

Research report

Synaptotagmin I and IV are differentially regulated in the brain by the recreational drug 3,4-methylenedioxymethamphetamine (MDMA)

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA or Ecstasy) is a widely abused drug. In brains of mice exposed to MDMA, we recently detected altered expression of several cDNAs and genes by using the differential display polymerase chain reaction (PCR) method. Expression of one such cDNA, which exhibited 98% sequence homology with the synaptic vesicle protein synaptotagmin IV, decreased 2 h after MDMA treatment. Herein, the effect of MDMA on expression of both synaptotagmin I and IV was studied in detail, since the two proteins are functionally interrelated. PCR analyses (semi-quantitative and real-time) confirmed that upon treatment with MDMA, expression of synaptotagmin IV decreased both in the midbrain and frontal cortex of mice. Decreases in the protein levels of synaptotagmin IV were confirmed by Western immunoblotting with anti-synaptotagmin IV antibodies. In contrast, the same exposure to MDMA increased expression of synaptotagmin I in the midbrain, a region rich in serotonergic neurons, but not in the frontal cortex. This differential expression was confirmed at the protein level with anti-synaptotagmin I antibodies. MDMA did not induce down- or up-regulation of synaptotagmin IV and I, respectively, in serotonin transporter knockout mice (–/–) that are not sensitive to MDMA. Therefore, psychoactive drugs, such as MDMA, appear to modulate expression of synaptic vesicle proteins, and possibly vesicle trafficking, in the brain.

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Theme: Neural basis of behavior

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1. Introduction

Synaptotagmins are vesicle-associated proteins, of which there are about a dozen isoforms [1,33]. Among the various activities of synaptotagmins are major role in vesicle trafficking and docking to the plasma membrane, and neurotransmitter secretion [30,40]. Depolarization of neurons and various stimulants transiently increase expression of synaptotagmin IV (syt IV) [8,41], and up regulation of syt IV by kainic acid was accompanied by a parallel down regulation of syt I [39]. Herschman, Baudry and

colleagues concluded therefore that syt IV has a role as an immediate early gene [8,41]. Both syt I and syt IV are also involved in neurite outgrowth [12,21], and the syt I/syt IV ratio regulates synaptic activity, possibly via formation of heteromultimers [11,23]. In PC12 cells, syt I and syt IV colocalize in common vesicles [8], whereas in the brain there are reports indicating colocalization [25] or presence in distinct vesicle populations [3].

Little is known about the effects of psychostimulating drugs on synaptotagmins. Cocaine, but not amphetamine, transiently increases syt IV mRNA in rat dorsal striatum [7]. This might result from activation of dopamine receptors, consequent to cocaine-induced dopamine release. In fact, the selective dopamine D1 receptor agonist SKF-82958 up regulates expression of syt IV in the striatum

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[15]. This is in agreement with the finding that a chronic treatment with the dopamine receptor antagonist haloperidol down regulates both syt IV and I in the nucleus accumbens [24].

MDMA, a methamphetamine analogue, has hallucinogenic, psychostimulant and multiple behavior activities, occasionally leading to death [16,19,28]. MDMA induces degeneration of certain serotonergic neuronal fibers in the brain [29,36], and programmed cell death (apoptosis) of human JAR cells [35] and cultured rat neocortical neurons [38]. Recently, we identified and cloned several cDNAs that are differentially expressed in the midbrain and frontal cortex of MDMA-treated mice [26]. Some of these cDNAs correspond to known genes, such as the mouse γ -aminobutyric acid (GABA) transporter 1 (mGAT1), whereas others are apparently novel [26]. Herein, we report that exposure to MDMA differentially affects the expression of two synaptic vesicle proteins, syt IV and I.

2. Materials and methods

2.1. Materials

Tri Reagent was purchased from MRC, (Cincinnati, OH), pGEM-T Easy Vector, RNasin and RNase-free DNase I from Promega (Madison, IL), Superscript™ amplification kit from Gibco-BRL, ethidium bromide, rabbit polyclonal anti-synaptotagmin I antibodies and Taq DNA polymerase from Sigma (St. Louis, MO), and DNA extraction kit from Biological Industries. LightCycler-Fast-Start DNA Master SYBR Green I was from Roche Molecular Biochemicals (Mannheim, Germany), BCA protein assay kit from Pierce (Rockford, IL), Kaleidoscope pre-stained standard proteins from Bio-Rad (Hercules, CA), Plasmid mini kit from Qiagen (Valencia, CA) and ^{33}P [dATP] (3000 Ci/mM), nitrocellulose membranes, horseradish peroxidase coupled to F(ab)₂ anti-rabbit IgG, and ECL western blotting detection reagents from Amersham (Buckinghamshire, UK). Syt IV antibodies were prepared as described [21]. MDMA was extracted as before [35] from pellets kindly supplied by Dr. R. Levy, Israel Police, Department of Forensic Identification. All other chemicals were of analytical grade.

