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Mss4 Gene Is Up-Regulated in Rat Brain after Chronic Treatment with Antidepressant and Down-Regulated When Rats Are Anhedonic

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ABSTRACT

Differential display reverse transcription-polymerase chain reaction was used to identify mRNAs that are differentially expressed in the brain of rats treated chronically with the reference tricyclic antidepressant, imipramine, in comparison with control rats. The gene encoding for a mutation suppressor for Sec4-8 yeast (*Mss4*) transcript is overexpressed in the amygdala of treated rats after 3 weeks of daily administration. This overexpression is also found in the hippocampus of rats treated chronically with either tianeptine or fluoxetine. Mss4 protein has the properties of a guanine nucleotide exchange factor, interacting with several members of the Rab family implicated in Ca^{2+}-dependent exocytosis of neurotransmitters. Mss4 was also overexpressed in other brain structures as judged by in situ hybridization. The kinetics of the up-regulation of Mss4 gene expression measured by Northern blot during the imipramine, tianeptine, or fluoxetine treatments are consistent with an antidepressant effect that occurs after 3 weeks. In rats in which anhedonia was induced by chronic mild stress during 3 weeks, Mss4 transcripts were specifically down-regulated in hippocampus and amygdala compared with control rats. It is proposed that Mss4 protein, which stimulates exocytosis in vivo, participates in the potentiation of the activity of neurotransmitter pathways implicated in the action of several antidepressants and constitutes one of the common functional molecules induced after chronic antidepressant treatment.

Imipramine is a representative member of the family of tricyclic antidepressant drugs and is currently used in the treatment of depression. The immediate effect of antidepressant drugs consists either in the inhibition of the reuptake of biogenic amines or in the decrease of monoamine oxidase activity; these effects form the basis of the monoamine hypothesis of depression. However, one of the strongest arguments against this theory is the slow onset of the action of antidepressant drugs: the beneficial therapeutic effects are seen only after 2 to 3 weeks of treatment. In addition, several drugs that neither inhibit the uptake of serotonin/noradrenaline nor decrease monoamine oxidase activity possess real antidepressant properties (Leonard, 1994). At the present time, the mechanism of mood disorders and the mode of action of antidepressants are far from being completely understood. The slow onset of action of these drugs probably reflects neuronal adaptive changes and plasticity of synaptic activity. Consequently, dysfunction of basic mechanisms underlying gene expression and neural plasticity is likely to play a significant role in the pathogenesis of depression. Antidepressant drugs may produce a therapeutic response by correcting or compensating these adaptive mechanisms (Duman et al., 1999). Up to now, several laboratories have demonstrated a role of chronic antidepressant treatment in up-regulating the cyclic AMP signal transduction cascade linked to the stimulation of many norepinephrine and serotonin receptors (Montminy, 1997). In this cascade, the transcription factor cyclic AMP response element binding (CREB), which controls expression of many genes, seems to play a pivotal role (Nibuya et al., 1996). However, other currently unidentified genes are probably involved in the mechanism of the therapeutic effect of antidepressants.

We have used differential display reverse transcriptase PCR (DD-RT-PCR) to identify changes in gene expression that occur in the amygdala, a crucial limbic structure of the brain, of rats treated for up to 3 weeks with a daily injection of a therapeutic dose of imipramine. The amygdala is thought to be largely involved in mood regulation and homeostasis and plays a fundamental role in the control of emotions (Maren, 1999). Differential display patterns were examined 21 days after chronic imipramine treatment and compared with saline-injected control rats.

ABBREVIATIONS: CREB, cyclic AMP response element binding; Dss4, dominant suppressor of Sec4; Mss4, mutation suppressor for Sec4-8 yeast; DD-RT-PCR, differential display reverse transcriptase polymerase chain reaction; DEPC, diethyl polycarbonate; DTT, dithiothreitol; SSC, standard saline citrate; bp, base pair; CSP, cysteine string protein; kb, kilobase pair(s).

A specific gene product, Mss4, involved in the regulation of exocytose in brain, was found to be overexpressed in amygdala of rats treated with imipramine. cDNA probes were used to study this specific gene expression in other brain regions, including the hippocampus. Limbic regions of the brain, particularly the hippocampus, have been reported to show atrophy and loss of neurons in response to chronic stress (Gould et al., 1992; Sheline et al., 1996). Moreover, the volume of the hippocampus is reduced in humans suffering from depression (Ongur et al., 1998; Bremner et al., 2000). Finally, given that the up-regulation of this gene by chronic imipramine administration indicates its possible implication in the regulation of mood, a study was undertaken to examine its level of expression in anhedonic rats. Interestingly, this subsensitivity to reward is reversed by chronic treatment with antidepressant drug, including imipramine (Willner et al., 1987), and the chronic mild stress model used fulfills many validating criteria as an animal model for depression (Willner, 1997). We demonstrate that, in this model, *Mss4* is down-regulated in limbic structures of the rat brain.

