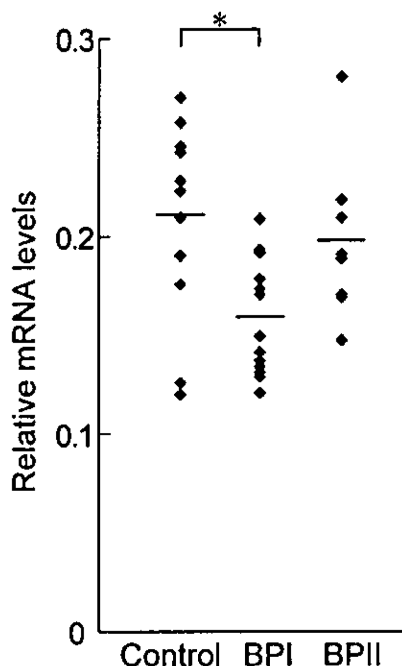

Fig. 2. Case control study of the *NDUFV2* mRNA expression. Relative mRNA levels of *NDUFV2* are compared with the *GAPDH* by real-time quantitative RT-PCR using SYBR/GREEN I dye. Each diamond indicates the relative value of mRNA of healthy control (n = 11), BPI (n = 13), and BPII (n = 8). Crossbars show average values. *$P = 0.006$ by the Student's *t*-test.

altered intracellular calcium dynamics through a decrease of the mitochondrial membrane potential, a driving force of calcium uptake by the mitochondria.

Our study has several limitations. The sensitivity of mutational detection by D-HPLC is not 100%. However, D-HPLC has been described as a very sensitive method in mutation screening (92.5–100%) [Liu et al., 1998; O'Donovan et al., 1998] and we repeated the experiments at three different conditions to minimize the false negative finding.

TABLE III. Haplotype Frequencies of the *NDUFV2* Gene in Patients With Bipolar Disorder and Controls

	Estimated haplotype frequency			
		Bipolar disorder		
Haplotype[a]	Controls (n = 222)	Total (n = 189)	BPI (n = 136)	BPII (n = 53)
CTAT	0.310	0.228	0.218	0.253
CTGT	0.253	0.336	0.321	0.375
GGAC	0.235	0.267	0.285	0.222
GGAT	0.124	0.135	0.143	0.115
GGGT	0.029	0.007	0.005	0.012
GGGC	0.025	0.003	0.004	0
CTAC	0.016	0.023	0.023	0.023
CTGC	0.009	0	0	0
P value (M)[b]		0.0001	0.001	0.124
P value (C)[c]		0.001	0.004	0.030

[a]Haplotypes consist of the −796C > G, −795T > G, −602G > A, and −233T > C polymorphisms.
[b]Differences in haplotype distributions by Monte Carlo method.
[c]Differences in major 2 haplotype (CTAT and CTGT) distributions by chi-square test.

The sample size in this study is not very large. The statistical power to detect the disorder-sensitive marker (genotype relative risk = 1.5) in this sample size at $P = 0.05$ is about 0.45. Furthermore, difference of the genotype distribution of −602G > A polymorphism is not significant after Bonferroni's correction. However, significant differences were confirmed in haplotype distributions.

In this study, we only used a case control association study to examine the pathophysiological significance of polymorphisms of *NDUFV2*. In spite that Japanese are relatively homogenous sample, population stratification cannot be completely ignored. However, we have examined other polymorphic markers, which showed no significant difference of frequencies between bipolar disorder and controls [Sasaki et al., 1998; Kunugi et al., 2002]. Therefore, this association, as well as the difference of *NDUFV2* expression, cannot be attributed to population stratification.

It is still unclear whether our findings in leukocyte reflect alterations in the brain. Although the symptoms of bipolar disorder arise from brain dysfunction, abnormalities in Ca^{2+}, phosphoinositides, and cAMP signaling systems have also been observed in lymphoblastoid cells or platelets, which are thought to be intermediate phenotypes of the disorder [Banks et al., 1990; Perez et al., 1995; Emamghoreishi et al., 1997].