2.2. Animal and drug treatment

Young adult male C57BI/6J mice (2–3 months old, two to four in each group) were injected intraperitoneally once with 0.25 ml saline (control) or saline containing MDMA (10 mg/kg). Unless otherwise indicated, animals were sacrificed 2 h later, and frontal cortex or midbrain were dissected and frozen on dry ice. This MDMA dose was selected after a set of preliminary experiments with 5–75 mg/kg. The 10 mg/kg used in the current report induced

the typical psychomotor hyperactivity, but did not cause acute toxicity or death. Serotonin transporter knockout mice [2] were used when indicated. Adequate measures were taken to minimise pain and discomfort to the animals.

2.3. RNA preparation, DD-PCR analysis and cDNA cloning

Frozen brain tissues were crushed, homogenized, 1 ml Tri Reagent was added and total RNA was isolated. RNA samples were treated with RNase-free DNase I for 30 min at 37 °C and RNA quality and yield was verified on 1.2% formaldehyde agarose gel. First-strand cDNA was synthesized using 0.5 μg total RNA as template and 0.8 μM dT₁₂CC as a primer, using Superscript preamplification kit. DD-PCR was carried out in 20 μl total volume, and included 0.8 μM each of the 3' primer dT₁₂CC and arbitrary 5' primer 5'-GGACAGCTTC-3', 2.5 μM dNTP, 2.5 mM MgCl₂, 2 μCi [α - ^{33}P]dATP (3000 Ci/mM), 2 μl cDNA prepared from 50 ng total RNA, and 2.5 units of Taq DNA polymerase. The DD-PCR reaction was started with a 3-min incubation at 94 °C, followed with 40 cycles of 30 s at 94 °C, 2 min at 40 °C and 30 s at 72 °C, and a final incubation of 5 min at 72 °C. Then, 10 μl of the reaction mixture were mixed with 10 μl of 95% formamide, containing 0.05% bromophenol blue and 0.05% xylene cyanol, incubated for 5 min at 75 °C and an aliquot of 6 μl was analyzed on a DNA sequencing gel. The gel was dried, exposed to Phosphorimage Analyzer (Fujix) or an X-ray film, and DNA bands expressed differentially upon MDMA treatment were identified, cut from the gel, and soaked in 100 μl H₂O for 10 min. Samples were then boiled for 15 min, centrifuged at 10 000 $\times g$, and 10 μl of the supernatant were used as a template to re-amplify the band of interest, with the same primers and PCR conditions described for DD-PCR, except that 25 μM dNTP and no isotopes were used. Ten μl of the PCR reaction were run on a 2% agarose gel. The band with predicted size was excised, purified on Agarose Gel DNA extraction Kit, and cloned into pGEM-T Easy Vector. Plasmid DNAs from successful clones were purified with Plasmid Mini Kit and sequenced (Biological Services, Weizmann Institute of Science). Sequence data were compared with GeneBank (NCBI).

2.4. RT-PCR: primers, conditions and semi-quantitation

After total RNA were isolated, treated with DNase I and quantified, the first-strand cDNA was synthesized from 0.2 to 1 μg RNA, using Superscript amplification kit. PCR was carried out in 20 μl total volume, including 0.5 μM of 3'- and 5'-primers, 25 μM dNTP, 2.5 mM MgCl₂, the cDNA from 10 to 100 ng total RNA synthesized, 5 units RNasin, and 2.5 units Taq DNA polymerase. PCR reaction

was as follows: 3 min incubation at 94 °C, 25–33 cycles of 30 s at 94 °C, 45 s at the annealing temperature, 30 s at 72 °C and finally 7 min at 72 °C. Specific primers for RT-PCR were designed according to syt IV and syt I gene sequence [13,20] (Accession Nos. U10355 and NM009306, respectively). Syt IV and syt I primers were: (F) 5'-ATGGCTCCTATCACCACCAG-3', (R) 5'-CCAT-TGAGGTCTCGCTTCTC-3'; and (F) 5'-GTGAGTGC-CAGTCGTCCTGAG-3' and (R) 5'-TTCATGGTCTTCC-CTAAGTC-3', respectively, with cDNA products corresponding to nucleotides 1716–2023 and 528–867 of the genes.

