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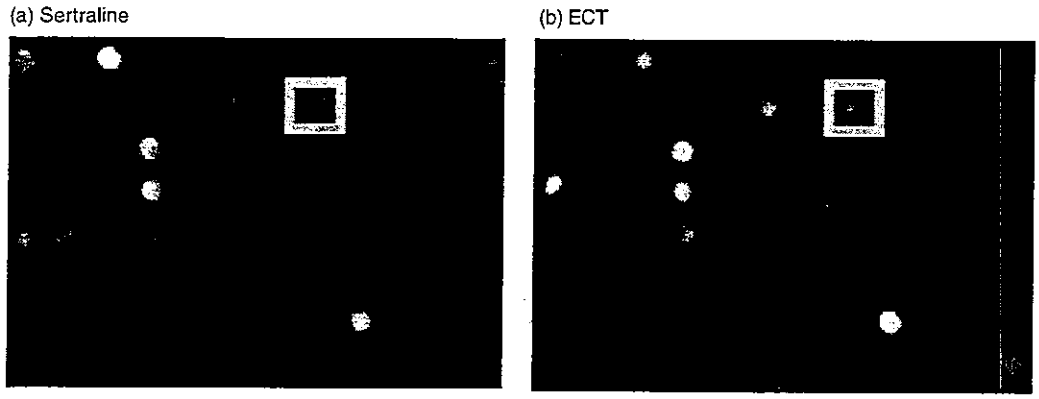

Figure 1. Image analysis of ADRG microarray after hybridization with fluorescent probes. Ninety-six spots representing ADRG97–192 are shown. (a) Merged pseudo-colour image of control group data (green) and chronic sertraline treatment group (red). As expected, we obtained low background and consistent 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.

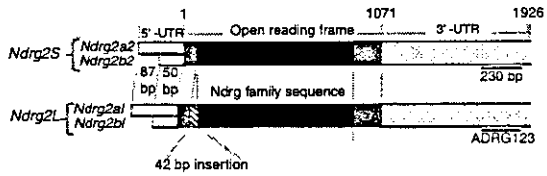

Figure 2. Schematic representations of rat Ndrg2. Rat Ndrg2 consists of four isoforms: Ndrg2a1, Ndrg2a2, Ndrg2b1, and Ndrg2b2. The 5'-UTR for Ndrg2a1/Ndrg2a2 was 87 bp, whereas the 5'-UTR for Ndrg2b1/Ndrg2b2 was 50 bp. In the translated region, Ndrg2a1/Ndrg2b1 has an additional 42-bp insertion compared 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%).

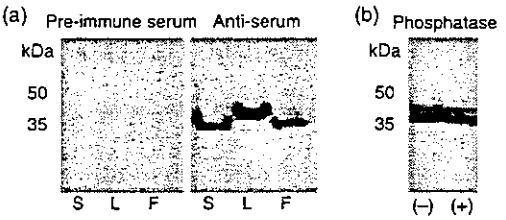

Figure 3. Specificity of anti-rat-Ndrg2 antiserum prepared by our group in the present study. To examine the specificity of the anti-rat-Ndrg2 antiserum, we immunostained HEK293 cells overexpressing rat Ndrg2S and Ndrg2L (a). The lysates from HEK293 cells (S, L) or rat frontal cortex (F) were electrophoresed 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 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. AQ5

