the effect of haloperidol in chronic schizophrenia patients,[19] whether or not the long-DUP patients' sluggish P300 recovery over the left temporo-parietal area would still persist after the administration of atypical antipsychotics needs to be clarified. Despite these limitations, the present study supports DUP as a key variable in future neurobiological studies of first-episode schizophrenia.

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REFERENCES

1. Lieberman JA, Perkins D, Belger A *et al.* The early stages of schizophrenia: Speculations on pathogenesis, pathophysiology, and therapeutic approaches. *Biol. Psychiatry* 2001; **50**: 884–897.
2. McGlashan TH. Duration of untreated psychosis in first-episode schizophrenia: Marker or determinant of course? *Biol. Psychiatry* 1999; **46**: 899–907.
3. Hirayasu Y, Asato N, Ohta H, Hokama H, Arakaki H, Ogura C. Abnormalities of auditory event-related potentials in schizophrenia prior to treatment. *Biol. Psychiatry* 1998; **43**: 244–253.
4. Asato N, Hirayasu Y, Hiramatsu KI, Ohta H. ERPs changes during neuroleptic treatment in schizophrenia: A vulnerability marker in schizophrenia. *Psychiatr. Neurol. Jpn.* 1999; **101**: 254–276 (in Japanese).
5. Coburn KL, Shillcutt SD, Tucker KA *et al.* P300 delay and attenuation in schizophrenia: Reversal by neuroleptic medication. *Biol. Psychiatry* 1998; **44**: 466–474.
6. Amminger GP, Edwards J, Brewer WJ, Harrigan S, McGorry PD. Duration of untreated psychosis and cognitive deterioration in first-episode schizophrenia. *Schizophr. Res.* 2002; **54**: 223–230.
7. Overall JE, Gorham DR. The brief psychiatric rating scale. *Psychol. Rep.* 1962; **10**: 799–812.
8. Wang J, Hirayasu Y, Hiramatsu K, Hokama H, Miyazato H, Ogura C. Increased rate of P300 latency prolongation with age in drug-naive and first episode schizophrenia. *Clin. Neurophysiol.* 2003; **114**: 2029–2935.
9. Ho BC, Andreasen NC, Flaum M, Nopoulos P, Miller D. Untreated initial psychosis: Its relation to quality of life and symptom remission in first-episode schizophrenia. *Am. J. Psychiatry* 2000; **157**: 808–815.
10. Ford JM, White PM, Csernansky JG, Faustman WO, Roth WT, Pfefferbaum A. ERPs in schizophrenia: Effects of antipsychotic medication. *Biol. Psychiatry* 1994; **36**: 153–170.
11. Duncan CC. Event-related brain potentials: A window on information processing in schizophrenia. *Schizophr. Bull.* 1988; **14**: 199–203.
12. Lieberman JA, Tollefson G, Tohen M *et al.* Comparative efficacy and safety of atypical and conventional antipsychotic drugs in first-episode psychosis: A randomized, double-blind trial of olanzapine versus haloperidol. *Am. J. Psychiatry* 2003; **160**: 1396–1404.
13. Gold S, Arndt S, Nopoulos P, O'Leary DS, Andreasen NC. Longitudinal study of cognitive function in first-episode and recent-onset schizophrenia. *Am. J. Psychiatry* 1999; **156**: 1342–1348.
14. McCarley RW, Salisbury DF, Hirayasu Y *et al.* Association between smaller left posterior superior temporal gyrus volume on magnetic resonance imaging and smaller left temporal P300 amplitude in first-episode schizophrenia. *Arch. Gen. Psychiatry* 2002; **59**: 321–331.
15. Kasai K, Shenton ME, Salisbury DF *et al.* Progressive decrease of left superior temporal gyrus gray matter volume in first-episode schizophrenia. *Am. J. Psychiatry* 2003; **160**: 156–164.
16. Kasai K, Shenton ME, Salisbury DF *et al.* Progressive decrease of left Heschl gyrus and planum temporale gray matter volume in first episode schizophrenia: A longitudinal magnetic resonance imaging study. *Arch. Gen. Psychiatry* 2003; **60**: 766–775.
17. Hirayasu Y, Tanaka S, Shenton ME *et al.* Prefrontal gray matter volume reduction in first episode schizophrenia. *Cereb. Cortex* 2001; **11**: 374–381.
18. Wiegand LC, Warfield SK, Levitt JJ *et al.* Prefrontal cortical thickness in first-episode psychosis: A magnetic resonance imaging study. *Biol. Psychiatry* 2004; **55**: 131–140.
19. Umbricht D, Javitt D, Novak G *et al.* Effects of clozapine on auditory event-related potentials in schizophrenia. *Biol. Psychiatry* 1998; **44**: 716–725.

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ARTICLE

Expression of Ndrg2 in the rat frontal cortex after antidepressant and electroconvulsive treatment

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Abstract

Although the therapeutic action of antidepressants most likely involves the regulation of serotonergic and noradrenergic signal transduction, no consensus has been reached concerning their precise molecular or cellular mechanisms of action. In the present study, we demonstrated that chronic treatment with a tricyclic antidepressant (imipramine) and a selective serotonin reuptake inhibitor (sertraline) reduced the expression of Ndrg2 mRNA and protein in the rat frontal cortex. Ndrg2 is a member of the N-Myc downstream-regulated genes. Interestingly, repeated ECT also significantly decreased Ndrg2 expression in this region of the brain. These data suggest that Ndrg2 may be a common functional molecule that is decreased after antidepressant treatment and ECT. Although, the functional role of Ndrg2 in the central nervous system remains unclear, our findings suggest that Ndrg2 may be associated with treatment-induced adaptive neural plasticity in the brain, a chronic target of antidepressant action. In conclusion, we have identified Ndrg2 as a candidate target molecule of antidepressants and ECT.

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Key words: Microarray, neural plasticity, pharmacogenomics, selective serotonin reuptake inhibitor.

Introduction

Antidepressants are very effective agents for preventing and treating depression and have been used clinically for more than 50 yr. Typical antidepressants significantly increase the synaptic concentration of norepinephrine and/or serotonin. However, a latency period of several weeks generally elapses before the therapeutic effects of antidepressants are observed. This delayed therapeutic action could result from either the indirect regulation of other neuronal signal transduction systems or the regulation of gene transcription following chronic treatment. Indeed, antidepressants have been shown to affect the expression of immediate early genes and transcription factors, including c-fos, FosB, junB, NGF1-A, and CREB (see review by Yamada and Higuchi, 2002). These regulatory proteins activate or repress genes that encode specific proteins, and may be involved in critical steps that mediate treatment-induced alterations of central nervous system function. We recently performed expressed-sequence tag (EST) analyses to identify some biological changes observed in rat brain after chronic treatment with antidepressants (Yamada et al., 2001). We developed our original ADRG microarray for high-throughput secondary screening of these candidate genes (Yamada et al., 2000). To date, we have cloned several cDNA candidates as ESTs from the rat brain and have named these antidepressant-related genes (ADRGs).

While antidepressant pharmaceuticals have been shown to be an effective treatment, another important therapy that is widely used for treating depression is repeated electroconvulsive treatment (ECT). Because of its safety, high efficacy, and rapid onset of action, ECT is well-suited for treating patients with severe psychotic depression, severe depression with suicidal ideation, drug-resistant depression, and for treating

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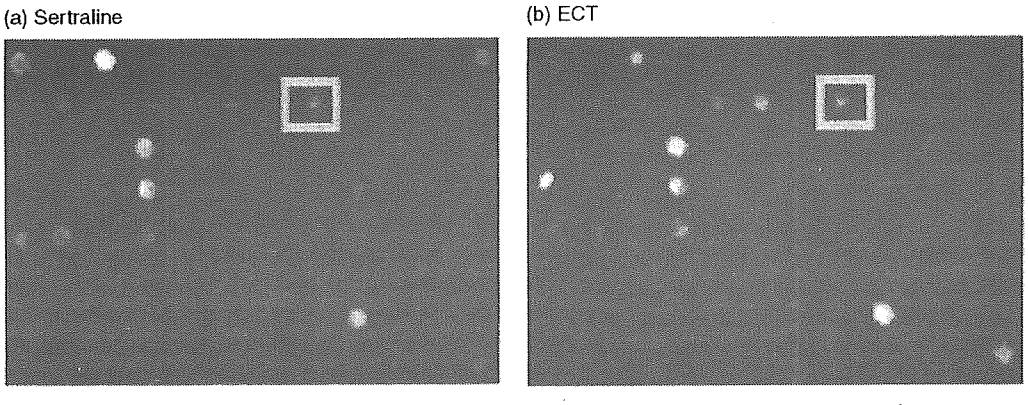


Figure 1. Image analysis of ADRG microarray after hybridization with fluorescent probes. Ninety-six spots representing ADRG97–192 are shown. (a) Merged pseudo-colour image of control group data (green) and chronic sertraline treatment group (red). As expected, we obtained low background and consistent results in duplicate experiments. (b) Merged pseudo-colour image of control group data (green) and repeated ECT group data (red). Blue rectangle demarcates ADRG123 (Ndrg2). Interestingly, the fluorescence intensities of the spots increased 0.63-fold in the sertraline group and 0.49-fold in the ECT group compared to controls.

geriatric patients and others with medical illnesses that contraindicate the use of antidepressants. Although ECT is an effective treatment for depression, the basis for its therapeutic mechanism remains unknown. An increasingly popular working hypothesis is that both antidepressants and ECT have therapeutic effects because they share some final common pathway regulating transcription of the same set of downstream genes. Indeed, we have recently reported that VAMP2 (Yamada et al., 2002) and kf-1 (Nishioka et al., 2003; Yamada et al., 2000) are expressed both after chronic antidepressant drug treatment and repeated ECT.

In the present study, we identified ADRG123 as rat Ndrg2 (Swiss-Prot/TrEMBL accession numbers Q8VBU2, Q8VI01). Ndrg2 is highly related to N-Myc downstream-regulated protein 1 (Ndrg1), which has been linked to stress responses, cell proliferation, and differentiation, although Ndrg2 itself is not repressed by N-Myc (Okuda and Kondoh, 1999). Thus far, four different isoforms of rat Ndrg2 have been identified (Figure 1; Boulkroun et al., 2002). The 5′ untranslated AQ1 region (5′ -UTR) for Ndrg2a1/Ndrg2a2 is 87 bp, whereas the 5′-UTR for Ndrg2b1/Ndrg2b2 is 50 bp. In the translated region, Ndrg2a1/Ndrg2b1 has an additional 42 bp insertion compared to Ndrg2a2/Ndrg2b2. Here, we denote Ndrg2a1/Ndrg2b1 and Ndrg2a2/Ndrg2b2 to represent Ndrg2L and Ndrg2S respectively. Comparison and alignment of amino-acid sequences indicated that Ndrg2L is longer than Ndrg2S by 14 amino acids and that both isoforms share the characteristic Ndrg family sequence. Here, we provide the first report that chronic antidepressant drug treatment and repeated ECT decreases the expression of Ndrg2 mRNA and protein in the rat frontal cortex.