2.5. Real-time quantitative PCR analysis

Real-time PCR was performed using the LightCycler System (Roche Molecular Biochemicals, Germany) and LightCycler-FastStart DNA Master SYBR Green I, according to the manufacturer's instructions. Reactions were performed in 20 µl with template cDNA (prepared from 0.1 to 0.5 µg total RNA), 3 mM MgCl₂ and 0.25–0.5 µM primers. Buffer, dNTP mixture (with dUTP instead of dTTP), Taq DNA polymerase, and SYBR Green I dye were included in the 'Hot start' reaction as recommended. A typical protocol was as follows. Ten minute denaturation at 95 °C followed by 40 cycles of amplification, starting with 15 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C. The extension period varied with each specific primer, depending on the product length (about 1 s/25 bp). Melting curve analysis was conducted by incubation at 95 °C for 0 s, 15 s at 65 °C and 0 s at 95 °C, followed by a cooling step for 30 s at 40 °C. Quantification relative to a housekeeping gene, with external standards, was used to determine the amount of a target gene. For each real-time PCR, homologous standards (of syt I or syt IV), house keeping gene (GAPDH), and target samples were prepared in separate capillaries, and amplified during the same run. Four 10-fold serial dilutions PCR products of syt I or syt IV with known concentrations were amplified under the same cycling conditions to create a standard curve for quantitation of the target gene (syt) and GAPDH in each DNA sample. To confirm amplification specificity, PCR products from each pair of primers were subjected to both melting curve analysis and agarose gel electrophoresis. Quantitation was performed with the LCDA (LightCycler Data Analysis) software by the Fit Points method.

2.6. Western immunoblotting with synaptotagmin antibodies

Protein was isolated by Tri Reagent, precipitated with iso-propanol, the pellet was washed three times with 0.3 M guanidine hydrochloride and 95% ethanol, followed by washing with ethanol, dried, and dissolved in 10 M urea containing 50 mM dithiothreitol (DTT). Samples were

kept at room temperature for 1 h before boiling for 3 min, sonicated on ice for 2 min, dissolved and stored at –20 °C. Protein concentration was determined with micro BCA protein assay kit. Samples of 100 µg run on SDS–PAGE (5% stacking gel and 8 or 10% separating gel) and Kaleidoscope pre-stained standard proteins were used. Proteins from the gel were transferred to nitrocellulose membranes. Membranes were blocked for 2 h in Tris-buffered saline (TBS) with 10% non-fat dry milk and 0.1% Tween 20, and incubated overnight at 4 °C or 2 h at room temperature with anti-Syt IV or anti-Syt I antibodies (1:200 and 1:1000, respectively, in TBS containing 5% non-fat dry milk). Membranes were then washed three times with TBS containing 0.1% Tween 20, incubated for 2 h at room temperature with horseradish peroxidase coupled to F(ab)₂ anti-rabbit IgG, washed four times with the same buffer, and subjected to ECL Western blotting detection reagents.

3. Results

3.1. Down-regulation of the expression of syt IV mRNA and protein in MDMA-treated mice

In an effort to identify changes in gene expression caused by a single treatment with MDMA, differential display PCR was performed on RNA from the frontal cortex and midbrain of mice treated with MDMA and extracted 2 h later [26]. We thus identified, cloned, and sequenced 11 cDNAs. The expression of one of these, a 356-bp cDNA fragment, decreased in the midbrain of MDMA-treated mice (Fig. 1A). Cloning and sequencing this cDNA indicated 98% sequence homology with syt IV, as recently reported [26]. The influence of MDMA on syt IV expression in the mouse brain was further verified by designing specific primers for syt IV (according to the published sequence; see Section 2), and using them in a RT-PCR assay. Upon an acute MDMA treatment, expression of syt IV decreased about 65% when compared with GAPDH expression, used as a reference (Fig. 1B). This was further verified by a quantitative real-time PCR analysis of RNA prepared from the frontal cortex and midbrain of mice treated with saline or MDMA for 2 h, along with GAPDH and four concentrations of an internal standard (Section 2). There was a decrease (1.73- and 1.96-fold) in the syt IV/GAPDH ratio (amount of syt IV mRNA) in the frontal cortex and midbrain, respectively, of mice treated with MDMA (Fig. 2). The melting curve of the syt IV DNA product of the real-time PCR, in control and MDMA-treated mice had single peak at 86.2±0.4 °C.

