 239 bp for GAPDH mRNA. The genome copy number of the Tg mice was estimated by the comparative Ct method using the amplification curve of the wild genome as a standard [51].

Physical examinations

We employed the primary behavioral screen SHIRPA developed by Rogers et al. [52] and estimate a behavioral and functional profile of NRG1 Tg mice by observational assessment. Parameters of undisturbed animals and animals submitted to battery of reflex tests are scored for quantitative analysis. The behavioral parameters assessed include posture, activity, gait, motor coordination, tremor, startle response, excitability and defecation as observed in a viewing jar and open field. Salivation, lacrimation, piloerection, placing and righting reflexes, muscle tone and other reflexes were scored by picking the animal up and eliciting the reflexes with specific equipment and manipulations [53], [54]. Naïve mice (i.e., mice not exposed to any other behavioral test) were used for these physical examinations.

Analysis of Locomotor Activity

Exploratory motor activity was measured in a novel environment under dim light. Mice were placed in an automated activity apparatus (27 cm L×27 cm W×20 cm H, MED Associates, St. Albans, VT, USA) equipped with infrared photosensors at 1.62 cm intervals, and we measured horizontal activity every 5 min for the first hour [27]. Horizontal activity was assessed via beam crossings, which were counted by a fully automated tracking system (Activity Monitor, Med Associates).

Measurement of acoustic startle response and prepulse inhibition

Mice were placed in a plastic cylinder and fixed in an automated startle chamber (SR-Lab Systems, San Diego, CA, USA) [27]. After a 5-min acclimation period with 70-dB-background noise (white noise), an 75-, 80-, 85-, 90-, 100-, 110-, or 120-dB white noise stimulus (40 msec duration) was given 8 times to each mouse in the same pseudo-random order at 15 sec intervals. Analysis for startle amplitudes was based on the mean of the seven trials (ignoring the first trial) for each trial type. PPI

responses were measured with 120 dB acoustic stimuli combined with four different prepulse intensities. Each mouse was placed in the startle chamber (SR-Lab) and initially acclimatized for 5 min with background noise alone (70 dB white noise). The mouse was then subjected to 48 startle trials, each trial consisting of one of six conditions: (i) a 40 msec 120 dB noise burst presented alone, (ii–v) a 40 msec 120 dB noise burst following prepulses by 100 msec (20 msec noise burst) that were 3-, 6-, 9-, or 12-dB above background noise (i.e., 73-, 76-, 79-, or 82-prepulse, respectively), or (vi) no stimulus (background noise alone), which was used to measure baseline movement in the chamber. These six trial types (i–vi) were each repeated 8 times in a pseudorandom order to give 48 trials. The inter-trial interval was 15 sec. Each trial type was presented once within a block of six trials and the order of 48 trial presentations was fixed for all mice. Analysis was based on the mean of the seven trials for each trial type. The percentage PPI of a startle response was calculated as: 100 − [(startle response on prepulse-pulse stimulus trials − no stimulus trials)/(pulse-alone trials − no stimulus trials)] ×100.

Context- and tone-dependent fear learning

The test paradigm for contextual fear conditioning was modified from procedures published in Frankland et al, 2004 [55]. Mice were placed in a shock chamber with a grid floor (10 cm L×10 cm W×10 cm H; Obaraika Ltd. Tokyo, Japan), and their baseline movement/freezing behavior was monitored for 2 min. The mice were then exposed to three rounds of 0.8 mA electric shocks (2 sec duration) with 180 sec tone cues (60 dB, 10 kHz). One day after conditioning, mice were returned to the chamber. The time spent freezing (i.e., no movement other than respiration) was recorded and scored at 30 sec intervals for 3 min. After 3 h, the mice were moved to a different chamber with a flat floor (10 cm L×10 cm W×10 cm H). In this chamber, the time spent freezing was recorded and scored for 3 min before and after the tone cue. Freezing behavior was monitored by a video camera during all sessions and analyzed by imaging software (Obaraika Ltd.).

