Title: AUTS2 in the nucleus accumbens is essential for heroin-induced behavioral sensitization

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Abbreviations

AUTS2: autism susceptibility candidate 2

CPu: Caudate-putamen

EGFP: enhanced green fluorescent protein

NAc: nucleus accumbens

PcG: polycomb group

PCGFs: polycomb group RING fingers

PRC: polycomb repressive complex

VTA: ventral tegmental area

ABSTRACT

Autism susceptibility candidate 2 (AUTS2) is a gene associated with autism and mental retardation. Recent studies have suggested an association of the *AUTS2* gene with heroin dependence, and reduced *AUTS2* gene expression may confer increased susceptibility to heroin dependence. However, the functional role of the AUTS2 protein in regulating enduring neuroadaptations in response to heroin exposure has not been established. Here, we investigated the effects of acute and chronic heroin exposure on AUTS2 mRNA and protein expression in the nucleus accumbens (NAc) and caudate-putamen (CPu) to determine whether changes in AUTS2 expression are associated with heroin-induced locomotor sensitization. Moreover, we explored whether AUTS2 knockdown affects heroin-induced locomotor sensitization. AUTS2 mRNA and protein expression in the NAc, but not the CPu, was decreased after chronic heroin (1 mg/kg) administration. In the NAc, the expression of heroin-induced locomotor sensitization was enhanced through the lentiviral-AUTS2-shRNA-mediated knockdown of AUTS2, while the overexpression of AUTS2 attenuated the locomotor-stimulant effects of heroin. Together, these results indicate that AUTS2 in the NAc, but not the CPu, suppresses the initiation and expression of heroin-induced behavioral sensitization, suggesting that AUTS2 may be a potential target for the treatment of heroin dependence.

Keywords: Autism susceptibility candidate 2; Nucleus accumbens; Behavioral sensitization

INTRODUCTION

Heroin abuse is a major health and social problem of modern society worldwide. From a neurobiological point of view, heroin addiction can be characterized by long-lasting changes in behavior that persist despite prolonged abstinence; however, the underlying mechanisms are complex. Extensive studies have suggested that alterations in gene expression in the reward circuitry contribute to the long-term adaptations in brain structure and function that accompany chronic exposure to heroin. In the past decade, epigenetic mechanisms, including alterations in the accessibility of genes within the native chromatin structure of these molecules induced through histone tail modifications and DNA methylation, have been implicated in mediating the long-term adaptations that underlie opiate addiction.

Polycomb group (PcG) proteins maintain repressive forms of chromatin and appropriate patterns of gene repression through epigenetic mechanisms. Alterations of PcG protein complexes, including Polycomb repressive complex 1 (PRC1) and 2 (PRC2), at *Bdnf* promoters in the rat ventral tegmental area (VTA) have been implicated in mediating neural and behavioral plasticity in response to chronic morphine administration (Koo et al., 2015). Previous studies have revealed a physical link between novel PRC1 complexes, comprising Polycomb group RING fingers (PCGFs) 3 and 5, and autism susceptibility candidate 2 (AUTS2) (Gao et al., 2012). A recent study indicated that PRC1-AUTS2 neutralizes PRC1 repressive activity, whereas AUTS2-mediated recruitment of P300 results in gene activation through promoter association (Gao et al., 2014). In addition, the conditional targeting of *Auts2*

in the mouse central nervous system (CNS) leads to various developmental impairments (Gao et al., 2014).