Despite these limitations, our study is the first report that suggested association of bipolar disorder with polymorphisms in 24-kDa nuclear-encoded mitochondrial complex I subunit gene, *NDUFV2*. Complex I consists of more than 40 subunits and other subunits may also relate to bipolar disorder. Possible pathophysiological role of other subunit genes in this complex might be worth studying further. Furthermore, the 18p11.2, on which the *NDUFV2* locates, is not only susceptibility region to be linked with bipolar disorder but with schizophrenia [Detera-Wadleigh et al., 1999; Reyes et al., 2002]. Recently, Dror et al. [2002] reported that the mRNA expression level of this gene and complex I activity changed with disease state in schizophrenia. These findings warrant further studies to examine a possible pathophysiological role of *NDUFV2* also in schizophrenia.

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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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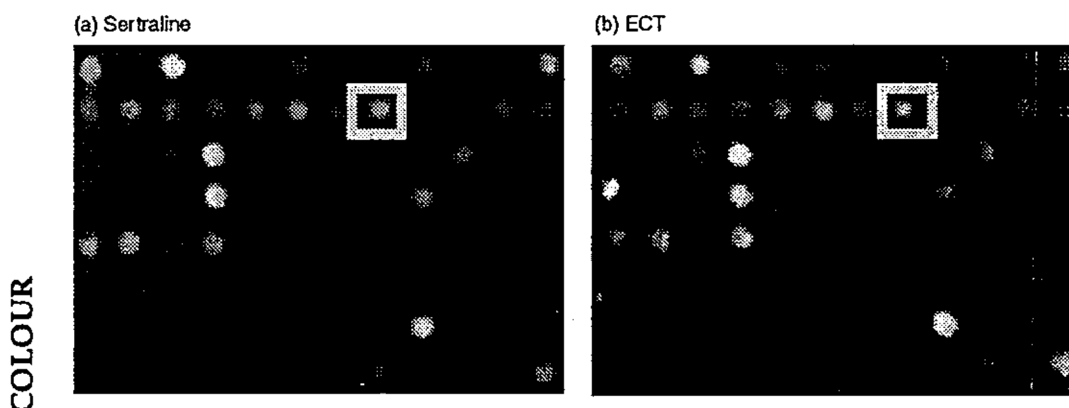

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Figure 1. Image analysis of ADRG microarray after hybridization with fluorescent probes. Ninety-six spots representing ADRG97–192 are shown. (a) Merged pseudo-colour image of control group data (green) and chronic sertraline treatment group (red).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

AQ1

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, 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 treated exactly like the ECT-treated rats but did not receive any electric current.

AQ4

AQ4

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). AQ4 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′-ATTCC ACCACGGCATCTTCA-3′) and β-actin (5′-TCGCTGA CAGGATGCAGAAGG-3′ and 5′-GCCAGGATAGAG CCACCAAT-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 AQ2 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., AQ4 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 AQ4 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 AQ4 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'-CTCGAGGCCACCATGGCAGAGC-3', 5'-GAATTCTCTCAACAGGAGACTTCCATGGTG-3' and high fidelity Platinum pfx DNA polymerase (Invitrogen). These primers contain either *XhoI* or *EcoRI* 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 AQ4 (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 AQ4 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.

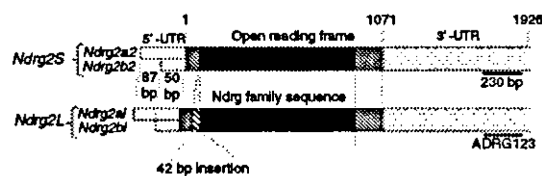

Figure 2. Schematic representations of rat Ndrg2. Rat Ndrg2 consists of four isoforms: Ndrg2a1, Ndrg2a2, Ndrg2b1, and Ndrg2b2. The 5'-UTR for Ndrg2a1/Ndrg2a2 was 87 bp, whereas the 5'-UTR for Ndrg2b1/Ndrg2b2 was 50 bp. In the translated region, Ndrg2a1/Ndrg2b1 has an additional 42-bp insertion compared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

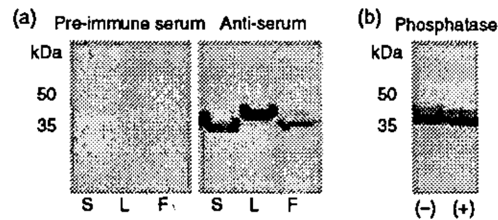

Figure 3. Specificity of anti-rat-Ndrg2 antiserum prepared by our group in the present study. To examine the specificity of the anti-rat-Ndrg2 antiserum, we immunostained HEK293 cells overexpressing rat Ndrg2S and Ndrg2L (a). The lysates from HEK293 cells (S, L) or rat frontal cortex (F) were electrophoresed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

