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DIFFERENTIAL EXPRESSION OF STRIATAL SYNAPTOTAGMIN mRNA ISOFORMS IN HEMIPARKINSONIAN RATS

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Abstract—Synaptotagmins (Syts) constitute a multi-gene family of 15 putative membrane trafficking proteins. The expression of some of the Syts in the brain might be dopaminergically controlled and thus affected by dopamine depletion in Parkinson's disease. We used hemiparkinsonian rats to investigate the effects of chronic striatal dopamine depletion and the acute effects of antiparkinsonic drug L-DOPA or D1 agonist (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF82958) on the levels of striatal Syt I, II, IV, VI, VII, X, XI mRNA isoforms.

On the 6-hydroxydopamine (6-OHDA)-lesioned side we observed a nearly total loss of tyrosine hydroxylase (TH), synaptotagmin I, Syt IV, Syt VII and Syt XI mRNA levels in the substantia nigra compacta (SNc). In dopamine-depleted striatum we also found a significant down-regulation Syt II and up-regulation of Syt X mRNA levels that could not be reversed by the acute treatment either with L-DOPA or SKF82958. By contrast, these two drugs induced an increase of Syt IV and Syt VII mRNA levels. A time-course study revealed the highest levels of Syt IV and VII mRNAs to occur at two hours and 12 hours after the treatment with SKF82958, respectively. D1 antagonist (±)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390) but not D2 antagonist haloperidol prevented the L-DOPA-driven increase of Syt IV and VII mRNAs.

These results imply that synaptic plasticity in response to chronic striatal dopamine depletion involves a complex pattern of changes in striatal Syt mRNA expression. The L-DOPA treatment does not reverse the changes in Syt II and Syt X gene expression, but recruits additional, D1 receptor-mediated changes in Syt IV and Syt VII gene expression. Whether these D1 receptor-mediated changes play a role in the alterations of synaptic transmission that results in the unwanted side effects of chronic L-DOPA treatment in Parkinson's disease remains to be determined. **© 2005 Published by Elsevier Ltd on behalf of IBRO.**

Key words: synaptotagmins, *in situ* hybridization, striatum, dopaminergic hypersensitivity, SKF82958, L-DOPA.

The Syts are a family of 15 isoforms of membrane proteins proposed to regulate membrane traffic in neuronal and non-neuronal cells. Different Syt isoforms mediate Ca^{2+}-dependent and Ca^{2+}-independent interactions with molecules involved in regulation of membrane fusion (Sudhof and Rizo, 1996). Syts were implicated both in regulated exocytosis and in the compensatory endocytosis of synaptic vesicles (Poskanzer et al., 2003). Syts are differentially localized either to secretory vesicles or to plasma membranes. Syts I and II are vesicular Ca^{2+} sensors (Sugita et al., 2001). They are probably responsible for fusion of docked synaptic vesicles with the plasma membrane and synchronous neurotransmitter release in response to stimulation (Sudhof and Rizo, 1996; Marqueze et al., 2000; Yoshihara et al., 2003; Tucker et al., 2003). Syt IV was originally described as an immediate early gene product induced by forskolin or membrane depolarization in PC12 cells (Vician et al., 1995) and may be associated with dense-core vesicles in forskolin-treated PC12 cells (Fukuda and Yamamoto, 2004). Syt VII is a plasma membrane Ca^{2+} sensor localized in the active zone opposite to synaptic vesicles (Sudhof, 2002). It has been suggested that Syt VII molecule resides on dense-core vesicles and functions as a vesicular Ca^{2+} sensor for exocytosis in endocrine cells (Fukuda et al., 2004). Syts VI and X are also localized on the plasma membrane, but are less abundant. Ca^{2+}-triggered exocytosis has been proposed to involve tandem Ca^{2+} sensors provided by distinct plasma membrane and vesicular Syts (Sugita et al., 2002).

