Changes in syntaxin-1B mRNA in the nucleus accumbens of amphetamine-sensitized rats

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Abstract

Repeated administration of amphetamine in animals induces persistent changes in dopamine (DA) functions and behaviour. These changes may be mediated by altered plasticity of the mesocorticolimbic DA system. We have previously reported changes in the content of axonal plasma membrane protein syntaxin-1 in the nucleus accumbens (NAc) of amphetamine-sensitized rats. In the present investigation, we investigated whether syntaxin-1 changes are derived from transcriptional events, i.e. mRNA changes, in the mesocorticolimbic DA regions of the brain. Behavioural sensitization was induced in adult Sprague–Dawley rats by repeated intermittent administration of d-amphetamine (1.5 mg/kg i.p. for 5 alternate days). The animals were sacrificed 24 h, 7 d or 14 d after the last injection and in-situ hybridization using oligonucleotide probes was employed to assess the expression of syntaxin-1A and -1B mRNAs. Results show that the expression of syntaxin-1B mRNA was significantly increased in the NAc shell (NAcS) region in the amphetamine-sensitized rats 14 d after the drug treatment compared to saline pretreated or untreated control animals. No significant change in syntaxin-1B mRNA was observed in animals sacrificed 24 h or 7 d after the sensitizing regimen of amphetamine in any brain region analysed, namely NAcS, NAc core (NAcC), caudate putamen (CPu), ventral tegmental area (VTA) or medial prefrontal cortex (mPFC). Levels of syntaxin1A mRNA were not significantly different from controls in any brain region at any time-point. These results suggest that syntaxin-1 protein changes in amphetamine-sensitized rats may be due to increased syntaxin-1B gene expression within local neurons of the NAcS that may lead to altered exocytosis from these neurons during the expression of sensitized response.

Keywords: Drug abuse, exocytosis, plasticity, psychostimulant, schizophrenia, sensitization, synaptic vesicle.

Introduction

Repeated administration of psychostimulants like amphetamine (Amph) or cocaine results in an increased behavioural response to the drugs at subsequent exposure (Pierce and Kalivas, 1997; Wolf et al., 2004). This phenomenon, called sensitization has been well characterized in experimental animals. The behavioural effects subject to sensitization include among others, locomotor activation (Pierce and Kalivas, 1997; Subramaniam et al., 2001), stereotypies (Fowler et al., 2003), drug self-administration (Pierre and Vezina, 1997) and acoustic startle response (Tenn et al., 2003). Sensitization is an enduring process as animals continue to display enhanced response to psychostimulants after months of abstinence, suggesting long-lasting and possibly irreversible neurobiological changes (for reviews see Pierce and Kalivas, 1997; Steketee, 2003; Vezina, 2004). The phenomenon is of interest since behavioural sensitization is suggested to be associated with drug addiction as well as some psychotic symptoms of schizophrenia (Castner and Goldman-Rakic, 2003; Laruelle, 2000; Robinson and Becker, 1986; Robinson and Berridge, 2000; Ujike, 2002).

Alteration within ventral tegmental area (VTA) dopamine (DA) neurons have been suggested to initiate the sensitization process whereas changes in DA release in the nucleus accumbens (NAc) appear necessary for the expression of the sensitized response (Kalivas and Stewart, 1991). The enduring nature of
sensitization suggests long-term adaptations in the property of VTA DA neurons, their terminals and/or post-synaptic cells in the NAc, as well as other brain regions that are connected to mesolimbic DA system (White and Kalivas, 1998). Repeated intermittent treatment with Amf can reorganize synapses at the site of DA–glutamate interaction in the striatum as revealed by long-lasting increased density of dendritic spines in medium spiny neurons of the NAc and caudate-putamen (CPu) in Amf-sensitized rats (Li et al., 2003). Further, there is evidence of alterations in the immediate early genes c-fos, transcription factor CREB phosphorylation in the striatum/accumbens of chronic Amf-treated rats (Turgeon et al., 1997; Vanderschuren et al., 2002). At the presynaptic end, there is evidence for changes in the DA terminal excitability and in-vitro enhanced Amf and K⁺-induced DA release mediated by Ca²⁺-calmodulin-dependent protein kinase II (CaM kinaseII) (Kantor et al., 1999). Enhanced phosphorylation of synapsin I and increased growth-associated protein-43 in the striatum in sensitized animals also argue for presynaptic adaptations that are geared towards facilitation of transmitter release (Iwata et al., 1996).