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

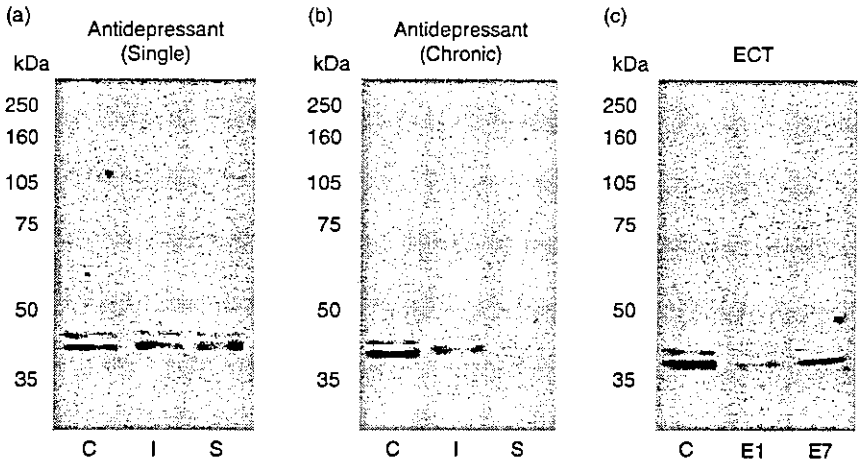

Figure 4. Western blot analysis of Ndrg2S and Ndrg2L in rat frontal cortex after a single antidepressant treatment (a), chronic antidepressant treatment (b), or ECT (c). A protein sample was prepared from rat frontal cortex and treated with either vehicle (control, lane 1), 10 mg/kg of imipramine (lane 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.

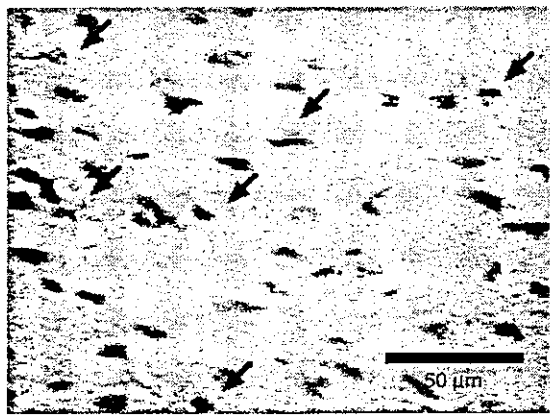

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Figure 5. Immunohistochemical identification of Ndrg2-expressing cells in the rat frontal cortex. Using the anti-rat-Ndrg2 antiserum prepared by our group, Ndrg2 immunoreactivity (brown) was observed in cells in the rat frontal cortex. Diaminobenzidine 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

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Statement of Interest

None.

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Repetitive transcranial magnetic stimulation induces kf-1 expression in the rat brain

Kentaro Kudo[a], Misa Yamada[b], Kou Takahashi[a], Gentaro Nishioka[a], Satoshi Tanaka[a], Tomo Hashiguchi[c], Hiroshi Fukuzako[c], Morikuni Takigawa[c], Teruhiko Higuchi[d], Kazutaka Momose[b], Kunitoshi Kamijima[a], Mitsuhiko Yamada[e,*]

[a]*Department of Psychiatry, Showa University School of Medicine, Tokyo 142-8666, Japan*
[b]*Department of Pharmacology, Showa University School of Pharmaceutical Sciences, Tokyo 142-8666, Japan*
[c]*Department of Neuropsychiatry, Faculty of Medicine, Kagoshima University, Kagoshima 890-8520, Japan*
[d]*Musashi Hospital, National Center of Neurology and Psychiatry, Tokyo 187-8551, Japan*
[e]*Division of Psychogeriatrics, National Institute of Mental Health, National Center of Neurology and Psychiatry, 1-7-3 Kohnodai, Ichikawa, Chiba 272-0827, Japan*

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive approach used for stimulating the brain, and has proven effective in the treatment of depression, however the mechanism of its antidepressant action is unknown. Recently, we have reported the induction of kf-1 in rat frontal cortex and hippocampus after chronic antidepressant treatment and repeated electroconvulsive treatment (ECT). In this study, we demonstrated the induction of kf-1 after rTMS in the rat frontal cortex and hippocampus, but not in hypothalamus. Our data suggest that kf-1 may be a common functional molecule that is increased after antidepressant treatment, ECT and rTMS. In conclusion, it is proposed that induction of kf-1 may be associated with the treatment induced adaptive neural plasticity in the brain, which is a long-term target for their antidepressant action.

Keywords: Depression; Antidepressant; Microarray

* Corresponding author. Tel.: +81 47 375 4742x1270; fax +81 47 375 4795.
E-mail address: mitsu@ncnp-k.go.jp (M. Yamada).