Syts have been implicated in adaptive changes of neurotransmission, such as induced by learning and memory (Ferguson et al., 2000), by drugs (Denovan-Wright et al., 1998; Nakahara et al., 1998; Glavan et al., 2000; Peng et al., 2002; Zhang et al., 2004) or observed in the models of different pathological states (Vician et al., 1995; Babity et al., 1997; Glavan et al., 2000). In Parkinson's disease there is a severe depletion of striatal dopamine due to the degeneration of dopaminergic nigrostriatal neurons. The resulting motor deficits could be reversed by the use of L-DOPA (Valldeoriola et al., 1997). One of the adaptations that follow chronic depletion of dopamine is the development of dopaminergic hypersensitivity. Dopaminergic hypersensitivity is the consequence of the up-regulation of dopamine receptors and/or their intracellular signaling pathways (Graham et al., 1990; Mileson et al., 1991). Dopaminergic hypersensitivity could play a role in the development of pharmacological complications of L-DOPA therapy, such as dyskinesia and psychosis (Calon et al., 2000).

Rats with 6-hydroxydopamine (6-OHDA) unilateral lesions (6-OHDA model) serve as an animal hemi-model of Parkinson's disease (Mendez and Finn, 1975). In this

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Abbreviations: ANOVA, analysis of variance; PENK, proenkephalin; PPT, preprotachykinin; ROD, relative optical density; SCH23390, (±)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; SKF82958, (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; Syt, synaptotagmin; TH, tyrosine hydroxylase; VTA, ventral tegmental area; 6-OHDA, 6-hydroxydopamine.

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model, dopaminergic agonists, such as D1 agonist SKF82958, or indirectly acting agonist, dopamine precursor L-DOPA, drive contralateral turning behavior (Ungerstedt and Arbuthnott, 1970), induce the expression of several immediate early genes (Berke et al., 1998), and modulate the expression of several genes encoding neuropeptide transmitters in dopamine-depleted striatum (Gerfen et al., 1990; Glavan et al., 2002).

We aimed to investigate whether chronic subtotal striatal dopamine depletion could affect the expression of striatal levels of Syt mRNA isoforms and whether dopaminergic agonists could reverse such changes. Striatal dopamine depletion was induced by unilateral injection of dopaminergic nigrostriatal neurons with 6-OHDA. We examined the effects of antiparkinsonic L-DOPA treatment in combination with peripheral decarboxylase inhibitor carbidopa on the expression of Syt I, II, IV, VI, VII, X, XI mRNA isoforms by semiquantitative autoradiographic *in situ* hybridization method. The dopamine receptor mechanisms involved in the effects of L-DOPA were analyzed using the selective D1 agonist SKF89258, D1 antagonist SCH23390 or D2 antagonist haloperidol.

EXPERIMENTAL PROCEDURES

Animals

We used female Wistar rats that were maintained on a 12-h light/dark cycle (light on: 07:00 AM–19:00 PM) in a temperature-controlled colony room at 22–24 °C with free access to rodent pellets and tap water. They were handled according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National Veterinary Institute Guide for the Care and Use of Laboratory Animals. Care was taken to minimize the number of experimental animals and their suffering.

Drugs

The following drugs were used: apomorphine hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline containing 0.02% ascorbic acid; (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF82958; RBI, Natick, MA, USA) was dissolved in 0.9% saline; L-DOPA and carbidopa (gift from LEK Pharmaceutical Company, Ljubljana, Slovenia) were dissolved in 0.3% ascorbate made in 100 mM Na phosphate buffer (pH=5), pH was then adjusted with 10 N NaOH to 6.5–7; (±)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390; RBI) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, Deisenhofen, Germany) and then diluted in 0.9% saline; haloperidol (Haldol; Krka-Janssen, Novo Mesto, Slovenia) was dissolved in 0.9% saline.

Unilateral 6-OHDA lesions of the nigrostriatal pathway

Stereotaxic lesions were created in female Wistar rats weighing between 150 and 200 g. The animals were anesthetized with the i.p. injection of 2% xylazine hydrochloride (8 mg/kg; Rompun®; Bayer, Leverkusen, Germany), ketamine hydrochloride (60 mg/kg; Ketanest®; Parke Davis, Wien, Austria), and atropine (0.6 mg/kg; Belupo, Koprivnica, Croatia), and placed in a stereotaxic frame (TrentWells, South Gate, CA, USA). 6-OHDA hydrobromide (8 μg of free base dissolved in 0.9% saline containing 0.02% ascorbic acid; RBI) was infused at a rate of 1 μl/min over 4 min into the right medial forebrain bundle at the following co-ordinates: anterior 3 mm from lambda, lateral 1.2 mm from the midline and ventral 7.3 mm from the surface of the dura (stereotaxic coordinates; Paxinos and Watson, 1998). At each injection site the cannula was left in place for 2 min before retraction.