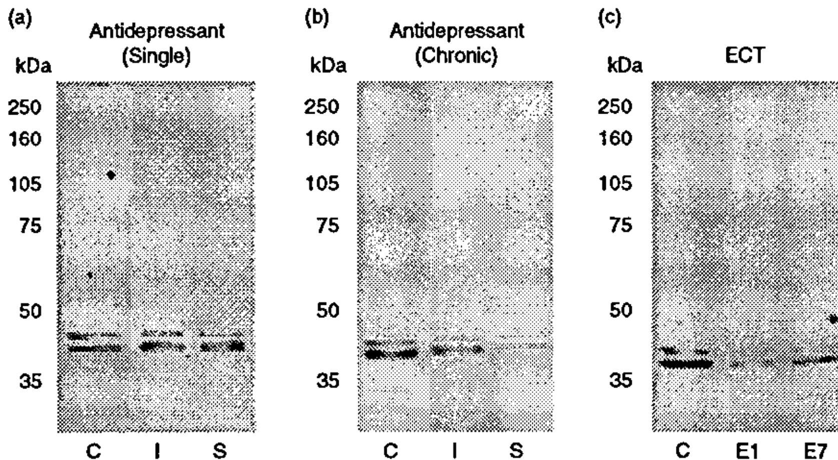

Figure 4. Western blot analysis of Ndrg2S and Ndrg2L in rat frontal cortex after a single antidepressant treatment (a), chronic antidepressant treatment (b), or ECT (c). A protein sample was prepared from rat frontal cortex and treated with either vehicle (control, lane 1), 10 mg/kg of imipramine (lane 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 ($55.0 \pm 18.5\%$ or $53.6 \pm 3.1\%$) 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.

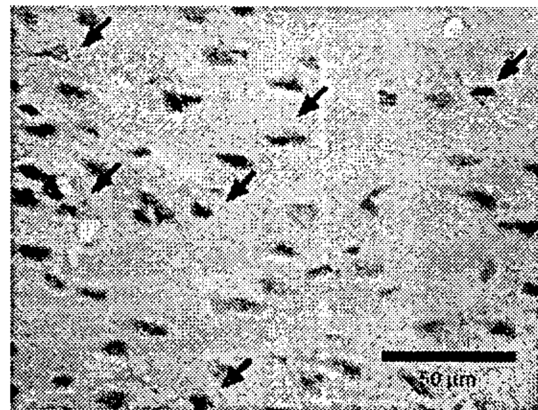

Figure 5. Immunohistochemical identification of Ndrg2-expressing cells in the rat frontal cortex. Using the anti-rat-Ndrg2 antiserum prepared by our group, Ndrg2 immunoreactivity (brown) was observed in cells in the rat frontal cortex. Diaminobenzidine was the chromogen, and the counterstain was haematoxylin.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

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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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Induction of kf-1 after repeated electroconvulsive treatment and chronic antidepressant treatment in rat frontal cortex and hippocampus

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Summary. It has been proposed that signaling pathways involved in adaptive neural plasticity are long-term targets for the action of electroconvulsive treatment (ECT), which is widely used in the treatment of drug-resistant depression. We have previously performed EST analysis to identify some molecular machinery responsible for antidepressant effect. One of the cDNA fragments identified as antidepressant related genes/ESTs was identified as kf-1 which has a RING-H2 finger motif at the carboxy-terminus. In the present study, we have demonstrated the induction of kf-1 in rat frontal cortex and hippocampus not only after chronic antidepressant treatment, but also after a single and repeated ECT. RING finger proteins are proposed to play some important roles in the ubiquitin-proteasome system. In conclusion, the current investigation has identified kf-1 as a novel molecular target for antidepressants and ECT.

Keywords: Imipramine, sertraline, SSRI, depression, EST analysis, cDNA microarray, ubiquitination.

