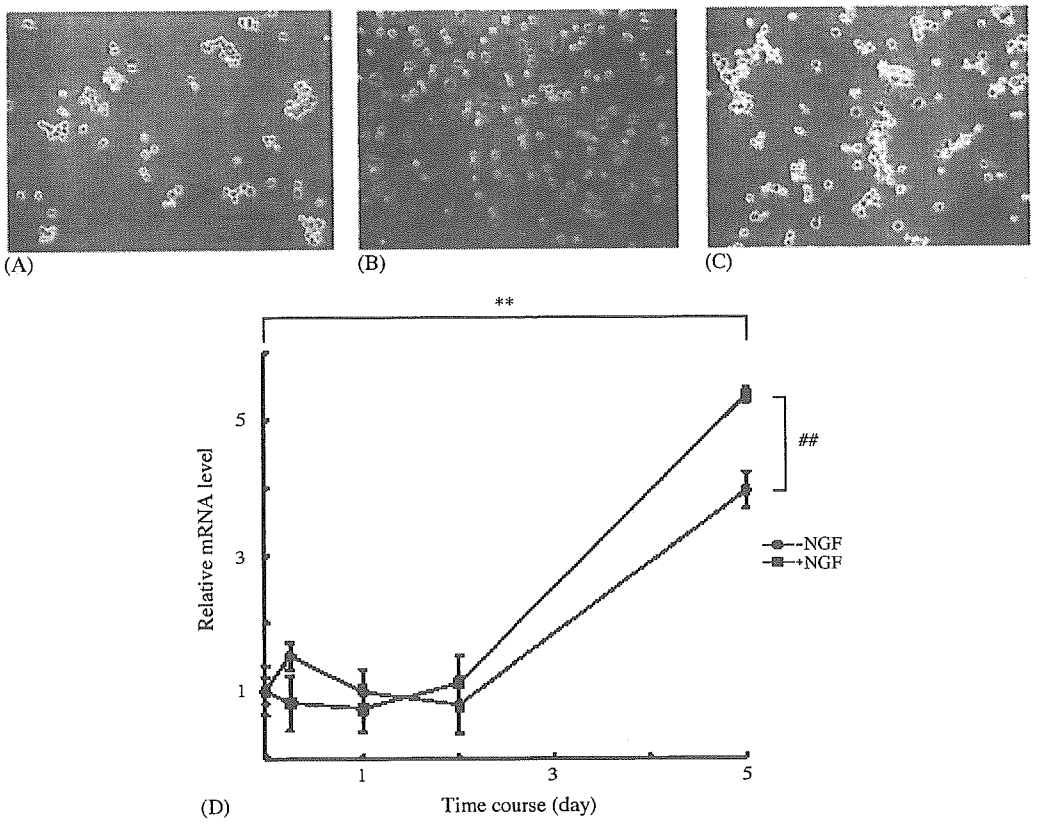

Fig. 1. Messenger RNA expression of *Ndrg2* during varying stages of neurite outgrowth in PC12 cells treated with (square) or without (circle) 50 ng/mL NGF. Images of PC12 cells cultured with NGF for 0 day (A), 2 days (B) and 5 days (C) were obtained from phase-contrast microscopy (scale bars = 200 μm). Neurites began to sprout 1–2 days after exposure to NGF; subsequent neurite growth continued for 5 days. During this time, the expression of *Ndrg2* mRNA was significantly increased in a time-dependent manner (D). Data are expressed as mean ± S.E.M. of three independent experiments. $^{**}p < 0.01$ ANOVA followed by the Dunnett's test, $^{\#\#}p < 0.01$ Student's *t*-test.

Leiden, Netherlands) to visualize F-actin filaments for double staining. For each dish, 20–30 fluorescent images were obtained using a laser scanning confocal microscopy imaging system (Bio-Rad). Only cells with GFP-immunofluorescence were regarded as transfectants. The length and the number of individual neurites from over one hundred cells were analyzed blindly using NIH image software running on an Apple Computer. 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/). Neurites sprouting more than 5 μm from the cell body were analyzed.

Data are presented as mean ± S.E.M. for each group. For the RTQ-PCR experiments, statistical differences were assessed using analysis of variance (ANOVA) followed by the Dunnett's test or Student's *t*-test. For the neurite outgrowth experiments, statistical differences were assessed using Bonferroni correction. A value of $p < 0.05$ was regarded as significant.

As expected, NGF induced PC12 cells to differentiate into neuron-like cells, the differentiation of which was manifested by neurite outgrowth (Fig. 1). Neurites began to sprout 1–2 days after exposure to NGF; subsequent neurite growth continued for 5 days (Fig. 1A–C). To determine more specifically the role of Ndrg2 in PC12 cell differentiation, we assessed *Ndrg2* mRNA expression during varying stages of neurite outgrowth in these cells. During this time, Ndrg2 mRNA expression was significantly increased in a time-dependent manner (Fig. 1D). Indeed, the mRNA expression was not changed for 2 days after the treatment with or without NGF. Interestingly, 5 days after the treatment, the mRNA expression was significantly increased and surpassed those found on day-0 by 5.4- and 3.9-fold, respectively (Fig. 1D). In addition, *Ndrg2* mRNA expressions on day-5 were significantly different between the cells treated with or without NGF (Fig. 1D). Although, a causal relationship between Ndrg2 expression and neurite outgrowth is not proved by our observation, our data may suggest that the increased levels of Ndrg2 is somehow related to differentiation of PC12 cells.

Interestingly, V5 immunoreactivity representing Ndrg2 expression localized on cell surface membranes and growth cones (Fig. 2A). Fluorescent signals were not detected in untransfected PC12 cells (data not shown). To verify the specificity of transfection, protein extracts derived from transfected PC12 cells were immunoblotted. As expected, Ndrg2S and Ndrg2L were detected as distinct ~39.3 and 40.8 kDa bands, respectively (Fig. 2B). In the present study,