Materials and Methods

Chronic Administration of Antidepressant. Six adult male Wistar rats (250–300 g) were injected i.p. with imipramine hydrochloride (10 mg/kg; Sigma, St. Louis, MO) or tianeptine sodium salt (15 mg/kg; batch 43275; Institut de Recherche International, SERVIER, France) or administered orally with fluoxetine (15 mg/kg; Sigma) at 10 AM each day for 21 days. A similar volume of 0.9% NaCl was administered to six control animals. All the animals were killed 24 h after the last injection. Their brains were removed and the amygdalae and hippocampi were rapidly dissected under sterile conditions and stored in liquid nitrogen until used.

RNA Isolation. Total cellular RNA was extracted from pooled frozen tissue obtained either from six treated or six control animals, using a previously described protocol (Chomczynski and Sacchi, 1987). The isolated RNA pellet was dissolved in 50 μl of diethyl polycarbonate (DEPC)-water and treated with DNase-I RNase-free (Promega, Madison, WI) to remove contaminating DNA.

DD-RT-PCR Analysis. DNA-free total RNA (3 μg) was used for RT reactions using 12 different oligo(dT)$_{11}$VN (V represents A, G, or C, and N represents A, T, G, or C), extended at their 5′ ends with a 17-mer oligonucleotide corresponding to the 3′ end of T7 promoter, as anchored primers. In combination with 20 different arbitrary primers, each constituted of a 10-mer core annealing sequence and a 16-base portion of an M13 reverse priming sequence, differential display analyses were performed in duplicate as described previously (Liang et al., 1993) modified according to the protocol from Genomyx/Beckman (Foster City, CA). Total amygdala RNA (1 μl; 250 ng) was added to each RT tube containing 3 μl of each 25 μM oligo(dT)$_{11}$VN primer and 9.5 μl of DEPC-water. The RNA was denatured at 70°C for 10 min and immediately cooled in ice. The RT master-mix (16.5 μl of 5× RT buffer containing 50 mM DTT and 250 μM concentrations of each dNTP in DEPC-water) and 1.5 μl of Moloney murine leukemia virus RT (300 U) were added to the sample and annealed for 10 min at room temperature before incubation at 40°C for 1 h. Samples were then heated at 95°C for 5 min to inactivate the RT and cooled in ice if used immediately or kept at −20°C for later use. Each DD-RT-PCR tube contained 1× PCR buffer containing 1.5 mM of $MgCl_2$, 50 μM concentrations of each dNTP, 0.5 μl of [α-^{33}P]dATP (10 μCi/μl), 4 μM oligo(dT)$_{11}$VN primer, 4 μM arbitrary primer, 1.5 μl of the corresponding RT product, and 0.05 units/μl of *Taq* polymerase in a total volume of 20 μl. PCR was started at 95°C for 2 min followed by four cycles at low annealing temperature (92°C for 15 s, 46°C for 30 s, 72°C for 2 min) and 35 cycles at high annealing temperature (92°C for 15 s, 60°C for 30 s, and 72°C for 2 min). The DD-RT-PCR was terminated by an elongation step of 7 min at 72°C. DD-RT-PCR products were separated on 6% denaturing Tris-borate-EDTA/polyacrylamide gel electrophoresis gels (HR-1000; Genomyx, Foster City, CA) for 2.5 h at 100 W, 2500 V, and 40°C. After the electrophoresis, the gel was dried and autoradiographed (BioMax MR, 33 × 61 cm; Eastman Kodak, Rochester, NY). After developing the film, the patterns of amplified cDNA bands were compared. The autoradiogram and the gel were oriented with needle punches and only DD-PCR bands with higher intensity, suggesting a significant overexpression of the gene represented in the band, were collected and PCR-reamplified. Because PCR is a semiquantitative method, modest differences in band intensity may not necessarily be attributable to differences in gene expression. The gel band PCR reamplification was performed with the full-length M13 reverse primer (AGCGGATAACAATTTCACACAGGA) and the T7 promoter sites (GTAAATACGACTCACTATAGGGC) as primers. This procedure provides experimentally useful amounts of each cDNA for sequencing without subcloning.

Probe Labeling. A PCR-amplified 184-bp fragment from Mss4 cDNA using 5′CAGCAATCCTGATGGTGATG3′ and 5′CTCCAAGGCCACATAGAAGC3′ as left and right primers was either ^{33}P- or ^{35}S-labeled by random priming. The amplified cDNA fragment was added to a mixture of hexanucleotide primers and heat-denatured for 5 min. The incubation was conducted in a 30-μl total volume in the presence of 3× random buffer containing dATP, dGTP, and dTTP, 1 μl of Klenow fragment (5 units/μl) and 5 μl of [α-^{33}P]dCTP (3000 Ci/mmol) or [α-^{35}S]dCTP (800 Ci/mmol) for 4 h at room temperature. The labeled probe was purified by centrifugation through a G-50 Sephadex column and stored at −70°C.