Materials and methods

Experimental animals and treatments

Male Sprague–Dawley rats (age 7–10 wk, Sankyo Labo Service Co., Tokyo, Japan) were housed in a temperature-controlled environment with a 12 h light/12 h dark cycle and were given free access to food and water. Rats were randomly separated into control and treated groups. Imipramine (Sigma-Aldrich, Inc., MO, USA) and sertraline (Pfizer Pharmaceuticals Inc., NY, AQ4 USA) were dissolved in 1.5% Tween-80. For the chronic antidepressant-treatment group, rats received daily intraperitoneal injections of vehicle, 10 mg/kg of imipramine, or 10 mg/kg sertraline for 21 d. For the ECT group, rats were anaesthetized with sevoflurane, then given either a single electric shock (90 mA, 1.0-s duration) via ear-clip electrodes (single-dose ECT group) or electric shocks (90 mA, 1.0-s duration) every other day for 14 d (repeated ECT group). ECT was delivered with a Ugo Basile Model 7801 unipolar square-wave electroconvulsive stimulation pulse generator (Stoelting Co., IL, USA). Control rats were AQ4 treated exactly like the ECT-treated rats but did not receive any electric current.

Twenty-four hours after the final antidepressant or ECT treatment, animals were euthanized by

decapitation. The brain was quickly removed, dissected, and immediately frozen in liquid nitrogen and stored at −80 °C until later use. All animal studies were carried out in accordance with National Institutes of Health guidelines in line with the OPRR Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Identification of Ndrg2 by ADRG microarray

Fabrication of the ADRG microarray and fluorescence image analysis was done as described previously (Yamada et al., 2000). Briefly, each of the ADRG cDNA inserts was amplified by vector primers and negative controls, and 10 different kinds of housekeeping genes were spotted in duplicate onto glass slides with a GMS417 Arrayer (Affymetrix Inc., CA, USA). Hybridization of fluorescent probes to the microarray was done competitively and in duplicate. After hybridization and washing, each slide was scanned with a GMS418 Array Scanner (Affymetrix Inc.). Gene expression levels were quantified and analysed with ImaGene software (Bio-Discovery Ltd, Swansea, UK). Preliminary assessment of the arrays (data not shown) indicated that the differences in fluorescence intensities (±2-fold) were significant. Sequence analysis of ADRG123 was performed by dideoxy sequencing methods. Homology search and sequence alignment was done using the FASTA search servers at the National Center for Biotechnology Information.

Expression analysis by real-time quantitative PCR

As described above, rat Ndrg2 protein consists of two splice variants, Ndrg2S and Ndrg2L. However, we previously demonstrated using conventional RT–PCR analyses that transcript processing into long and short forms of Ndrg2 does not appear to be significantly regulated after antidepressant treatments (data not shown). Therefore, we performed mRNA expression analysis of Ndrg2 with real-time quantitative PCR; total levels of Ndrg2S and Ndrg2L mRNA were examined in the present study.

Total RNA was extracted from samples using Isogen reagent (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. Total RNA samples treated with RNase-free DNase I were used to synthesize the first strand cDNA via reverse transcriptase and oligo-dT primer. We quantified Ndrg2 expression in the rat frontal cortex with real-time quantitative PCR using an ABI PRISM 7000 instrument (Applied Biosystems, Foster City, CA, USA). PCR primers were designed using Primer Express Software (Applied Biosystems). A quantity of cDNA corresponding to 20 pg of total RNA was amplified by PCR in duplicate. For each sample, three distinct amplifications were carried out in parallel. The following primers were used for rat Ndrg2 (5′-AACTTTGAGCGAGGTGGTGAGA-3′ and 5′-ATTCCACCACGGCATCTTCA-3′) and β-actin (5′-TCGCTGACAGGATGCAGAAGG-3′ and 5′-GCCAGGATAGAGCCACCAAT-3′). The SYBR® Green PCR Core Reagents kit (Applied Biosystems) was utilized for fluorescence detection of cDNA. For quantification, we used the Standard Curve Method (User Bulletin, ABI PRISM 7000 Sequence Detection System). Briefly, for rat Ndrg2 and β-actin, an absolute standard curve was obtained by plotting the threshold cycle following PCR amplification of serial dilutions of control cDNA template.

Expression analysis by Western blotting

Anti-rat-Ndrg2 antiserum was prepared as follows. Synthetic rat Ndrg2 peptides (CSLTSAASIDGSRSR, RDLNFERGGEMTLKC, and CEVQITEEKPLLPGQ) were coupled to activated keyhole limpet haemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, then injected into Hartley guinea pigs (Takara). Immune serum was then collected and used for Western blot analysis and immunohistochemistry.

Frontal cortices from control and treated rats were homogenized in ice-cold sucrose–Tris buffer (250 mM sucrose, 50 mM Tris–HCl, 5 mM EDTA, 10 mM EGTA, 0.3% mercaptoethanol; pH 7.4). Three rats were used for each treatment group. The protein concentration was determined by the Bradford method and a Bio-Rad protein assay kit. Each fraction (20 μg protein) was separated by 7.5% SDS–PAGE after solubilization and boiling in Laemmli buffer. Electrophoretically separated proteins were transferred from gels onto nitrocellulose membranes via standard techniques. To examine the expression of Ndrg2 in HEK293 cells overexpressing rat Ndrg2S and Ndrg2L respectively, Western blot analyses were performed on protein extracts derived from the transfected cells. Pre-immune serum was used as negative control.

Non-specific immunostaining was blocked by incubating the membranes in blocking buffer comprised of 5% skim milk. The membranes were sequentially incubated in blocking buffer with anti-rat-Ndrg2 antiserum (1:500), followed by HRP-conjugated goat anti-guinea pig antibody (1:2000; ICN Biomedicals Inc., CA, USA). Immunoreactive bands were visualized on film via the ECL system. To ensure the fidelity of this analysis, we assayed only film exposed in the linear

range. The optical density of the digitized bands was quantified using NIH Image. NIH Image is a public domain program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Phosphatase digestion

For the phosphatase digestion study, a protein sample from the rat frontal cortex was incubated with lambda protein phosphatase, a Mn^{2+}-dependent protein phosphatase that acts on phosphorylated serine, threonine, and tyrosine residues. The protein aliquot was incubated for 1 h at 30 °C in 50 μl of lambda-protein phosphatase reaction buffer [50 mM Tris–HCl (pH 7.5), 5 mM dithiothreitol, 0.1 mM Na_2EDTA, 0.01% Brij 35, and 2 mM $MnCl_2$] with or without 1 μl lambda-protein phosphatase (400 000 U/ml; New England Biolabs Inc., USA). The proteins were then analysed by Western blot together with an identically treated aliquot incubated without phosphatase.

Cell culture and transfection of Ndrg2S and Ndrg2L in HEK293 cells

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum, 0.1 mM MEM non-essential amino-acid solution (Invitrogen, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere comprised of 5% CO_2.

The coding regions for Ndrg2S and Ndrg2L were obtained by RT–PCR of rat brain mRNA with the following set of primers: 5′-CTCGAGGCCACCAT GGCAGAGC-3′, 5′-GAATTCTCTCAACAGGAGACT TCCATGGTG-3′ and high fidelity Platinum pfx DNA polymerase (Invitrogen). These primers contain either *Xho*I or *Eco*RI sites (underlined) to facilitate subcloning. Each of the PCR products were then ligated into pCR II-TOPO vectors (Invitrogen) and transformed into competent DH5α *E. coli* cells. The resulting plasmid vectors were subcloned into pIRES-EGFP (Clontech, CA, USA) for transfection. In this study, we used the pIRES-EGFP vector, which can express GFP and target molecules separately. HEK293 cells were then transfected with 3 μg of recombinant plasmid in serum-free medium using 4 μl Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Immunohistochemistry

Rats were anaesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4). The brains were then cryoprotected and quickly frozen. The brain was sectioned (40 μm) using a cryostat CM-501 (Sakura, Tokyo, Japan), and floating sections were further fixed with 4% paraformaldehyde overnight. Sections were boiled in phosphate buffer containing 0.9% NaCl (PBS) for 1 h, permeabilized with 0.1% Triton X-100 in PBS (PBST) for 20 min, and then blocked with PBS containing 1.5% normal goat serum for 20 min. Sections were incubated with anti-rat-Ndrg2 antiserum (1:500) in PBST for 24 h at 4 °C, washed three times with PBST, and incubated with biotinylated anti-guinea pig antibody (1:250, Vector Laboratories, CA, USA) for 30 min at room temperature. Sections were washed three times with PBST, treated with 0.3% hydrogen peroxide for 30 min, washed three times with PBST again, and incubated with avidin-biotin peroxidase complex (Vector Laboratories) for 30 min. Visualization of the peroxidase was performed with 0.01% hydrogen peroxide and 0.01% diaminobenzidine as a chromogen. The slides were counterstained with haematoxylin and analysed with an Olympus BX-60 light microscope (Olympus Optical, Tokyo, Japan).

Statistical analysis

Data are presented as means ± S.E.M. for each group. For antidepressant or ECT experiments, differences were assessed using analysis of variance (ANOVA) followed by the Dunnetts test. A value of $p < 0.05$ was regarded as significant.

Results

Identification of Ndrg2 as ADRG123

Figure 1 shows a pseudo-colour image of the ADRG microarray after hybridization with frontal cortex samples obtained from sertraline- or ECT-treated rats. As expected, we obtained low background and consistent results in duplicate experiments. After normalization of the signals with both negative and positive controls, fluorescence intensities representing ADRG123 decreased 0.63-fold in the sertraline group and 0.49-fold in the ECT group. These data were reproducible and inter-assay variability was negligible. As shown in Figure 2, the ADRG123 fragment obtained from the initial EST analysis was 230 bp (starting at the 3′-end containing poly-A^+ sequences). Homology search of the EMBL/GeneBank database revealed that ADRG123 perfectly matches the full-length cDNA sequence of the rat *Ndrg2* gene (Swiss-Prot/TrEMBL accession numbers Q8VBU2, Q8VI01).

Table 1. Real-time RT–PCR analysis of *Ndrg2* mRNA expression in the rat frontal cortex after antidepressant treatment or ECT

	Ndrg2
Single antidepressant treatment	
Control	100±2.3
Imipramine	101±13.2
Sertraline	86.7±2.7
Chronic antidepressant treatment	
Control	100±7.9
Imipramine	65.3±2.6*
Sertraline	65.3±13.2*
ECT	
Control	100±6.1
Single-dose ECT	71.5±9.3*
Chronic ECT	47.2±6.8**

Data are expressed as means±S.E.M. $*p<0.05$, $**p<0.01$, ANOVA followed by Dunnetts test.