Apomorphine test

To assess the development of nigrostriatal degeneration, 6-OHDA-lesioned animals were treated with directly acting mixed agonist of dopamine receptors apomorphine (0.5 mg/kg, *s.c.*) in the sixth post-operative week. Turning response was recorded by placing the rats in plastic cylindrical chambers (40 cm diameter) of the Lablinc automated rotometer system (Colbourn Instruments, Allentown, PA, USA). Only the 6-OHDA rats that responded with peak turning frequency of at least seven contralateral turns per minute were used in subsequent experiments.

Drug treatment and brain preparation

One week after the treatment with apomorphine, 6-OHDA rats (n=58) received saline (1 ml/kg, s.c.) (n=6), D1 receptor agonist SKF82958 (1 mg/kg, s.c.) (n=28) or combined L-DOPA/carbidopa treatment (30 mg/kg, i.p./25 mg/kg, s.c.) (n=24). The animals that received SKF82958 were divided into seven groups of four animals and were killed at different times, i.e. immediately, 1 h, 2 h, 4 h, 8 h, 12 h or 24 h after the injections of SKF82958. The animals that received L-DOPA/carbidopa treatment were divided into six groups of four animals. Groups 1 and 2 were pretreated with saline (1 ml/kg, s.c.) and carbidopa 20 min before the injection of L-DOPA. Groups 3 and 4 were pretreated with SCH23390 (1 mg/kg, s.c.) and with carbidopa 20 min before the injection of L-DOPA. Groups 5 and 6 were pretreated with haloperidol (0.5 mg/kg, s.c.) and with carbidopa 20 min before the injection of L-DOPA. The animals from groups 1, 3 and 5 were killed 4 h; the animals groups 2, 4 and 6 were killed 12 h after the L-DOPA injection.

The brains were rapidly removed and quickly frozen on dry ice. Coronal sections (10 μm) were cut through the neostriatum (between 2.2 mm and −0.3 mm from bregma) and substantia nigra (between −4.8 and −5.8 mm from bregma). The sections were fixed in 4% phosphate-buffered paraformaldehyde, washed in phosphate-buffered saline, dehydrated in 70% ethanol and stored in 95% ethanol at +4 °C until processed for *in situ* hybridization histochemistry.

Oligonucleotide probes

We used oligodeoxyribonucleotide 'antisense' probes (45 bases long) complementary to the rat Syt I mRNA (bases encoding 601–645, sequence 5′-GGA AAA GGC ATC TTC CTT CCC TTC CCC AGG ACT GGC TGG CTC AGT-3′), rat Syt II mRNA (bases encoding 148–192, sequence 5′-GGG TGC CAG AGG CAT TGT GGC AGT GGT GGT GGC CGG AGC CAC AAT-3′), rat Syt IV mRNA (bases encoding 1082–1126, sequence 5′-CAG AGG GAG ACC AGA AGT TCA CCC CGT CCA GAA GAC TTC TTA GCA-3′), rat Syt VI mRNA (bases encoding 518–562, sequence 5′-TCC TTG TTC CTC CAG GGC ATC CAG CAC AGC TTC CAA AAG AGA AAG-3′), rat Syt VII mRNA (bases encoding 300–344, sequence 5′-CCG AGT CTG GCG TGC CCA CCG TCT CCA AGG AGT TCT TGT AGC GTT-3′), rat Syt X mRNA (bases encoding 1509–1553, sequence 5′-TCA GCG TCT AGT CCA GTT CGA CAC ACA CCT ATG ACC TCA TTG TGT-3′), rat Syt XI mRNA (bases encoding 1249–1293, sequence 5′-TTA GTA CTC GTC CAG ACT GTG CCA CTT GGC TAC AGG CTT GCG GGG-3′), rat tyrosine-hydroxylase (TH) mRNA (bases encoding 471–515, sequence 5′-AAC CAA ACC AGG GCA CAC AGG GAG AAC CAT GCT GGA CTT CCT AAG-3′), rat preprotachykinin (PPT) mRNA (bases encoding 136–180, sequence 5′-TCG GGC GAT TCT CTG AAG AAG ATG CTC AAA GGG CTC CGG CAT

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