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Introduction

Antidepressants are widely used in the treatment of depression. On the other hand, electroconvulsive treatment (ECT) is also an important therapy that is believed to have a rapid onset of antidepressant activity. In addition, newer findings regarding the mechanisms of action of ECT have led to novel developments in treatment technique to further improve this highly effective treatment for major depression. These new approaches include new methods for inducing more targeted seizures (eg, transcranial magnetic stimulation, TMS). TMS was introduced by Barker et al in 1985 as a new method for noninvasive and almost painless stimulation of the central nervous system (George et al., 2002). The development of stimulators capable of delivering stimulation frequencies of up to 60 Hz (repetitive TMS, rTMS) increased the potential clinical applications of rTMS. It is reported that rTMS over the prefrontal cortex is as effective as ECT in the treatment of nondelusional major depressive disorder (Grunhaus et al., 2003). Despite some differences in the physical properties of magnetic and electrical stimulation, rTMS shares many of the behavioral and biochemical actions of ECT and other antidepressant treatments. For example, rTMS reduces immobility in the Porsolt swim task and enhances apomorphine-induced stereotypy, as does ECT (Lisanby et al., 2000). However, the mechanism of the antidepressant action of rTMS is unknown.

By inducing electric currents in brain tissue via a time-varying strong magnetic field, rTMS has the potential to either directly or trans-synaptically modulate neuronal circuits thought to be dysfunctional in depression. Recent animal studies have broadened our understanding of how rTMS affects brain functioning. There is compelling evidence that rTMS causes changes in neuronal circuits as reflected by behavioural changes (Post and Keck, 2001). These alterations suggest regional changes in neurotransmitter release, transsynaptic efficiency, signaling pathways and in gene transcription. Indeed, it is demonstrated that rTMS stimulates subcortical dopamine release, modulates cortical beta-adrenergic receptors, reduces frontal cortex 5-HT_2 receptors, increases 5-HT_{1A} receptors in frontal cortex and cingulate, and increases N-methyl-D-aspartate receptors in the ventromedial hypothalamus, basolateral amygdala, and parietal cortex (Lisanby et al., 2000; Padberg and Moller, 2003). However, in order to optimize rTMS for therapeutic use, it is necessary to understand the neurobiological mechanisms involved, particularly the nature of the changes induced and the brain regions affected. Previously, we investigated neuronal response to rTMS and ECT in terms of c-Fos expression (Doi et al., 2001). In rats rTMS sessions induced widespread nuclear c-Fos-like immunoreactivity in frontal cortex, lateral orbital cortex, striatum, lateral septal nucleus, piriform cortex, dentate gyrus, Ammon's horn, cingulate cortex, parietal cortex, thalamus, occipital cortex, and amygdala; this reactivity was greater than with control rats, which were treated as the rTMS-treated rats but without magnetic stimulation. ECT produced even stronger c-Fos expression than rTMS in all regions except thalamus (no difference) and striatum (stronger with rTMS). Thus, functional modification of neuroanatomical substrates as demonstrated by c-Fos expression may partially differ between rTMS and ECT (Doi et al., 2001). Studies to further characterize the neuronal circuitry of these brain regions will help elucidate the neuroanatomical substrates of antidepressive effects by rTMS.

An increasingly popular working hypothesis is that chronic treatment with drugs of various classes, ECT or rTMS have common antidepressant effects because they regulate transcription of the same set of downstream genes. Indeed, antidepressants and ECT have been shown to affect the expression of immediate early genes and transcription factors, including c-Fos, FosB, NGF1-A and CREB (see review by Yamada and Higuchi, 2002). These proteins activate or repress genes that encode specific proteins by binding to DNA regulatory elements, and they may be involved in critical steps that mediate treatment-

induced neural plasticity. Interestingly, we recently demonstrated the induction of kf-1 in rat frontal cortex and hippocampus after chronic antidepressant treatment and repeated ECT (Yamada et al., 2000, Nishioka et al., 2003). Therefore, in the present study, to identify a novel candidate target molecule of rTMS, we have examined the expression of kf-1 after rTMS in the rat brain, and compared the effects to those of chronic antidepressant treatment and repeated ECT. Here, we first demonstrated the induction of kf-1 after rTMS in rat frontal cortex and hippocampus.