Neurotransmitter release from synaptic vesicles is a complicated and elaborately regulated process, involving a cascade of protein–protein interactions, a critical component of which consists of the SNARE core complex. The SNARE core complex itself consists of synaptobrevin/VAMP (a synaptic vesicle protein), syntaxin and SNAP-25 (synaptic membrane-associated proteins). It acts in synaptic vesicle exocytosis by docking vesicles at release sites adjacent to voltage-gated calcium channels and thereby priming them for calcium-mediated membrane fusion and exocytosis (Lin and Scheller, 1997). Syntaxins are a large (>20) family of targeting proteins (sharing 23–84% homology) that have well-defined roles in exocytosis as part of the SNARE core complex (Bennett et al., 1993) and are also strongly implicated in synaptic plasticity underlying learning and long-term potentiation (LTP) (Hicks et al., 1997; Mochida, 2000). Syntaxin-1 (35 kDa) is a presynaptic plasma membrane protein which exists in two isoforms, syntaxin-1A and -1B, with 84% homology, that are present in neuronal and secretory cells (for review Teng et al., 2001). While both isoforms are involved in vesicle exocytosis, syntaxin-1B in particular has been implicated as a marker for synapses undergoing LTP (Davis et al., 1998, 2000; Hicks et al., 1997) and is up-regulated within discrete neural circuits in animals performing memory tasks (Davis et al., 1996, 1998). It has been seen to be up-regulated not only at synapses undergoing LTP but also at synapses downstream of them within neural circuits leading to speculation of a role for syntaxin-1B in trans-synaptic plasticity and the laying down of a memory trace in neural circuits (Davis et al., 1996, 1998; Helme-Guizon et al., 1998; Hicks et al., 1997). Systemic administration of psychostimulants has been shown to enhance the learning of striatum-dependent tasks (Packard et al., 1994). Similarly psychostimulant-induced increase in DA release is also reported to modify hippocampal and striatum LTP (for review see Davis et al., 2000; Mochida, 2000) and mechanisms of sensitization to psychostimulants are believed to involve LTP and long-term depression (LTD) (Ungless et al., 2001; Wolf et al., 2004).

We have previously reported reciprocal changes in the protein levels of syntaxin-1 in the core and shell subregions of NAc of Amf-sensitized rats (Subramaniam et al., 2001). In this study, we investigated whether the increase in protein levels were related to changes in mRNA levels of syntaxin-1A or -1B in the VTA, NAc and CPu as well as medial prefrontal cortex (PFC), a structure relevant to sensitization, using an in-situ hybridization technique.

**Materials and methods**

**Animals**

Ninety adult male Sprague–Dawley rats (250–300 g) were obtained from Charles River Canada and housed in a temperature- and humidity-controlled environment on a 12:12 h light/dark cycle with free access to food and water. Upon arrival, the animals were left in the animal housing centre for a period of 72 h before use.

**Sensitization**

The procedures were approved by the Animal Care Committee of Douglas Hospital/McGill University, and were carried out in accordance with the guidelines of the Canadian Council of Animal Care. The protocol for the sensitization has been described by us previously (Subramaniam et al., 2001). Briefly, the spontaneous locomotor activity of animals was measured over 3 h for 3 d in locomotor boxes under dim light. Each box (30 × 40 × 40 cm) was equipped with two photoelectric switches and connected to a personal computer with actanal software (Concordia University). Locomotor activity was measured as consecutive light beam interruptions monitored for 3 h in 10-min time bins.
The animals were then assigned to one of the three groups (control, saline or d-Amph, n = 8–10 animals per group), in such a way that each group showed comparable mean total spontaneous locomotor activity over all three habituation days. The Amph group of animals received a sensitizing regimen of d-amphetamine sulphate (Sigma, St. Louis, MO, USA) (1.5 mg/kg free base i.p.) consisting of a total of five injections, one injection every alternate day for a total of 10 d. The injections were made in the locomotor boxes after which the activity was monitored for 3 h. The saline group received an equal volume of saline vehicle (0.9% i.p.) and their locomotor activity was monitored in the same way as the Amph group. The control group consisted of animals that were not injected with anything but handled the same way as the other two groups described above. It was ensured that each animal went to the same testing chamber at the same time throughout the duration of activity recording. All animals were returned to their home cage after completing activity testing.

