Neuroscience 137 (2006) 483–492

RHES, THE RAS HOMOLOG ENRICHED IN STRIATUM, IS REDUCED UNDER CONDITIONS OF DOPAMINE SUPERSENSITIVITY

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Abstract—Striatal dopamine receptors become supersensitive when dopaminergic input is removed through either surgical denervation or pharmacological depletion. Although alterations such as increased D2 receptor binding and increased receptor-G protein coupling have been described in supersensitive striatal tissue, their roles in the mechanism of supersensitivity remain uncertain. The *R*as *H*omolog *E*nriched in *S*triatum (Rhes) is expressed in brain areas that receive dopaminergic input, and here we test whether alterations in its expression accompany treatments that promote dopamine receptor supersensitivity in rats. Removal of dopamine input to the striatum by surgical denervation with 6-hydroxydopamine resulted in a decrease in *rhes* mRNA expression throughout striatum, as measured with quantitative *in situ* hybridization. The decrease was detected as early as two weeks and as late as seven months after surgery. Furthermore, a decrease in *rhes* mRNA was evident after repeated or acute reserpine treatment. Chronic daily injection of rats with the D2 antagonist eticlopride, which is known to up-regulate D2 receptors without inducing profound receptor supersensitivity, did not alter the expression of *rhes* mRNA in striatum. Thus, changes in *rhes* mRNA expression are strictly correlated with receptor supersensitivity, perhaps as a result of continuous removal of dopaminergic input. These findings suggest that *rhes* mRNA expression is maintained by dopamine and may play a role in determining normal dopamine receptor sensitivity. **© 2005 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: GTP-binding protein, denervation, gene expression, 6-OHDA, reserpine.

Dopamine receptors can exhibit profound differences in sensitivity, depending on the physiological state of the animal. For example, dopaminergic denervation of striatum in rats with the neurotoxin 6-hydroxydopamine (6-OHDA) (Ungerstedt, 1971) results in 10-40-fold increases in behavioral sensitivity to dopamine agonists (Mandel and Randall, 1985; Marshall and Ungerstedt, 1977). This supersensitivity may be a reflection of the loss of requisite D1/D2 synergism (LaHoste and Marshall, 1992), whereby stimulation of either subtype alone can induce responses (Arnt, 1985; Arnt and Hyttel, 1984) that under normal conditions require concomitant stimulation of both D1-type and D2-type receptors (Clark and White, 1987). Conditions which promote receptor supersensitivity, such as 6-OHDA lesion and reserpine treatment, involve the removal of dopamine from postsynaptic sites. Thus, dopamine itself may normally limit its own response. In agreement with this hypothesis, dopamine-deficient mutant mice are hypersensitive to the locomotor activating effects of exogenous agonist (Kim et al., 2000) and do not exhibit the dampening of dopamine receptor signaling that occurs with the advent of dopaminergic synaptic transmission in wild type mice during ontogeny (Kim et al., 2002). The implication of these studies is that dopamine transmission itself induces some factor which maintains a certain level of sensitivity in postsynaptic neurons, and in the absence of this factor, postsynaptic receptors become supersensitive.

Although the exact mechanism of receptor supersensitivity is unknown, several biochemical and molecular changes in striatal neurons accompany dopamine depletion. For example, striatal D2 receptor binding is increased by 25–50% upon denervation (Creese et al., 1977), but this increase appears after the onset of receptor supersensitivity (Neve et al., 1982). Other proposed mechanisms for receptor supersensitivity include increased dopamine receptor-G protein coupling (Cai et al., 2002), G protein activation (Geurts et al., 1999; Cai et al., 2002), and stimulation of adenylyl cyclase (Mishra et al., 1974). Changes in expression of certain genes, including regulators of G protein signaling (RGS; Geurts et al., 2003), ΔFosB (Doucet et al., 1996; Tekumalla et al., 2001), $G\alpha_{olf}$ (Corvol et al., 2004), and preproenkephalin (Sivam et al., 1986; Schneider et al., 1999; Harrison et al., 2001; Westin et al., 2001) have also been demonstrated after dopamine depletion. However, there is still uncertainty as to the contributions of each of these changes to receptor supersensitivity.