Introduction

Depression is one of the major neuropsychiatric disorders. It has been demonstrated that typical antidepressants acutely inhibit the monoamine reuptake in nerve terminals resulting in significant increase in synaptic concentrations of monoamines, noradrenaline or serotonin. However, there is a latency period of several weeks before the onset of clinical effect of antidepressants. Electro-

convulsive treatment (ECT) is another important therapy that is widely used in the treatment of depression. ECT may be used in severe psychotic depression, severe depression with suicidality, resistant depression, and in patients intolerant of antidepressant medications, geriatric patients and those with medical illnesses which contraindicate the use of antidepressants (e.g. renal, cardiac or hepatic disease), because its safety, high efficacy and rapid onset of action are suited to the special needs of this population. Although ECT is believed to have a rapid onset of antidepressant activity, the basis for its therapeutic mechanism is still unknown.

It is proposed that ECT would alter regulation of the monoamine neurotransmitters. Repeated ECT significantly increases the levels of 5-HT2 receptor mRNA (Butler et al., 1993) and induces β-adrenergic receptor downregulation in rat frontal cortex (Hosoda and Duman, 1993). Messenger RNA levels of tyrosine hydroxylase, which is the rate-limiting enzyme of catecholamine synthesis, is significantly increased in rat locus coeruleus after ECT (Kapur et al., 1993). In addition, it has been proposed that signaling pathways involved in adaptive neural plasticity are long-term targets for the action of ECT in the brain. Indeed, ECT induces a rapid and transient increase in mRNA levels of immediate early genes in rat brain. These genes, zif268, c-fos, c-jun, jun-B (Cole et al., 1990), NGFI-A (Jensen et al., 2000; Bjartmar et al., 2000) and cAMP response element binding protein (CREB) (Nibuya et al., 1996), encode sequence specific DNA binding proteins thought to act as transcription regulatory factors. Electrical stimulation also induces Arc, activity regulated cytoskeleton associated protein (Wallace et al., 1998). In addition, it is raised the possibility that among the many long-term targets of antidepressant treatments, including ECT, may be regulation of neurotrophic factors. Electrical or chemical-induced seizures increase the expression of BDNF and its receptor, trkB, in the rodent brain (Ernfors et al., 1991; Isackson et al., 1991). Induction of these genes after ECT may be important in long-term changes of neuronal activity, morphology, receptor distribution and antidepressant effect. However, the detailed mechanisms underlying ECT-induced adaptive neuronal changes are not yet known.

On the other hand, it is proposed that some common or shared effects on neural transduction systems or adaptive neuronal changes are involved with the therapeutic action of ECT and antidepressants. Indeed, it is reported that repeated administration of ECT and several antidepressant significantly increased BDNF and trkB mRNA in hippocampus (Lindefors et al., 1995; Nibuya et al., 1995; Zetterstrom et al., 1998). Also, repeated ECT and SSRI administration have equivalent effects on hippocampal synaptic plasticity (Stewart and Reid, 2000).

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). Previously, we developed our original cDNA microarray

(ADRG microarray) using these ADRG genes (Yamada et al., 2000). By gene expression analysis using ADRG microarray and fluorescence-labeled probes, we identified several interesting candidate genes and ESTs. One of the spots, ADRG34, was significantly increased in rat frontal cortex chronically treated with antidepressant, sertraline. Then, 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 of ADRG34 with the EMBL/GeneBank database indicated that ADRG34 is a putative rat homologue of mouse and human kf-1 gene (Yasojima et al., 1997). However, the physiological function of kf-1 protein in the central nervous system is still not clear.

In the present study, we investigated whether the expression of kf-1 is also induced after repeated administration of ECT in rat frontal cortex, hippocampus and hypothalamus. Theses regions of the brain are suggested to play some critical roles in mediating antidepressant effects.