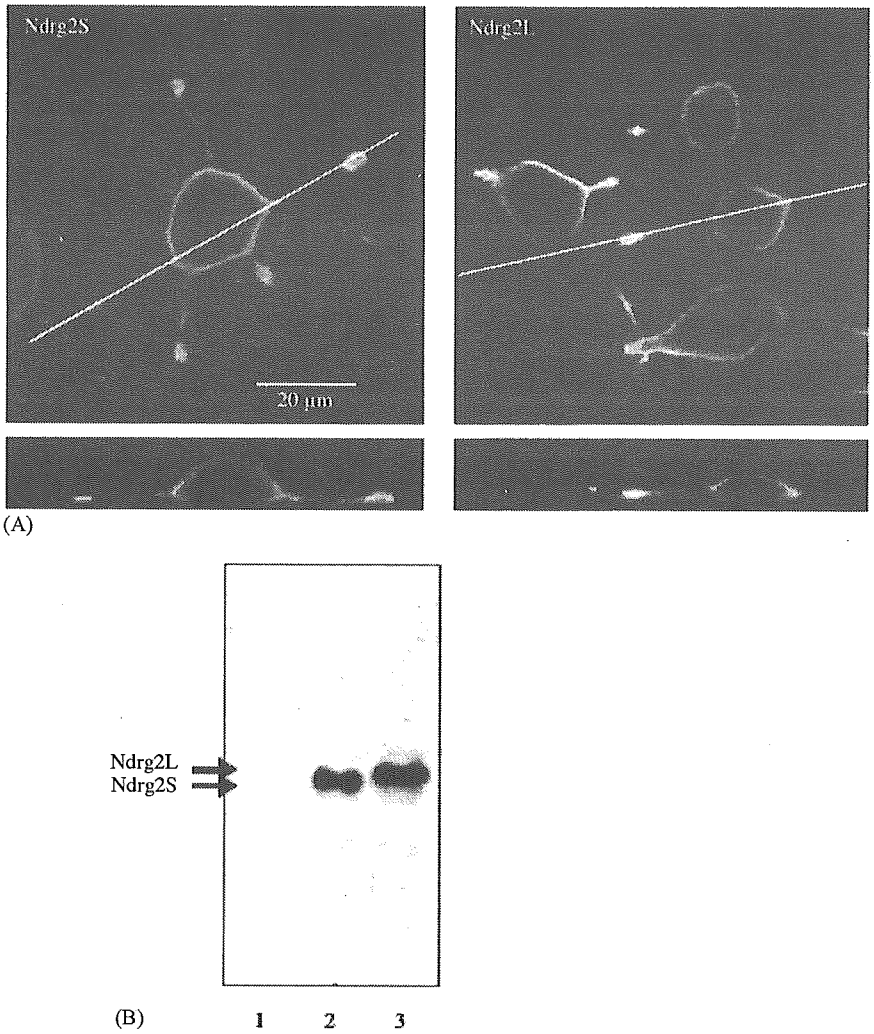

Fig. 2. Immunocytochemical localization of transfected Ndrg2S and Ndrg2L in NGF-differentiated PC12 cells. V5 immunoreactivity for both V5/HIS tagged Ndrg2S (left panel) and Ndrg2L (right panel) proteins localized on cell surface membranes and growth cones (A). Typical longitudinal section (A, up) and cross-section (A, bottom) images of Ndrg2S and Ndrg2L transfected PC12 cells were demonstrated. To verify the specificity of transfection, protein extracts derived from non-vector (lane 1), Ndrg2S (lane 2) or Ndrg2L (lane 3) transfected PC12 cells were immunoblotted (B). As expected, Ndrg2S and Ndrg2L were detected as distinct ~39.3 and 40.8 kDa bands, respectively.

we found Ndrg2S and Ndrg2L to specifically localize to cell surface membranes and growth cones, suggesting that these proteins are transported to the tip of neurites in these cells. Interestingly, human Ndrg2-GFP fusion protein has been localized to the cytosol in transfected COS-7 cells [9]. In addition, Ndrg2-V5 fusion protein has been expressed at the perinuclear region in the oligodendrocytic 6E12 cells [5]. The distribution of Ndrg2, however, may vary depending on the type of cell lines used in different transfection experiments. Together, our results suggest two possibilities: (1) Ndrg2 is growth cone components, and/or (2) Ndrg2 is a mediator of neurite outgrowth.

Indeed, 2 days after transfection, the neurites of transfected cells were clearly longer than those found in control cells (Fig. 3A). The mean length of the longest neurite of each cell was significantly longer in transfected cells (control, 27.1 ± 1.9 μm; Ndrg2S transfectant, 34.9 ± 2.5 μm; Ndrg2L transfectant, 37.8 ± 3.2 μm; Bonferroni correction $p < 0.05$; Fig. 3B). On the other hand, the mean number of neurites per cell did not vary between the groups (control, 2.38 ± 0.13; Ndrg2S transfectant, 2.15 ± 0.11; Ndrg2L transfectant, 2.27 ± 0.10; Fig. 3C). As discussed above, the expression of endogenous Ndrg2 mRNA was not influenced by NGF-differentiation in PC12 cells at this time point. Together, our data demonstrated that overexpression of Ndrg2S and Ndrg2L promoted elongation of neurites in NGF-differentiated PC12 cells, even though the number of neurites remained unchanged.

Molecules localized within growth cones have been shown to function in synaptic transduction and in neurite sprouting, extension, and guidance. Thus, since Ndrg2 mainly localized to cell surface membranes and growth cones of PC12 cells, Ndrg2 may also play a role in the mechanisms underlying such synaptic events. Previously, we found that chronic

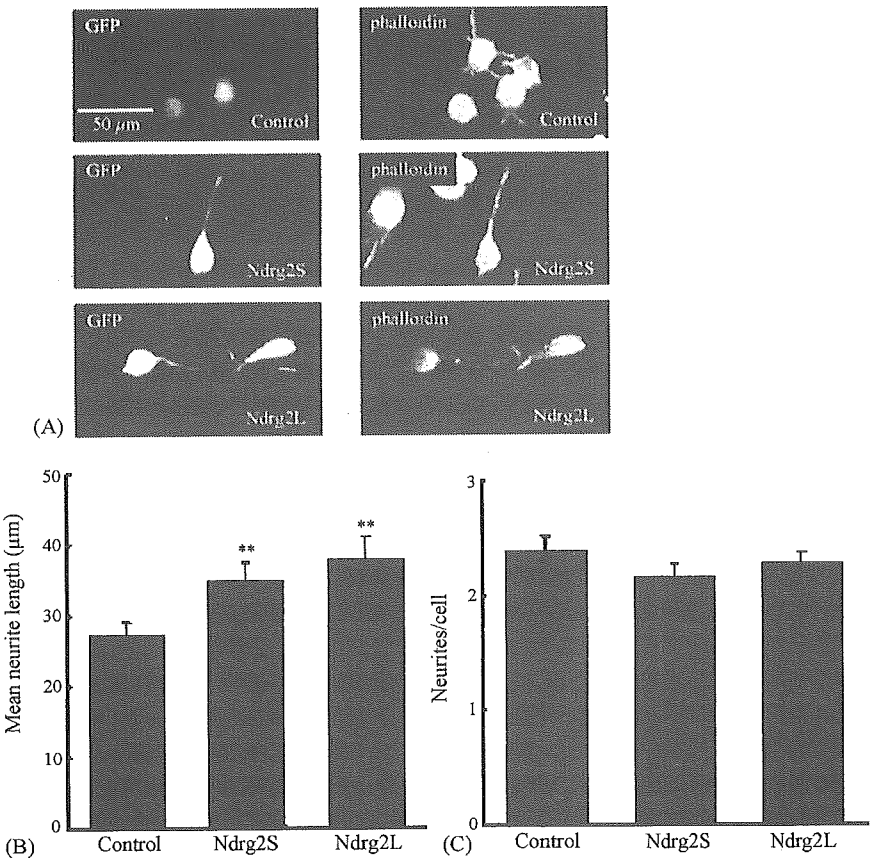

Fig. 3. Neurite outgrowth of NGF-differentiated PC12 cells after Ndrg2S and Ndrg2L transfection. The images of GFP-expressing cells were collected using confocal microscopy (A, left). F-actin was visualized in the identical field with Alexa Fluor 568-conjugated phalloidin (A, right). Two days after transfection, the mean length of the longest neurite of each cell was significantly longer in transfected cells (B). On the other hand, the mean number of neurites per cell did not vary between the groups (C). Data are expressed as mean ± S.E.M. of 130–150 cells analyzed. $^{**}p < 0.01$, Bonferroni correction.