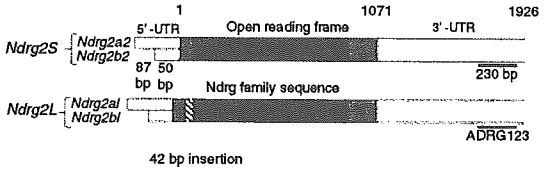


Figure 2. Schematic representations of rat Ndrg2. Rat Ndrg2 consists of four isoforms: Ndrg2a1, Ndrg2a2, Ndrg2b1, and Ndrg2b2. The 5′-UTR for Ndrg2a1/Ndrg2a2 was 87 bp, whereas the 5′-UTR for Ndrg2b1/Ndrg2b2 was 50 bp. In the translated region, Ndrg2a1/Ndrg2b1 has an additional 42-bp insertion compared to Ndrg2a2/Ndrg2b2; both isoforms contained the characteristic Ndrg family sequence in the middle of their sequences. In this study, Ndrg2S (upper) and Ndrg2L (lower) correspond to Ndrg2a2/Ndrg2b2 and Ndrg2a1/Ndrg2b1 respectively. The ADRG123 fragment obtained from the initial EST analysis was part of rat Ndrg2 (230 bp, starting at the 3′-end containing poly-A^+ sequences). UTR, untranslated region.

Messenger RNA expression analysis by real-time quantitative PCR

Using real-time quantitative RT–PCR, we confirmed the significantly decreased expression of total *Ndrg2* mRNA in the frontal cortex that resulted from chronic treatment with either imipramine or sertraline (65.3±2.6% or 65.3±13.2%, Table 1). On the other hand, single-dose treatments of either antidepressant failed to affect the expression of total Ndrg2 mRNA (101±13.2% or 86.7±2.7%). Interestingly, as shown in Table 1, not only repeated ECT but also single-dose ECT significantly decreased total Ndrg2 mRNA expression in rat frontal cortex (71.5±9.3% or 47.2±6.8%).

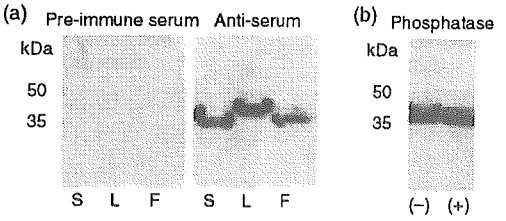


Figure 3. Specificity of anti-rat-Ndrg2 antiserum prepared by our group in the present study. To examine the specificity of the anti-rat-Ndrg2 antiserum, we immunostained HEK293 cells overexpressing rat Ndrg2S and Ndrg2L (a). The lysates from HEK293 cells (S, L) or rat frontal cortex (F) were electrophoresed on a 7.5% acrylamide gel and analysed using pre-immune serum [(a), left panel] or anti-rat-Ndrg2 antiserum [(a), right panel]. As expected, immunoblotting of protein extracts from HEK293 cells showed a single band corresponding to rat Ndrg2S and Ndrg2L proteins, while pre-immune serum (control) showed no bands. The effect of phosphatase digestion on Ndrg2 immunoreactivity in the rat frontal cortex was also examined. Undigested rat frontal cortex showed two major immunoreactive bands when stained with anti-rat-Ndrg2 antiserum [(b), lane 1]. The double bands persisted, even after phosphatase digestion, and did not show a mobility shift in a gel [(b), lane 2].

Expression analysis of Ndrg2S- and Ndrg2L-protein by Western blot analysis

Immunoblotting of protein extracts from control frontal cortex demonstrated two Ndrg2-immunoreactive ~39.3 and ~40.8 kDa bands (Figure 4). To examine AQ5
the specificity of the anti-rat-Ndrg2 antiserum, we immunostained HEK293 cells overexpressing rat Ndrg2S and Ndrg2L. As expected, immunoblotting of protein extracts from these HEK293 cells showed a single band corresponding to rat Ndrg2S and Ndrg2L proteins (Figure 3a), while immunoblotting with pre-immune serum showed no staining.

To determine whether the antidepressant-associated decrease of Ndrg2S and Ndrg2L mRNAs also affected protein levels, we examined Ndrg2S and Ndrg2L protein expression in the rat frontal cortex with Western blot analysis. As expected (Figure 4), chronic treatment with either imipramine or sertraline decreased Ndrg2S (82.9±14.1% or 60.2±5.7%) and Ndrg2L (80.1±18.5% or 59.8±5.5%) immunoreactivity. In contrast, single-dose treatments with either antidepressant failed to affect Ndrg2S and Ndrg2L immunoreactivity (Table 2, Figure 4). Moreover, both single-dose and repeated ECT significantly decreased Ndrg2S (57.3±14.3% or 60.2±12.2%) and

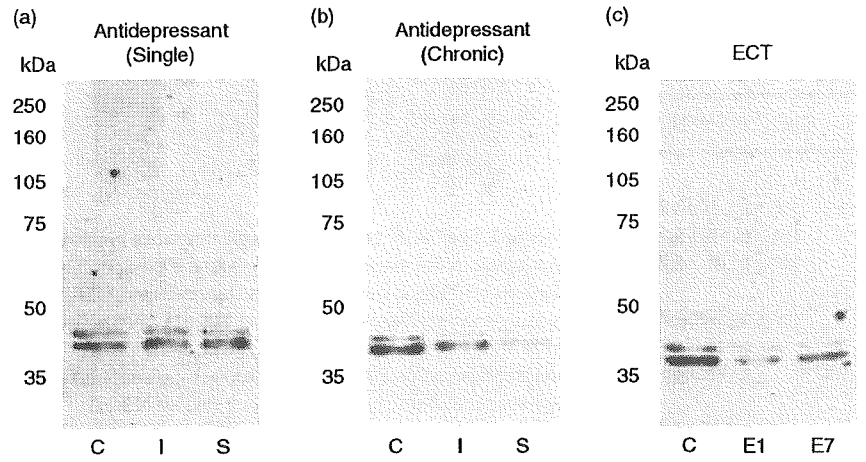


Figure 4. Western blot analysis of Ndrg2S and Ndrg2L in rat frontal cortex after a single antidepressant treatment (a), chronic antidepressant treatment (b), or ECT (c). A protein sample was prepared from rat frontal cortex and treated with either vehicle (control, lane 1), 10 mg/kg of imipramine (lane 2) or sertraline (lane 3). A protein sample was also prepared from frontal cortices from rats that received a sham operation (control, lane 1), a single dose of ECT (lane 2) or repeated ECT treatments (lane 3). Immunoblotting confirmed that NDRG2-S and NDRG2-L proteins (~39.3 and ~40.8 kDa) exist in the frontal cortex. As expected, chronic treatment with either imipramine or sertraline decreased NDRG2-S and NDRG2-L immunoreactivity. This figure represents typical results from three independent experiments. AQ3

Ndrg2L (55.0±18.5% or 53.6±3.1%) immunoreactivity (Table 2, Figure 4).

Phosphatase digestion

The insulin-dependent phosphorylation of Ndrg2 has been reported to occur in skeletal muscle of Wistar rats as well as in mouse C2C12 skeletal muscle cells (Burchfield et al., 2004). These findings prompted us to determine whether Ndrg2 is also phosphorylated in the central nervous system. As described above immunoblotting of undigested frontal cortex with anti-rat-Ndrg2 antiserum revealed two major immunoreactive bands (Figure 3b, lane 1). In these experiments, these two bands remained immunoreactive even after phosphatase digestion; moreover, they did not shift in mobility in a gel (Figure 3b, lane 2). Taken together, these findings indicate that these bands do not represent phosphorylated forms of Ndrg2S or Ndrg2L.

Immunohistochemical localization of Ndrg2 in the rat frontal cortex

To confirm Ndrg2 protein expression in the central nervous system, we examined anti-rat-Ndrg2 immunostaining in the rat frontal cortex. We observed Ndrg2-immunoreactivity throughout the frontal cortex. Figure 4 presents a typical image of Ndrg2-immunoreactive cells found in the external pyramidal layer (layer III). Interestingly, we also observed small Ndrg2-immunoreactive astrocyte-like cells. Their entire soma and proximal processes were immunostained.

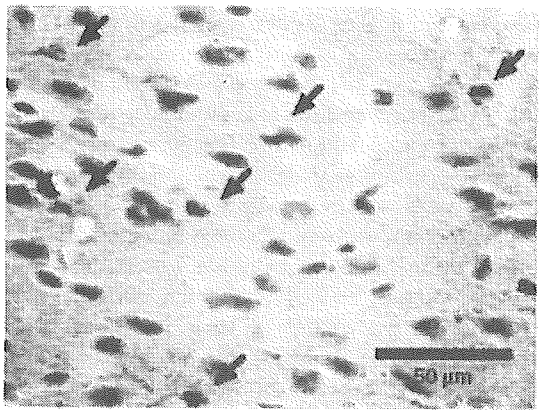
Discussion

We identified an EST, ADRG123, the expression of which decreased after chronic antidepressant treatment and repeated ECT. Sequence and homology comparisons using the EMBL/GeneBank database showed that ADRG123 perfectly matches rat Ndrg2. Ndrg2 is a member of the Ndrg family; thus far, four members of this family, Ndrg1-4, have been identified (Zhou et al., 2001). Although Ndrg members do not possess a clear functional motif, they do share a high level of sequence homology. Phylogenetic analysis of Ndrg1-4 revealed that Ndrg1 and Ndrg3 belong to one subfamily, while Ndrg2 and Ndrg4 belong to another (Qu et al., 2002). In the present study, we demonstrated that chronic treatment with the tricyclic antidepressant imipramine and the selective serotonin reuptake inhibitor sertraline reduced both Ndrg2 mRNA and protein levels in the rat frontal cortex. The frontal cortex is one of several brain regions that may contribute to the endocrine, emotional, cognitive, and vegetative abnormalities observed in depressed patients. This is supported by findings showing that glucose metabolism, blood flow, and

Table 2. Ndrg2 immunoreactivity in the rat frontal cortex after antidepressant treatment and ECT analysed by Western blot analysis

	Ndrg2S	Ndrg2L
Single antidepressant treatment		
Control	100±7.2	100±13.2
Imipramine	104±6.0	90.6±12.0
Sertraline	107±27.7	80.9±7.5
Chronic antidepressant treatment		
Control	100±10.9	100±8.4
Imipramine	82.9±14.1	80.1±18.5
Sertraline	60.2±5.7*	59.8±5.5*
ECT		
Control	100±6.0	100±11.3
Single-dose ECT	57.3±14.3*	55.0±18.5*
Chronic ECT	60.2±12.2*	53.6±3.1*

Data are expressed as means±S.E.M. *$p<0.05$, ANOVA followed by Dunnetts test.