The antisense and sense mRNA probes were generated by in vitro transcription reaction. PCR was first run using the combination of the 5′ end 10-mer oligonucleotide of the 184-bp cDNA fragment extended with a 16-mer oligonucleotide corresponding to the 3′ end of T3 promoter (5′GGGAGACTAGTGTCAGCAGCAATCCT3′) and the 3′ end 10-mer oligonucleotide of the 184-bp cDNA extended with a 17-mer oligonucleotide corresponding to the 3′ end of the T7 promoter (5′ACGACTCACTATAGGGCCTCCAAGGCC3′) as primers. The PCR product was then reamplified with the complete sequences of T3 and T7 promoter. In vitro transcription reaction was performed in a total volume of 25 μl [containing 5 μl of 5× transcription buffer, 40 mM dithiothreitol (DTT), 1 μl of recombinant RNasin ribonuclease inhibitor, 1 μg of PCR-extended cDNA, 1 μl of 10 mM rATP, 1 μl of 10 mM rUTP, 1 μl of 10 mM rGTP, 1 μl of 1 mM rCTP, 1 μl of 1 M DTT, 1 μl of RNase block inhibitor, 5 μl of [α-^{35}S]CTP (800 Ci/mmol), 10 units of T3 or T7 RNA polymerase, and DEPC-treated water] and incubated at 37°C for 1 h. The DNA template was removed by incubating the transcript with 10 units of RNase-free DNase/μg of DNA at 37°C for 15 min. The RNA probe was extracted with phenol/chloroform and precipitated with ethanol in the presence of 3 M sodium acetate, resuspended in 20 μl of water and stored at −70°C.

Northern Blot. Pooled hippocampus total RNA (10 μg) from six treated rats and six controls was subjected to electrophoresis in 1.0% agarose-formaldehyde gel and transferred to Hybond membranes (Amersham Biosciences). Hybridization and washing conditions were carried out as described previously (Kalinyak and Perlman, 1987).

In Situ Hybridization on Frozen Brain Sections. Three rats were injected with imipramine or saline as described above for 3 weeks. Twenty-four hours after the last injection, animals were intracardially perfused with paraformaldehyde. The brains were collected, frozen in nitrogen liquid, and cryostat-sectioned (20 μm sections). Frozen slides with sections were immediately immersed in an acetone bath for 3 to 5 min to remove lipids, incubated in a solution of 0.1 M triethanolamine, pH 8.0, and finally, in a triethanolamine/0.25% anhydride acetic acid bath. After two rinses with 2× SSC, the slides were prehybridized and hybridized with the appro-

priate probe as described previously (Andriamampandry et al., 1998). Slides were incubated in prewarmed 1× SSC containing 50% formamide at 60°C for 10 min and dehydrated successively with 50, 75, and 100% cold ethanol solutions. The slides were finally dried at room temperature.

A ^{35}S-labeled probe (25,000 cpm/μl) was diluted with the following medium: 50% formamide, 0.6 M NaCl, 10 mM Tris buffer, pH 7.5, 10 mM Denhardt's solution, 1 mM EDTA, 500 μg/ml t-RNA, 1 mM DTT, 500 mg/ml dextran sulfate, and salmon sperm DNA and incubated at 50°C for 2 h. Fifty microliters of this dilution was added to each slide and incubation was performed at 52°C overnight. The slides were washed twice with 1× SSC containing 50% formamide at 55°C for 1 h followed by two rinses at room temperature for 5 min. They were then incubated in a 1× NaCl-Tris-EDTA solution in the presence of 3 μg/ml of RNase at 37°C for 30 min. Finally, the slides were washed twice with 2× SSC, 50% formamide at 55°C for 1 h, 0.1× SSC at 55°C for 15 min and dehydrated successively with 50%, 75%, and chilled absolute ethanol solution. Once the slides were dried, they were autoradiographed at room temperature for 3 to 7 days. Autoradiographic microscales were used for calibration of autoradiographs.

Chronic Mild Stress Model of Depression in Rats. The protocol of Willner et al. (1987) was used for these experiments. Briefly, seven adult male Wistar rats (250–300 g) were submitted to chronic sequential exposure of a variety of mild unpredictable stressors for 21 days (food or water deprivation, continuous lighting, cage tilt, grouped housing, etc.). Chronic mild stress-induced anhedonia was estimated by measuring the decrease in the consumption of a palatable (1%) sucrose solution, compared with seven control rats housed under normal conditions. After 3 weeks, the stressed rats presented a significant decrease in sucrose consumption and were sacrificed together with their corresponding controls. The brains were removed and several brain regions of interest (including amygdalae and hippocampi) were rapidly dissected under sterile conditions and stored in liquid nitrogen until use.

Sequence Analysis. The sequences were analyzed by using the GCG Winsconsin Software package including Fasta, Bestfit, and Fetch (Genetic Computer) and the GenBank/EMBL database.

Results

Differential Expression of Mss4 mRNA in the Amygdalae of Rats Undergoing Long-Term Treatment with Imipramine. Differential display analysis was performed in duplicate on total RNA from amygdala to identify specific changes in gene expression in response to chronic imipramine administration to rats. We systematically compared mRNA display patterns between saline-injected rats, as controls, and imipramine-treated rats. Thirty-nine bands exhibited consistent differential expression between control and treated rats; 18 bands indicated mRNA overexpression, but only those that showed at least 2-fold higher relative densitometric intensity were selected. Five bands with these selection criteria were reamplified and sequenced. Under these conditions, a 330-bp cDNA fragment was identified (Fig. 1) and the sequence analysis revealed 100% homology with the *Rattus norvegicus* mRNA for Mss4 protein (Fig. 2). This cDNA fragment was barely detectable in control animals injected with saline. In imipramine-treated rats, two bands were seen to be up-regulated (Fig. 1A). The upper band corresponded to Mss4, whereas the lower one did not correspond to a known sequence and was therefore not selected for further analysis. To confirm the overexpression of *Mss4* gene by long-term imipramine treatment, we performed Northern blot analyses using a ^{35}S-labeled antisense probe generated from the isolated cDNA fragment (Fig. 1B).