antidepressant treatment or ECT decreased Ndrg2 expression [11]. Our findings suggest that gene expression-dependent alterations of synaptic function, which includes Ndrg2, may be an important component of the pharmacological action of antidepressants and ECT. In conclusion, our findings offer novel insights into the physiological roles of Ndrg2 in the central nervous system.

References

[1] S. Boulkroun, M. Fay, M.C. Zennaro, B. Escoubet, F. Jaisser, M. Blot-Chabaud, N. Farman, N. Courtois-Coutry, Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene, J. Biol. Chem. 277 (2002) 31506–31515.

[2] Y. Deng, L. Yao, L. Chau, S.S. Ng, Y. Peng, X. Liu, W.S. Au, J. Wang, F. Li, S. Ji, H. Han, X. Nie, Q. Li, H.F. Kung, S.Y. Leung, M.C. Lin, N-Myc downstream-regulated gene 2 (NDRG2) inhibits glioblastoma cell proliferation, Int. J. Cancer 106 (2003) 342–347.

[3] L.A. Greene, A.S. Tischler, Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, Proc. Natl. Acad. Sci. USA 73 (1976) 2424–2428.

[4] L. Kalaydjieva, D. Gresham, R. Gooding, L. Heather, F. Baas, R. de Jonge, K. Blechschmidt, D. Angelicheva, D. Chandler, P. Worsley, A. Rosenthal, R.H. King, P.K. Thomas, N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom, Am. J. Hum. Genet. 67 (2000) 47–58.

[5] C. Mitchelmore, S. Buchmann-Moller, L. Rask, M.J. West, J.C. Troncoso, N.A. Jensen, NDRG2: a novel Alzheimer's disease associated protein, Neurobiol. Dis. 16 (2004) 48–58.

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

[7] T. Okuda, H. Kondoh, Identification of new genes ndr2 and ndr3 which are related to Ndr1/RTP/Drg1 but show distinct tissue specificity and response to N-myc, Biochem. Biophys. Res. Commun. 266 (1999) 208–215.

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

[9] X. Qu, Y. Zhai, H. Wei, C. Zhang, G. Xing, Y. Yu, F. He, Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family, Mol. Cell. Biochem. 229 (2002) 35–44.

[10] K. Salnikow, T. Kluz, M. Costa, D. Piquemal, Z.N. Demidenko, K. Xie, M.V. Blagosklonny, The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible

factor 1 in response to hypoxia, Mol. Cell Biol. 22 (2002) 1734–1741.

[11] K. Takahashi, M. Yamada, H. Ohata, K. Momose, T. Higuchi, K. Honda, M. Yamada, Expression of Ndrg2 in the rat frontal cortex after antidepressant and electroconvulsive treatment, Int. J. Neuropsychopharmacol. 8 (2005) 381–389.

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

[13] D. Zhou, K. Salnikow, M. Costa, Cap43, a novel gene specifically induced by Ni^{2+} compounds, Cancer Res. 58 (1998) 2182–2189.

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

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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

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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′-TCCGA-GAAGCTGCATGGGC-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:

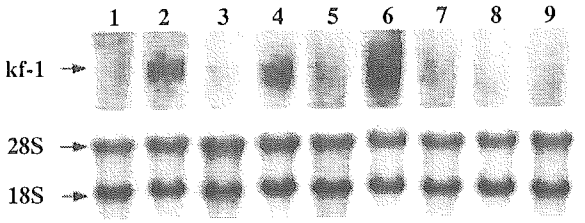

Fig. 1. Expression of kf-1 in rat brain tissues. Complimentary DNA fragment of kf-1 obtained from EST analysis was labeled with [^{32}P]dCTP and used as a probe. Rat brain tissues (1 olfactory, 2 cerebral cortex, 3 hippocampus, 4 thalamus, 5 hypothalamus, 6 midbrain, 7 cerebellum, 8 pons with m. oblongata, 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).

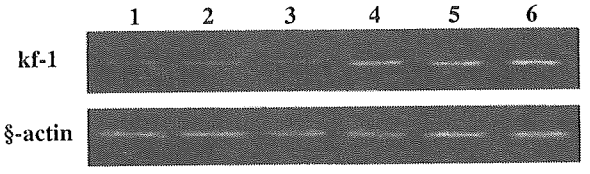

Fig. 2. A typical image of gel electrophoresis after RT-PCR. RNA was extracted from rat frontal cortex obtained from control rats (lane 1–3), or rTMS-treated rats (lane 4–6), and used for RT-PCR analysis. Control rats were treated as the rTMS-treated rats but without magnetic stimulation. The PCR products 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.

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

	Frontal Cortex	Hippocampus	Hypothalamus
Repetitive transcranial magnetic stimulation, rTMS			
Control	100 ± 5.7	100 ± 16.9	100 ± 12.2
rTMS	142.4 ± 8.4*	156.7 ± 15.8*	101.5 ± 9.7^{ns}
Repeated electroconvulsive treatment, ECT[1]			
Control	100 ± 8.2	100 ± 14.1	100 ± 6.1
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 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).

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

	Frontal Cortex	Hippocampus	Hypothalamus
Chronic haloperidole treatment			
Control	100 ± 6.1	100 ± 19.9	100 ± 8.3
0.5 mg/kg	96.1 ± 9.8^{ns}	108.9 ± 2.8^{ns}	98.8 ± 11.8^{ns}
2.0 mg/kg	94.3 ± 5.9^{ns}	120.2 ± 19.2^{ns}	102.1 ± 13.7^{ns}
Chronic antidepressant treatment[1]			
Control	100 ± 9.3	100 ± 3.7	100 ± 18.8
Imipramine	165.6 ± 9.9*	204.0 ± 20.8*	112.9 ± 8.9^{ns}
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 $*p < 0.05$, 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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References

Doi, W., Sato, D., Fukuzako, H., Takigawa, M., 2001. c-Fos expression in rat brain after repetitive transcranial magnetic stimulation. Neuroreport 12, 1307–1310.