In a search for factors contributing to receptor supersensitivity, we have used differential display to identify genes selectively altered in denervated striatum (LaHoste and Marshall, unpublished observations). One gene which was found to be decreased upon dopamine denervation is identical to the *rhes* (*R*as *h*omolog *e*nriched in *s*triatum) gene identified by Falk et al. (1999) that is relatively enriched in striatum. This gene encodes a 266-amino acid guanosine triphosphate (GTP) binding protein that, along with its close homolog Dexras1 (Kemppainen and Behrend, 1998), defines a novel group of intermediate size G proteins (Graham et al., 2001; Vargiu et al., 2004). Here

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Abbreviations: AGS, activator of G protein signaling; CPu, caudate-putamen; DL, dorsolateral; DM, dorsomedial; DTT, dithiothreitol; ERK, extracellular signal regulated kinase; GTP, guanosine triphosphate; NAc, nucleus accumbens; NHS, normal horse serum; PB, phosphate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RGS, regulator of G protein signaling; Rhes, Ras homolog enriched in striatum; *rhes*, mRNA encoding Rhes; SSC, saline sodium citrate; TH, tyrosine hydroxylase; VL, ventrolateral; VM, ventromedial; 6-OHDA, 6-hydroxydopamine.

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doi:10.1016/j.neuroscience.2005.08.017

we use surgical and pharmacological treatments that perturb dopamine systems in order to demonstrate that *rhes* expression is selectively decreased by treatments that induce dopamine receptor supersensitivity.

EXPERIMENTAL PROCEDURES

Animal surgery and behavioral testing

Male Sprague–Dawley rats weighing 175–200 g were used for dopamine denervation surgery. Animals were anesthetized with a ketamine (80 mg/kg)/xylazine (8 mg/kg) mixture and placed in a stereotaxic frame. Rats were administered the neurotoxin 6-OHDA (8 μg free base in a volume of 4 μl; Sigma-Aldrich; St. Louis, MO, USA) into the medial forebrain bundle of the left hemisphere in order to cause unilateral denervation of the striatum. Uptake of the toxin by noradrenergic neurons was prevented by injection of desmethylimipramine (15 mg/kg, i.p.) 30 min before 6-OHDA injection. Two weeks post-surgery, animals were screened for contralateral rotation to apomorphine (0.25 mg/kg i.p.; Sigma-Aldrich), an indication of >95% dopamine depletion and dopamine receptor supersensitivity. Animals which displayed at least 100 rotations in 20 min were used for further study. All animal experiments were performed in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Experiments were designed and conducted in a manner that minimized the number of animals used and their suffering.

Reserpine treatment

Acute. Male Sprague–Dawley rats weighing 300–325 g were injected s.c. with either reserpine (5 mg/kg; RBI/Sigma) or vehicle (5% acetic acid, 4% 1 N NaOH in distilled water) and killed by decapitation 24 h later. Brains were removed, bisected in the midsagittal plane, frozen in liquid isopentane, and stored at −80 °C for later determination of *rhes* mRNA expression.

Repeated. Rats were injected s.c. once daily for four consecutive days with either vehicle or reserpine (1 mg/kg) and killed 24 h after the last injection. Brains were processed as above except that they were frozen whole rather than bisected.

Eticlopride treatment

Male Sprague–Dawley rats weighing 225–250 g were injected s.c. with either the D2 receptor antagonist eticlopride (0.5 mg/kg; Sigma) or vehicle (0.9% saline) once daily for 21 consecutive days. Four days after the last injection, rats were killed, and the brains were bisected in the midsagittal plane, frozen in isopentane, and stored at −80 °C for later determination of *rhes* mRNA expression by *in situ* hybridization. Previous work has shown that this regimen of eticlopride treatment maximally up-regulates D2 receptor binding following a 96-h drug withdrawal period (LaHoste and Marshall, 1991).

In situ hybridization

Tissue preparation. For acute reserpine and chronic eticlopride studies, hemispheres from vehicle- and drug-treated rats were combined just before sectioning and “glued” together with tissue mounting medium in a counterbalanced manner. This procedure was done in order to overcome slide-to-slide variability when comparing treatments in different brains, as opposed to comparing treatments in the same brain after unilateral 6-OHDA lesions. Twenty-micrometer coronal sections through the striatum were cut on a cryostat at −14 °C and adhered to Vectabond-treated (Vector Laboratories; Burlingame, CA, USA) slides. Slide-mounted sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored at −80 °C until use.