Whether MDMA-induced decreases in the expression of syt IV mRNA were accompanied by decreases in syt IV protein were examined using affinity-purified anti-syt IV antibodies [10] and Western immunoblotting. The single treatment with MDMA did indeed decrease the immuno-

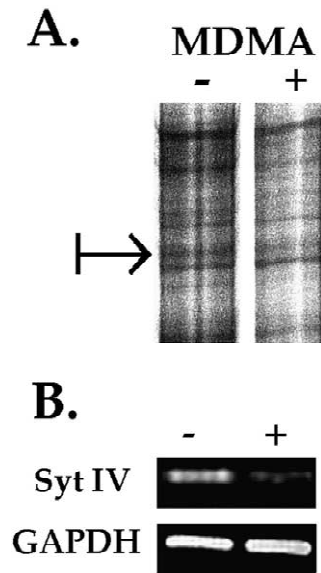


Fig. 1. Differential display PCR (A) and RT-PCR (B) analysis of syt IV in the midbrain of mice treated once with MDMA (+) or saline (–) and extracted 2 h later. (A) ^{33}P -labeled DNA sequencing acrylamide gel showing decreased expression of a 356-bp cDNA fragment. The primers used for DD-PCR are indicated in Section 2. RT-PCR (B) of midbrain RNA was performed with specific syt IV primers designed according to sequence reported by others [13,20]. GAPDH was used as control.

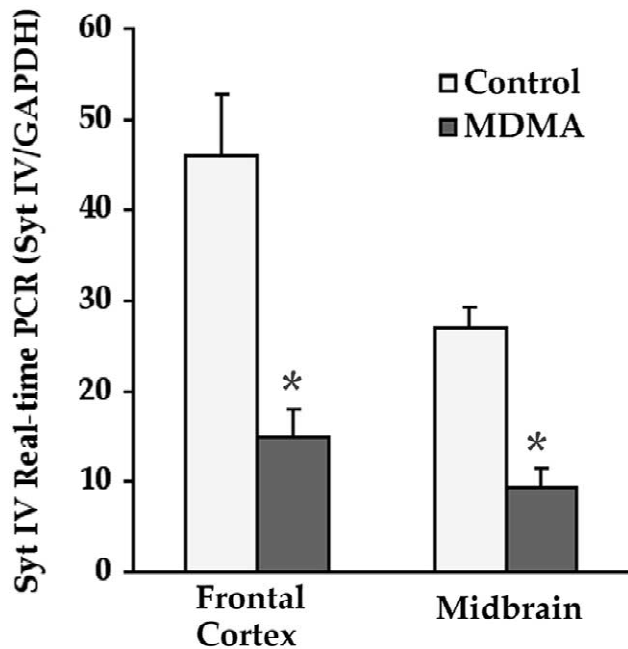


Fig. 2. Quantitative Real-time PCR analysis of syt IV expression in frontal cortex and midbrain of mice treated once with MDMA (black bars) or saline (white bars) and extracted 2 h later. Data are mean $\pm$ S.D. of duplicates, and presented as the ratio expression of syt IV/GAPDH in pmol/ 10^6 . GAPDH was used as a reference as described in Materials and methods. The * indicates significant difference from saline treatment, $P < 0.01$.

