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Insulin Receptor Substrate-2 in the Ventral Tegmental Area Regulates Behavioral Responses to Cocaine

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Abstract

Neurotrophic factor signaling modulate cellular and behavioral responses to drugs of abuse. Chronic exposure to abused drugs, among other biochemical adaptations, decreases the expression of Insulin Receptor Substrate-2 (IRS-2; a protein involved in neurotrophic signaling) in the ventral tegmental area (VTA), a neural substrate for many drugs of abuse. Using viral-mediated gene transfer to locally alter the activity of IRS-2, we show that overexpression of IRS-2 in the VTA results in an enhanced preference for environments previously paired with cocaine, as measured by the place conditioning paradigm, while blockade of IRS-2 activity results in avoidance of cocaine-paired compartments. In addition, IRS-2 overexpression leads to enhanced cocaine-induced locomotor activity, while blockade of IRS-2 expression significantly blunts behavioral responses to cocaine. These results demonstrate that levels of IRS-2 in the VTA regulate responsiveness to the behavioral effects of cocaine.

Keywords

growth factors; neural plasticity; viral-mediated gene transfer; drug addiction; behavioral sensitization

Neurotrophic factors (NF) and their signaling pathways are best understood for promoting growth, differentiation and survival of neurons during development. More recently, these factors have been implicated as mediators of neuronal maintenance and plasticity in the adult nervous system (Lindsay, Weigand, Altar, & DiStefano, 1994; Lu & Figurov, 1997). Neurotrophins are a family of neurotrophic factors that are highly expressed in brain. Neurotrophins, acting in several brain regions, have been implicated in the modulation of neuronal excitability and synaptic transmission (Poo, 2001; Schuman, 1999), learning and memory (Alonso *et al.*, 2002), mood disorders (Duman, 2002; Nestler *et al.*, 2002; Russo-

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Neustadt, 2003) and the cellular and behavioral responses to drugs of abuse (Bolaños & Nestler, 2004).

Repeated exposure to drugs of abuse causes long-lasting cellular, molecular, and behavioral adaptations in the mesolimbic dopamine system, adaptations that have been implicated, at least in part, in the transition from drug abuse to addiction (Koob, Sanna, & Bloom, 1998; Nestler, 2001). This neural circuit is comprised of dopaminergic neurons in the ventral tegmental area (VTA) and their target regions in the limbic forebrain such as the nucleus accumbens (NAc), and is a major substrate for motivated behavior and responses to natural and drug reinforcers (Di Chiara & North, 1992; Kelley & Berridge, 2002).

Chronic exposure to morphine, among other adaptations, induces higher levels of tyrosine hydroxylase (TH) in the VTA, impairs the structural integrity of VTA dopamine neurons (Russo *et al.*, 2007; Sklair-Tavron *et al.*, 1996), and alters levels of several NF signaling proteins, including the down-regulation of the Insulin Receptor Substrate 2/phosphatidylinositol-3'-kinase (IRS2-PI3K) signaling pathway in the VTA (Nestler, Berhow, & Brodtkin, 1996; Wolf, Numan, Nestler, & Russell, 1999). The functional consequences of changes in IRS-2 levels in the VTA are yet to be fully elucidated; however, we have shown that viral-mediated changes in expression of IRS-2 in the VTA regulates cell morphology and morphine reward (Russo *et al.*, 2007) including responses to emotion-eliciting stimuli (Krishnan *et al.*, 2008). Here we further investigated the influence of IRS2 in the VTA on behavioral responses to drugs of abuse by studying interactions between IRS-2 and cocaine, using locomotor activity and conditioned place preference (CPP) paradigms, after selectively increasing or blocking IRS-2 activity in the VTA by microinjecting a herpes simplex virus (HSV) vector encoding a wild type (HSV-IRS2_{wt}) or a dominant negative mutant form (HSV-IRS2_{dn}) of this protein.

Adult male Sprague-Dawley rats (Charles River, Raleigh, NC), weighing 350–375 g at the start of the experiment, were used in this study. All rats were habituated to the animal facility for at least one week before experimental manipulation. Rats were double housed in clear polypropylene boxes containing wood shavings, in an animal colony maintained at 23–25°C on a 12-hr light/dark cycle in which lights were on between 07:00 and 19:00 hr. All rats were provided with food and water ad libitum. Experiments were conducted in compliance with the Guidelines for the Care and Use of Laboratory Animals (National Institute of Health, 1996), and approved by Florida State University animal care and use committee.