Overexpression of *Mss4* Gene in the Hippocampus: Effects of Imipramine, Tianeptine and Fluoxetine. These studies aimed to demonstrate that the *Mss4* gene was also overexpressed in the hippocampus after long-term imipramine, tianeptine, or fluoxetine treatment for 3 weeks. For each compound, total RNA were prepared from three control rats and three treated rats and Northern blots were run using the isolated ^{33}P-labeled Mss4 cDNA fragment as probe. In Fig. 3, densitometry analysis showed that the expression of Mss4 mRNA was increased by +104% with imipramine, +220% with tianeptine, and +189% with fluoxetine compared with untreated rats. These results indicate that the up-regulation of *Mss4* gene expression also occurs in the hippocampus of treated rats. However, fluoxetine also potentiated Mss4 expression in the olfactory bulbs (+35%), whereas no significant effect was noted in the amygdala (data not shown).

The pattern of expression of *Mss4* gene was also studied during the time course of imipramine or tianeptine effects, in the hippocampus of rats treated for 7, 14, and 21 days. Under these conditions, the expression of Mss4 mRNA was similar to the control values after the first week of treatment and increased after the second and the third week [+67 and +104% with imipramine, respectively; +180% and +220% with tianeptine, respectively, as judged by densitometry analysis (Fig. 4)].

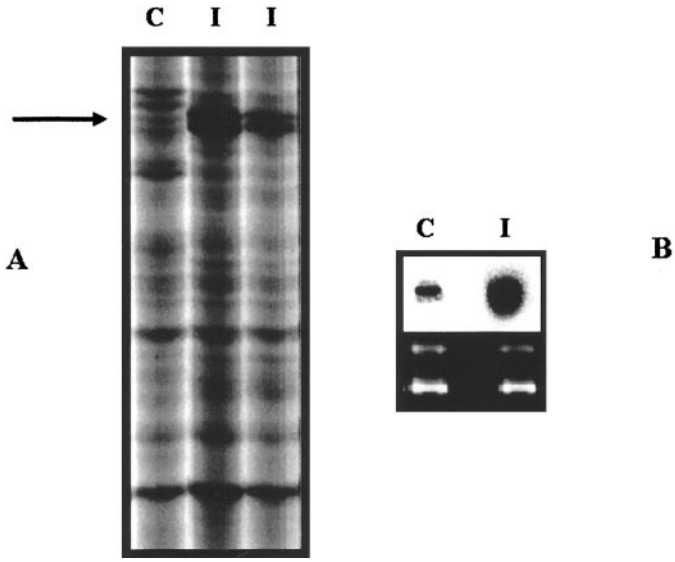

Fig. 1. A, representative autoradiogram resulting from PCR differential display of Mss4 gene expression in rat brain amygdala between chronically imipramine treated animals (Im) and controls (C). Differential displays were performed in duplicate using ACGACTCACTATAGGGCTTTTTTTTTTTTAA as the oligo(dT) anchored primer and ACAATTTCACACAGGAGACCATTGCA as arbitrary primer. The reaction products were run on a denaturing gel and bands showing intensity differences were excised, eluted from the gel. The arrow indicates two reproducible PCR-amplified cDNA fragments that seem to be induced in imipramine treated rats. The upper band, which corresponds to Mss4 cDNA, was subjected to PCR reamplification, whereas the lower band, which represents an unknown gene, was not selected for further analysis. B, Northern blot analysis of amygdala total RNA samples from control and imipramine-treated rats confirm the overexpression of Mss4 gene in treated rats. An equal amount (10 μg) of total RNA from each sample was loaded on the gel as shown by 28S (4.9 kb) and 18S (1.9 kb) rRNA bands and transferred to a Hybond membrane. The antisense ^{35}S-labeled probe was obtained by in vitro transcription using the complete T7 promoter sequence in the PCR-amplified cDNA fragment.

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                                                10        20        30
dd17.s                                  CTAGTGTCAGCCGAAGGCCGGAACCGGAAG
                                        ||||||||||||||||||||||||||||||
rnMss4 GCAATGGAACCCTGCGAGCTGCAGAACGAGCTAGTGTCAGCCGAAGGCCGGAACCGGAAG
              730       740       750       760       770       780

               40        50        60        70        80        90
dd17.s GCGGTGCTGTGCCAGCGTTGTGGCTCTCGGGTGCTGCAGCCAGGGACTGCTCTTTTCTCT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
rnMss4 GCGGTGCTGTGCCAGCGTTGTGGCTCTCGGGTGCTGCAGCCAGGGACTGCTCTTTTCTCT
              790       800       810       820       830       840

              100       110       120       130       140       150
dd17.s CGCCGGCAGCTTTTCCTCCCCTCCATGAGGAAGAAGCCGGATCTGGTGGACGGCAGCAAT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
rnMss4 CGCCGGCAGCTTTTCCTCCCCTCCATGAGGAAGAAGCCGGATCTGGTGGACGGCAGCAAT
              850       860       870       880       890       900