Drevets, W., Videen, T., Price, J., Preskorn, S., Carmichael, S., Raichle, M., 1992. A functional anatomical study of unipolar depression. Journal of Neuroscience 12, 3628–3641.

George, M.S., Nahas, Z., Kozel, F.A., Li, X., Denslow, S., Yamanaka, K., Mishory, A., Foust, M.J., Bohning, D.E., 2002. Mechanisms and state of the art of transcranial magnetic stimulation. Journal of ECT 18, 170–181.

Grunhaus, L., Schreiber, S., Dolberg, O.T., Polak, D., Dannon, P.N., 2003. A randomized controlled comparison of electroconvulsive therapy and repetitive transcranial magnetic stimulation in severe and resistant nonpsychotic major depression. Biological Psychiatry 53, 324–331.

Levkovitz, Y., Grisaru, N., Segal, M., 2001. Transcranial magnetic stimulation and antidepressive drugs share similar cellular effects in rat hippocampus. Neuropsychopharmacology 24, 608–616.

Lisanby, S.H., Luber, B., Perera, T., Sackeim, H.A., 2000. Transcranial magnetic stimulation, applications in basic neuroscience and neuropsychopharmacology. International Journal of Neuropsychopharmacology 3, 259–273.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S., Weissman, A.M., 1999. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proceedings of the National Academy of Sciences of the United States of America 96, 11364–11369.

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

Padberg, F., Moller, H.J., 2003. Repetitive transcranial magnetic stimulation, does it have potential in the treatment of depression? CNS Drugs 17, 383–403.

Post, A., Keck, M.E., 2001. Transcranial magnetic stimulation as a therapeutic tool in psychiatry, what do we know about the neurobiological mechanisms? Journal of Psychiatry Research 35, 193–215.

Sachdev, P.S., McBride, R., Loo, C., Mitchell, P.M., Malhi, G.S., Croker, V., 2002. Effects of different frequencies of transcranial magnetic stimulation (TMS) on the forced swim test model of depression in rats. Biological Psychiatry 51, 474–479.

Stewart, C.A., Reid, I.C., 2000. Repeated ECS and fluoxetine administration have equivalent effects on hippocampal synaptic plasticity. Psychopharmacology 148, 217–223.

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

Yamada, M., Kiuchi, Y., Nara, K., Kanda, Y., Morinobu, S., Momose, K., Oguchi, K., Kamijima, K., Higuchi, T., 1999. Identification of a novel splice variant of heat shock cognate protein 70 after chronic antidepressant treatment in rat frontal cortex. Biochemical and Biophysical Research Communications 261, 541–545.

Yamada, M., 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.

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

Yasojima, K., Tsujimura, A., Mizuno, T., Shigeyoshi, Y., Inazawa, J., Kikuno, R., Kuma, K., Ohkubo, K., Hosokawa, Y., Ibata, Y., Abe, T., Miyata, T., Matsubara, K., Nakajima, K., Hashimoto-Gotoh, T., 1997. Cloning of human and mouse cDNAs encoding novel zinc finger proteins expressed in cerebellum and hippocampus. Biochemical and Biophysical Research Communications 231, 481–487.

Young, E.A., Haskett, R.F., Murphy-Weinberg, V., Watson, S.J., Akil, H., 1991. Loss of glucocorticoid fast feedback in depression. Archives of General Psychiatry 48, 693–699.

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Review article

Antidepressant-elicited changes in gene expression Remodeling of neuronal circuits as a new hypothesis for drug efficacy

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Abstract

Although antidepressants have been used clinically for more than 50 years, no consensus has been reached concerning their precise molecular mechanism of action. Pharmacogenomics is a powerful tool that can be used to identify genes affected by antidepressants or by other effective therapeutic manipulations. Using this tool, others and we have identified as candidate molecular targets several genes or expressed sequence tags (ESTs) that are induced by chronic antidepressant treatment. In this article, we review antidepressant-elicited changes in gene expression, focusing especially on the remodeling of neuronal circuits that results. This refocusing motivates our hypothesis that this plasticity represents the mechanism for drug efficacy, and thus a causal event for clinical improvement. Defining the roles of these molecules in drug-induced neural plasticity is likely to transform the course of research on the biological basis of antidepressants. Such detailed knowledge will have profound effects on the diagnosis, prevention, and treatment of depression. Consideration of novel biological approaches beyond the "monoamine hypothesis" of depression is expected to evoke paradigm shifts in the future of antidepressant research.

Keywords: Antidepressant; Depression; Microarray; Neural plasticity; Pharmacogenomics

Contents

Abbreviations: ADRG, antidepressant-related gene; BDNF, brain-derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; CSP, cysteine string protein; ECT, electroconvulsive therapy; EST, expressed sequence tag; GAP-43, growth-associated-protein 43; HPA, hypothalamus–pituitary–adrenocortical; LAL, long attack latency; Mss4, mutation suppressor for the Sec4–8 yeast; NGF, nerve growth factor; RDA, representational difference analysis; SAGE, serial analysis of gene expression; SAL, short attack latenc; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TOGA, total gene expression analysis.

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1. Introduction

Depression is one of the major psychiatric diseases and is estimated to affect 12–17% of the population at some point during the lifetime of an individual (Wittchen et al., 1994). Therefore, there is a pressing need to develop new and better antidepressants.

Antidepressants are very effective agents for preventing and treating depression and have been used clinically for more than 50 years. Although the therapeutic action of antidepressants most likely involves the regulation of serotonergic and noradrenergic signal transduction pathways, to date, no consensus has been reached concerning the precise molecular and cellular mechanism of action of these drugs. Many antidepressants acutely regulate monoaminergic signal transduction, resulting in a significant increase in synaptic concentrations of the monoamine noradrenaline or serotonin within a few hours of initial treatment. But at the same time, the onset of the clinical effect of these drugs lags by several weeks. A satisfying explanation for the discrepancy in the acute increase of synaptic monoamines and delayed clinical effect remains elusive. Theories that postulate long-term changes in receptor sensitivity have unsuccessfully tried to bridge this gap (Siever and Davis, 1985). Consequently, the monoamine hypothesis does not fully explain this clear discrepancy. This delayed therapeutic action of antidepressants could result from either the indirect regulation of other neuronal signal transduction systems or the regulation of gene transcription following chronic treatment. An increasingly popular working hypothesis is that drugs of various classes have common antidepressant effects after chronic use because they regulate transcription of the same set of downstream genes.

In this article, we review antidepressant-elicited changes in gene expression, especially focusing on the remodeling of neuronal circuits as a new hypothesis for drug efficacy.

2. Antidepressant-elicited changes in gene expression

Hyman and Nestler (1996) proposed an "initiation and adaptation" model to describe the drug-induced neural plasticity associated with the long-term actions of antidepressants in the brain. However, the detailed mechanisms underlying such drug-induced adaptive neuronal changes are as of yet unknown.