COLOUR

Figure 5. Immunohistochemical identification of Ndrg2-expressing cells in the rat frontal cortex. Using the anti-rat-Ndrg2 antiserum prepared by our group, Ndrg2 immunoreactivity (brown) was observed in cells in the rat frontal cortex. Diaminobenzidine was the chromogen, and the counterstain was haematoxylin. Interestingly, Ndrg2 immunoreactivity was observed in small astrocyte-like cells and their proximal processes in the rat frontal cortex (arrows). Scale bar, 50 μm.

electroencephalograph activity are altered in the frontal cortices of depressed patients (Drevets et al., 1992). It is reasonable, therefore, to hypothesize that alterations of mood, neurovegetative signs, or even social behaviour of depressed patients may reflect changes in physiological functions within this important brain region. In addition, repeated ECT treatment also decreased Ndrg2 mRNA expression. Although single-dose ECT treatments also significantly decreased Ndrg2 expression, single-dose antidepressant treatments failed to do so. The relatively rapid effect of ECT on Ndrg2 expression may explain the rapid onset of its antidepressant effects in clinical settings. The detailed mechanisms underlying antidepressant-induced adaptive changes are as of yet unknown. However, our findings may suggest that Ndrg2 expression-dependent alterations of the frontal cortex may be an important component of the pharmacological action of antidepressants and ECT.

Phosphorylation of Ndrg proteins has been studied very little, although protein kinase A-dependent phosphorylation of Ndrg1 has been described previously (Agarwala et al., 2000). In addition, Ndrg1 is a multiphosphorylated protein in mast cells, and the kinetics of increased Ndrg1 phosphorylation has been shown to parallel signalling events leading to exocytosis (Sugiki et al., 2004). More recently, it was reported that insulin-dependent phosphorylation of Ndrg2 occurs in skeletal muscle of Wistar rats and in mouse C2C12 skeletal muscle cells (Burchfield et al., 2004). However, in the present study, we demonstrated that two Ndrg2-immunoreactive bands found in the rat frontal cortex remained immunoreactive even after phosphatase digestion; moreover, they did not shift in mobility in a gel. These findings indicate that these bands do not represent phosphorylated forms of Ndrg2S or Ndrg2L, suggesting possible differential regulation of Ndrg2 phosphorylation in the central nervous system.

Ndrg family members may be intimately involved in cellular differentiation and development. Indeed, Ndrg1 expression is induced by hypoxia and has been implicated in cell growth regulation and Schwann cell signalling for axonal survival (Kalaydjieva et al., 2000; Piquemal et al., 1999; Salnikow et al., 2002; Zhou et al., 1998). In human leukaemia cells, Ndrg1 expression is up-regulated by differentiation-related retinoids and vitamin D3 (Piquemal et al., 1999). Suppression of Ndrg4 expression by Ndrg4 antisense transfection inhibits neurite outgrowth in PC12 cells (Ohki et al., 2002). Stable expression of human Ndrg2 in glioblastoma cell lines decreases cell growth rates (Deng et al., 2003). More recently, Ndrg2 mRNA and protein has been shown to be up-regulated in Alzheimer's disease brains (Mitchelmore et al., 2004). Taken together, these findings indicate that Ndrg's may be critically involved in developmental processes, and Ndrg2 in particular, may be involved in neural and/or glial development and plasticity. Interestingly, in the present study, we observed Ndrg2 immunoreactivity in small astrocyte-like cells in the rat frontal cortex.

There have now been reports showing that glial cell density is reduced in the prefrontal cortex of patients with major depressive disorders (see review by Cotter et al., 2001). These findings suggest that, in addition to examining neuronal or glial pathology, neuronal–glial interactions associated with the pathophysiology of depression also requires in-depth study.

In conclusion, we have identified Ndrg2 as a novel candidate target molecule of antidepressants and ECT in the rat frontal cortex. Although, the functional role of Ndrg2 in the central nervous system remains unclear, our findings suggest that Ndrg2 expression-dependent alterations of the frontal cortex may be an important component of the pharmacological action of antidepressants and ECT. Additional work is necessary to test this hypothesis.

Acknowledgements

Sertraline was kindly supplied by Pfizer Pharmaceuticals Inc., NY, USA. This work was in part supported by Health Science Research Grants from the Ministry of Health, Labour and Welfare; Ministry of Education, Culture, Sport, Science, and Technology; the Japan Society for the Promotion of Science; Showa University School of Medicine Alumni Association; and the Mitsubishi Pharma Research Foundation. Misa Yamada was supported by a fellowship from the Japan Foundation for Aging and Health.

Statement of Interest

None.

References

Agarwala KL, Kokame K, Kato H, Miyata T (2000). Phosphorylation of RTP, an ER stress-responsive cytoplasmic protein. *Biochemical and Biophysical Research Communications 272*, 641–647.

Boulkroun S, Fay M, Zennaro MC, Escoubet B, Jaisser F, Blot-Chabaud M, Farman N, Courtois-Coutry N (2002). Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene. *Journal of Biological Chemistry 277*, 31506–31515.

Burchfield JG, Lennard AJ, Narasimhan S, Hughes WE, Wasinger VC, Corthals GL, Okuda T, Kondoh H, Biden TJ, Schmitz-Peiffer C (2004). Akt mediates insulin-stimulated phosphorylation of Ndrg2: evidence for cross-talk with protein kinase C theta. *Journal of Biological Chemistry 279*, 18623–18632.

Cotter DR, Pariante CM, Everall IP (2001). Glial cell abnormalities in major psychiatric disorders: the evidence and implications. *Brain Research Bulletin 55*, 585–595.

Deng Y, Yao L, Chau L, Ng SS, Peng Y, Liu X, Au WS, Wang J, Li F, Ji S, Han H, Nie X, Li Q, Kung HF, Leung SY, Lin MC (2003). N-Myc downstream-regulated gene 2 (NDRG2) inhibits glioblastoma cell proliferation. *International Journal of Cancer 106*, 342–347.

Drevets WC, Videen TO, Price JL, Preskorn SH, Carmichael ST, Raichle ME (1992). A functional anatomical study of unipolar depression. *Journal of Neuroscience 12*, 3628–3641.

Kalaydjieva L, Gresham D, Gooding R, Heather L, Baas F, de Jonge R, Blechschmidt K, Angelicheva D, Chandler D, Worsley P, Rosenthal A, King RH, Thomas PK (2000). N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. *American Journal of Human Genetics 67*, 47–58.

Mitchelmore C, Buchmann-Moller S, Rask L, West MJ, Troncoso JC, Jensen NA (2004). NDRG2: a novel Alzheimer's disease associated protein. *Neurobiology of Disease 16*, 48–58.

Nishioka G, Yamada M, Kudo K, Takahashi K, Kiuchi Y, Higuchi T, Momose K, Kamijima K, Yamada M (2003). Induction of kf-1 after repeated electroconvulsive treatment and chronic antidepressant treatment in rat frontal cortex and hippocampus. *Journal of Neural Transmision 110*, 277–285.

Ohki T, Hongo S, Nakada N, Maeda A, Takeda M (2002). Inhibition of neurite outgrowth by reduced level of NDRG4 protein in antisense transfected PC12 cells. *Brain Research. Developmental Brain Research 135*, 55–63.

Okuda T, Kondoh H (1999). Identification of new genes ndr2 and ndr3 which are related to Ndr1/RTP/Drg1 but show distinct tissue specificity and response to N-myc. *Biochemical and Biophysical Research Communications 266*, 208–215.

Piquemal D, Joulia D, Balaguer P, Basset A, Marti J, Commes T (1999). Differential expression of the RTP/Drg1/Ndr1 gene product in proliferating and growth arrested cells. *Biochimica et Biophysica Acta 1450*, 364–373.

Qu X, Zhai Y, Wei H, Zhang C, Xing G, Yu Y, He F (2002). Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family. *Molecular and Cellular Biochemistry 229*, 35–44.

Salnikow K, Kluz T, Costa M, Piquemal D, Demidenko ZN, Xie K, Blagosklonny MV (2002). The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. *Molecular and Cellular Biology 22*, 1734–1741.

Sugiki T, Murakami M, Taketomi Y, Kikuchi-Yanoshita R, Kudo I (2004). N-Myc downregulated gene 1 is a phosphorylated protein in mast cells. *Biological and Pharmaceutical Bulletin 27*, 624–627.

Yamada M, Higuchi T (2002). Functional genomics and depression research. Beyond the monoamine hypothesis. *European Neuropsychopharmacology 12*, 235–244.

Yamada M, Takahashi K, Tsunoda M, Nishioka G, Kudo K, Ohata H, Kamijima K, Higuchi T, Momose K (2002). Differential expression of VAMP2/synaptobrevin-2 after antidepressant and electroconvulsive treatment in rat frontal cortex. *Pharmacogenomics Journal 2*, 377–382.

Yamada M, Yamazaki S, Takahashi K, Nara K, Ozawa H, Yamada S, Kiuchi Y, Oguchi K, Kamijima K, Higuchi T, Momose K (2001). Induction of cysteine string protein after chronic antidepressant treatment in rat frontal cortex. *Neuroscience Letters 301*, 183–186.

Yamada M, Yamazaki S, Takahashi K, Nishioka G, Kudo K, Ozawa H, Yamada S, Kiuchi Y, Kamijima K, Higuchi T, Momose K (2000). Identification of a novel gene with RING-H2 finger motif induced after chronic antidepressant treatment in rat brain. *Biochemical and Biophysical Research Communications 278*, 150–157.

Zhou D, Salnikow K, Costa M (1998). Cap43, a novel gene specifically induced by Ni2+ compounds. *Cancer Research 58*, 2182–2189.

Zhou RH, Kokame K, Tsukamoto Y, Yutani C, Kato H, Miyata T (2001). Characterization of the human NDRG gene family: a newly identified member, NDRG4, is specifically expressed in brain and heart. *Genomics 73*, 86–97.

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Full Paper

Identification and Expression of Frizzled-3 Protein in Rat Frontal Cortex After Antidepressant and Electroconvulsive Treatment

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Abstract. The biological basis for the therapeutic mechanisms of depression are still unknown. While performing EST (expressed sequence tag) analysis to identify some molecular machinery responsible for the antidepressant effect, we determined the full-length nucleotide sequence of rat frizzled-3 protein (Frz3) cDNA. Interestingly, Northern blot analysis demonstrated that elevated levels of Frz3 were expressed continually from embryonic day 20.5 to postnatal 4 weeks in developing rat brain. In adult rat brain, Frz3 mRNA was expressed predominantly in the cerebral cortex and hypothalamus and moderately in the hippocampus. Using real-time quantitative PCR, we demonstrated that chronic treatment with two different classes of antidepressants, imipramine and sertraline, reduced Frz3 mRNA expression significantly in rat frontal cortex. Electroconvulsive treatment (ECT) also reduced Frz3 expression. In contrast, antidepressants and ECT failed to reduce Frz2 expression. Additionally, chronic treatment with the antipsychotic drug haloperidol did not affect Frz3 expression. Recently, the Frz/Wingless protein pathway has been proposed to direct a complex behavioral phenomenon. In conclusion, the Frz3-mediated signaling cascade may be a component of the molecular machinery targeted by therapeutics commonly used to treat depression.

Keywords: gene expression, mRNA fingerprinting, imipramine, sertraline

Introduction

Depression is one of the most prominent psychiatric diseases. Typical antidepressants acutely inhibit monoamine reuptake in nerve terminals, resulting in significant increases in the synaptic concentration of either noradrenaline or serotonin. However, a latency period of several weeks generally lapses before the clinical effects of antidepressants emerge. Repeated electroconvulsive treatment (ECT) is another therapy that is widely used, particularly in the treatment of drug-resistant depression. Although it has been known as an efficient treatment modality for decades, the basis of its therapeutic effects remains unknown. Its clinical effects also emerge over time.