              160       170       180       190       200       210
dd17.s CCTGATGGTGATGTCCTCGAGGAACACTGGCTGGTTAATGACATGTTCATCTTTGAGAAC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
rnMss4 CCTGATGGTGATGTCCTCGAGGAACACTGGCTGGTTAATGACATGTTCATCTTTGAGAAC
              910       920       930       940       950       960

              220       230       240       250       260       270
dd17.s GTGGGCTTTACCAAGGACGTGGGCAACGTCAAGTTTCTAGTCTGTGCAGACTGTGAGATT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
rnMss4 GTGGGCTTTACCAAGGACGTGGGCAACGTCAAGTTTCTAGTCTGTGCAGACTGTGAGATT
              970       980       990      1000      1010      1020

              280       290       300       310       320       330
dd17.s GGACCCATTGGCTGGCATTGCCTAGATGACAAGAACAGCTTCTATGTGGCCTTGGAGCGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
rnMss4 GGACCCATTGGCTGGCATTGCCTAGATGACAAGAACAGCTTCTATGTGGCCTTGGAGCGA
             1030      1040      1050      1060      1070      1080
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Fig. 2. Search for homology by FASTA in Genbank/EMBL database. The 330-bp cDNA (dd17.s) isolated by DD-RT-PCR showed 100% identity with *R. norvegicus* mRNA for Mss4 (2.5 kb; gb_ro:rnMss4).

In Situ Hybridization. In situ hybridization experiments were performed to explore the distribution of Mss4 expression in the brain of rats treated for 3 weeks with imipramine rats versus controls using the 184-bp cDNA or sense mRNA as probes. As shown in Fig. 5i, the ^{35}S-sense mRNA probe did not label any specific region. On the other hand, a quantitative autoradiography analysis showed significant increases in Mss4 expression in olfactory bulbs (+550%), striatum (+400%), cerebellum (+377%), ventral hippocampus (+345%), dorsal hippocampus (+311%), amygdala (+165%), and cortex (+160%). These values represent the percentage increases of *Mss4* gene expression in imipramine-treated rats (Fig. 5, a–d) compared with nontreated rats (Fig. 5, e–h) using four independent measures per brain region. The S.E.M. on each percentage was always below 35%. These experiments confirmed the overexpression of *Mss4* gene in hippocampus (dorsal and ventral) and in the amygdala. However, this overexpression was also important in the cortex, olfactory tractus, striatum, and cerebellar cortex. The other brain regions exhibited a very low level of *Mss4* gene expression.

***Mss4* Gene Expression in Anhedonic Rats.** The level of expression of Mss4 was studied in several brain regions of rats submitted to mild repetitive stress for 3 weeks. These rats exhibited an anhedonic behavior (subsensitivity to rewards) characterized by a decrease in the consumption of sucrose (Monleon et al., 1995). Anhedonia is the core symptom of depressive illness and the chronic mild stress model of depression possesses a good validity (Willner et al., 1997), in

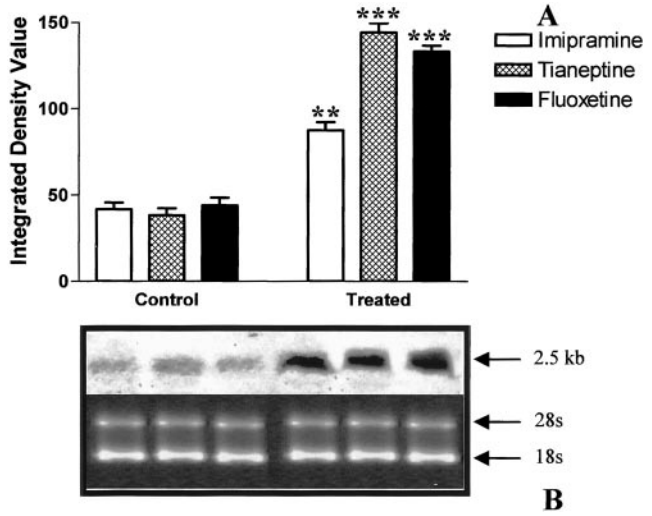

Fig. 3. Effects of imipramine, tianeptine or fluoxetine on *Mss4* gene expression. An equal amount (10 μg) of total RNA from each sample was loaded on the gel as shown by 28S (4.9 kb) and 18S (1.9 kb) rRNA bands and transferred to a Hybond membrane. A, spot densitometry of the blot (percentage integrated density ± S.D.). B, Northern blot of pooled hippocampus total RNA from control rats and rats treated for 3 weeks with imipramine, tianeptine, or fluoxetine, labeled with [^{33}P]Mss4 cDNA probe. Three control rats and three rats treated with imipramine, tianeptine, or fluoxetine were used for each time point and experiments were performed in triplicate. Student's *t* test was applied for the significance of data. ***, $p < 0.001$; **, $p < 0.01$.

