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Dynamic downregulation of Nogo receptor expression in the rat forebrain by amphetamine

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ABSTRACT

Nogo receptors (NgRs) are a family of cell surface receptors that are broadly expressed in the mammalian brain. These receptors could serve as an inhibitory element in the regulation of activity-dependent axonal growth and spine and synaptic formation in the adult animal brain. Thus, through balancing the structural response to changing cellular and synaptic inputs, NgRs participate in constructing activity-dependent morphological plasticity. Psychostimulants have been well documented to induce morphological plasticity critical for addictive properties of stimulants, although underlying molecular mechanisms are poorly understood. In this study, we initiated a study to investigate the response of NgRs to a stimulant. We tested the effect of acute administration of amphetamine on protein expression of two principal NgR subtypes (NgR1 and NgR2) in the rat striatum, medial prefrontal cortex (mPFC) and hippocampus. We found that a single injection of amphetamine induced a rapid and time-dependent decrease in NgR1 and NgR2 expression in the striatum and mPFC. A relatively delayed and time-dependent decrease in expression of the two receptors was seen in the hippocampus. The drug-induced decrease in NgR1 and NgR2 expression in the three forebrain regions was dose-dependent. A behaviorally active dose of the drug was required to trigger a significant reduction in NgR1 and NgR2 expression. These data indicate that NgRs are subject to the regulation by the stimulant. Amphetamine exposure exerts the inhibitory modulation of basal NgR1 and NgR2 expression in the key structures of reward circuits *in vivo*.

1. Introduction

Nogo receptors (NgRs), also known as Nogo-66 receptors and reticulon 4 receptors (RTN4R), belong to a glycosylphosphatidylinositol-anchored and membrane-bound family of cell surface receptors (Zhang et al., 2008; McDonald et al., 2011). In the mammalian brain, these receptors are distributed in broad areas, including the striatum, medial prefrontal cortex (mPFC), and hippocampus (Laurén et al., 2003; Hasegawa et al., 2005; Barrette et al., 2007). At the cellular level, NgRs are distributed densely in axonal, dendritic and spine membranes and in both pre- and post-synaptic density fractions (Lee et al., 2008; Raiker et al., 2010). Upon binding to myelin-associated neurite outgrowth inhibitory proteins (e.g., Nogo), NgRs restrict activity-dependent structural alterations at the synaptic level by blocking axonal outgrowth, dendritic arborization, spinogenesis, and/or synaptogenesis (Lee et al., 2008; Raiker et al., 2010; Zagrebelsky et al., 2010; Wills et al., 2012). Through actively regulating spinogenesis and synaptogenesis in the adult brain, NgRs play an important role in synaptic plasticity (Raiker et al., 2010) and memory formation (Karlén et al., 2009).

NgR expression is subject to the activity-dependent modulation. In cultured hippocampal neurons, application of the agents that elevate neuronal activity, including KCl, NMDA and the GABA receptor antagonist, consistently reduced NgR mRNA and protein expression (Wills et al., 2012). In contrast, the agents that inhibit neuronal activity (the Na^+ channel blocker and the NMDA receptor antagonist) enhanced NgR expression (Wills et al., 2012). In adult animals, kainate-induced seizure and enriched environment stimulation decreased NgR1 expression in the hippocampus *in vivo* (Josephson et al., 2003; Wills et al., 2012). It is suggested that enhanced neuronal activity may relieve the NgR-dependent barrier to synaptic growth and thus facilitate synaptogenesis during development and plasticity in the adult brain (Wills et al., 2012).

Psychostimulants are known to induce robust morphological changes at the spine and synaptic level in multiple brain regions (Robinson and Kolb, 2004). This long-lasting structural plasticity contributes to the enduring remodeling of excitatory synapses

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and stimulant addiction (Abraham, 2008; Shen et al., 2009). As an experience-dependent disorder involving reward and learning and memory, drug addiction is believed to be closely linked to neural activities in the striatum, mPFC and hippocampus (Belujon and Grace, 2011). In fact, substantial structural changes occur in these regions in response to stimulant exposure. For instance, prenatal cocaine exposure increased the dendritic spine density in medium spiny neurons of the striatum, layer II/III of the mPFC, and CA1 pyramidal neurons of the hippocampus in postnatal rats (Frankfurt et al., 2009). Chronic cocaine or amphetamine (AMPH) also induced similar changes in the rat striatum and mPFC (Robinson and Kolb, 1999). While these morphological changes are thought to mediate stimulant effects, molecular mechanisms underlying these changes are poorly understood. NgRs are key regulators of morphological plasticity. Their adaptive changes in response to stimulant exposure may constitute an attractive metaplastic basis for the morphological effect of stimulants.