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The delayed clinical effects of antidepressants and ECT could result from either indirect regulation of neural signal transduction systems or regulation of gene transcription. Indeed, selective effects of antidepressants on specific immediate early genes and transcription factors have been described (1). These molecules may be important for adaptive neuronal changes resulting from chronic antidepressant treatment. Region-specific effects of chronic antidepressant treatment on the DNA-binding activities of CRE-, SP1-, and GRE-binding elements in rat hippocampus and frontal cortex are known (2). Taken together, these data demonstrate the possible role of gene expression alternations in the mechanism underlying antidepressant action. Therefore, quantifying these alterations occurring after chronic antidepressant treatment can help to identify novel molecular markers useful in the diagnosis and treatment

of depression. Using differential cloning techniques, we and other groups have isolated genes that are differentially expressed in rat brain after chronic antidepressant treatment. To date, we have cloned several cDNA fragments as expressed sequence tags (ESTs), termed antidepressant-related genes, ADRGs (3 – 6).

In the present study, we focused on ADRG#78, an ADRG that encodes rat frizzled-3 protein (Frz3). The frizzled protein (Frz) family is a group of receptor proteins with seven putative transmembrane helixes (7). The N-terminal extracellular cysteine-rich domain of Frzs has been identified to be the ligand-binding domain that binds wingless proteins (Wnts), a family of secreted cysteine-rich glycosylated ligands (8). The Frz/Wnt pathway, first described in *Drosophila*, is a highly conserved developmental pathway involved in cell fate determination in the central nervous system of virtually all-eukaryotic organisms (9, 10). Recently, the Frz/Wnt pathway has been proposed to direct a complex behavioral phenomenon, and molecules participating in this pathway are encoded by candidate genes thought to be involved in neuropsychiatric disorders.

Here, we provide the first report identifying Frz3 in rat frontal cortex and describing its down-regulation after chronic treatment with antidepressants and ECT.

Materials and Methods

Experimental animals

Male Sprague Dawley rats (age: 7 – 10 weeks; Sankyo Labo Service Co., Tokyo) were housed in a temperature-controlled environment with a 12-h light / 12-h dark cycle. They had free access to food and water. Rats were randomly separated into control and treated groups. Six rats were used for each treatment group. Experimental animals for antidepressant treatment received daily intraperitoneal injections of either vehicle, 10 mg/kg of imipramine (Sigma Chemical, St. Louis, MO, USA), or sertraline (Pfizer Pharmaceuticals, Inc., New York, NY, USA) dissolved in saline containing 1.5% Tween-80; this injection protocol lasted for either 1 day or 21 days. As previously reported by our group, chronic treatment with 10 mg/kg of imipramine or sertraline reduced immobility in the water wheel test (3). Thus, it is proposed that both imipramine and sertraline administration in our protocol is adequate to induce an antidepressive-like effect in rats. Animals in the antipsychotic drug group were treated with haloperidol (0.5 mg/kg, Sigma Chemical) for 21 days. Animals in the ECT group were anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka), and then they were given either a single electric shock (90 mA, 1.0-s duration) via ear-clip electrodes (single ECT group) or a series of electric shocks (90 mA, 1.0-s duration) every other day for 14 days (repeated ECT group). ECT was delivered with an Ugo Basile Model 7801 Unipolar, square-wave, electroconvulsive stimulation pulse generator (Stoelting Co., Wood Dale, IL, USA). Control rats were treated exactly as ECT-treated rats but received no electric current through the electrodes. Twenty-four hours after administration of the final antidepressant treatment or ECT, animals were euthanized by decapitation. The brain was quickly removed, dissected, and immediately frozen in liquid nitrogen and stored at –80°C until use. All animal studies were carried out in accordance with protocols approved by the Showa University Ethics Committee in line with the OPRR Public Health Service Policy on Humane Care and Use of Laboratory Animals.

EST analysis and mRNA fingerprinting

Total RNA from rat frontal cortex was extracted by Isogen reagent (Nippon Gene Co., Tokyo) according to the manufacturer's instructions. Isolated total RNA was dissolved in RNase-free water, and the concentration was estimated by UV spectrometry. Total RNA samples were treated with RNase-free DNase I (Nippon Gene Co.) for 30 min at 37°C and purified by phenol-chloroform extraction. The first strand cDNA was synthesized with reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 1 μM of oligo-dT primer, and 2 μg of total RNA treated with DNase I and then diluted to a final volume of 100 μL. EST analysis was then carried out in the presence of [^{33}P]dATP (Life Science Products, Inc., Boston, MA, USA) with an mRNA fingerprinting kit (Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. Radiolabeled PCR products on denaturing 6% polyacrylamide gels were analyzed by electrophoresis. Three individual samples from each drug treatment group were applied side-by-side and visualized by autoradiography.

Subcloning and sequence analysis

The band of interest (ADRG#78) was cut out of the dried gel, and the cDNA fragment was re-amplified using the same primer set used for mRNA fingerprinting (ATTAACCCTCACTAAATGCTGTATG, CATTATGCTGAGTGATATCTTTTTTTTTAC). The PCR conditions were as follows: denaturation at 94°C for 3 min followed by 40 cycles at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The re-amplified product was ligated into a pCR II-TOPO vector (Invitrogen) and transformed into competent TOP 10F' *E. coli* cells (Invitrogen). Sequence analysis was performed by standard dideoxy sequencing methods. Homology searches and sequence alignment were done using the FASTA search servers at the National Center

Table 1. Oligonucleotides used for real-time quantitative PCR

mRNA	Forward primer	Reverse primer
rat Frz3	GACACAGCAGCATCCGAGACG	GGTTCATGCTGGTGCCAT
rat Frz2	TGCACTCGTGGAGGAAGTTCT	CGCTTCACACGGTGGTCTCT
rat Frz1	GCGCACCTGGATAGGCAT	TACTAGGTACGTGAGCACCGTGA
rat β-actin	TCGCTGACAGGATGCAGAAGG	GCCAGGATAGAGCCACCAAT

for Biotechnology Information. Additional cDNA sequences were determined by 3'- and 5'-RACE-PCR using primer sequences derived from the partial cDNA sequences obtained from EST analysis.

Northern blot analysis

The cDNA fragment of rat Frz3 was amplified by PCR using a pair of [^{32}P]dCTP-primers (5'-CGTCAC AAGATTCCGTAACCC-3' and 5'-CGGCATTATATC CCTAAAC-3') (Amersham Pharmacia Biotech, Tokyo). This amplified product was used as a probe for Northern blot analysis. Hybridization was carried out in ExpressHyb hybridization solution according to the manufacturer's instructions (Clontech). After hybridization, the membrane was exposed to X-ray film for 24 h. To study the expression of Frz3, Rat Adult Tissue Blots (pre-made Northern blot nylon membrane from Seegene, Del Mar, CA, USA) were used for the Northern blot analysis. Total RNA isolated from tissues of SD rats, aged 8 – 12 weeks, was transferred onto the nylon membrane to make the "Rat Adult Tissue Blot". These tissues included brain, heart, lung, liver, spleen, kidney, stomach, small intestine, skeletal muscle, thymus, testis, nonimpregnated uterus, and placenta (20.5 days post-coitus). To study the developmental expression of rat Frz3 in the brain, Rat Brain Aging Blots (pre-made Northern blot nylon membrane from Seegene) were used. Total RNA from late embryonic and postnatal rat brain (20.5 days post-coitus and 1 day, 3 days, 1 week, 2 weeks, 4 weeks, 2 months, 3 months, 6 months, and 12 months postnatal) were isolated and transferred onto the nylon membrane to make the "Rat Brain Aging Blot". To study the expression pattern of Frz3 in the adult rat brain, Rat Brain Tissue Blots (pre-made Northern blot nylon membrane from Seegene) were used. Total RNA was isolated from olfactory bulb (including tubercle), cerebral cortex, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata, and spinal cord were transferred onto the nylon membrane to make the "Rat Brain Tissue Blot".

Real-time quantitative PCR

Quantification of Frz expression in rat frontal cortex was performed with the real-time quantitative PCR method; this was carried out with an ABI PRISM 7000 instrument (Applied Biosystems, Foster City, CA, USA). PCR primers were designed by Primer Express Software (Applied Biosystems). A quantity of cDNA corresponding to 20 pg of total RNA was amplified by PCR in duplicate. For each sample, three distinct amplifications were carried out in parallel. The primers used for rat Frz3, Frz2, Frz1, and β-actin, a reference for gene amplification, are indicated in Table 1. The SYBR® Green PCR Core Reagents Kit (Applied Biosystems) was utilized for fluorescent detection of cDNA. For quantification, we used the Standard Curve Method (User Bulletin, ABI PRISM 7000 Sequence Detection System). Briefly, for rat Frzs and β-actin, an absolute standard curve was obtained by plotting the cycle of threshold (Ct) following PCR amplification of serial dilutions of the control cDNA template. Data are presented as percentages of the control value (mean ± S.E.M.). Differences were assessed using Student's *t*-test. A value of $P<0.05$ was regarded as statistically significant.

Results

Identification and sequence analysis of rat Frz3

We determined the nucleotide sequence of full-length ADRG#78, one of the ADRGs we obtained from EST analysis. The original 395-bp cDNA fragment occupied positions 1518 – 1913 in the full-length cDNA. Its open reading frame comprised 1998 nucleotides encoding a 666-residue polypeptide with a predicted molecular mass of 76.2 kDa. Homology analysis using the EMBL/GeneBank database revealed ADRG#78 shares 95.2% and 88.5% homology with mouse and human Frz3 (11, 12), respectively, suggesting that ADRG#78 is a rat homologue of mouse and human Frz3. The nucleotide sequence of full-length rat Frz3 cDNA can be found in the EMBL/GeneBank (accession number AF323956). Alignment of the deduced amino acid sequence of rat Frz3 with those of mouse Frz3, human Frz3, rat Frz1, and rat Frz2 is shown in Fig. 1. The deduced amino acid sequence of rat Frz3 was 99.4% and 97.7% identical to those of mouse and human