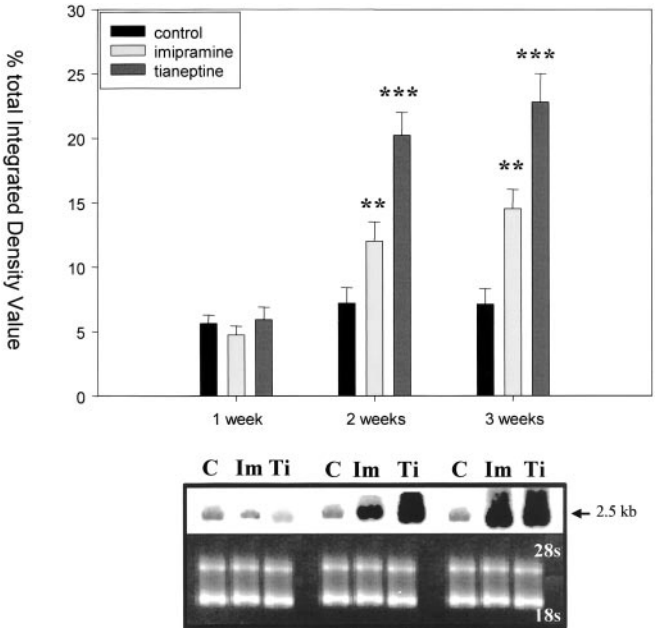

Fig. 4. Kinetics of *Mss4* gene expression. A, spot densitometry of the blot (percentage integrated density ± S.D.). B, Northern blot of pooled hippocampus total RNA from control rats (C) and rats treated with imipramine (Im) and tianeptine (Ti) for 1, 2, and 3 weeks labeled with [^{33}P]Mss4 cDNA probe. Three control rats, three rats treated with imipramine, and three rats treated with tianeptine were used for each time point and experiments were performed in duplicates. An equal amount (10 μg) of total RNA from each sample was loaded on the gel as shown by 28S (4.9 kb) and 18S (1.9 kb) rRNA bands. Student's *t* test was applied for the significance of data. ***, $p < 0.001$; **, $p < 0.01$.

particular because it is sensitive to imipramine at the doses we used; i.e., the attenuation of sucrose consumption in mice by chronic mild stress is restored by long-term administration of several antidepressants, including imipramine (Monleon et al., 1995). Under our conditions, the stressed rats exhibited a decrease of body weight and of sugared water consumption of 20 and 35%, respectively, compared with control rats, but the total intake of water remained stable (Fig. 6). A significant decrease in the signal obtained with the Mss4 probe was detected by Northern blotting of total RNA from the amygdalae and hippocampi of anhedonic rats (minus 55 and 50%, respectively). Other brain regions tested show no significant changes (Fig. 7). Thus, Mss4 expression is down-regulated in the hippocampi and amygdalae of anhedonic rats, whereas long-term administration of the antidepressant drug imipramine induced overexpression of this gene in the same brain regions.

Discussion

Mss4, a homolog of the yeast dominant suppressor of Sec4 (Dss4), was cloned from a rat brain cDNA library on the basis of its ability to suppress Sec4 defects when expressed in the sec4-8 yeast secretory mutant (Burton et al., 1993). Dss4 was identified as a spontaneous suppressor of temperature sensitive, secretory defects in Sec4 protein, a yeast Rab required for vesicle transport from the Golgi to the plasma membrane (Moya et al., 1993). Rab GTPases comprise a large family regulating discrete steps in exocytic and endocytic trafficking pathways (Novick and Zerial, 1997; Olkkonen and Stenmark, 1997) and their activation is tightly regulated by guanine nucleotide exchange factors, which promote exchange of GTP for GDP in response to extracellular and intracellular signals (Südhof, 1997). More generally, members of the Rab branch of the Ras GTPase superfamily are required for late event in the exocytic process, probably to facilitate attachment of the synaptic vesicle at the active site of the presynaptic membrane, followed by docking and by an ATP-dependent priming step (Geppert et al., 1997; Zhu et al., 2001). It has been shown that Mss4 coimmunoprecipitates with Rab3a in rat brain extracts and that a tight interaction exists between Mss4 and Rab3a that includes a conserved subdomain containing the invariant cysteine residues required for Zn^{2+} binding as well as the residues implicated in the interactions with Rab GTPases (Burton et al., 1994). Mss4 stimulates GDP release activity for exocytic Rab GTPases (Rab1, Rab3a, Rab8 and Rab10, Sec4, and Ypt1) and facilitates neurotransmitter release as shown in giant squid nerve termini (Convit et al., 1997). The up-regulation of Mss4 after chronic administration of imipramine in rats could then be one of the major steps in the regulation of the release of neurotransmitters and therefore could contribute to the adaptation of synaptic