Rats were anesthetized with an intramuscular injection of a ketamine/xylazine cocktail (80/10 mg/kg) and given atropine (0.25 mg/kg) subcutaneously to minimize bronchial secretions; afterward, rats were given unilateral microinjections (2.0 µl over 10 min of either HSV-GFP (control vector expressing green fluorescent protein), HSV-IRS2_{wt}, or HSV-IRS2_{dn}, or Sham surgery (lowered needle to targeted area but no volume injected), into the rostral region of VTA (AP: -4.9, Lat: +2.2, DV: -7.6 mm below dura) (Paxinos & Watson, 1997) using a 32 gauge Hamilton syringe angled at 10° from the midline, to avoid piercing the sinus system. Construction of the viral vectors has been described previously (Neve, Howe, Hong, & Kalb, 1997). All behavioral experiments were commenced 3 days after viral surgery, a time at which maximal transgene expression is observed (Barrot *et al.*, 2002; Carlezon *et al.*, 1997). No detectable IRS-2 or GFP expression was seen in either efferent (e.g., the NAc) or afferent (e.g., the dorsal raphe) regions of the injected area, nor a week after viral infusion consistent with earlier findings (Barrot *et al.*, 2002). Histological assessment of microinjections and transgene detection was performed as described previously (Russo *et al.*, 2007). Half the rats utilized for these experiments received viral infusion in the left or right side of the VTA. Separate statistical analyses were done to control for side of infusion; no differences were detected. Only needle

placements ranging from -4.9 and -5.5 mm from Bregma (i.e., rostral VTA) were included in this study.

Place conditioning was carried out exactly as described (Bolaños *et al.*, 2003). Briefly, a three-compartment apparatus (FSU Psychology engineering group) was used, where compartments differed in floor texture, wall coloring, and lightning. On the preconditioning day (day 0), rats were allowed to freely explore the entire apparatus for 30 min to obtain baseline preference to any of the three compartments (side compartments: 35 × 27 × 25 cm; middle compartment: 10 × 27 × 25 cm). Only rats showing no spontaneous preference to either compartment were used (unbiased procedure); this accounted for more than 90% of all of the animals tested. Rats then received unilateral viral infusions, including Sham surgery, into the rostral VTA, and were allowed to recover for two days. After recovery, conditioning trials (two per day) were given on two consecutive days (day 3 and 4). During the conditioning trials, rats received an intraperitoneal (IP) saline injection (1 ml/kg) and were confined to one of the large size compartments of the apparatus for 1 hr. Then, 3 hr after the first conditioning session, rats received cocaine (10 or 20 mg/kg, IP) and were confined to the opposite side compartment for 1 hr. Conditioning trials were counterbalanced such that half the rats received drug in one compartment and the other half received the drug in the opposite compartment. On the final day (day 5), rats were again allowed to freely explore the entire apparatus for 30 min.

Locomotor activity was measured each day for 2 hr in automated (75 cm diameter × 15 cm wide, 4 photocell beams) circular activity chambers (Med Associates, St. Albans, VT). Rats were exposed to the chambers after one IP saline injection each day for 3 consecutive days and underwent surgery at the end of day 3 (as described above). On day 5, rats received an acute injection of saline to assess whether surgery itself changed baseline locomotion (see Fig. 2A). Rats were then treated with cocaine (5 or 15 mg/kg in 1 ml/kg, IP; Sigma-Aldrich, St. Louis, MO) once daily for 5 consecutive days (starting on day 6). One day after the last cocaine injection (day 11), rats were given an injection of saline to assess whether they were responding to the injection itself or to cocaine, and were rested for one week. At the end of the rest period (day 18), rats were challenged with either 5 or 15 mg/kg cocaine. Statistical significance was measured using mixed-design (between and within variables) analysis of variance (ANOVAs) followed by Tukey *post-hoc* tests. Data are expressed as the mean ± SEM. Statistical significance was defined as $P < 0.05$.

Figure 1A (left panel) shows the region of the VTA to which Sham surgeries or microinjections of HSV vectors (HSV-GFP, HSV-IRS-2_{wt}, or HSV-IRS-2_{dn}) were aimed. As previously reported, we found that expression of GFP (data not shown) and IRS-2 was maximal between days 3 and 4 after virus injection, significantly declining thereafter, and non-detectable 1 week after the microinjection (Barrot *et al.*, 2002; Carlezon *et al.*, 1997). In addition, no change in IRS-2 immunoreactivity was present in rats receiving Sham or HSV-GFP microinjections (Russo *et al.*, 2007). Confocal microscopy (Fig. 1A; right panel) revealed that the percentage of TH-positive neurons overexpressing IRS-2 in the VTA (~50%) was similar to previous findings (Olson *et al.*, 2005; Russo *et al.*, 2007).