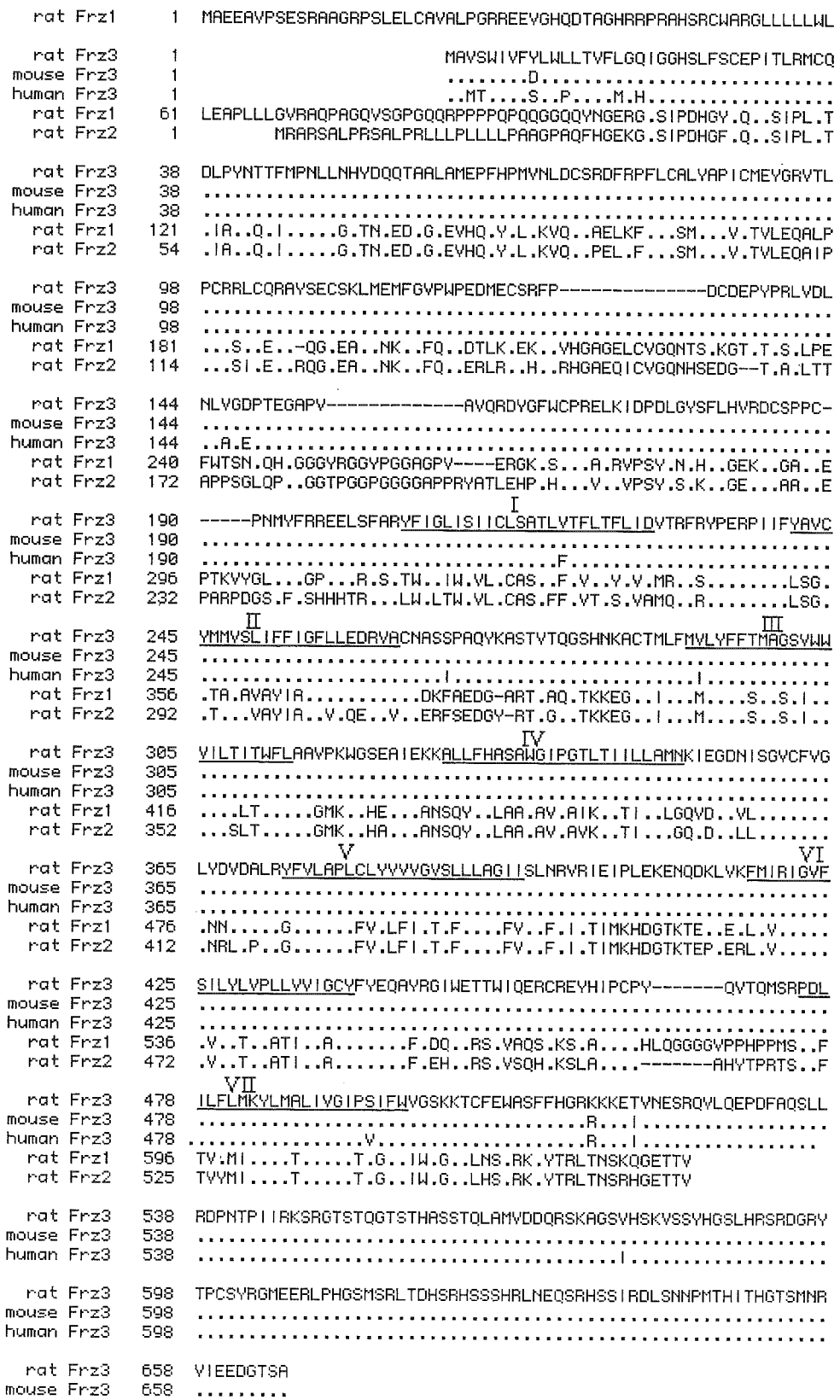

Fig. 1. Alignment of deduced amino acid sequences of rat Frz3, mouse Frz3, human Frz3, rat Frz1, and rat Frz2. Amino acid positions are numbered on the left. DDBJ/EMBL/GeneBank accession numbers are AF323956, U43205, AY005130, L02529, and L02530 for rat Frz3, mouse Frz3, human Frz3, rat Frz1, and rat Frz2, respectively. Dots represent identical amino acid residues found in rat Frz3. Predicted transmembrane domains are underlined.

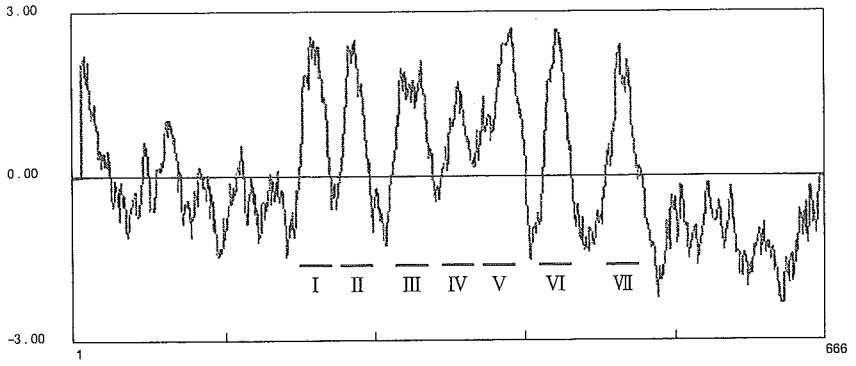

Fig. 2. Hydrophilicity/hydrophobicity analysis for rat Frz3 determined by the method of Kyte and Doolittle. Positive values denote regions of increased hydrophobicity. Putative transmembrane domains are indicated by solid lines. The presence of seven hydrophobic membrane-spanning stretches suggests Frz3 would be a member of the G-protein coupled receptor superfamily. These stretches are underlined in Fig. 1.

Frz3, respectively. On the other hand, the amino acid sequence homology of rat Frz3 to rat Frz1 and rat Frz2 (13) was only 50.6% and 44.5%, respectively. As shown in Fig. 2, hydrophilicity/hydrophobicity analysis for rat Frz3 revealed the presence of seven hydrophobic membrane-spanning stretches, suggesting that it is a member of the G-protein coupled receptor superfamily. These stretches are underlined in Fig. 1.

Expression of Frz3 mRNA in the rat brain

In adult rats, Frz3 mRNA was expressed widely in various tissues but to different degrees. Northern blot analysis with a [^{32}P]-Frz3 probe demonstrated that the 11.0-kb transcript was in several tissues, including brain, kidney, and uterus. The 1.7-kb transcript was primarily found in testis (Fig. 3A). The expression of Frz3 in the brain varied during development and aging. Expression levels from E20.5 to postnatal 4 weeks were relatively elevated. During postnatal development, Frz3 mRNA expression was present until at least 12 months of age and progressively diminished with age (Fig. 3B). In adult rats, Frz3 mRNA was expressed predominantly in the cerebral cortex and hypothalamus; moderately in the olfactory bulb and tubercle, hippocampus, thalamus, cerebellum, pons and medulla oblongata, and spinal cord; and weakly in the midbrain (Fig. 3C) . Expression of Frz2 mRNA in the rat brain was previously reported by another group (13).

Rat Frz3 mRNA expression determined by real-time quantitative PCR

We assessed the differential expression of Frz3 in antidepressant-treated and ECT-treated rats using real-time quantitative PCR and specific primers (Table 1). All expression data were normalized to the expression level of the housekeeping gene β-actin. As shown in Table 2, chronic treatment with either the tricyclic antidepressant imipramine or the selective serotonin reuptake inhibitor sertraline decreased Frz3 mRNA expression in the frontal cortex compared to control

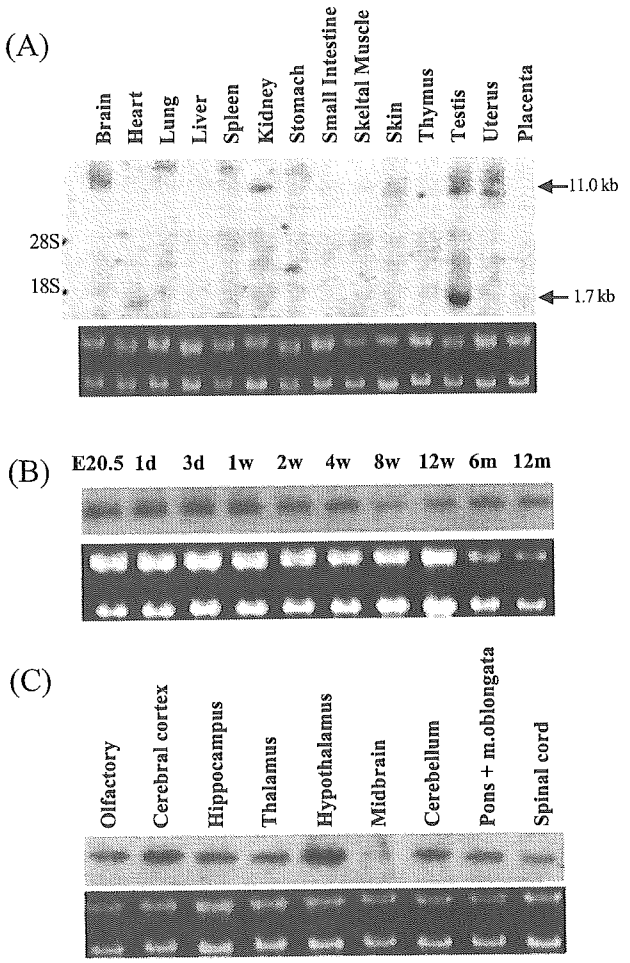

Fig. 3. Expression of Frz3 in rat tissues. Northern blot nylon membrane (Seegene) containing 20 μg of total RNA extracted from various rat tissues (A), aged rat brain (B), and adult rat brain (C) were hybridized with [^{32}P]dCTP-labeled Frz3-specific probe. Lower panels contain photographs of the ethidium bromide-stained gel, demonstrating similar levels of 18S and 28S ribosomal RNA as a loading control.

samples. Single-dose treatments with imipramine failed to affect Frz3 expression, whereas those with sertraline decreased expression (Table 2). This decrease, however,

Table 2. Expression of rat Frz3 and Frz2 in the frontal cortex following antidepressant treatment, as determined by real-time quantitative PCR

		Vehicle	Imipramine	Sertraline
rat Frz3	acute	100.0 ± 9.0	107.3 ± 19.8	72.2 ±10.0*
	chronic	100.0 ± 17.1	49.7 ± 10.1*	62.4 ±16.1*
rat Frz2	acute	100.0 ± 16.5	87.7 ± 12.8	91.3 ±15.5
	chronic	100.0 ± 10.9	95.0 ± 11.2	86.8 ±9.3

Total RNA was extracted from frontal cortices of rats treated with either vehicle or 10 mg/kg of imipramine or sertraline for either 1 day or 21 days. Total RNA used for real-time quantitative PCR is described in the Materials and Methods. Data are percentages of control values (mean ± S.E.M.). Differences were assessed by Student's *t*-test. A value of $*P<0.05$ was regarded as significant.