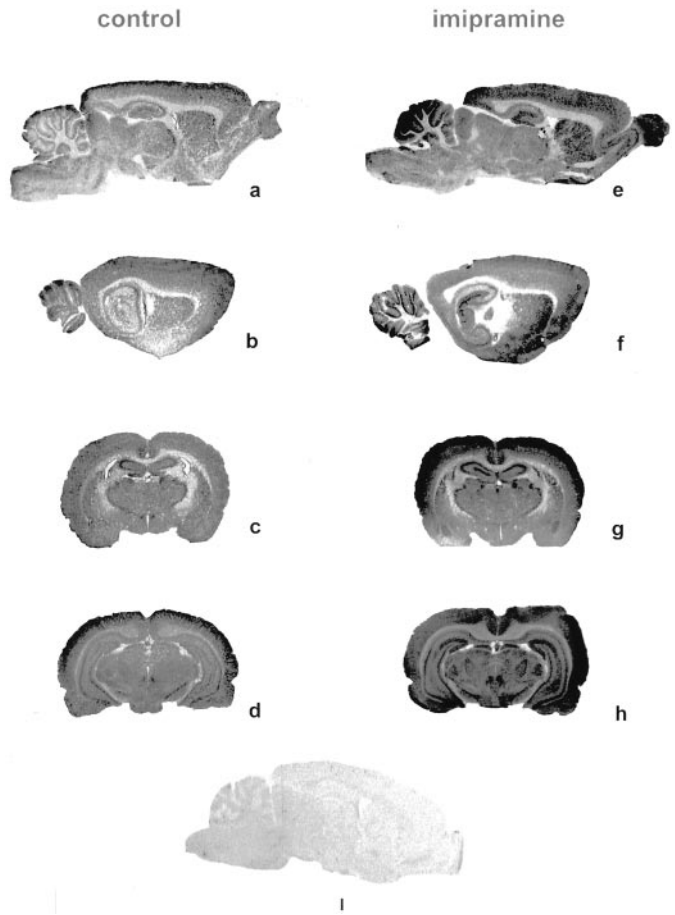

Fig. 5. Brain regional distribution of Mss4 overexpression. In situ hybridization was performed on brain sections of untreated rats (a–d) and rats treated for 3 weeks with imipramine (e-g). A ^{35}S-labeled, PCR-amplified cDNA fragment specific to Mss4 was used as probe. Films were analyzed by quantitative autoradiography. A ^{35}S-labeled sense mRNA probe was used as control (i).

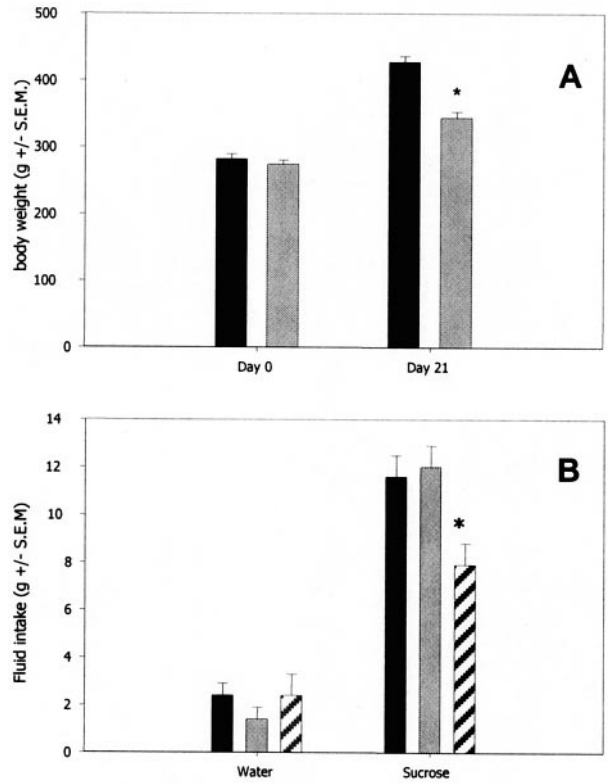

Fig. 6. A, effects of chronic mild stress on body weight (■, control rats; $n = 7$; ▨, stressed rats; $n = 7$). Results are mean ± S.E.M. (unpaired t test; ***, $p < 0.001$; significantly different from control). B, effects of chronic mild stress on water and sucrose consumption (■, control rats, day 0; $n = 14$; ▨, nonstressed rats, day 21; $n = 7$; ▨, stressed rats day 21; $n = 7$). Results are means ± S.E.M. (analysis of variance followed by Dunnett multiple comparison test; *, $p < 0.05$; significantly different from control).