Materials and methods

Experimental animals

Male Sprague-Dawley rats (age 7–10 weeks, Sankyo Labo Service Co. Tokyo, Japan) were housed in a temperature-controlled environment with 12 h light/ 12 h dark cycle with free access to food and water. Although a sexual difference of kf-1 expression in the brain is not clear, only the male rats were used to minimize the possible variability naturally found in the female rats. Rats were randomly separated into control and treated groups. It is reported that rTMS reduces immobility time in the Forced Swim Test model of depression, suggesting an antidepressant effect, which is evident at a range (1–25 Hz) of frequencies (Sachdev et al., 2002). With repeated administration, it is suggested that the antidepressant effect is likely to be sustained. In this study, rTMS was administered with the figure-eight magnetic coil in contact with the head pointed to the bregma, 1.9 tesla, 20 Hz/train, 3.5 sec, 70 pulses, once daily for 10 days (Magstim 200 rapid stimulator, Magstim Company, Whitland, UK). Control rats were treated as the rTMS-treated rats but without magnetic stimulation. Experimental animals for chronic haloperidol treatment (antipsychotic agent, as non-antidepressant control drug) received vehicle, 0.5 mg/kg or 2.0 mg/kg of haloperidol (Sigma Chemical Co., St. Louis, MO, USA) dissolved in saline, by daily intraperitoneal injection for 21 days. Three or four rats were used for each treatment group.

Twenty-four hours after the final rTMS or haloperidol injection, 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 Ethic Committee in line with the OPRR Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Northern blot analysis

Complimentary DNA fragment of kf-1 was cut out from PCR II-TOPO vector and labeled with [α-^{32}P]dCTP, and then used as a probe for northern blot analysis. Rat multiple tissue Northern blot nylon membrane (Clontech, Palo Alto, CA, USA) was used for the experiment. Hybridization procedure was carried out following the manufacture's instructions. After the hybridization, the membrane was exposed to X-ray firm for 24 h.

Fabrication of cDNA Microarray and fluorescence image analysis

Fabrication of cDNA microarray was described by our group previously (Yamada et al., 2000). Briefly, cDNA inserts were amplified by vector primers and spotted in duplicated on the glass slide

using GMS417 Arrayer (Affymetrix, Inc., CA, USA). To make the fluorescence-labeled probe for hybridization, total RNA samples obtained from rat hippocampus from control or sertraline group was extracted by Isogen reagent (Nippon gene Co., Tokyo, Japan) following the manufacture's instruction. Then, three independent total RNA samples from each group were pooled and used for the next procedure. Poly A^+ RNA was then purified from pooled total RNA with oligo-dT columns (Takara, Tokyo, Japan). One μg of poly A^+ RNA from control or rTMS samples was converted to cDNA in the presence of Cy-5 or Cy-3-dUTP respectively to make fluorescence-labeled probes. Hybridization of probes to microarray was done competitively. The probes were mixed and placed on an array, overlaid with coverslip, and hybridized for 16.5 h at 65°C. After hybridization and washing procedure, each slide was scanned with GMS418 Array Scanner (Affymetrix, Inc., CA, USA). Then, gene expression levels were quantified and analyzed using ImaGene software (Bio-Discovery Ltd. Swansea, UK).

Messenger RNA expression analysis with RT-PCR

The first strand cDNA was synthesized with reverse transcriptase and 1 μM of oligo-dT primer, from 2 μg of total RNA samples treated with RNase-free DNase I, and diluted to a final volume of 100 μL. One μL of each cDNA sample was added to 24 μL of PCR reaction mixture containing 0.5 μM of a pair of primers for kf-1, 5′-GGAATACGGACAGGACTTTC-3′ and 5′-TCCGAGAAGCTG-CATGGGC-3′ (Amersham Pharmacia Biotech, Tokyo, Japan). A pair of primers for ß-actin, 5′-TCCTGACCCTGAAGTACCCCATTG-3′, 5′-GGAACCGCTCCATTGCCGATAGT-3′ was also used for normalization. To ensure the fidelity of this analysis, we assayed several cycles of PCR to determine the liner range for amplification of PCR product in each region of the brain. Amplification of kf-1 was performed as follows: 3 min at 94°C for initial denaturation, an appropriate cycles of 94°C denaturing for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 1 min, followed by a final extension at 72°C for 7 min. Typically, 36, 24, or 30 cycles were used for samples obtained from frontal cortex, hippocampus or hypothalamus, respectively. Amplification of ß-actin for normalization was performed as follows: 3 min at 94°C for initial denaturation, 18–20 cycles of 94°C denaturing for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 1 min, followed by a final extension at 72°C for 7 min. The PCR products were electrophoresed in a 1% agarose gel containing SYBR green, a nucleic acid gel stain reagent GelStar (Takara, Tokyo, Japan). The optical density of the digitized image was quantified using a fluorescence image analyzer, FM-bio II (Hitachi, Tokyo, Japan).