Isolation-induced resident-intruder test

The isolation-induced resident-intruder test to estimate social behaviors was modeled after the procedure described by Mohn et al, 1999 [56]. For one week before testing, male wild-type (WT) and NRG1-Tg mice were housed individually (resident) or in groups (intruder) of three or four mice. We note that the bedding was changed in all cages one day prior to testing. On the test day, an intruder was placed in the home cage of resident mice, and their behavior was video-recorded for 10 min. The duration and frequency of 1) anogenital sniffing, 2) sniffing of any part of resident mice excluding anogenital area (non-anogenital sniffing) and 3) non-agonistic social behaviors (grooming and lying down next to each other of resident mice) were scored by observers blind to the experimental conditions. In addition, aggressive behaviors; 1) aggressive following (resident mice rapidly follow intruder mice from behind and force it to retreat, fiercely tugging hair or tail), and 2) attacks (biting and pinning), and 3) threats (upright posture and tail rattling) were scored. Scores for each behavior were then averaged for each genotype. The experimental groups included 10 WT residents and 11 NRG1-Tg residents in the Tg5 line experiment, and 7 WT and 7 NRG1-Tg residents in the Tg7 line experiment. We purchased and used novel adult male C57BL/6 NCr mice (same age) as unfamiliar intruders.

Quantification of dopamine and its metabolites

We measured the tissue contents of dopamine, DOPAC and HVA as described previously [57]. The prefrontal cortex, hippocampus, and striatum were dissected and frozen on dry ice. The tissue was homogenized in monoamine extraction buffer [0.1 M perchloric acid, 0.1 mM EDTA, 50 nM isoproterenol (internal standard)], incubated on ice for 30 min, and then centrifuged at 10,000×g for 10 min. Precipitates were homogenized in 0.5 N NaOH for protein determination.

The high performance liquid chromatography (HPLC) system consisted of a pump (model LC-10ADVP; Shimadzu, Kyoto, Japan), an automatic sample injector (model SIL-10ADVP; Shimadzu), and an electrochemical detector (ECD) with a glassy carbon-working electrode (model ECD-300; Eicom, Kyoto, Japan). Tissue contents of dopamine, DOPAC and HVA were measured using a C18 column (model CA-5ODS, 4.6×150 mm; Eicom). The mobile phase consisted of 50 mM trisodium citrate, 25 mM NaH_2PO_4, 0.03 mM EDTA, 10 mM diethylamine, 3 mM octanesulfonic acid sodium salt, 6% methanol, and 1% dimethylacetamide, pH 3.2.

Statistical analysis

Health and physical conditions (39 parameters) were analyzed using a multiple analysis of variance (MANOVA) with genotype (two levels), line (two levels) and gender (two levels). Behavioral scores were initially analyzed using a three-way analysis of variance (ANOVA) with genotype (two levels) and gender (two levels) as the between-subjects factors and time or prepulse (four levels) as the within-subjects factors. Because the initial ANOVAs did not yield any significant results with gender, the variable was collapsed and the analysis rerun. Univariate data for the social behavioral scores, protein expression levels and monoamine contents were analyzed using an unpaired two-tailed *t* test. For post hoc testing, Fisher's LSD was used to detect differences in the absolute behavioral values. A *P*-value of less than 0.05 was regarded as statistically significant, and "N" values represent the number of animal used in the analysis. These statistical analyses were performed using SPSS 11.0 for Windows.

Supporting Information

Table S1. Statistical values and results of MANOVA in SHIRPA test. N, animal number; AVE, average; SD, standard deviation.
Found at: doi:10.1371/journal.pone.0014185.s001 (0.03 MB DOC)

Table S2. Physical and health conditions of NRG1-Tg mice in SHIRPA test. N, animal number; AVE, average; SD, standard deviation.
Found at: doi:10.1371/journal.pone.0014185.s002 (0.05 MB DOC)

Acknowledgements

The authors are grateful to Minoru Tanaka and Sachiko Hirokawa for mouse breeding.

Author Contributions

Conceived and designed the experiments: HN. Performed the experiments: TK AK MM LF MY. Analyzed the data: TK AK SM. Contributed reagents/materials/analysis tools: NS SM MY MO. Wrote the paper: TK HN.

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