We therefore initiated the present study to investigate the response of NgRs to the stimulant AMPH. We investigated possible changes in basal protein expression of two major subtypes of NgRs (NgR1 and NgR2) in the striatum, mPFC, and hippocampus of adult rat brains in response to acute AMPH administration *in vivo*. Both time-course and dose–response experiments were carried out to characterize the effect of AMPH.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 250–300 g from Charles River (New York, NY) were individually housed in a controlled environment at a constant temperature of 23 °C and humidity of 50 ± 10% with food and water available *ad libitum*. The animal room was on a 12-h/12-h light/dark cycle. Rats were allowed 5–6 days of habituation to the animal colony. All animal use and procedures were in strict accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Systemic drug injection

D-amphetamine sulfate was purchased from Sigma–Aldrich (St. Louis, MO). The drug was freshly prepared by dissolving it into 0.9% saline solution before experiments. In time-course experiments, rats received a single intraperitoneal (i.p.) injection of AMPH at a dose of 5 mg/kg. The rats were then sacrificed at a different time point (0.5, 1, 2, or 4 h) after drug injection for the following immunoblot analysis of changes in NgR expression. A separate time-course study was added to test the effect of AMPH (5 mg/kg, i.p.) at 8 h after the drug injection. In dose–response experiments, rats were given a single injection of AMPH at different doses (0.2, 1, or 5 mg/kg, i.p.). These rats were sacrificed 4 h after drug injection for detecting the effect of AMPH on NgR expression. Rats treated with saline (1 ml/kg, i.p.) served as controls. To harvest brain tissue for immunoblot analysis, rats were anesthetized with equithesin (5 ml/kg, i.p.) and decapitated. Brains were removed and cut into coronal sections. Three forebrain regions, including the striatum, mPFC and hippocampus, were dissected. Brain tissue was homogenized in the isotonic sucrose homogenization buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, and a protease inhibitor cocktail (Thermo Scientific, Rochester, NY) in a glass grinding vessel with a motor driven Teflon pestle at 700 rpm. The homogenate was centrifuged at 800*g* for 10 min at 4 °C. The supernatant was centrifuged at 12,000*g* for 15 min at 4 °C. Pellets were dissolved in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) with a protease and phosphatase inhibitor cocktail. Protein concentrations were determined with a Pierce BCA assay kit.

2.3. Western blot analysis

Western blot was performed as described previously (Guo et al., 2010). Briefly, the equal amount of proteins was separated on SDS NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA). Proteins were transferred to the polyvinylidene fluoride membrane (Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20) for 1 h. Blots were washed and incubated in the blocking buffer containing an indicated primary antibody. Antibodies used in the present study include rabbit antibodies against β-actin (1:4000, Sigma), and mouse antibodies against NgR1 (1:1000, R&D Systems, Minneapolis, MN) or NgR2 (1:1000, R&D Systems). Both NgR1 and NgR2 antibodies have been pre-validated for their specificity for the targets (Wills et al., 2012). The primary antibody incubation was carried out overnight at 4 °C and followed by 1 h incubation in a horseradish peroxidase-linked secondary antibody against rabbit (1:5000) or mouse (1:3000). Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software.

2.4. Data analysis

The results were presented as means ± S.E.M. Data were analyzed using Student's *t* test or one-way analysis of variance (ANOVA), followed by a Bonferroni comparison of groups. Probability levels of <0.05 were considered statistically significant.

3. Results

3.1. Time-dependent effects of AMPH on NgR1 and NgR2 expression in the striatum

To determine whether acute AMPH administration time-dependently alters basal expression of NgR1 and NgR2 in the striatum, we subjected rats to a single dose of AMPH (5 mg/kg, i.p.) and we then sacrificed rats at a different time point (0.5, 1, 2, or 4 h) after drug injection. Changes in striatal NgR1 and NgR2 protein abundance were analyzed using Western blot. AMPH induced a significant decrease in NgR1 levels at an early time point (0.5 h after drug injection) (70.9 ± 3.9% of saline, $P < 0.05$; Fig. 1A). However, no statistically significant decrease in NgR1 expression was seen at 1 or 2 h, respectively. At 4 h, AMPH again produced a marked reduction of NgR1 (63.9 ± 9.2% of saline, $P < 0.05$). Due to this reduction, we carried out a separate experiment to evaluate the effect of AMPH at a longer time point. As shown in Fig. 1B, AMPH at 8 h after injection caused no evident changes in NgR1 expression. Like NgR1, NgR2 expression in the striatum was reduced 0.5 h after AMPH administration (64.5 ± 8.7% of saline, $P < 0.05$; Fig. 1C). This reduction became no significant at 1 and 2 h. A significant reduction reoccurred at 4 h (59.3 ± 9.5% of saline, $P < 0.05$; Fig. 1C) and then returned to the control level at 8 h (Fig. 1D). These data indicate that both NgR1 and NgR2 expression in striatal neurons is sensitive to AMPH. These receptors noticeably undergo two phases of downregulation (early and late components) in response to the drug.