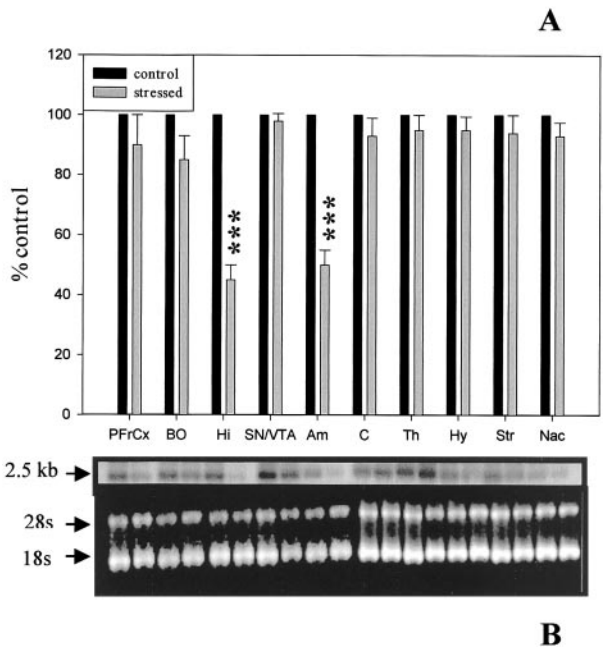

Fig. 7. Brain regional distribution of *Mss4* gene expression in chronically stressed rats. A, spot densitometry (percentage of control ± S.D.). B, Northern blot of the following brain regions: prefrontal cortex (PFrCx), olfactory bulb (BO), hippocampus (Hi), substantia nigra and ventral tegmental area (SN/VTA), amygdala (Am), cerebellum (C), thalamus (Th), hypothalamus (Hy), striatum (Str), and nucleus accumbens (Nac). Seven control rats and seven imipramine-treated rats were used. Total RNA from each region was pooled and 10 μg was loaded on the gel as shown by 28S (4.9 kb) and 18S (1.9 kb) rRNA bands. Experiments were run in duplicate. Student's t test was applied for the significance of data (***, $p < 0.001$).

events underlying the mechanism of action of many antidepressants, including imipramine, tianeptine, and fluoxetine. The expression of the cysteine string protein (CSP), which is localized to synaptic vesicles in the neurons, was reported to be induced in the rat frontal cortex after chronic imipramine and sertraline treatment (Yamada et al., 2001). Given that the CSP binds to the secretory vesicle and enhances the activity of presynaptic calcium channels upon docking of the vesicle, it is suggested that CSP could activate the neurotransmitter release at nerve terminal (Chen et al., 2002). Moreover, it is reported that the CSP N-terminal J-domain, which is involved in the interaction with chaperone proteins of the heat-shock protein family and central cystine-rich region may serve to anchor the protein to membranes and intervene in the late events of exocytosis (Magga et al., 2000). All steps in the synaptic vesicle life cycle are regulated by a cascade of protein-protein and lipid-protein interactions and Mss4 may play a central role in the events controlled by the SNARE proteins VAMP-2, SNAP-25, and syntaxin, which are essential for membrane fusion; synaptotagmin is the major Ca^{2+} sensor for Ca^{2+}-regulated exocytosis at the synapse. Other antidepressant-induced changes in the expression of specific genes in selected brain areas have been described. These include the cAMP phosphodiesterase (Takahashi et al., 1999), mineralo- and glucocorticoid receptors (Vedder et al., 1999; Semont et al., 2000), dopamine receptors (Lammers et al., 2000), serotonin or norepinephrine transporters (Benmansour et al., 1999), immediate early gene transcription factors [including c-fos (Torres et al., 1998), zif268 (Dahmen et al., 1997), NGF1-A (Bjartmar et al., 2000)], and expression/phosphorylation of CREB as mentioned earlier (Thome et al., 2000). It is tempting to postulate that the potentiation of serotonin and/or norepinephrine release via sustained activity of Mss4 could represent a phenomenon that participates in the plastic adaptation of the cAMP cascade and CREB with specific target genes, such as brain-derived nerve growth factor. Moreover, the effects of Mss4 overexpression may be not limited to monoaminergic terminals and could concern other neurotransmitters and pathways in brain, including those that release trophic substances. Depression and chronic stress could involve dystrophic responses that occur in brain limbic regions involved in the control of basic affective functions. Synaptic plasticity has been reported to be implicated in the atrophy of the human hippocampus in mild cognitive impairment in aging (Gurvits et al., 1996), post-traumatic stress disorders (Sheline et al., 1999), and recurrent depressive illness (Duman and Charney, 1999). Hippocampal atrophy in depression may be associated with repeated stress (Andersen and Soleng, 1998). Decreases or increases in expression of a protein that regulates the efficiency of synaptic transmission may be very important in long-term effects on atrophy and depression, because neurotrophic effects may be activity-dependent (Duman et al., 1997).

Our results provide new data that support a role of Mss4 overexpression in the mechanism of action of imipramine. Interestingly, the present studies also showed a stimulatory effect of tianeptine and fluoxetine on Mss4 expression in hippocampus. This suggests that this phenomenon is common for a tricyclic antidepressant (imipramine), a serotonin uptake inhibitor (fluoxetine), and an atypical antidepressant (tianeptine). Possible links between Mss4 up-regulation and the therapeutic benefits induced by chronic antidepressant treatment can be drawn from the present experiments. First, at least in the hippocampus, the time course of Mss4 mRNA increase is consistent with a delay of 3 weeks before the appearance of a therapeutic effect. Secondly, chronic imipramine treatment, which leads to the induction of Mss4 in several regions of the brain of normal rats, reverses the depression induced by chronic mild stress in rats (Willner et al., 1987). In fact, all clinically effective antidepressants tested in the chronic mild stress model reverse the induced reduction in sweet intake. The face and predictive validity of this model seem to be high and it is usually considered a valid model of depression. In our present experiments, the anhedonic rats exhibit a down-regulation of Mss4 in their amygdala and hippocampus. Thus, it may be assumed that chronic administration of imipramine or fluoxetine may produce some elements of the therapeutic response by correcting Mss4 underexpression, which could reflect some part of the adaptive mechanisms to chronic stress. However, the potential relevance of Mss4 in the pathophysiology and treatment of depression does not exclude the probable involvement of a variety of molecular disorders in different forms of depression (Duman and Charney, 1999).

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