Material 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 a 12 h light/12 h dark cycle and free access to food and water. Rats were randomly separated into control and treated groups. Six rats were used for each treatment group. Rats for ECT were anesthetized with sevoflurane and received a 90 mA, 1.0 sec electric shock via ear-clip electrodes once (acute ECT group), or every other day for 14 days (repeated ECT group). ECT employed the Ugo Basile Model 7801 Unipolar square-wave electroconvulsive stimulation pulse generator (Stoelting Co., IL, USA). The control group was treated exactly the same as ECT-treated rats, but without the administration of the electric current. Experimental animals for antidepressant experiments received either vehicle, 10 mg/kg of imipramine (Sigma Chemical., MO, USA) or sertraline (Pfizer Pharmaceuticals Inc., NY, USA), dissolved in 1.5% tween 80, by daily intraperitoneal injection for 21 days. Animals were killed by decapitation 24 hours after the final antidepressant administration or ECT treatment, and the brain was quick removed, dissected and then frozen in liquid nitrogen immediately and stored at −80°C until use. All studies using animals were carried out in accordance with animal protocols approved by the National Institutes of Health, OPRR Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Fabrication of cDNA microarray and fluorescence image analysis

Fabrication of the ADRG microarray was done as described by our group previously using a GMS417 Arrayer (Affymetrix, Inc., CA, USA) (Yamada et al., 2000). To make the fluorescence-labeled probe for hybridization, poly A^+ RNA was purified from total RNA that was pooled with three independent control or treated groups. One µg of poly A^+ RNA from control or treated 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 the microarray was done competitively in duplicate. The probes were mixed and placed on an array, overlaid with a coverslip, and hybridized for 16.5 h at 65°C. After the hybridization and washing procedure, each slide was scanned with a GMS418 Array Scanner (Affymetrix, Inc., CA, USA). 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 rat kf-1, 5′-GGAATA CGGACAGGACTTTC-3′ and 5′-TCCGAGAAGCTGCATGGGC-3′ (Amersham Pharmacia Biotech, Tokyo, Japan). Amplification of rat 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. For RT-PCR experiments with antidepressant-treated samples, a pair of primers for GAPDH, 5′-TGAAGGTCGGTGTCA ACGGATTTGGC-3′ and 5′5CATGTAGGCCATGAGGTCCACCAC-3′ was used for normalization. Amplification of GAPDH 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, 16, 25 or 22 cycles were used for samples obtained from frontal cortex, hippocampus or hypothalamus, respectively. In our preliminary experiments, the expression of a housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was significantly increased in rat hippocampus after repeated ECT (data not shown). Therefore, data were normalized with starting RNA content for ECT experiments in this study. To ensure the fidelity of the analysis, we assayed several cycles of PCR to determine the liner range for amplification of PCR product in each region of the brain. 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). Data are given as mean ± s.e.m for the group. Differences were assessed using Student's t-test. A value of $P < 0.05$ was regarded as significant.

Results

Messenger RNA expression analysis with ADRG microarray

In the present study, we used an ADRG microarray for high-throughput, secondary screening to identify genes commonly affected by antidepressant and ECT. The pseudo-color image of the ADRG microarray after hybridization with samples obtained from chronic sertraline-treated rat frontal cortex had been published previously (Yamada et al., 2000). As expected, we obtained low background and consistent results in duplicated experiments. With samples obtained from rat frontal cortex, the fluorescence intensities of the spots for ADRG34 (rat kf-1) were increased 3.9 times in the chronic sertraline-treated group (Yamada et al., 2000) and 2.0 times in the repeated ECT group, when compared to controls after normalization of the signals for both negative and positive controls. These data were reproducible and interassay variation was negligible, when the differences of fluorescence intensities (±2-fold) were regarded as significant.

Messenger RNA expression analysis with RT-PCR

The differential expression of kf-1 after antidepressant treatment or ECT was also investigated by RT-PCR analysis. A typical image of gel electrophoresis

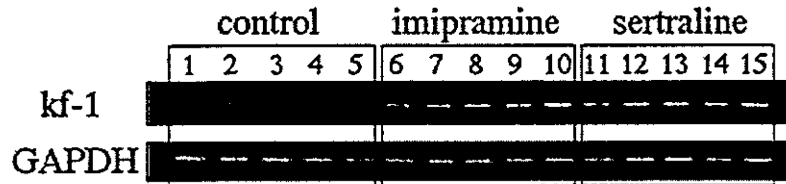

Fig. 1. A typical image of gel electrophoresis after RT-PCR. RNA was extracted from rat frontal cortex treated either with vehicle (control, lane 1–5), 10 mg/kg of imipramine (lane 6–10) or 10 mg/kg of sertraline (lane 11–15) for 21 days, and used for RT-PCR analysis. The PCR products were electrophoresed in a 1% agarose gel. The reproducible single band corresponding to rat kf-1 or GAPDH was observed on a gel, respectively. This figure represents a typical result of three independent experiments

after RT-PCR is shown in Fig. 1. As shown here, the reproducible band corresponding to kf-1 at the size of 199 bp existed on a gel.