2.1. "Reverse" pharmacological approaches

As shown in Fig. 1, three research directions (i.e., brain system and circuits, neural transduction, and transcriptional regulation) need to be considered to understand biochemical, neurophysiological, and/or morphological changes that underlie the long-term actions of antidepressants. Functional genomics is one powerful tool that can be used to reveal the basic molecular constituents of neurons at transcription level and can help explain how these constituents work together in the brain. Therefore, so-called "reverse" pharmacological approaches, armed with functional genomics/proteomics techniques, are thought to be very effective strategies for researching the mode of actions of antidepressants. Understanding the functional role of these molecules will shed light on the pathogenic mechanisms underlying the development of depression. Such detailed and fundamental knowledge about the brain will have profound effects on the way depression is diagnosed, prevented, and treated. Therefore, it is very

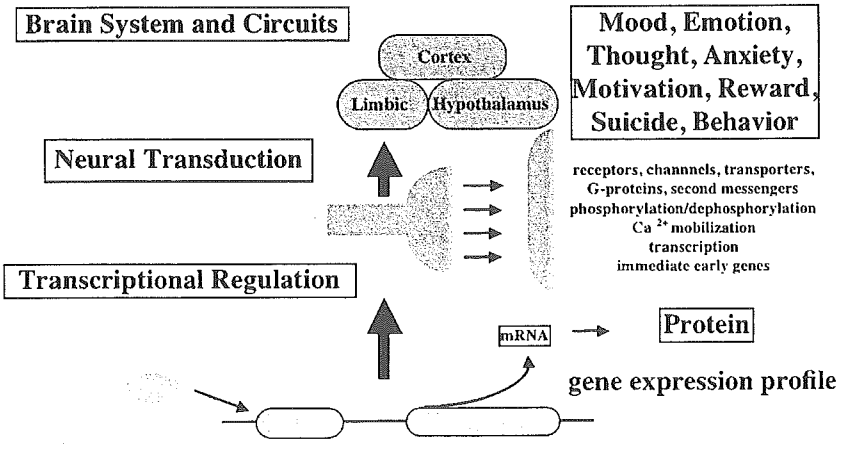

Fig. 1. Three research directions necessary to understand the neural plasticity underlying the long-term actions of antidepressants.

important to understand how antidepressants change the brain in depressed patients.

Interestingly, antidepressants selectively affect certain immediate early genes and transcription factors, including c-fos (Dahmen et al., 1997; Torres et al., 1998), zif268 (Dahmen et al., 1997), NGFI-A (Bjartmar et al., 2000; Johansson et al., 1998), and Arc (activity-regulated role of cytoskeleton-associated protein) (Pei et al., 2000). In addition, the cyclic adenosine monophosphate (cAMP) second messenger system is another pathway that could be involved in antidepressant action. Chronic administration of antidepressants up-regulates the cAMP pathway at several levels, including at points that increase expression and phosphorylation of the cAMP response element binding protein (CREB) (Thome et al., 2000). These molecules may be important for adaptive neuronal changes that occur as a result of chronic antidepressant treatment. Region-specific effects of chronic antidepressant treatment on the DNA-binding activities of CRE-, SP1- and GRE-binding elements have been previously reported in rat hippocampus and frontal cortex (Frechilla et al., 1998). In addition, chronic treatment with citalopram (a selective serotonin reuptake inhibitor, SSRI) induces time-dependent changes in the expression and DNA-binding activity of transcription factor AP-2 in rat brain (Berggard et al., 2003; Damberg et al., 2000).

Together, these data suggest that changes in gene expression may have a role in the mechanism underlying antidepressant action. Therefore, the identification and quantitation of changes in gene expression associated with chronic antidepressant treatment can pave the way for the discovery of novel molecular markers that would be useful for the diagnosis and treatment of depression. For example, Chardenot et al. (2002) recently described significantly enhanced expression of a protein associated with the peripheral-type benzodiazepine receptor (PRAX-1) in rat hippocampus after chronic antidepressant treatment.

2.2. High-throughput methods for gene expression analysis

Recent developments in molecular neurobiology provide new conceptual and experimental tools (i.e., "the open target screen approaches") to investigate, and facilitate understanding of the mechanisms by which antidepressants produce long-lasting alterations in brain function. The emerging techniques and powerful tools derived from the relatively new subfields of genomics and proteomics hold great promise for the identification–independent of any preconceived hypotheses–of genes and gene products that are altered by chronic antidepressant treatment or other effective therapeutic manipulations, such as electroconvulsive therapy (ECT). Pharmacogenomic tools, such as differential display PCR, serial analysis of gene expression (SAGE), total gene expression analysis (TOGA), representational difference analysis (RDA), cDNA microarrays, and GeneChip® (ffymetrics, Santa Clara, CA), are now being used to study antidepressant-elicited changes in gene expression (Table 1). Because these methods are unbiased, the potential exists for identifying completely new classes of drug targets. Novel biological approaches tapping novel hypotheses beyond the "monoamine hypothesis" will definitely cause paradigm shifts to emerge in future antidepressant research.

2.2.1. cDNA microarray and GeneChip®

The recently introduced microarray technique (e.g., cDNA microarray and GeneChip®) permits us to efficiently perform large-scale, coordinated monitoring of gene expression during different functional states found in control and treated animals. The ability to monitor changes in gene expression at the genomic scale would be valuable for identifying factors that contribute to complex neuronal processes associated with antidepressant treatment. This technology also enhances efforts to characterize the structure and function of these genes.

Using GeneChip®, Rausch et al. (2002) studied whole brain kinase mRNA expression in rats treated with antidepressants. Interestingly, protein kinase C (PKC)-delta, PKC-gamma, stress-activated protein kinase, cAMP-dependent protein kinase beta isoform, Janus protein kinase, and phosphofructokinase M are all chronically down-regulated in rats treated with two SSRIs citalopram and fluoxetine. In addition, Landgrebe et al. (2002) reported antidepressant-induced changes in gene expression using cDNA microarrays containing 3624 expressed sequence tags (ESTs); these represented murine genes expressed in the brain. They found that two different antidepressants, paroxetine (a SSRI) and mitrazepine (a noradrenergic and specific serotonergic antidepressant), down-regulated four common genes (a ribosomal protein and three genes of unknown function). These drugs, however, induced very different gene expression profiles. Taken together, these findings suggest that antidepressants with different pharmacologies can have the same molecular targets, but may act on these targets via different primary pathways.