Riboprobe. A 285 base-pair fragment of the rat *rhes* gene (AF134409) was amplified by polymerase chain reaction (PCR) from a rat brain cDNA library (Clontech; Palo Alto, CA, USA) with Pfu Turbo Hotstart polymerase (Stratagene; La Jolla, CA, USA). This segment corresponds to bases 1000–1284 of the mRNA, which fall within the open reading frame. The following primer pair was used (underlined bases are complementary bases for directional insertion into the vector): forward 5′-CCACCATCGAG-GACTTTCAT-3′, reverse 5′-CACCTGTGGTCATTCTTGTTC-CCA-3′ (for antisense orientation). The PCR product was subcloned directionally into pcDNA3.1D/V5-His-TOPO® (Invitrogen; Carlsbad, CA, USA) by a topoisomerase-catalyzed reaction according to the manufacturer’s instructions. Sequence and orientation of the insert were confirmed by dideoxynucleotide sequencing. Plasmids were linearized by cutting with *Xba*I, and radiolabeled mRNA was generated by *in vitro* transcription from this plasmid with ^{35}S-UTP (>1000 Ci/mmol, Amersham/GE Health Care; Piscataway, NY, USA). The transcription reaction (15 μl volume) consisted of the following components: transcription buffer, dithiothreitol (DTT) (7 mM final concentration), rNTP mix (ATP, CTP, GTP; 500 μM each final concentration), 1.5 μg purified plasmid, 125 μCi ^{35}S-UTP, RNase-free water, RNasin RNase inhibitor (20 U), and T7 RNA polymerase (6 U). After a 1 h incubation at 37 °C, plasmid DNA was digested with RNase-free DNase, and the riboprobe was column-purified (RNeasy, Qiagen; Valencia, CA, USA).

Hybridization. Prior to hybridization, slide-mounted sections were exposed to the following solutions in succession: 0.1 M glycine in 0.1 M phosphate buffer (PB), 0.1 M PB, 0.25% acetic anhydride in 0.1 M triethanolamine, 2× saline sodium citrate (SSC), ethanol (50%, 70%, 95%, 100%), chloroform, and ethanol (100%, 95%). 50 μl of hybridization solution (Sigma) containing 1×10^6 cpm *rhes* riboprobe were applied to each air-dried slide, and slides were coverslipped and sealed with DPX mounting medium (Electron Microscopy Sciences; Hatfield, PA, USA). Sections were hybridized for 12–16 h at 55 °C. Following hybridization, sections were washed with 4× SSC/DTT, treated with RNase A, and washed in descending concentrations of SSC/DTT (1×, 2×, 0.5×, 0.1×). Sections were dried and exposed to Kodak Biomax film (Rochester, NY, USA), along with a set of ^{14}C standards (American Radiolabeled Chemicals; St Louis, MO, USA) for quantification of radioactivity, for 1–7 days. Films were developed with Kodak GBX developer and fixed with Kodak GBX fixer.

Tyrosine hydroxylase (TH) immunohistochemistry

Immunohistochemistry was performed on slide-mounted, postfixed striatal sections adjacent to sections used for *rhes in situ* hybridization. Following H_2O_2 pretreatment, sections were washed three times in PBS and blocked for 1 h in PBS with 5% normal horse serum (NHS) and 0.2% Triton X-100. Sections were then incubated overnight at room temperature with mouse anti-TH monoclonal antibody (1:5000; Chemicon; Temecula, CA, USA) in PBS with 1% NHS and 0.1% Triton X-100. Following three washes in PBS, sections were incubated for 1 h in horse anti-mouse biotinylated IgG (1:200; Vector Laboratories) in PBS with 1% NHS. Sections were washed three times in PBS, and immunoreactivity was visualized by incubation of sections with ABC reagent (Vector) followed by diaminobenzidine.

Data analysis

Autoradiograms were digitized and analyzed with MCID Elite software (Imaging Research, Inc.; St. Catharines, Ontario, Canada). For each hybridization run, optical density readings were taken from the images of the ^{14}C standards, and a standard curve was established based on these values. Caudate-putamen (CPu) was divided into anterior (11.08-10.08, AP coordinates in mm anterior to IA, from Paxinos and Watson, 1998), middle (10.08-9.08), and

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