Figure 1B shows the effects of HSV treatments on a model of drug-seeking behavior, namely the cocaine CPP paradigm. Place preference conditioning has been widely utilized to assess the rewarding or aversive properties of drugs. In this behavioral assay, animals learn to prefer environments previously associated with rewarding, while avoiding environments associated with aversive drug effects (Carlezon, 2003; Hoffman, 1989). As can be seen in Figure 1B, time spent in the cocaine-paired compartments varied by viral treatment and drug dose (virus X drug dose interaction: $F_{(6,66)} = 4.40$; $p < 0.0009$). Rats receiving the various viral-vector treatments and conditioned to saline did not show preferences for either side of the CPP compartments. Conversely, rats receiving HSV-IRS2_{wt} microinjections into the VTA spent

significantly more time in environments paired with a moderate dose (10 mg/kg) of cocaine ($p < 0.05$), while rats with HSV-IRS2_{dn} microinjections did not consistently approach the cocaine-paired environments. In fact, microinjecting HSV-IRS2_{dn} into the VTA resulted in avoidance of the cocaine-paired compartments ($p < 0.05$). The Sham, HSV-GFP, and HSV-IRS2_{wt} groups treated with 20 mg/kg cocaine showed robust place conditioning, while IRS2_{dn} expressing rats did not show reliable conditioning to the cocaine-paired compartments.

We next assessed the effect of IRS-2 manipulation on locomotor activity using a behavioral sensitization paradigm. Figure 2A shows that baseline locomotion was similar between the groups before and 2 days after surgery, thus indicating that surgery alone, or the virus itself, did not affect spontaneous locomotor activity. Data presented in Figure 2B compares behavioral responding to 5 or 15 mg/kg cocaine on day 6 (first cocaine exposure), day 10 (last day of cocaine before rest period) and day 18 (challenge day) in all groups. These rats also received a saline injection on day 11 to assess for potential conditioned locomotion effects. No differences in behavioral responding to saline in any of the groups were revealed (data not shown). A three-factor ANOVA yielded significant main effects of viral treatment ($F_{(3,180)} = 17.9$; $p < 0.0001$), drug ($F_{(1,180)} = 309.4$; $p < 0.0001$), and day ($F_{(2,238)} = 27.9$; $p < 0.0001$). Behavioral responses to cocaine also varied as a function of viral treatment and drug (virus X drug dose interaction: $F_{(3,180)} = 5.7$; $p < 0.0009$), and day (drug dose X day interaction: $F_{(3,180)} = 9.8$; $p < 0.0001$). Post hoc analyses revealed significant differences for 5 mg/kg cocaine dose on day 6, indicating that behavioral responses were significantly higher for the HSV-IRS2_{wt}-treated, and lower for the HSV-IRS2_{dn}-treated rats ($p < 0.05$). As expected for the higher cocaine dose, analysis indicated that the Sham and HSV-GFP groups had enhanced locomotor responding on days 10 and 18 when compared to day 6 ($p < 0.05$), indicating the development and expression of locomotor sensitization, respectively. Further analysis revealed that rats receiving HSV-IRS2_{wt} had greater overall behavioral responding to cocaine than the Sham, HSV-GFP, and HSV-IRS2_{dn} groups on day 6, findings which indicate that overexpression of IRS2_{wt} increases behavioral sensitivity to acute cocaine. In addition, these rats showed enhanced sensitivity to the stimulant on days 10, and 18, as compared to their behavioral responding on day 6 ($p < 0.05$), thus indicating that overexpression of IRS2_{wt} in the VTA results in enhanced behavioral responding. However, the magnitude of their behavioral responding did not differ from that observed in the Sham and HSV-GFP groups on day 18. Moreover, no significant differences in behavioral responding were evident in the HSV-IRS2_{wt} group between day 10 and 18. Conversely, a different behavioral profile was observed in rats microinjected with IRS2_{dn} (Fig. 2B). For instance, no significant differences were apparent amongst IRS2_{dn} expressing rats when their behavioral responding was compared on days 6 and 10 ($p > 0.05$). In fact, these rats showed significantly blunted responses to cocaine when compared to the other groups on days 6 and 10. However, a significant increase in locomotor activity was observed in these animals on day 18 ($p < 0.05$), although their behavioral responding to the cocaine challenge injection was still significantly lower than that observed in the other treatment groups (Fig. 2B).