In contrast, tricyclic antidepressants (TCAs) appear to have multiple molecular targets in vitro and in vivo. For example, chronic treatment with the TCA desipramine increases the expression of six genes and decreases the expression of two genes in primary cultures of rat hippocampal cells (Chen et al., 2003). One of the up-regulated genes encodes the neuronal growth cone marker, growth-associated-protein 43 (GAP-43). Interestingly, in situ hybridization also revealed that desipramine increased GAP-43 gene expression in dentate gyrus but not other

Table 1
Open target screen approaches for gene expression analysis

Differential display PCR
Serial analysis of gene expression (SAGE)
Total gene expression analysis (TOGA)
Representational difference analysis (RDA)
cDNA microarrays/GeneChip®

brain regions (Chen et al., 2003). Because GAP-43 regulates growth of axons and modulates the formation of new connections, these findings suggest that desipramine may affect neuronal plasticity in the central nervous system.

Interestingly, noradrenaline also may have multiple molecular targets. Laifenfeld et al. (2002) previously found that noradrenaline treatment increased GAP-43 expression and progressively decreased Oct4 expression in human neuroblastoma SH-SY5Y cells. Using cDNA microarrays, this group also reported increased expression of two neurite-outgrowth promoting genes: neural cell adhesion molecule L1 and laminin. In addition, noradrenaline-treated SH-SY5Y cells display elongated, granule-rich somata and an increased number of neurites. Moreover, in the presence of noradrenaline, cell survival is enhanced, while proliferation is inhibited. Taken together, these results support a role for noradrenaline in processes associated with synaptic connectivity and in mediating the hypothesized neuronal plasticity associated with antidepressant treatment.

By combining genomic tools with sophisticated animal model systems, it will be possible to identify patterns of altered gene expression that are associated with particular features of antidepressant-induced and adaptative changes in the brain. Using SAGE and GeneChip® analysis, Feldker et al. (2003) reported differential expression of several genes in the hippocampus of genetically selected long attack latency (LAL) and short attack latency (SAL) mice. LAL and SAL mice differ in a wide variety of behavioral traits and display differences in the serotonergic system and the hypothalamus–pituitary–adrenocortical (HPA) axis. LAL mice exhibit elevated expression of numerous cytoskeleton genes, such as cofilin and several tubulin isotypes. LAL mice also show elevated expression of several calmodulin-related genes and genes encoding components of the MAPK cascade (e.g., raf-related oncogene and ERK2). These results suggest that differential regulation of the raf/ERK pathway may be related to structural differences in the hippocampus of LAL and SAL mice. As stress-related disorders, such as depression, are also linked to differential regulation of the HPA-axis and the serotonergic system and are associated with altered hippocampal, morphology, differential regulation of these genes may be involved in the pathogenesis of these diseases.

Recently, more and more large-scale expression analyses have been performed or are underway in several laboratories. In the next few years, pharmacogenomics will yield huge amounts of data about antidepressant-associated changes or depression-associated changes in gene expression that are relevant for the development of novel therapeutics. Therefore, it is very important to apply standardized criteria for experimental design and make primary data freely available for comparison and analysis.

2.2.2. *Differential display PCR*

Most known neuronal proteins (i.e., receptors, ion channels, and enzymes) were discovered previously either through biochemical isolation or traditional cloning methods, or through the examination of random sequences of cDNAs. Using a differential cloning strategy, we and other groups have isolated genes that are differentially expressed in the brain after chronic antidepressant treatment (Huang et al., 1997; Nishioka et al., 2003; Wong et al., 1996; Yamada et al., 1999, 2000, 2001, 2002). Independent of any preconceived hypothesis, these genes and proteins have been implicated in a physiological or pathophysiological process. For example, in the amygdala of rats that received daily treatment with the TCA imipramine for 3 weeks, the gene encoding a mutation suppressor for the Sec4–8 yeast (Mss4) transcript was overexpressed (Andriamampandry et al., 2002). This overexpression was also found in the hippocampus of rats treated chronically with two antidepressants having opposite molecular mechanisms of action, the serotonin reuptake enhancer tianeptine and the SSRI fluoxetine.

Using the RNA fingerprinting technique, a modified differential display PCR, we continue our efforts to identify biochemical changes induced by chronic antidepressant treatments. To date, we have cloned hundreds of cDNA candidates as ESTs from rat frontal cortex and hippocampus. Some of these candidate cDNAs should be affected by antidepressants and are thus named antidepressant-related genes (ADRGs).

The benefits and technical limitations of differential display PCR and cDNA microarrays are shown in Table 2. In addition to gene expression monitoring, the cDNA microarray method is useful for the identification of genes because it facilitates the screening of enriched libraries derived from experiments using differential cloning techniques. Therefore, to identify molecular machinery that mediate the therapeutic action of antidepressants, we developed an original cDNA microarray for ADRGs (ADRG microarray) (Yamada and Higuchi, 2002). To develop the ADRG microarray, each of the ADRGs were first amplified using PCR and then spotted in duplicate onto glass slides (Fig. 2) using the modified method of Salunga et al. (1999). Messenger RNA was purified from pooled total RNA using oligo-dT columns, and then converted to cDNA in the presence of either Cy-5 or Cy-3-dUTP to make fluorolabeled probes. Hybridization of the probes to the microarray was done competitively. The probes were mixed and placed on an array, overlaid with coverglass, and hybridized. After the hybridization and washing procedures, each slide was analyzed and gene expression levels were quantified using

Table 2
Comparison of two differential cloning techniques

(1) Differential display PCR
High rate of false positives, "needle-in-a-haystack" approach
Powerful tool to discover completely unknown genes/ESTs
Possible to detect novel splice variants
(2) cDNA microarrays
Ultra-high throughput analysis
Costly, limited accessibility for patented genes
Only genes/ESTs "spotted" on the microarray can be analyzed

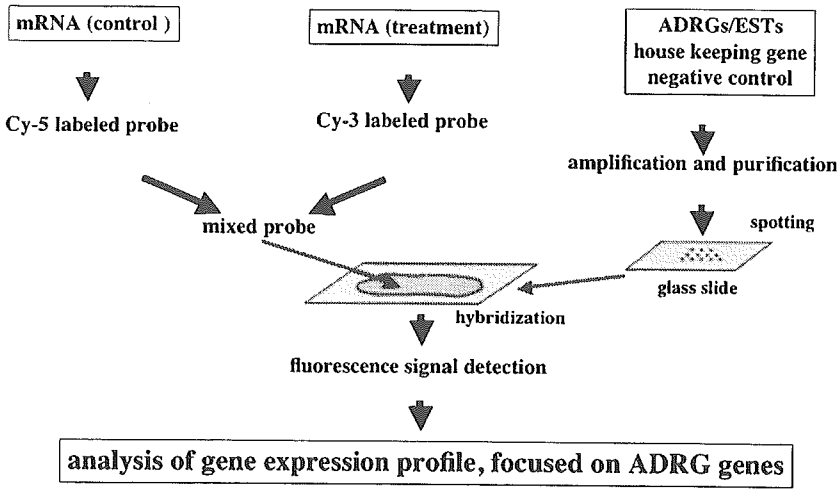

Fig. 2. Fabrication and analysis of ADRG microarray with fluorescent probes.

a PC and ImaGene software (Bio-Discovery Ltd., Swansea, UK). As expected, we obtained low background and consistent results in duplicate experiments. To date, we have identified several interesting genes and ESTs using the ADRG microarray and fluorolabeled probes. A pseudo-color image of an ADRG microarray is shown in Fig. 3.