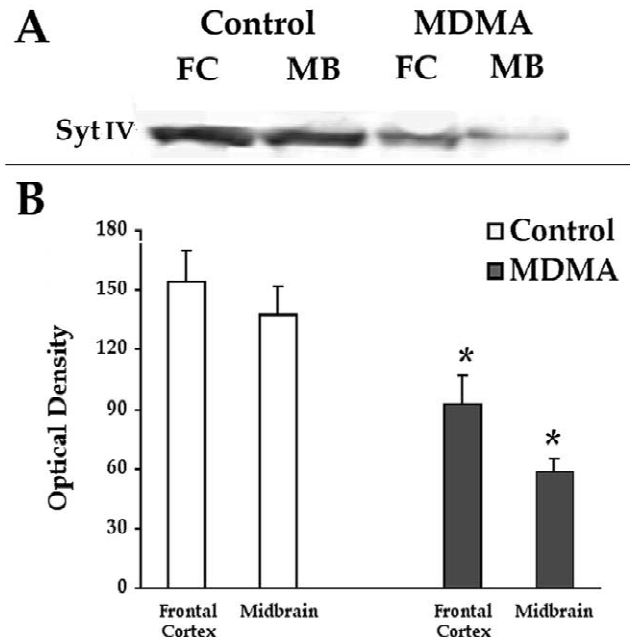


Fig. 3. Western immunoblotting of syt IV with anti-syt IV antibodies after an acute MDMA treatment for 2 h. (A) Syt IV immunoreactive. (B) Optical density $\pm$ S.D. of two scanned bands. FC, frontal cortex; MD, midbrain. The * indicates significant difference from saline treatment, $P < 0.05$.

reactivity, i.e., amount of syt IV protein in the frontal cortex and midbrain of mice by 1.66- and 2.36-fold, respectively (Fig. 3).

3.2. MDMA-enhanced expression of syt I

Since there is a relationship between syt IV and syt I proteins (see Section 1) the effect of MDMA (2 h after a single treatment) on expression of syt I was also examined. RT-PCR analysis with primers designed for syt I showed a distinct effect of MDMA on its expression; in the frontal cortex the levels of syt I mRNA were not affected significantly, but in the midbrain they increased 4.4-fold (Fig. 4A). This was confirmed by real-time PCR (Fig. 4B), which revealed a 3.8-fold increase in the levels of syt I mRNA in the midbrain (0.28×10^{-5} and 1.09×10^{-5} pmol in saline and MDMA-treated mice, respectively). In agreement with previous reports, RT-PCR and real-time PCR detected higher content of syt I mRNA in the frontal cortex than midbrain (6.2- and 16-fold, respectively).

The kinetics of MDMA-induced changes in syt I expression at the RNA and protein levels was determined using RT-PCR and Western immunoblotting with anti-syt I antibodies. In the midbrain, the highest increase in syt I mRNA (Fig. 5A) and protein (Fig. 5B) levels was observed 1 day after treatment, followed by a decrease to control levels at 7 days. In contrast, in the frontal cortex,

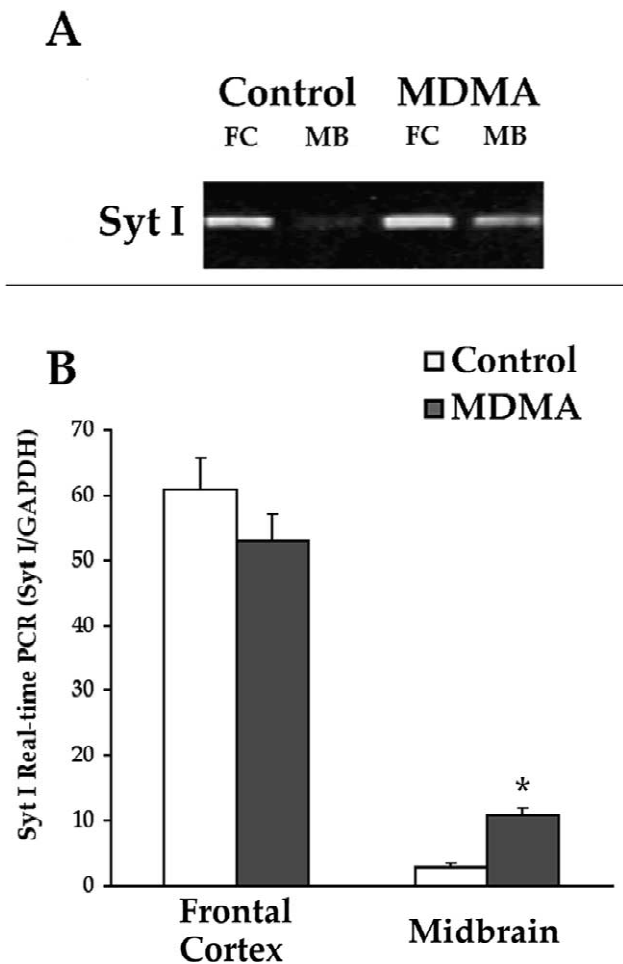


Fig. 4. RT-PCR (A) and real-time PCR (B) of syt I expression in untreated and MDMA treated mice (2 h). The real-time PCR data are mean $\pm$ S.D. of duplicates, and presented as the ratio expression of syt I/GAPDH in pmol/10⁶. The * indicates significant difference from saline treatment, $P < 0.01$. Note that in both assays MDMA had no effect in frontal cortex (FC), but did increase syt I expression in the midbrain (MD).

the levels of syt I mRNA and protein did not change significantly from 2 h to 7 days (Fig. 5A,B).