To better understand the role IRS-2 plays in mediating enhanced behavioral responding to cocaine, we conducted a third experiment (Figure 2C) in which rats received the drug regimen as described above. Briefly, rats were placed in the locomotor chambers after receiving a saline injection for 5 days. Starting on day 6, rats received one daily cocaine injection (15 mg/kg) for 5 consecutive days and were placed in the locomotor chambers. After the last cocaine injection, rats were rested for a week. On day 15, rats were anesthetized and received viral infusions as described above. Only rats showing enhanced behavioral responding to cocaine on the last day of cocaine treatment (day 10) were assigned to the various viral conditions. Three days after surgery (day 18) rats were challenged with 15 mg/kg cocaine. Figure 2C shows that rats treated with HSV-IRS_{dn} had a diminished response to cocaine, whereas the other groups showed the expected behavioral responding to the cocaine challenge ($F_{(3,32)} = 10.39$; $p < 0.0001$). Further

tests confirmed that blockade of IRS2 activity significantly blunted the cocaine-induced behavioral effects (HSV-IRS2_{dn} versus HSV-IRS2_{wt}, $p = 0.007$; HSV-IRS2_{dn} versus HSV-GFP, $p = 0.0009$).

Previous reports have implicated neurotrophins in the cellular and behavioral adaptations occurring in the VTA after prolonged exposure to drugs of abuse (see Introduction). In the present study, we increased or decreased levels of IRS-2, a protein known to mediate and amplify PI3K activity (Fisher & White, 2004), in the VTA by using viral-mediated gene transfer to locally increase or block IRS-2 activity levels in this brain region. We demonstrate here that increased levels of IRS-2 in the VTA leads to enhanced reward and locomotor sensitivity to cocaine, while decreasing the levels of this protein caused opposite effects. Together, these data suggest that IRS-2 activity in the VTA regulates behavioral responsiveness to cocaine.

Enhancing IRS-2 activity resulted in increased sensitivity to the rewarding effects of cocaine because rats treated with HSV-IRS2_{wt} reliably approached environments previously paired with doses of cocaine that did not induce place conditioning in control rats. In contrast, HSV-IRS2_{dn}-treated rats avoided environments paired with a threshold dose of cocaine, while showing no reliable preference or avoidance to environments paired with the higher dose of cocaine. These findings are in agreement with demonstrations that molecular manipulations that reduce the rewarding effects of cocaine often lead to cocaine-induced place aversions (Barot, Ferguson, & Neumaier, 2007; Carlezon *et al.*, 1997; Olson *et al.*, 2005). The biphasic properties of cocaine are well documented, and it is possible that the aversion is caused by a decrement in cocaine's rewarding effects (i.e., tolerance to the rewarding effects of the drug), which, in turn, unmasks other aversive effects of the drug (Ettenberg, 2004; Koob & Le Moal, 2008; Solomon & Corbit, 1974). A parallel pattern of behavioral reactivity was observed when the effects of IRS-2 activity levels on locomotor activity were assessed. Increasing IRS-2 activity lead to an enhancement in the locomotor-activating effects induced by acute and repeated cocaine exposure, while blocking IRS-2 activity blocked the behavioral responding to cocaine. However, HSV-IRS2_{dn}-treated rats still showed a modest, though significant, increase in locomotor activity, on the challenge day. Given that no viral expression can be detected a week after virus infusion (Barrot *et al.*, 2002; Carlezon *et al.*, 1997), these data suggest that IRS-2 activity may be important for the expression of drug-induced behavioral sensitization (Izzo, Martin-Fardon, Koob, Weiss, & Sanna, 2002; Russo *et al.*, 2007). This is likely the case because rats previously sensitized to the drug and treated with HSV-IRS2_{dn} three days before the drug challenge showed a significantly blunted response to cocaine.