One, 7 or 14 d after the last injection, animals were sacrificed by rapid decapitation and their brains frozen in isopentane/dry ice and stored at -80°C. Coronal sections (15-μm-thick) at the levels of medial prefrontal cortex (mPFC) (plates 9–10 of the atlas of Paxinos and Watson, 1986), CPu and NAc (plates 12–13 and VTA (plates 43–44) were sectioned using a cryostat (Cryomicrotome, Leica Microsystems Inc., Bannockburn, IL, USA) and obtained on poly 1-lysine coated slides and kept at -80°C. For each animal, two slides of each region containing three sections each were studied. To confirm the expression of the sensitization following 14 d of drug withdrawal, an independent cohort of Amph, saline or control animals were injected with a challenge dose of Amph (0.5 mg/kg i.p.) and their locomotor activity monitored for 90 min.

**In-situ hybridization**

In-situ hybridization was performed essentially according to our previously described procedure (Wood et al., 1997) using oligonucleotide probes complementary to rat syntaxin-1A (5'-TCC AAA GAT GCC CCC GAT GGT GGA GGC GAT GAT GAT GCC CAG AAT) and syntaxin-1B (5'-TCA ACC CCT TGT GCT TCA TCG TCT ACT) that have been used previously (Hicks et al., 1997). Sense oligonucleotides from the same sequences were also synthesized as controls. The probes were 3'-end labelled with [α-35S]dATP (1500 Ci/mmol) using terminal transferase (Roche Diagnostics, Indianapolis, IN, USA). Briefly, brain sections were quickly thawed and dried at room temperature and fixed in a solution of 4% paraformaldehyde in phosphate buffer. The sections were then treated with 0.25% acetic anhydride and 0.1 M triethanolamine followed by hybridization with 1–2 × 10^6 cpm of labelled oligonucleotide probes in hybridization buffer containing 50% formamide, 10% dextran sulphate, 5× SSC, 10 μM dithiothreitol, 1% salmon sperm DNA, 1% yeast tRNA, 2× Denhardt’s solution and 1% BSA at 42°C in humidified chambers for 16–18 h. After the hybridization, the slides were washed for 5 min with 2× SSC at room temperature, 2× 30 min in 2× SSC at 55°C and finally 1× 30 min in 1× SSC at 37°C. Slides were dried and apposed to Beta Max hyperfilm for 2 wk. The level of syntaxin-1 mRNA was quantified using a computerized image analysis system (MCID-4, Imaging Research, St. Catherine, Ontario, Canada). Optical density of the films was analysed and the results are expressed as relative optical density of syntaxin-1 mRNA.

**Statistical analysis**

Locomotor activity was analysed by a two-way ANOVA with Amph treatment as independent factor and day (time-points after the last injection) as repeated measure followed by Newman–Keuls post-hoc test. The data from the locomotor scores after the challenge drug administration was analysed using simple ANOVA followed by post-hoc Newman–Keuls test. The data on mRNA expression at different time-points was analysed by two-way ANOVA with Amph treatment as independent factor and brain region as repeated measure.

**Results**

**Behavioural sensitization to Amph**

The total locomotor counts for each group (measured over the 180 min) for each of the 5 d is illustrated in Figure 1a. Two-way ANOVA showed a significant main effect of treatment [F(2, 84) = 164.94, p < 0.0001], but no significant effect of day [F(4, 84) = 1.47, p = 0.21] or treatment × day interaction [F(8, 84) = 1.10, p = 0.37]. As reported earlier (Subramaniam et al., 2001), the post-hoc analysis showed that the mean locomotor activity of the animals treated with Amph on each treatment day was significantly greater than the saline-treated (p < 0.001) or control animals (p < 0.001). The data from the animals after the challenge drug administration indicated a significant main effect of treatment [F(2, 24) = 23.34, p < 0.0001]. Following
Amph challenge injection (0.5 mg/kg) after 14 d of withdrawal, animals pretreated with Amph displayed significantly higher locomotor activity compared to both saline pretreated (\(p < 0.001\)) and untreated control (\(p < 0.001\)) animals (Figure 1b).

Syntaxin-1A mRNA expression

The schematic representation of the anatomical boundaries of the brain regions sampled for image analysis of in-situ hybridization signals were followed according to the plates 9–10 for PFC, plates 12–13 for CPu and NAc and plates 43–44 for VTA according to the atlas of Paxinos and Watson (1986). Representative film autoradiograms from syntaxin-1B [column (b)] from rats sacrificed 14 d after the last injection.

Amph challenge injection (0.5 mg/kg) after 14 d of withdrawal, animals pretreated with Amph displayed significantly higher locomotor activity compared to both saline pretreated (\(p < 0.001\)) and untreated control (\(p < 0.001\)) animals (Figure 1b).

