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

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

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

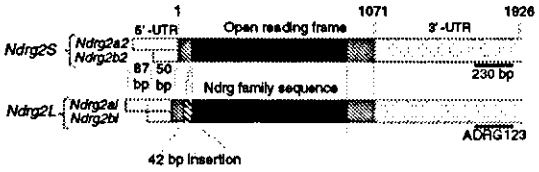


Figure 2. Schematic representations of rat Ndrg2. Rat Ndrg2 consists of four isoforms: Ndrg2a1, Ndrg2a2, Ndrg2b1, and Ndrg2b2. The 5'-UTR for Ndrg2a1/Ndrg2a2 was 87 bp, whereas the 5'-UTR for Ndrg2b1/Ndrg2b2 was 50 bp. In the translated region, Ndrg2a1/Ndrg2b1 has an additional 42-bp insertion compared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

Messenger RNA expression analysis by real-time quantitative PCR

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

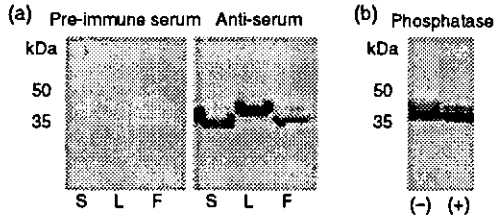


Figure 3. Specificity of anti-rat-Ndrg2 antiserum prepared by our group in the present study. To examine the specificity of the anti-rat-Ndrg2 antiserum, we immunostained HEK293 cells overexpressing rat Ndrg2S and Ndrg2L (a). The lysates from HEK293 cells (S, L) or rat frontal cortex (F) were electrophoresed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

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

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

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

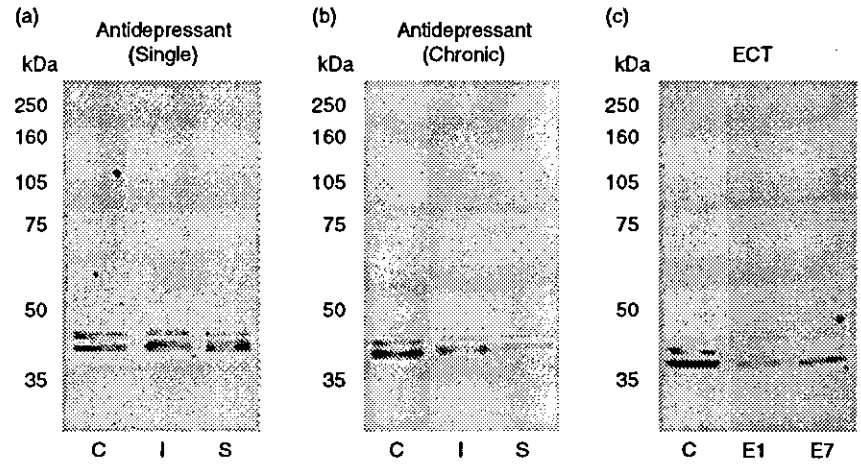


Figure 4. Western blot analysis of Ndrg2S and Ndrg2L in rat frontal cortex after a single antidepressant treatment (a), chronic antidepressant treatment (b), or ECT (c). A protein sample was prepared from rat frontal cortex and treated with either vehicle (control, lane 1), 10 mg/kg of imipramine (lane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

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

Phosphatase digestion

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

Immunohistochemical localization of Ndrg2 in the rat frontal cortex

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

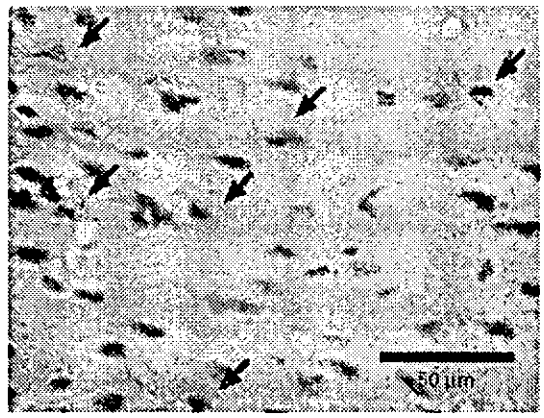
Discussion

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

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

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

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



COLOUR

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

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

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

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

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

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

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Statement of Interest

None.

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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