After normalizing the fluorescent signals using both negative and positive controls, several spots of interest on our ADRG microarray showed increased or decreased fluorescence intensities following chronic antidepressant treatment (Nishioka et al., 2003; Yamada et al., 2000, 2001, 2002). The functional implications of these gene expression changes are currently under investigation.

2.3. Technical limitations and target validation

While rapid technical development of gene expression analysis raises high expectations for the future, many problems and limitations related to these methods need to be considered when interpreting data resulting from such analyses. Microarray analysis can typically identify relatively large differences (>2.0 fold). Because the brain is the most complex organ in our body with many different areas, nuclei, and cell types, the sensitivity of an expression array analysis may be insufficient to detect less abundant but physiologically important changes. Differential gene expression in the brain may also be masked if the mRNA is expressed by a large number of neurons but is regulated only in a subpopulation of these neurons. Furthermore, neuronal populations responsive to antidepressant treatment may be sparse or dispersed in brain. Therefore, the accuracy in dissection of specific brain regions for expression analysis is crucial to determine genuinely altered gene expression profiles. Attempts to reduce variation by examining a single well-defined brain nucleus, or even a single neuron, using laser capture microscopy have been introduced. These methods will have obvious advantages to reduce this limitation.

Gene expression analysis permits the identification of multiple regulated targets. Each target molecule must then be evaluated with traditional approaches (e.g., Northern analysis, quantitative RT-PCR, in situ hybridization, immunoblotting analysis, immunohistochemistry, etc.). Furthermore, data obtained by methods other than expression profiling

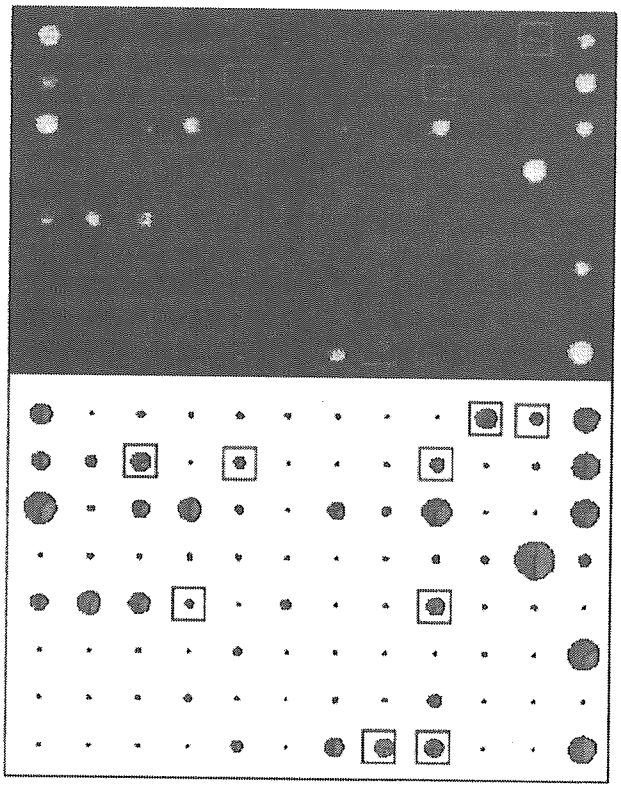

Fig. 3. Pseudo-color image of ADRG microarray after hybridization with fluorescent probes. Ninety-six spots representing ADRG 1–96 are shown here. The pseudo-color images of control group data (green) and antidepressant-treated group (red) were overlapped. The spots within the blue rectangles represent genes up-regulated by chronic antidepressant treatment. The spots within the pink rectangles represent genes down-regulated by chronic antidepressant treatment.

(such as genome sequence data, single nucleotide polymorphism data, homology data, molecular pathway data, etc.) must be combined with gene expression data to achieve a better understanding of protein function or drug treatments. In addition to pharmacological validations, novel drug targets can be validated by characterizing the neurobiological function of a target under normal conditions and by studying the target in behavioral models of depression. Unfortunately, animal models for depression are less straightforward when compared to those of other physical illnesses. Thus, despite all the best preclinical validation of a putative target of an antidepressant, it remains unclear whether "a drug aimed at the novel molecular target" will have the desired clinical effects in patients.

3. Neural plasticity, remodeling of neuronal circuits, and a new hypothesis

Many of the previous reports describing mechanisms of antidepressant action have focused on acute changes in synaptic pharmacology, especially on neurotransmitter turnover and neurotransmitter receptor changes. To advance our understanding of the therapeutic actions of antidepressants, we must now extend our efforts beyond theories based on the simple pharmacology of the synapse. This new effort must seek a deeper understanding of cellular and molecular neurobiology as well as examine the architecture and function of relevant neural systems. Many now believe that changes in brain gene expression, which are elicited after chronic antidepressant treatment, might underlie the drug-induced neural plasticity associated with the long-term actions of antidepressants in the brain and their clinical effects. Table 3 summarizes possible neuroplastic changes induced after chronic antidepressant treatment.

3.1. Survival of neurons and neurogenesis in the hippocampus

Although depression involves many psychological and social factors, it also represents a biological process: the effects of repeated exposure to stress on a vulnerable brain.

Table 3
Possible neuroplastic changes induced after chronic antidepressant treatment

(1) Functional neuroplastic changes
Vesicular docking/fusion/exocytotic machinery
Neurotransmitter release
Post-synaptic signal transduction system
(2) Morphological neuroplastic changes (remodeling of the neural circuits)
Vesicular docking/fusion/exocytotic machinery
Sprouting, neurite outgrowth
Neurotrophic factors
Neuronal death and survival
Axon guidance
Neurogenesis and new neural circuits

Preclinical and clinical research has focused on the interactions between stress and depression and their effects on the hippocampus (Duman et al., 1999; McEwen, 2000). The hippocampus is one of several brain regions that, when exposed to stressful stimuli, can contribute to the emotional, cognitive, and vegetative abnormalities found in depressed patients. This region of the brain is also involved in the feedback regulation of the hypothalamus–pituitary–adrenal axis, the dysfunction of which is associated with depression (Young et al., 1991). Studies suggest that stress-induced atrophy and loss of hippocampal neurons may contribute to the pathophysiology of depression. Interestingly, hippocampal volume is decreased in patients with stress-related psychiatric illnesses, including depression and post-traumatic stress disorder (Sapolsky and Duman, 2000; Sheline et al., 1996).