Figure 1. Locomotor activity counts measured for each of the three groups (\(n = 30\)) over 5 alternative days of treatment. (a) The amphetamine-treated rats displayed significantly increased locomotor activity compared to the saline-treated (+) and control (*) groups of rats over all tested days. (b) Indicates that following the challenge injection 14 d after the last treatment, the amphetamine-pretreated rats display hyper-responsiveness to amphetamine compared to other groups (*, + \(p < 0.001\)). □, Control group; ■, saline group; ■■, amphetamine group.

Figure 2. Schematic representation of the rat brain regions analysed for syntaxin-1A and -1B mRNA expression. Specific brain regions sampled for analysis are shown as shaded areas (modified from the atlas of Paxinos and Watson, 1986).
or treatment × region interaction \( F(8, 108) = 0.84, p = 0.57 \) but a significant effect of brain region \( F(4, 100) = 2.90, p = 0.03 \) 14 d after the last Amph injection (Figure 3c).

**Syntaxin-1B mRNA expression**

Representative film autoradiograms of syntaxin-1B oligonucleotide labelling are shown in Figure 2b. A two-way ANOVA of the data in animals sacrificed 24 h after the last injection (Figure 4a) showed no significant effect of treatment \( F(2, 108) = 2.13, p = 0.14 \) or treatment × region interaction \( F(8, 108) = 1.12, p = 0.36 \) but a significant effect of brain region \( F(4, 108) = 37.44, p < 0.0001 \). Data from animals sacrificed 7 d after the last treatment (Figure 4b) indicated no significant effect of treatment \( F(2, 108) = 0.39, p = 0.68 \) or treatment × region interaction \( F(8, 108) = 1.99, p = 0.05 \) but a significant effect of brain regions \( F(4, 108) = 15.92, p < 0.0001 \). Two weeks after the last treatment (Figure 4c), analysis of the data showed 24 h after the last injection (Figure 4a) showed no significant effect of treatment \( F(2, 108) = 2.13, p = 0.14 \) or treatment × region interaction \( F(8, 108) = 1.12, p = 0.36 \) but a significant effect of brain region \( F(4, 108) = 37.44, p < 0.0001 \). Data from animals sacrificed 7 d after the last treatment (Figure 4b) indicated no significant effect of treatment \( F(2, 108) = 0.39, p = 0.68 \) or treatment × region interaction \( F(8, 108) = 1.99, p = 0.05 \) but a significant effect of brain regions \( F(4, 108) = 15.92, p < 0.0001 \). Two weeks after the last treatment (Figure 4c), analysis of the data showed
a significant main effect of brain region [F(4, 108) = 49.63, \( p < 0.0001 \)] and treatment \( \times \) region interaction [F(8, 108) = 3.12, \( p = 0.03 \)] but no significant main effect of treatment [F(2, 108) = 1.79, \( p = 0.19 \)]. Post-hoc analysis indicated significant increase in syntaxin-1B mRNA expression in nucleus accumbens shell (NAcS) of Amph-sensitized animals compared to saline pretreated (\( ^+ p < 0.05 \)) or control rats (\( ^* p < 0.05 \)).

**Discussion**

Animals pre-administered with repeated d-Amph displayed significantly higher levels of locomotion compared to controls upon administration of challenge dose of Amph following 14 d of withdrawal. Thus this paradigm involving repeated intermittent administration of low doses of Amph followed by 2 wk of withdrawal induced long-lasting behavioural sensitization to Amph.

The major finding of this study is that sensitized rats show an increased expression of syntaxin-1B mRNA in the NAcS region compared to control or saline pretreated rats which is apparent after 2 wk of drug withdrawal but not after 1 wk or 24 h of withdrawal. No significant changes were seen in the mRNA expression level of syntaxin-1B in the VTA, mPFC, CPu, or nucleus accumbens core (NAcC) at any time-point. Syntaxin-1A mRNA levels were not changed in any assay performed. The increase in syntaxin-1B mRNA in the NAcS is in agreement with our previous findings in which we saw increased levels of syntaxin-1 protein in this region (Marcotte and Srivastava, unpublished observations; Subramaniam et al., 2001). As the antibody used in the previous study did not discriminate between the two isoforms of syntaxin-1, while the oligoprobes used in this report do, it remains possible that the increased protein seen in the previous study reflected an increase in levels of syntaxin-1A.