Results

Northern blot analysis

Previously, we have demonstrated the presence of a single transcript of about 3.5 kb in size for mRNA prepared from several rat tissue regions, which hybridized to the [^{32}P]-labeled kf-1 probe. These regions included brain, lung and kidney, liver and heart, but at much lower levels in spleen and muscle (Yamada et al., 2000). In the present study, northern blot analysis also demonstrated the presence of a single transcript of about 3.5 kb in size for mRNA prepared from several rat brain tissues (1: olfactory, 2:

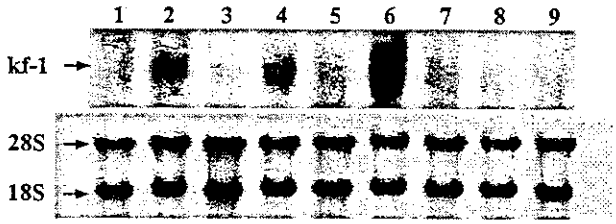

Fig. 1. Expression of kf-1 in rat brain tissues. Complimentary DNA fragment of kf-1 obtained from EST analysis was labeled with [^{32}P]dCTP and used as a probe. Rat brain tissues (1 olfactory, 2 cerebral cortex, 3 hippocampus, 4 thalamus, 5 hypothalamus, 6 midbrain, 7 cerebellum, 8 pons with m. oblongata, 9 spinal cord) were analyzed by Northern blot analysis. Photograph of the gel demonstrated that the total RNA samples used in this study contained intact and similar levels of 28S and 18S ribosomal RNA bands as determined by denaturing gel electrophoresis. Northern blot analysis demonstrated the presence of a single transcript of about 3.5 kb in size for mRNA prepared from several rat brain tissues, which hybridized to the kf-1 probe. Note: The print, made for the review process, was taken directly from the high-quality image file, which will be used in preparing the publication, if the manuscript is found acceptable.

cerebral cortex, 3: hippocampus, 4: thalamus, 5: hypothalamus, 6: midbrain, 7: cerebellum, 8: pons with m. oblongata, 9: spinal cord), which hybridized to the [^{32}P]-labeled kf-1 probe. As shown in Fig. 1, the total RNA samples used in this study contained intact and similar levels of 28S and 18S ribosomal RNA bands as determined by denaturing gel electrophoresis. The expression of kf-1 was at higher levels in the midbrain, thalamus and cerebral cortex, but at relatively lower levels in the olfactory, cerebellum, pons with m. oblongata and spinal cord (Fig. 1).

Messenger RNA expression analysis with ADRG microarray

As expected, we obtained low background and consistent results in duplicated experiments. After normalization with the signals for both negative and positive controls, several spots of our interest on the ADRG microarray showed increased or decreased fluorescence intensities after rTMS (data not shown). The expression of kf-1 (ADRG34) in ADRG microarray was 2.39 times increased in rTMS group when compared to controls. While, the expression of kf-1 in ADRG microarray was 2.17 times in antidepressant treatment group (Yamada et al., 2000) and 1.97 times in ECT group (Nishioka et al., 2003).