Here, we first report the induction of kf-1 in rat frontal cortex and hippocampus after repeated ECT. Interestingly, as shown in Table 1, the expression of kf-1 was significantly increased in single or repeated ECT-treated rat frontal cortex (131.0 ± 2.0% and 126.9 ± 5.6%, respectively) and hippocampus (142.1 ± 9.9% and 140.4 ± 6.6%, respectively). On the other hand, the expression of kf-1 was not changed in rat hypothalamus (105.7 ± 6.6% or 96.3 ± 3.8%, respectively) after single or repeated ECT (Table 1).

In rat frontal cortex, we have previously demonstrated that the chronic treatment with either imipramine or sertraline significantly induced the expression of kf-1 mRNA levels (165.0 ± 9.9% or 182.2 ± 8.8%, respectively) (Table 2, Yamada et al., 2000). Interestingly, similar to ECT, the chronic treatment with these antidepressants also significantly induced the expression of kf-1 mRNA levels in rat hippocampus (204.0 ± 20.8% or 173.4 ± 13.6%, respectively). On the other hand, in rat hypothalamus, the chronic treatment with these antidepressants did not induce significant effect on the expression of kf-1 (112.9 ± 8.9%, 129.2 ± 14.6%, respectively). A single administration of these antidepressants did not affect kf-1 expression in all regions of the brain investigated in this study (Table 2).

Discussion

The biological basis for the therapeutic mechanisms of depression are still unknown. Many of the previous reports have focused on synaptic pharmacol-

Table 1. The expression of kf-1 after single or repeated ECT

Brain region	Control	Single ECT	Repeated ECT
Frontal cortex	100 ± 7.8	131.0 ± 2.0*	126.9 ± 5.6*
Hippocampus	100 ± 8.9	142.1 ± 9.9*	140.4 ± 6.6*
Hypothalamus	100 ± 7.5	105.7 ± 6.6$^{n.s.}$	96.3 ± 3.8$^{n.s.}$

Data are expressed as % of the control data (means ± s.e.m.) of three independent experiments. $^{n.s}p > 0.05$ and $^{*}p < 0.05$, Student's t-test

Table 2. The expression of kf-1 after acute or chronic antidepressant treatments

Brain region		Control	Imipramine	Sertraline
Frontal cortex	acute	100 ± 7.3	106.7 ± 2.3$^{n.s}$	109.1 ± 10.9$^{n.s.}$
	chronic[1]	100 ± 9.3	165.6 ± 9.9*	182.2 ± 8.8*
Hippocampus	acute	100 ± 4.2	115.4 ± 11.6$^{n.s}$	115.2 ± 16.4$^{n.s}$
	chronic[1]	100 ± 3.7	204.0 ± 20.8*	173.4 ± 13.6*
Hypothalamus	acute	100 ± 8.6	110.4 ± 12.3$^{n.s}$	101.4 ± 5.1$^{n.s}$
	chronic[1]	100 ± 18.8	112.9 ± 8.9$^{n.s}$	129.2 ± 14.6$^{n.s}$

Data are expressed as % of the control data (means ± s.e.m.) of three independent experiments. $^{n.s}p > 0.05$ and $^{*}p < 0.05$, Student's t-test. [1]Data for chronic antidepressant treatments were in part reported by our group previously (Yamada et al., 2000)

ogy, especially on monoamine neurotransmitter turnover and their receptors. However, to understand the therapeutic actions of antidepressants and ECT, we must now extend our efforts beyond the monoamine neurotransmitters, to an understanding of cellular and molecular neurobiology. Identification of quantitative changes in gene expression that occur in the brain after repeated ECT or chronic antidepressant treatment can yield novel molecular markers that may be useful in the diagnosis and treatment of major depression. Using differential cloning strategy, we and other groups have reported the isolation of some genes that are differentially expressed in the rodent brain after chronic antidepressant treatment (Huang et al., 1997; Wong et al., 1996; Yamada et al., 1999, 2000).