Table 3. Expression of rat Frz3 and Frz2 in the frontal cortex following ECT, as determined by real-time quantitative PCR

	Control	Single ECT group	Repeated ECT group
rat Frz3	100.0 ± 10.8	59.9 ± 9.7*	54.5 ± 7.2*
rat Frz2	100.0 ± 11.1	138.1 ± 10.3*	153.5 ± 18.0*

Total RNA was extracted from frontal cortices of sham rats or rats receiving either a single dose of ECT (90 mA, 1.0 s) or repeated ECT (90 mA, 1.0 s, every other day for 14 days). Real-time quantitative PCR was performed as described in the Materials and Methods. Data are percentages of control values (mean ± S.E.M.). Differences were assessed by Student's *t*-test. A value of $*P<0.05$ was regarded as significant.

was less than that observed in rats receiving chronic sertraline treatments. Interestingly, single-dose and repeat ECT significantly decreased Frz3 expression in the frontal cortex (Table 3). Although neither chronic nor single-dose administration of antidepressants affected Frz2 expression (Table 2), both single-dose and repeat ECT increased Frz2 expression (Table 3). We did not detect Frz1 in either treatment condition (data not shown), perhaps because Frz1 expression in the frontal cortex is too weak to detect in adult rats (13). To determine the pharmacological specificity of these drugs, we examined the effect of a non-antidepressant antipsychotic drug, haloperidol, on Frz3 expression. Chronic haloperidol treatment failed to induce significant changes in Frz3 expression in the frontal cortex (data not shown), suggesting that the effects of imipramine and sertraline are specific.

Discussion

In the present study, we determined the full-length nucleotide sequence of rat Frz3 cDNA. The pattern of rat Frz3 expression was similar to the patterns of mouse Frz3 and human Frz3 expression (11, 14). In adult rats, we found Frz3 mRNA in various tissues, but in different amounts. In the rat, Frz3 is transcribed into two major transcripts: an 11.0-kb mRNA in brain, kidney, and uterus and a 1.7-kb mRNA in testis (Fig. 3A). In humans, the Frz3 is also transcribed into two major transcripts, a 14.0-kb mRNA in brain and a 1.8-kb mRNA in pancreas (12). They concluded that the 1.8-kb transcript encodes truncated Frz3 protein containing a N-terminal cysteine-rich domain and four transmembrane domains, which permit the alternative polyadenylation of the mRNA at an internal AATAAA signal in the coding region. This internal signal also exists in rat Frz3 and is located in a similar region as that in

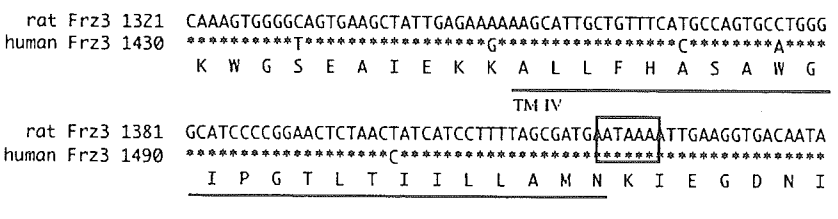

Fig. 4. Alignment of the nucleotide sequence for the fourth transmembrane domain and surrounding region of rat Frz3 and human Frz3. Nucleotide positions are numbered on the left. Asterisks represent identical nucleotides found in rat Frz3. The predicted fourth transmembrane domain is underlined. An alternative polyadenylation site is enclosed within a rectangle.

human Frz3 (Fig. 4). Examination of rat Frz3 expression in brain during development and aging revealed that Frz3 is expressed continually at high levels from late embryogenesis (E20.5) to early postnatal development (postnatal 4 weeks) (Fig. 3B). This expression diminished with age. These findings suggest that Frz3 exerts profound effects on growth and development of the central nervous system.

In adult rat brain, Frz3 mRNA was expressed predominantly in the cerebral cortex and the hypothalamus and moderately in the hippocampus. Using real-time quantitative PCR, we demonstrated that Frz3 expression in rat frontal cortex decreased after chronic treatment with either imipramine or sertraline. Our data indicate that Frz3 would be one of the common functional molecules in the frontal cortex affected by the different types of chronic antidepressant treatments. Although it is possible that the therapeutic action on a single brain region underlies antidepressant treatment, it is also possible that pharmacological effects on multiple brain regions are responsible for the real therapeutic action of antidepressants and ECT. Studies to further characterize the neuronal circuitry of these brain regions will help elucidate the neuroanatomical substrates of antidepressive effects.

Interestingly, we also observed this altered pattern of Frz3 gene expression after both single-dose and repeated ECT. Single-dose treatments with imipramine failed to affect Frz3 expression, whereas those with sertraline decreased expression (Table 2). This decrease, however, was less than that observed in rats receiving chronic sertraline treatments. Together, the decreased Frz3 expression may initiate the adaptive neuronal changes resulting from chronic antidepressant treatment.

On the other hand, the non-antidepressant antipsychotic drug haloperidol, however, did not affect Frz3 expression. Moreover, Frz2 expression failed to decrease after chronic or single-dose administration of antidepressants, and rather, it increased after ECT. These results suggest that therapeutic treatments for depression specifically decrease Frz3 expression. The physiological role of the Frz2 induction after ECT is yet to be elucidated.

Interactions between Frz and certain Wnt ligands mediate specific downstream signals. Activation of the Frz/Wnt pathway leads to inactivation of glycogen synthase kinase (GSK-3β) through the Dishevelled-mediated pathway. GSK-3β normally promotes the turnover of β-catenin, a protein involved in synaptogenesis (15, 16), by direct phosphorylation, marking it for ubiquitylation and degradation in proteasomes. Therefore, the inhibition of GSK-3β leads to accumulation of β-catenin (10). Some researchers propose that β-catenin then enters the nucleus and binds LEF/TCF transcription factors; the β-catenin-LEF/TCF complex then acts as a transcriptional activator of Frz/Wnt target genes (17). In brain, β-catenin inactivation leads to structural malformation and craniofacial development failure (18). Dysfunction in the pathway also affects behavior. Transgenic mice overexpressing GSK-3β exhibit spatial learning deficits (19). It was recently proposed that the Frz/Wnt pathway participates in a complex behavioral phenomenon (20). Moreover, molecules involved in this pathway are encoded by candidate genes thought to be involved in certain neuropsychiatric disorders. Gene-targeting of dishevelled-1, a cytoplasmic signaling protein that regulates β-catenin levels, shows that it influences social behavior and sensorimotor gating in mice (20).

Several pharmaceuticals commonly used to treat depression target components of this pathway. The direct inhibition of GSK-3β by lithium, an effective mood stabilizer for the treatment and prophylaxis of manic-depressive illness, leads to β-catenin stabilization both in vivo and in vitro (21, 22). Valproate, another mood stabilizer, regulates GSK-3β-mediated axonal remodeling in developing neurons (22). GSK-3β is inhibited by phosphorylation on serine-9. It was recently demonstrated that serotonergic activity regulates the phosphorylation of GSK-3β in mouse brain. These results raise the possibility that impaired inhibitory control of GSK-3β may occur in conditions where serotonergic activity is dysregulated, such as in mood disorders (23). More recently, it is reported that a selective GSK-3 inhibitor AR-A014418 induces behavioral changes in rats, which are consistent with the effects of antidepressant medications (24). In addition, chronic ECT up-regulates β-catenin and induces β-catenin-associated cell division and differentiation in adult rat hippocampus (25). Detection of changes in the expression levels of molecules participating in the Frz/Wnt cascade may aid the diagnosis of depression or the ability to alter expression levels may contribute to the therapeutic efficacy of drugs used to treat depression.

In conclusion, we demonstrated here that Frz3 is commonly down-regulated following chronic antidepressant treatment or by ECT in rat frontal cortex. It is interesting to investigate the effect of non-monoaminergic candidates for antidepressants, including the non-peptidic selective delta-opioid receptor agonist SNC80 (26), on Frz3 expression. Also, further studies with a specific and sensitive antibody for rat Frz3 will be necessary to clarify the relationship between the Frz3-mediated pathway and the therapeutic efficacy of antidepressant.

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References

1 Yamada M, Higuchi T. Functional genomics and depression research. Beyond the monoamine hypothesis. Eur Neuropsychopharmacol. 2002;12:235–244.
2 Frechilla D, Otano A, Del Rio J. Effect of chronic antidepressant treatment on transcription factor binding activity in rat hippocampus and frontal cortex. Prog Neuropsychopharmacol Biol Psychiatry. 1998;22:787–802.
3 Yamada M, Yamada M, Yamazaki S, Takahashi K, Nishioka G, Kudo K, et al. Identification of a novel gene with RING-H2 finger motif induced after chronic antidepressant treatment in rat brain. Biochem Biophys Res Commun. 2000;278:150–157.
4 Yamada M, Yamada M, Yamazaki S, Takahashi K, Nara K, Ozawa H, et al. Induction of cysteine string protein after chronic antidepressant treatment in rat frontal cortex. Neurosci Lett. 2001;301:183–186.
5 Yamada M, Takahashi K, Tsunoda M, Nishioka G, Kudo K, Ohata H, et al. Differential expression of VAMP2/synaptobrevin-2 after antidepressant and electroconvulsive treatment in rat frontal cortex. Pharmacogenomics J. 2002;2:377–382.
6 Takahashi K, Yamada M, Ohata H, Momose K, Higuchi T, Honda K, et al. Expression of Ndrg2 in the rat frontal cortex after antidepressant and electro-convulsive treatment. Int J Neuropsychopharmacol. 2005;8:1–9.
7 Vinson CR, Conover S, Adler PN. A Drosophila tissue polarity locus encodes a protein containing seven potential transmembrane domains. Nature. 1989;338:263–264.
8 Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ. Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. Nature. 2001;412:86–90.
9 McMahon AP, Bradley A. The Wnt-1 proto-oncogene is required for development of a large region of the mouse brain. Cell. 1990;62:1073–1085.
10 Dale TC. Signal transduction by the Wnt family of ligands. Biochem J. 1998;329:209–223.
11 Wang Y, Macke JP, Abella BS, Andreasson K, Worley P, Gilbert DJ, et al. Large family of putative transmembrane receptors homologous to the product of the Drosophila tissue polarity gene frizzled. J Biol Chem. 1996;271:4468–4476.
12 Kirikoshi H, Koike J, Sagara N, Saitoh T, Tokuhara M, Tanaka K, et al. Molecular cloning and genomic structure of human frizzled-3 at chromosome 8p21. Biochem Biophys Res Commun. 2000;271:8–14.
13 Chan SD, Karpf DB, Fowlkes ME, Hooks M, Bradley MS, Vuong V, et al. Two homologs of the Drosophila polarity gene frizzled (fz) are widely expressed in mammalian tissues. J Biol Chem. 1992;267:25202–25207.
14 Sala CF, Formenti E, Terstappen GC, Caricasole A. Identification, gene structure, and expression of human frizzled-3. Biochem Biophys Res Commun. 2000;273:27–34.
15 Nagafuchi A, Takeichi M, Tsukita S. The 102 kd cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. Cell. 1991;65:849–857.
16 Nishimura W, Yao I, Iida J, Tanaka N, Hata Y. Interaction of synaptic scaffolding molecule and β-catenin. J Neurosci. 2002;22:757–765.
17 Willert K, Nusse R. β-catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev. 1998;8:95–102.
18 Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, et al. Inactivation of the β-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development. 2001;128:1253–1264.
19 Hernandez F, Borrell J, Guaza C, Avila J, Lucas JJ. Spatial learning deficit in transgenic mice that conditionally overexpress GSK-3βeta in the brain but do not form tau filaments. J Neurochem. 2002;83:1529–1533.
20 Lijam N, Paylor R, McDonald MP, Crawley JN, Deng CX, Herrup K, et al. Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. Cell. 1997;90:895–905.
21 Williams RS, Harwood AJ. Lithium therapy and signal transduction. Trends Pharmacol Sci. 2000;21:61–64.
22 Hall AC, Brennan A, Goold RG, Cleverley K, Lucas FR, Gordon-Weeks PR, et al. Valproate regulates GSK-3-mediated axonal remodeling and synapsin I clustering in developing neurons. Mol Cell Neurosci. 2002;20:257–270.
23 Li X, Zhu W, Roh MS, Friedman AB, Rosborough K, Jope RS. In vivo regulation of glycogen synthase kinase-3beta by serotonergic activity in mouse brain. Neuro-psychopharmacol. 2004;29:1426–1431.
24 Gould TD, Einat H, Bhat R, Manji HK. AR-A014418, a selective GSK-3 inhibitor, produces antidepressant-like effects in the forced swim test. Int J Neuropsycho-pharmacol. 2004;26:1–4.
25 Madsen TM, Newton SS, Eaton ME, Russell DS, Duman RS. Chronic electroconvulsive seizure up-regulates β-catenin expression in rat hippocampus: role in adult neurogenesis. Biol Psychiatry. 2003;54:1006–1014.
26 Saitoh A, Kimura Y, Suzuki T, Kawai K, Nagase H, Kamei J. Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents. J Pharmacol Sci. 2004;95:374–380.