3.3. Effects of MDMA on syt IV and syt I expression in serotonin transporter knockout mice

Whether the MDMA-induced down- and up-regulation of syt IV and syt I expression, respectively, results from interaction of the drug with serotonin transporters was examined in serotonin transporter knockout mice (–/–), previously shown not to be behaviorally affected by the drug [2]. Western immunoblotting with anti-syt IV and anti syt I antibodies (Fig. 6) indicated that in the serotonin transporter knockout mice (–/–), an the single treatment with MDMA induced a small but insignificant changes in these proteins.

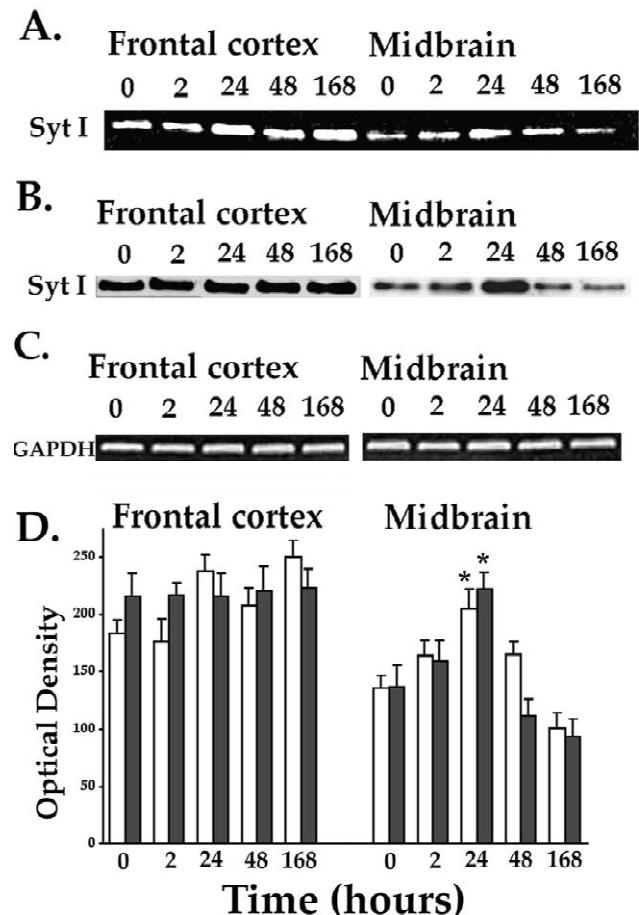


Fig. 5. Time course (hours) change in syt I expression in MDMA-treated mice determined by RT-PCR (A) and Western immunoblotting (B). GAPDH expression (RT-PCR) was used a reference (C). (D) Optical density $\pm$ S.D. of the scanned bands shown in (A; open bars) or (B; black bars). The * indicates significant difference from zero time, $P < 0.01$.

4. Discussion

Several proteins control neurotransmitter release, including those associated with synaptic vesicles, such as synaptotagmins [23,30,33]. Neuronal stimulation triggers binding of synaptotagmins with other vesicle proteins in a calcium-dependent fashion, which in turn induces synaptic vesicle trafficking to the plasma membrane, fusion, and release of neurotransmitters [9,23,30]. In this study, a single treatment with MDMA was shown to alter the brain content of two synaptotagmins, I and IV. The effect of MDMA was selective and differential, depending on the synaptotagmin and the brain region. Exposure of mice to MDMA resulted in a decrease in the expression of syt IV mRNA and protein in their frontal cortices and midbrain. In contrast, the levels of syt I mRNA and protein increased in the midbrain, and were unaffected in the frontal cortex. Since the midbrain is the predominate site at which serotonergic neuronal cell bodies reside, up regulation of syt I in this region may reflect a close interaction of