In the present study, we demonstrated that kf-1 is induced after repeated ECT or chronic antidepressant treatment in rat frontal cortex and hippocampus. Interestingly, a single administration of antidepressant did not affect kf-1 mRNA expression in these regions of the brain. Clinically, there is a latency period of several weeks before the onset of therapeutic effect of antidepressants. Our results may support the hypothesis that kf-1 mediated neural systems or adaptive neuronal changes in rat hippocampus and frontal cortex are involved with therapeutic action of ECT and antidepressants. The frontal cortex is one of the several brain regions that would be involved in the endocrine, emotional, cognitive, and vegetative abnormalities found in depressed patients. In the frontal cortex, glucose metabolism, blood flow, and electroencephalograph activity are altered in depressed patients (Drevets et al., 1992). The hippocampus is another region of the brain that is implicated in the pathophysiology of depression. It is demonstrated that chronic stress causes atrophy of hippocampal neurons and that the volume of hippocampus is decreased in depressed patients (Magarinos et al., 1996; Sapolsky et al., 1990; Sheline et al., 1996; Smith et al., 1995). Stress-induced disfunction may contribute to the loss of hippocampal control of the hypothalamic-pituitary-adrenal axis (HPA) (Sapolsky et al., 1990; Young et al., 1991). Interestingly, it is recently reported that ECT increases the number of new born cells and neurogenesis in hippocampus (Madsen et al., 2000). On the other hand, in rat hypothalamus, the chronic treatment with either imipramine or sertraline did

not induce significant effect on the expression of kf-1 mRNA levels. Also, the expression of kf-1 was not changed in repeated ECT-treated rat hypothalamus. 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 contribute the real therapeutic action of antidepressants. Studies to further characterize the neuronal circuitry of these brain regions will help elucidate the neuroanatomical substrates of antidepressive effects.

In this study, a single ECT also significantly induced the expression of kf-1 mRNA levels in rat frontal cortex and hippocampus. Previously, Segman et al. confirmed the clinical impression that ECT is a rapidly effective treatment for major depression than generally reported for antidepressant drugs (Segman et al., 1995). Although the eventual effect of antidepressants on kf-1 expression appears greater than that of repeated ECT (Table 1–2), our results may support the hypothesis that rapid induction of kf-1 in hippocampus and frontal cortex are involved with the relatively rapid onset of the therapeutic action of ECT. Further characterization of kf-1 as a functional protein in the central nervous system is needed to test this hypothesis.

Clinically, repeated ECT is necessary to obtain continuous clinical effects in depressed patients. Sustained increases in kf-1 levels in hippocampus and frontal cortex after repeated ECT may be related to the continuous clinical effects. A single ECT may induce "short-term" therapeutic effect that are reinforced by repeated ECT. On the other hand, it is still possible that a single ECT induces "long-term" changes in the kf-1 expression that persist. To differentiate between these two possibilities, we need to investigate the gene expression at various time intervals following either single or repeated ECT in our future study.

Kf-1 was originally identified as the gene with RING-H2 finger motif whose expression has been augmented in the cerebral cortex of a sporadic Alzheimer's disease patient (Yasojima et al., 1997). Subsequently, the same group identified mouse kf-1. RING finger proteins were assessed for their ability to facilitate E2-dependent ubiquitination (Lorick et al., 1999). The conserved protein ubiquitin (Ub) functions as a covalent proteolytic signal. The Ub-proteasome system plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, endocytosis, long-term facilitation and long-term memory in the bain. Therefore, the modification of Ub-proteasome system may induce various pathological conditions in the brain. Indeed, a mutant form of ubiquitin, termed Ub(+1), has been selectively observed in the brains of Alzheimer's patients. Expression of Ub(+1) in aging brain could result in dominant inhibition of the Ub-proteasome system, leading to neuropathologic consequences (Lam et al., 2000). The physiological role of kf-1 is still not clear. However, the induction of kf-1 after chronic antidepressant treatment and repeated ECT may suggest that the relationship between Ub-proteasome system and mechanism of the alleviation of depression.

In conclusion, the current investigation has identified kf-1 as a novel molecular target for antidepressants and ECT. Our findings offer novel

insights into the actions of antidepressants and ECT that may be of both basic and clinical significance. Furthermore, the ADRG microarray we developed seemed to be a powerful tool for the discovery of novel therapeutic targets for future drug development with a new class of action in the brain.

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