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Ndrg2 promotes neurite outgrowth of NGF-differentiated PC12 cells

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Abstract

Ndrg2 is a member of the N-myc downstream-regulated genes. Thus far, two different isoforms of rat *Ndrg2* protein, Ndrg2S and Ndrg2L, have been identified. Recently, we have identified rat Ndrg2 as a novel target molecule of antidepressants and ECT. The functional role of Ndrg2 in the central nervous system, however, remains unclear. In the present study, we examined the expression of endogenous Ndrg2, cellular localization of transfected Ndrg2 protein, and morphological changes resulting from overexpression of Ndrg2 in NGF-differentiated PC12 cells. Neurites began to sprout 1–2 days after exposure to NGF; subsequent neurite growth continued for 5 days. During this time, we evaluated *Ndrg2* mRNA expression by real-time quantitative PCR and found that expression significantly increased in a time-dependent manner. Interestingly, V5-conjugated Ndrg2S and Ndrg2L proteins expressed in NGF-differentiated PC12 specifically localized to cell surface membranes and growth cones. Moreover, Ndrg2S and Ndrg2L overexpression promoted neurite elongation in NGF-differentiated PC12 cells. In conclusion, our findings offer novel insights into the physiological roles of Ndrg2 in the central nervous system.

Keywords: N-Myc downstream-regulated genes; Neurites elongation; Differentiation

Ndrg2 is a member of the N-myc downstream-regulated genes, the Ndrg family; thus far, four members of this family, Ndrg1–4, have been identified [12]. Ndrg family members may be intimately involved in cellular differentiation and development. Indeed, Ndrg1 expression is induced by hypoxia and has been implicated in cell growth regulation and Schwann cell signaling for axonal survival [4,8,10,13]. In human leukemia cells, Ndrg1 expression is upregulated by differentiation-related retinoids and Vitamin D3 [8]. Suppression of Ndrg4 expression by Ndrg4 antisense transfection inhibits neurite outgrowth in PC12 cells [6]. Stable expression of human Ndrg2 in glioblastoma cell lines decreases cell growth rates [2]. More recently, *Ndrg2* mRNA and protein has been shown to be upregulated in Alzheimer's disease brains [5].

Although Ndrg members do not possess a clear functional motif, they do share a high level of sequence homology. Phylogenetic analysis of Ndrg1–4 revealed that Ndrg1 and Ndrg3 belong to one subfamily, while Ndrg2 and Ndrg4 belong to another [9]. Interestingly, Ndrg2 is highly related to Ndrg1, which has been linked to stress responses, cell proliferation, and differentiation, although *Ndrg2* itself is not repressed by N-Myc [7]. Thus far, four different isoforms of rat *Ndrg2* have been identified [1]. The 5′-untranslated region (UTR) for *Ndrg2a1/Ndrg2a2* is 87 bp, whereas the 5′-UTR for *Ndrg2b1/Ndrg2b2* is 50 bp. In the translated region, *Ndrg2a1/Ndrg2b1* has an additional 42 bp insertion compared to *Ndrg2a2/Ndrg2b2*. In this study, we denote *Ndrg2a1/Ndrg2b1* and *Ndrg2a2/Ndrg2b2* to represent *Ndrg2L* and *Ndrg2S*, respectively. Comparison and alignment of amino acid sequences indicated that Ndrg2L is longer than Ndrg2S by 14 amino acids and that both isoforms share the characteristic Ndrg family sequence.

Recently, we have identified rat Ndrg2 as a novel target molecule of antidepressants and electroconvulsive treatment (ECT) [11]. However, the functional role of Ndrg2 in the central nervous system remains unclear. In the present study,

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we examined the expression of endogenous Ndrg2, cellular localization of transfected Ndrg2 protein, and morphological changes resulting from overexpression of Ndrg2 in nerve growth factor (NGF)-differentiated neural crest-derived rat phenochromocytoma (PC12) cells. PC12 cells have been used extensively for examining neurite outgrowth in response to NGF treatment [3], and thus provide a good model for assessing Ndrg2 effects in a neural context.

PC12 cells were cultured in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, Inc. MO, USA) supplemented with 10% fetal calf serum, 5% horse serum (Invitrogen, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37 °C in a humidified atmosphere comprised of 5% CO_2. Cells were treated with NGF in uncoated 6 cm dishes. Neuronal differentiation of PC12 cell was generated by culturing the cells in DMEM with or without 50 ng/mL NGF for various lengths of time (0, 6, 24 h and 2, and 5 days). At each of these time points, the cells were assessed with a phase contrast microscope, and then RNA was extracted. As described above, rat Ndrg2 protein consists of two splice variants, Ndrg2S and Ndrg2L. However, we previously demonstrated using conventional reverse transcriptase-polymerase chain reaction (RT-PCR) that transcript processing into long and short forms of *Ndrg2* does not appear to be significantly regulated in NGF-differentiated PC12 cells (data not shown). Therefore, we performed mRNA expression analysis of *Ndrg2* with real-time quantitative polymerase chain reaction (RTQ-PCR) using an ABI PRISM 7000 instrument (Applied Biosystems, Foster City, CA, USA); total levels of *Ndrg2S* and *Ndrg2L* mRNA were examined in the present study. Total RNA was extracted from samples using Isogen reagent (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. Total RNA samples treated with RNase-free DNase I was used to synthesize the first strand cDNA via reverse transcriptase and oligo-dT primer. PCR primers were designed using Primer Express Software (Applied Biosystems). A quantity of cDNA corresponding to 20 pg of total RNA was amplified by PCR. The following primers were used for rat *Ndrg2* (5′-AACTTTGAGCGAGGTGGTGAGA-3′ and 5′-ATTCCACCACGGCATCTTCA-3′) and β-actin (5′-TCGCTGACAGGATGCAGAAGG-3′ and 5′-GCCAGGATAGAGCCACCAAT-3′). The SYBR® Green PCR Core Reagents Kit (Applied Biosystems) was utilized for fluorescence detection of cDNA. For quantification, we used the Standard Curve Method (User Bulletin, ABI PRISM 7000 Sequence Detection System). Briefly, for rat Ndrg2 and β-actin, an absolute standard curve was obtained by plotting the threshold cycle following PCR amplification of serial dilutions of control cDNA template.

To determine the localization of Ndrg2S and Ndrg2L protein in PC12 cells, V5-conjugated Ndrg2S and Ndrg2L were transfected and overexpressed in NGF-differentiated PC12 cells, and V5 was detected immunochemically with an anti-V5 antibody. The coding regions for *Ndrg2S* and *Ndrg2L* were obtained from rat brain via PCR with high fidelity Platinum pfx DNA polymerase (Invitrogen) and the following set of primers: 5′-CTCGAGGCCACCATGGCAGAGC-3′, 5′-GAATTCTCTCAACAGGAGACTTCCATGGT G-3′. These primers contain either *Xho*I or *Eco*RI sites (underlined) to facilitate subcloning. Electrophoresis of agarose gels containing PCR products resulted in two reproducible bands that corresponded to *Ndrg2S* and *Ndrg2L*. Both of the amplified products were ligated into pCR II-TOPO vectors (Invitrogen) and transformed into competent DH5α *Escherichia coli* cells. The resulting plasmid vectors were recombined into pIRES-EGFP (Clontech, CA, USA) and pEF-DEST 51 (V5/His-tagged C-terminal region) vectors via GATEWAY cloning technology (Invitrogen) and adaptive attB1 and attB2 site primers. PC12 cells (2×10^5 cells) were seeded onto collagen-coated 25 mm diameter glass coverslips and transfected with 2 μg of recombinant plasmid in serum-free medium using 2 μL Lipofectamine reagent (Invitrogen). Three hours after transfection, 50 ng/mL of NGF (Sigma-Aldrich) was added to the dishes to initiate cell differentiation. To examine the specificity of transfection, Western blot analysis were performed on protein extracts derived from the transfected PC12 cells. The cells were homogenized in ice-cold Sucrose-Tris buffer, protein concentration was determined, 20 μg fractions of protein were separated by 7.5% SDS-PAGE, then blotted onto nitrocellulose membranes. The membranes were assessed immunochemically for Ndrg2-V5 fusion proteins by incubating them in blocking buffer (5% skim milk), anti-V5 antibody (1:500; Invitrogen) in blocking buffer, followed by anti-mouse IgG (1:20,000; American Qualex). The immunoreactive bands were visualized on film by the ECL system. Three days after transfection, NGF-differentiated PC12 cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer for 15 min at room temperature, washed with PBS three times, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 1% fetal calf serum prepared in PBS for 30 min. PC12 cells transfected with either V5-conjugated Ndrg2S or Ndrg2L were incubated with anti-V5 antibody (1:200), followed by a fluorescein-labeled anti-mouse IgG (1:100; American Qualex) for 1 h at room temperature. For each dish, 10–20 fluorescent images in the identical viewing field were captured at an excitation wavelength of 488 nm with a Bio-Rad MRC-500 laser scanning confocal attachment (Bio-Rad, MA, USA) mounted on a Nikon Diaphot inverted microscope (Nikon, NY, USA).

To determine whether Ndrg2 promotes neurite elongation, we analyzed morphological changes in PC12 cells that overexpressed either Ndrg2S or Ndrg2L. In this study, we used the pIRES-EGFP vector, which can express green fluorescent protein (GFP) and target molecules separately. Two days after transfection, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min at room temperature, washed with PBS three times, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 1% fetal calf serum for 30 min. PC12 cells transfected with pIRES-EGFP containing either Ndrg2S or Ndrg2L were incubated with Alexa Fluor 568-conjugated phalloidin (Molecular Probes,