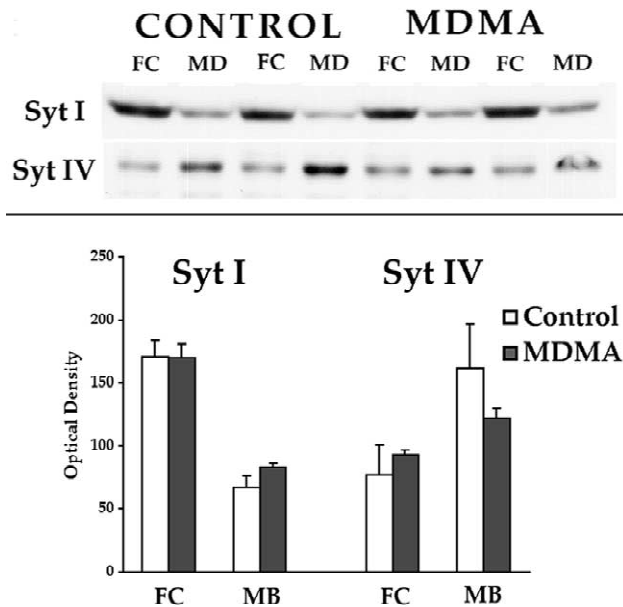


Fig. 6. Syt I and IV expression in serotonin transporter knockout mice (–/–): effect of saline (control) or a single MDMA treatment for 2 h. FC-frontal cortex, MD-midbrain. The lower panel shows the optical density $\pm$ S.D. of the scanned bands. No significant differences were observed upon MDMA treatment.

MDMA with serotonergic neurons themselves, or adjacent cells. As for syt IV, the similar MDMA-induced decrease in its levels in the frontal cortex and midbrain suggests a more global effect of the drug on expression of this protein, possibly one that affects serotonergic fibers and terminals, which are widely distributed throughout the brain. MDMA exhibits a variety of psychoactive and physiological effects, including hallucination, psycho-stimulation, memory impairment, neurotoxic activity, and increasing body temperature [4,16,18,29,31,37]. This multiplicity reflects the influence of MDMA on several other neuronal pathways, besides the serotonergic system, such as dopamine, glutamate, and GABA [16,17,43]. Exposure to MDMA activates therefore non-serotonergic neurons, and thus could contribute to the general decrease in syt IV. The effect of chronic application of MDMA was not addressed in the current report.

Peak increase in expression of syt I was observed within 24 h after treatment with MDMA, and then subsided, which suggests a transient effect of the drug. Denovan-Wright et al. have found a transient change in the expression of syt IV in rats treated with cocaine, however cocaine increased rather than decreased expression of syt IV [7]. Activation of dopamine D-1 receptors also increases expression of syt IV [15], whereas chronic treatment with haloperidol, a dopamine receptor antagonist, causes an opposite effect [24]. Apparently, activation of the dopamine receptor D-1 modulates expression of syt IV, but in the opposite direction as MDMA. Yet, it has been documented that MDMA treatment caused stimulation of

dopaminergic neurons, release of dopamine, and activation of both D-1 [22,34] and D-2 dopamine receptors [32]. Whether the observed down regulation of syt IV by MDMA is due to activation of serotonin receptors, activated upon MDMA-induced serotonin release, or other (possibly non-dopaminergic) receptors may become clear from further studies on defined brain regions. Interestingly, chronic treatment of rats with three antidepressive drugs, which also increase serotonin neurotransmission via inhibition of serotonin transporters, increased phosphorylation of synaptotagmin I, but not its protein level [27]. Development of desensitization after such chronic treatment has not been ruled out.

Besides major role in neurotransmission, synaptotagmins act in the brain as immediate early genes [39,41], calcium regulator of release probability [9], neurite growth [12,21], and possibly other processes [5,10,42]. Fluctuations in syt I and syt IV levels upon MDMA treatment might therefore initiate expression of different genes or alter neuronal growth. The possibility that these synaptotagmins are involved in the acute or long-term (adaptive) molecular, cellular or behavioral effects of abused drugs such as MDMA (and cocaine) is therefore worth further investigations. As immediate early genes, synaptotagmins may also be involved in the programmed cell death induced by MDMA in cultured cells [35,38].

It is of interest that syt I knockout mice die after birth, even though the development of the CNS is normal [14], suggesting that different synaptotagmin isoforms apparently have a unique function. Synaptotagmins possess binding sites for the presynaptic plasma membrane protein syntaxin and SNAP-25 [5,30,33]. Syntaxin 1A interact with and regulate the activity of GABA I transporter [6]. Whether the MDMA-induced changes in expression of syt I and syt IV observed herein are associated with activation of GABA neurotransmission is of interest.

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