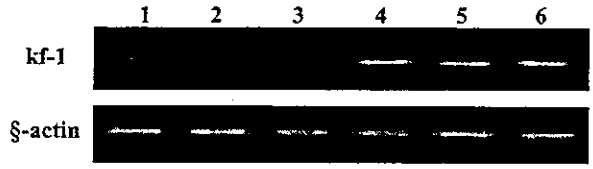

Fig. 2. A typical image of gel electrophoresis after RT-PCR. RNA was extracted from rat frontal cortex obtained from control rats (lane 1–3), or rTMS-treated rats (lane 4–6), and used for RT-PCR analysis. Control rats were treated as the rTMS-treated rats but without magnetic stimulation. The PCR products were electrophoresed in a 1% agarose gel. The reproducible single band corresponding to rat kf-1 (upper) or β-actin (under) was observed on a gel, respectively. Note: The print, made for the review process, was taken directly from the high-quality image file, which will be used in preparing the publication, if the manuscript is found acceptable.

t1.1 Table 1
t1.2 The mRNA expression of kf-1 after rTMS and repeated ECT

t1.3		Frontal Cortex	Hippocampus	Hypothalamus
t1.4	*Repetitive transcranial magnetic stimulation, rTMS*			
t1.5	Control	100 ± 5.7	100 ± 16.9	100 ± 12.2
t1.6	rTMS	142.4 ± 8.4*	156.7 ± 15.8*	101.5 ± 9.7^{ns}
t1.7				
t1.8	*Repeated electroconvulsive treatment, ECT*[1]			
t1.9	Control	100 ± 8.2	100 ± 14.1	100 ± 6.1
t1.10	ECT	161.7 ± 11.5*	172.8 ± 22.3*	96.3 ± 3.1^{ns}

[1]Data for repeated ECT were reported by our group previously (Nishioka et al., 2003). Briefly, rats for ECT were anesthetized and received a 90 mA, 1.0 sec electric shock via ear-clip electrodes every other day for 14 days. Data are expressed as % of the
t1.11 control data (means ± s.e.m.) of three independent experiments. $^{ns}p > 0.05$ and $*p < 0.05$, Student's t-test.

Messenger RNA expression analysis with RT-PCR

The induction of kf-1 after rTMS was also confirmed by RT-PCR analysis. The reproducible band corresponding to kf-1 at the size of 199 bp existed on a gel. A typical image of gel electrophoresis after RT-PCR is shown in Fig. 2. As shown in Table 1, the mRNA levels of kf-1 after rTMS were significantly increased in the frontal cortex (100 ± 5.7% for control and 142.4 ± 8.4% for rTMS samples, respectively) and hippocampus (100 ± 16.9% for control and 156.7 ± 15.8% for rTMS samples, respectively), but not in the hypothalamus (100 ± 12.2% for control and 101.5 ± 9.7% for rTMS samples, respectively) after normalization by ß-actin expression.

On the other hand, to determine the pharmacological specificity of this antidepressant action, the effect of non-antidepressant antipsychotic drug, haloperidol, on rat kf-1 expression was investigated. Interestingly, the mRNA level of kf-1 after chronic haloperidol treatment was not changed in the frontal cortex, hippocampus and hypothalamus (Table 2).

t2.1 Table 2
t2.2 The mRNA expression of kf-1 after chronic treatments with antidepressants and antipsychotic agent, haloperidole

t2.3		Frontal Cortex	Hippocampus	Hypothalamus
t2.4	*Chronic haloperidole treatment*			
t2.5	Control	100 ± 6.1	100 ± 19.9	100 ± 8.3
t2.6	0.5 mg/kg	96.1 ± 9.8^{ns}	108.9 ± 2.8^{ns}	98.8 ± 11.8^{ns}
t2.7	2.0 mg/kg	94.3 ± 5.9^{ns}	120.2 ± 19.2^{ns}	102.1 ± 13.7^{ns}
t2.8				
t2.9	*Chronic antidepressant treatment*[1]			
t2.10	Control	100 ± 9.3	100 ± 3.7	100 ± 18.8
t2.11	Imipramine	165.6 ± 9.9*	204.0 ± 20.8*	112.9 ± 8.9^{ns}
t2.12	Sertraline	182.2 ± 8.8*	173.4 ± 13.6*	129.2 ± 14.6^{ns}

[1]Data for chronic antidepressant treatments were reported by our group previously (Yamada et al., 2000). Briefly, rats for chronic antidepressant treatment received either vehicle, 10 mg/kg of imipramine or sertraline by daily intraperitoneal injection for 21 days. Data are expressed as % of the control data (means ± s.e.m.) of three independent experiments. $^{ns}p > 0.05$ and $*p <$
t2.13 0.05, Student's t-test.