In vitro and in vivo data provide direct evidence that brain-derived neurotrophic factor (BDNF) is one of the key mediators of the therapeutic response to antidepressants (D'Sa and Duman, 2002). BDNF promotes the differentiation and survival of neurons during development and in the adult brain, as well as in cultured cells (Memberg and Hall, 1995; Palmer et al., 1997; Takahashi et al., 1999). Stress decreases the expression of BDNF, and reduced levels could contribute to the atrophy and compromised function of stress-vulnerable hippocampal neurons. In contrast, antidepressant treatment increases the expression of BDNF in the hippocampus, and could thereby reverse the stress-induced atrophy of neurons or protect these neurons from further damage (Duman, 1998; Duman et al., 1997). These findings have resulted in the development of a novel model of the mechanism of antidepressant action and have suggested new targets for the development of therapeutic agents.

While hippocampal volume can decrease in disease, the hippocampus is also one of only a few brain regions where the production of neurons normally occurs throughout the lifetime of several species of animals, including humans (Eriksson et al., 1998). Hippocampal neurogenesis is influenced by several environmental factors and stimuli (Gould and Tanapat, 1999; Nilsson et al., 1999; van Praag et al., 1999). For example, both acute and chronic stress cause decreases in cell proliferation (Fuchs and Flugge, 1998). On the other hand, administration of several different classes of antidepressant, as opposed to non-antidepressant, agents increases the number of BrdU-labeled cells, indicating that this is a common and selective action of antidepressants (Malberg et al., 2000). In addition, recent evidence indicates that electroconvulsive seizures (an animal model of ECT in humans) can also enhance neurogenesis in rat hippocampus (Hellsten et al., 2002; Madsen et al., 2000; Scott et al., 2000). These findings raise the possibility that increased cell proliferation and increased neuronal number may be a mechanism by which antidepressant treatment mitigates stress-induced atrophy and loss of hippocampal neurons, and thus may contribute to the therapeutic actions of antidepressant treatment. Furthermore, increased formation

of new neurons in the hippocampus related to antidepressant treatment may lead to altered expression of genes specifically expressed in immature neurons. Therefore, observed changes in gene expression may reflect alterations in cell composition of the tissue rather than changes in individual neurons.

3.2. Neurotransmitter release and vesicular exocytotic machinery

Considerable evidence indicates that VAMP-2 is a key component of the synaptic vesicle docking/fusion machinery that forms the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex (Bennett et al., 1992; Oyler et al., 1989; Trimble et al., 1988; Weis and Scheller, 1998). As shown in Fig. 4, the SNARE complex consists of proteins on the target membrane, called t-SNARE, and proteins on the vesicular membrane, called v-SNARE. SNAP-25 and syntaxin are t-SNARE proteins, whereas VAMP-2 is a v-SNARE protein. Fusion of vesicles with the plasma membrane leads to exocytosis, which mediates the release of neurotransmitter into the synapse. Previously, we demonstrated a significant increase of both VAMP2 mRNA and protein levels in rat frontal cortex after chronic treatment with two different classes of antidepressants, imipramine and sertraline, and also with repeated ECT (Yamada et al., 2002). In this context, pharmacological modulation of VAMP2 gene expression would also be predicted to alter neurotransmitter release. Our data suggest that VAMP2 may be one of the common functional molecules induced after chronic antidepressant treatment. Interestingly, the work of others shows that acute and chronic administration of antidepressants diminishes the release of glutamate and aspartate, and inhibits veratridine-evoked 5-HT release (Golembiowska and Dziubina, 2000). An important feature of the action of antidepressants and ECT is that they do not globally alter the expression of other membrane-trafficking proteins. In contrast to the enhanced expression of VAMP2, we detected no significant change in the expression of other synaptic vesicle proteins (syntaxin-1 and SNAP-25). Although there are more than a dozen synaptic vesicle proteins (Sudhof, 1995), we chose to investigate the expression of syntaxin-1 and SNAP-25 because they make a SNARE-complex with VAMP2 and mediate the synaptic vesicle docking/fusion machinery. We reasoned that a coordinated change of VAMP2 and the expression of syntaxin-1 and SNAP-25 might signal a change in the overall number of SNARE complexes. An antidepressant-induced change in the expression of syntaxin-1 and SNAP-25, associated predominantly with the presynaptic plasma membrane, would have been indicative of more complex changes in the transmitter secretory pathway, such as an increase in the number of active zones. Instead, the absence of such a coordinated change in syntaxin-1 and SNAP-25 expression indicates that antidepressants or ECT produces a more selective modification of the regulated secretory machinery. Additional work will be necessary to understand the role of selective VAMP2 induction in rat frontal cortex.

As mentioned above, the CREB phosphorylation pathway could be involved in antidepressant action. Among the multiple target genes that could be regulated by CREB is BDNF (Duman, 1998; Duman et al., 1997). BDNF promotes long-term potentiation at hippocampal CA1 synapses via a presynaptic enhancement of synaptic transmission during high-frequency stimulation (HFS). Pozzo-Miller et al. (1999) showed that heterozygous mice with BDNF knockout display more pronounced synaptic fatigue

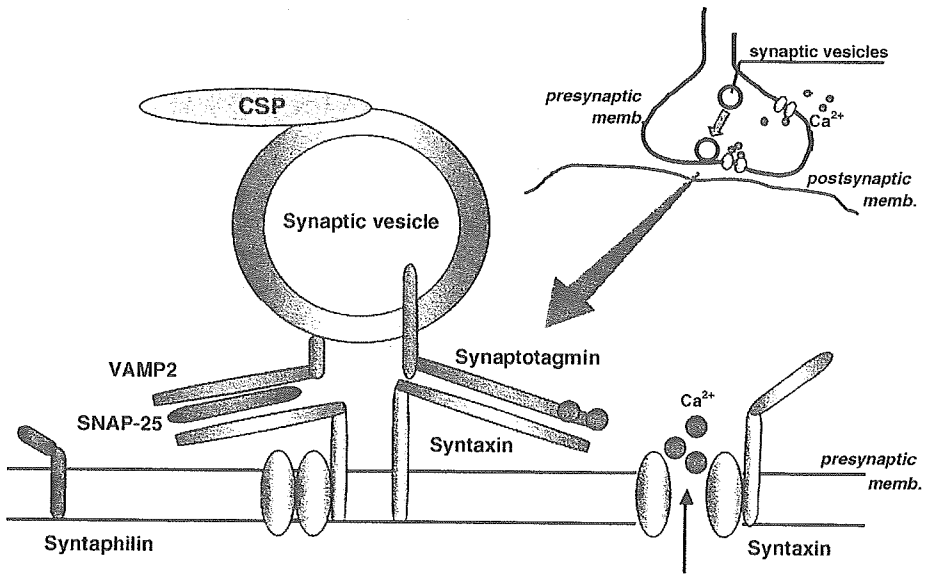

Fig. 4. SNARE complex at synapses.