The effects of manipulating IRS-2 expression in the VTA on measures of cocaine-induced reward and locomotor reactivity are in agreement with previous evidence demonstrating PI3K activity as necessary for enhancement of opiate and natural reward (Krishnan *et al.*, 2007; Russo *et al.*, 2007), as well as for the expression of behavioral sensitization to cocaine (Izzo *et al.*, 2002). The mechanism(s) underlying these effects are not fully known. The initiation of behavioral sensitization occurs at the level of the VTA, while its expression is mediated at the level of the NAc (Pierce & Kalivas, 1997). In the Izzo *et al.* study (2002), rats received intracerebroventricular infusions of the PI3K inhibitor LY294002 and, thus, it is conceivable that the inhibitor was acting in any of numerous brain regions (e.g., NAc) to block the expression of behavioral sensitization, while in the present study, we specifically targeted rostral portions of the VTA to regulate IRS-2 activity. Given that topographical differences within the VTA have been shown to mediate differential responding to the rewarding and locomotor-activating properties of drugs (Bolaños, Neve, & Nestler, 2005; Bolaños *et al.*, 2003; Carlezon *et al.*, 2000; Ikemoto, Kohl, & McBride, 1997; Olson *et al.*, 2005), it is conceivable that distinct populations of dopamine neurons within the VTA might mediate the effects observed in this study (Carr & Sesack, 2000; Di Chiara, 1997; Ikemoto, 2007; Olson &

Nestler, 2007). Though anatomical characterization of the VTA is not yet complete, this assumption is consistent with findings suggesting that dopamine neurons from rostral portions of the VTA innervate primarily, but not exclusively, the NAc shell (i.e., mesolimbic), while dopamine neurons from more caudal VTA regions project predominantly, but not exclusively, to cortical areas (i.e., mesocortical regions) (Emson & Koob, 1978). These neural projections also show differential responding to drugs of abuse by increasing extracellular dopamine levels in the NAc shell as compared to prefrontal cortical areas (Bassareo, Tanda, Petromilli, Giua, & Di Chiara, 1996). Thus, it is conceivable, within this framework, that increasing IRS-2 activity within dopamine neurons in rostral VTA increases sensitivity to cocaine, resulting in increased reward and locomotor responding, while resulting in opposite effects when IRS-2 activity is blocked in this brain region.

Together, these results are in agreement with studies showing that other NF signaling pathways (e.g., MAP/ERK, bFGF, PI-3-K, PLC γ 1, Akt) also regulate behavioral responding to drugs of abuse (Bolaños & Nestler, 2004; Pierce & Bari, 2001). Results from the present study further establish the functional importance of drug-induced regulation of IRS-2 expression in the VTA. Further assessment of the mechanisms underlying this IRS2-induced behavioral effects will lead to a better understanding of the neural and molecular basis of drug addiction.

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Fig. 1A

Fig. 1B

Figure 1.

Viral-mediated gene transfer in the VTA. (A) *Left panel*: Rostral region of the VTA to which microinjections of HSV vectors and Sham surgery were targeted. *Right panel*: Merged confocal photomicrograph (magnification, 400X) of a representative brain slice from the rostral VTA (~5 mm caudal to Bregma) double-labeled for TH (green: Cy2) and IRS-2 (red: Cy3) fluorescence. Dual-labeled cells represented by *orange* fluorescence. *Arrows* indicate colabeled cells. (B): IRS-2 activity in the VTA regulates responses to cocaine CPP (N = 78). HSV-IRS2_{wt} overexpression enhanced sensitivity to 10 mg/kg of cocaine (**p* < 0.05), whereas expression of HSV-IRS2_{dn} resulted in place aversion (**p* < 0.05). Sham, HSV-GFP, and HSV-IRS2_{wt} rats treated with 20 mg/kg cocaine showed reliable place conditioning, while the HSV-IRS2_{dn} rats did not show place conditioning or aversion to cocaine.

Figure 2.

IRS-2 activity in the VTA regulates cocaine-induced locomotion. (A) Baseline locomotor activity for 3 consecutive days prior to and following 2 days after surgery. (B) Data representing all groups on day 6, 10, and 18 (N = 68). Repeated administration of cocaine enhanced behavioral responding in Sham, GFP, and IRS-2 groups (* $p < 0.05$; compared to day 6). Blockade of IRS-2 activity decreased sensitivity to cocaine ($\sqrt{p} < 0.05$; compared to Sham, HSV-GFP, and HSV-IRS2_{wt} on day 6; † $p = 0.07$; compared to day 10). HSV-IRS2_{dn}-treated rats show behavioral sensitization to cocaine on day 18 (* $p < 0.05$; compared with the group's responsiveness on day 6 and 10). (C) HSV-IRS2_{dn} significantly blunted behavioral responses to the cocaine challenge on day 18 (* $p < 0.05$; N = 36)