Discussion

Although the therapeutic action of antidepressants most likely involves the regulation of serotonergic and noradrenergic signal transduction, to date, no consensus has been reached concerning their precise molecular and cellular mechanism(s) of action. With expressed-sequence tag (EST) analysis, we had been continuing our effort to elucidate the involvement of some common biochemical changes induced after chronic treatment with two different classes of antidepressants, imipramine (a tricyclic antidepressant) or sertraline (a serotonin selective reuptake inhibitor, SSRI). Until now, we have molecularly cloned several cDNA fragments as ESTs, which we named them antidepressant related genes, ADRGs (Nishioka et al., 2003; Yamada et al., 1999; Yamada et al., 2002; Yamada et al., 2001; Yamada et al., 2000). Previously, we developed our original cDNA microarray (ADRG microarray) using ADRG genes. By gene expression analysis using ADRG microarray and fluorescence-labeled probes, we identified several interesting candidate genes and ESTs (Yamada and Higuchi, 2002). One of the spots, ADRG34, was significantly increased in sertraline treated rat hippocampus on the ADRG microarray. Moreover, we have determined the nucleotide sequence of the full-length cDNA for ADRG34 (Yamada et al., 2000). This cDNA encoded 685 amino acid residues yielding a mass of 79 kDa, containing a RING-H2 finger motif at the carboxy-terminus. Homology analysis with the EMBL/GeneBank database indicated that ADRG34 is a putative rat homologue of mouse and human kf-1 gene (Yasojima et al., 1997). Kf-1 was originally identified as the gene whose expression has been augmented in the cerebral cortex of a sporadic Alzheimer's disease patient (Yasojima et al., 1997). The RING-H2 finger motif of rat kf-1 was identical to those of mouse and human kf-1 (Yamada et al., 2000). RING finger proteins were assessed for their ability to facilitate E2-dependent ubiquitination and ubiquitinqtion was observed (Lorick et al., 1999). The abnormality of Ub-proteasome system may induce various pathological conditions. A common induction of kf-1 after chronic antidepressant treatment, repeated ECT and rTMS might indicate that the relationship between ubiquitin system and mechanism of the alleviation of depression is significantly important.

In the present study, northern blot analysis demonstrated the presence of a single transcript of about 3.5 kb in size for mRNA prepared from several rat brain tissues (Fig. 1). Interestingly, the expression of kf-1 was at higher levels in the midbrain, thalamus and cerebral cortex, but at relatively lower levels in the olfactory, cerebellum, pons with m. oblongata and spinal cord. Gene expression analysis using ADRG microarray demonstrated that the expression of kf-1 was 2.39 times increased in rat frontal cortex treated with rTMS, when compared to controls. While, the expression of kf-1 in ADRG microarray was 2.17 times in antidepressant treatment group (Yamada et al., 2000) and 1.97 times in ECT group (Nishioka et al., 2003). The induction of kf-1 after rTMS was also confirmed by RT-PCR analysis. As shown in Table 1, the mRNA levels of kf-1 after rTMS were significantly increased in the frontal cortex and hippocampus after normalization by ß-actin expression. Interestingly, we previously demonstrated the induction of kf-1 in rat frontal cortex and hippocampus after chronic antidepressant treatment and repeated ECT (Nishioka et al., 2003). On the other hand, the mRNA level of kf-1 after chronic haloperidol treatment was not changed in the frontal cortex, hippocampus and hypothalamus (Table 2). Thus, it is proposed that the induction of kf-1 is specific to antidepressive treatments.