In our previous study, we also reported reduced levels of syntaxin-1 protein levels in the NAcC subregion using an antibody that recognized both syntaxin-1A and -1B isoforms. However, in the present study no decrease was seen in mRNA expression of either syntaxin-1A or -1B in NAcC or brain regions which send terminals to the NAcC (e.g. VTA and mPFC). A possible reason for this discrepancy could be that syntaxin-1 change in the NAcC of sensitized animals is due to altered translation/degradation of the protein, and not due to transcriptional change in mRNA expression.

The relationship between syntaxin-1B and Amph sensitization is not clear from the present study. Alterations in synaptic plasticity in the mesolimbic system may be a possible link. Drugs of abuse are reported to result in alterations in synaptic plasticity in the NAc and VTA mediated by mechanisms underlying LTP and LTD (Li and Kauer, 2004; Thomas et al., 2001; Thomas and Malenka, 2003; Wolf et al., 2004). Syntaxin-1B plays a role in plasticity and learning. The protein is increased in many limbic areas including NAcS but not NAcC of rats performing memory tasks (Davis et al., 1996, 1998; Richter-Levin et al., 1998) while its mRNA is up-regulated in cells of the dentate gyrus following induction of LTP (Hicks et al., 1997; Richter-Levin et al., 1998). It is of interest that in another model of behavioural sensitization induced by chronic morphine administration, increased mRNA of syntaxin isoforms 5 and 13 in the NAcS has been reported (Hemby, 2004).

There is no evidence here to support a connection between syntaxin-1B and the initiation of the sensitization process. The increase in syntaxin-1B mRNA in the NAcS does not occur until 2 wk after the end of the sensitization regimen and no change in syntaxin-1B mRNA was seen at any time-point in the VTA, the structure most implicated in initiating sensitization (Kalivas and Stewart, 1991; Pierce and Kalivas, 1997; Wolf et al., 2004). Thus, we can assume that changes in this protein seen at the NAcS are the result of the process of expression of sensitization or withdrawal from psychostimulants. Expression of sensitization has been hypothesized to be largely mediated in the NAc, however, the relative contributions of the core and shell subregions remain controversial. Injections of Amph (Pierce and Kalivas, 1995) or cocaine (Filip and Siwanowicz, 2001) into shell but not core in cocaine-sensitized rats leads to sensitized locomotion and sensitized accumbens DA release (Pierce and Kalivas, 1995); however, another study found sensitized accumbens DA release in the core and not shell after systemic Amph challenge (Cadoni et al., 2000).

The identity of cell types showing changes in syntaxin-1B is speculative; however, based on the results presented here and in our previous paper, a possibility is that one population of cells is mediating both the increase in cell body expression of syntaxin-1B mRNA and the increase in nerve terminal expression of syntaxin-1 protein occurring in the NAcS. Such a population would have to be intrinsic neurons of the NAcS, either GABAergic or cholinergic interneurons.

The cholinergic interneurons of the NAcS are heavily implicated in sensitization. Drug-induced...
behavioural sensitization has been associated with increased acetylcholine (ACh) release from rat NAc and other limbic brain regions after acute (Vanderschuren et al., 1999) and chronic (Bickerdike and Abercrombie, 1997; Nelson et al., 2000) Amph exposure. It is tempting to speculate that increased ACh release might be associated with changes in syntaxin-1B since this molecule is thought to promote transmitter release (Helme-Guizon et al., 1998). The reported alterations in the firing pattern of NAcS cholinergic interneurons following Amph sensitization (de Rover et al., 2004) might be a result of plastic changes marked by syntaxin-1B. Double-labeling experiments using antibodies against both syntaxin-1B and markers of cholinergic nerve terminals would allow us to investigate whether cholinergic interneurons are in fact mediating syntaxin-1B changes in response to repeated Amph. It could also be that syntaxin-1B changes are occurring on GABAergic interneurons of NAcS; a possibility which is interesting in light of the role of syntaxin-1 in regulating surface expression and function of the GABA transporter GAT1 (Hansra et al., 2004; Horton and Quick, 2001), although this role has been associated with the syntaxin-1A isoform.

Conclusion

Our current and previous data indicate that Amph sensitization leads to the increased expression of syntaxin-1B mRNA in the NAcS 2 wk following cessation of Amph. The increase in syntaxin-1B mRNA could be an indication of plastic processes involved in the expression of behavioural sensitization to psychostimulants and may be associated with local neurons of the NAcS.

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Statement of Interest

None.

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