The frontal cortex is one of the several brain regions that is implicated in the pathophysiology of depression (Drevets et al., 1992). The hippocampus is another brain region that would be involved in the endocrine, emotional, cognitive, and vegetative abnormalities found in depressed patients. It is reported that repeated ECT and SSRI administration have equivalent effects on hippocampal synaptic plasticity

(Stewart and Reid, 2000). Previous study by other group indicated that TMS has a long-lasting effect on neuronal excitability in the hippocampus (Levkovitz et al., 2001). They compared the effects of chronic TMS with those of the antidepressant drugs desipramine and mianserin. Interestingly, these treatments enhanced the expression of long-term potentiation in the perforant path synapse in the dentate gyrus. Thus, TMS, mianserin, and desipramine are likely to affect the same neuronal populations, which may be relevant to their antidepressant action. Stress-induced atrophy, and, in extreme cases, cell death, may contribute to the loss of hippocampal control of the hypothalamus-pituitary-adrenal (HPA) axis and hypercortisolism often exhibited in depression. Hippocampus is involved in feedback regulation of the HPA axis, and depression is associated with dysfunction of this neuroendocrine axis (Young et al., 1991). There is compelling evidence that rTMS causes changes in neuronal circuits as reflected by decreases in the activity of the HPA axis. However, mRNA level of kf-1 was not changed in rat hypothalamus after rTMS (Table 2). Interestingly, the mRNA level of kf-1 after chronic antidepressant treatment and repeated ECT was not changed in this region of the brain (Nishioka et al., 2003; Yamada et al., 2000).

In conclusion, we have identified kf-1 as a novel candidate target molecule of antidepressants, ECT and rTMS. Although mouse, rat and human kf-1 gene had been molecularly cloned, the physiological function of kf-1 protein the central nervous system is still not clear. Our findings suggest that induction of kf-1 may be associated with the treatment induced adaptive neural plasticity in the brain, which is a long-term target for their antidepressant action. Further characterization of kf-1 in the central nervous system is needed to test our hypothesis.

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Linkage disequilibrium in *aquaporin 4* gene and association study with schizophrenia

Tatsuyuki Muratake[a], Naoki Fukui[a], Naoshi Kaneko,[a], Hideki Amagane[a], Toshiyuki Someya[a]

[a]Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

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Correspondence to;

Tatsuyuki Muratake, Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi, Niigata 951-8510, Japan

mura@med.niigata-u.ac.jp

Phone; +81-25-227-2213, Fax; +81-25-227-0777

Running Title: *AQP4* and schizophrenia

Abstract

Aquaporin 4 (AQP4) has an important role in water homeostasis of human brain and a dysfunction of AQP4 could induce pathological conditions in neuronal activity. Several genome scan studies for schizophrenia found a suggestive linkage on 18q, where human *AQP4* (18q11.2-12.1) is located nearby. We performed case-control study comprised 261 of schizophrenia and 277 of controls in Japanese population with four SNP markers. We found strong linkage disequilibrium (LD) and an LD block in *AQP4* gene but found no association between *AQP4* and schizophrenia both single SNP and haplotype analyses. This study shows that *AQP4* is not directory associated with schizophrenia in our Japanese patients.

(105 words)

Key words:

Aquaporin; Schizophrenia; Case-control study; Single nucleotide polymorphism; Haplotype

Introduction

Water homeostasis in the brain is important in both physiological and pathological conditions. Neuronal activity and ion water homeostasis are coupled. Water channel proteins, aquaporins, are membrane proteins that mediate the selective efficient movement of water across the cell membrane. In eleven isoforms of the aquaporin family, aquaporin 4 (AQP4) is most highly expressed in brain, including ependymal cells, pial cells, paraventricular hypothalamic nucleus, hippocampus, and cortex of the cerebrum[1-5]. Since AQP4 is supposed to play an important role in potassium buffering in a neuronal environment[6,7], a dysfunction of AQP4 could induce pathological conditions in neuronal activities.

Schizophrenia is a complex genetic disorder with high heritability (=~ 80%) and shows a wide variety of behavioral and cognitive symptoms caused by brain dysfunctions. Although genetic factors of schizophrenia still remain to be elucidated, several genome scan studies for schizophrenia revealed that a suggestive linkage is on chromosome 18q [8,9], which is in the vicinity of human *AQP4* (18q11.2-12.1). Williams et al found a multipoint MLS of 1.62 near D18S450 which is located on 18q12 [8], and Paunio et al also found a Zmax score of 2.37 at D18S877 on 18q12 in Finnish population[9]. Hence, we examined whether AQP4 is involved in susceptibility to schizophrenia performing an association study between *AQP4* gene and schizophrenia.