The *Auts2* gene, located on chromosome 7q11.2, encodes a nuclear protein and is frequently associated with multiple neurological diseases, including autism. In addition, *Auts2* has been implicated as an important gene in human-specific evolution (Sultana et al., 2002, Oksenberg and Ahituv, 2013). A recent functional analysis of this gene revealed a potential role for AUTS2 in the regulation of alcohol consumption and heroin addiction (Schumann et al., 2011, Chen et al., 2013, Chojnicka et al., 2013), as well as neurodevelopment (Beunders et al., 2013, Oksenberg et al., 2013, Gao et al., 2014); however, the precise function and signaling pathways associated with AUTS2 in adult brains and opiate addiction have not been established. Here, we report that decreased AUTS2 mRNA and protein expression in the nucleus accumbens (NAc), but not the caudate-putamen (CPu), is associated with repeated heroin exposure. Furthermore, AUTS2 expression levels in the NAc are reduced during the initiation and expression of heroin-induced behavioral sensitization. The specific overexpression of AUTS2 in the mouse NAc blocks the locomotor-stimulant effects of heroin, while AUTS2 knockdown enhances heroin-induced hyperlocomotion.

MATERIALS AND METHODS

Subjects

Male C57BL/6J mice (weighing 18-20 g upon arrival) were obtained from the Laboratory Animal Center, Xi'an Jiaotong University. The mice were housed in groups of four in a temperature-controlled ($23 \pm 2^{\circ}\text{C}$) and humidity-controlled ($50 \pm 5\%$) room with food and water provided *ad libitum* in the home cage. The animals were maintained on a reverse 12-h/12-h light/dark cycle (lights off at 8:00 AM). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the local Animal Care and Use Committee.

Drugs

Heroin hydrochloride (purity 80%) was provided by the Drug Intelligence and Forensic Center, Ministry of Public Security from PR China. The drugs were dissolved in a vehicle (saline with 0.005% acetic acid), and an injection volume of 10 ml/kg body weight was freshly prepared prior to each experiment.

Acute and repeated heroin treatment paradigm

For the acute injections, the mice were subcutaneously (s.c.) injected with 1 mg/kg of heroin ($n = 8$) or vehicle (vehicle, $n = 8$) at 1 h prior to sacrifice. For repeated injections, the mice ($n = 8$ per group) were injected twice a day (at 11:00 h and 16:00 h) for five consecutive days with heroin (1 mg/kg, s.c.). The mice were sacrificed 1 h after the last injection.

Heroin-induced behavioral sensitization

Heroin-induced behavioral sensitization was conducted according to previous studies, with some modifications (D'Este et al., 2002, Ranaldi et al., 2009, Wu et al., 2015). Briefly, heroin (HRI; 1.0 mg/kg, s.c.) or vehicle (SAL) was injected twice a day (at 8:00 h and 20:00 h) for five consecutive days (Day 1 to Day 5). Three days after the last injection (Day 8), the mice were challenged with an injection of heroin (0.5 mg/kg, s.c., at 20:00 h), and the locomotor activity was measured for 60 min after the challenge injection. To examine the influence of AUTS2 on the development of behavioral sensitization, lentiviral-AUTS2-shRNA was administered 1 week prior to the twice-daily heroin regimen.

Locomotor responses were determined in individual activity chambers (43 X 43 X 43 cm) as previously described (Zhu et al., 2012). Following a 2-hour habituation period, the mice were injected, and locomotor activity was monitored using a Smart Video Tracking System (version 2.5; Palnlab Technology for Bioresearch, Spain). The cages were carefully cleaned with a 0.1% acetic acid solution to prevent any odor traces.

Plasmid constructions and preparation of viral stocks

For lentivirus expression, cDNAs encoding AUTS2-shRNA or control-shRNA (Hori et al., 2014) and a fusion AUTS2 protein with the V5 tag at the C-terminal (AUTS2 overexpression) were ligated into the lentiviral vector plenti6/V5-topo (Life Technologies, UK), bicistronically expressing enhanced green fluorescent protein (EGFP). Recombinant lentiviruses were produced using the ViraPower Lentiviral Expression System (Invitrogen, K4970-00). The virus was concentrated 10-15 times through centrifugation in a Centricon Plus-20 filter (Millipore) according to the

manufacturer's instructions. Aliquots were stored at -80°C . All virus preparations were titered according to the Virapower protocol and contained 4×10^8 TU/ml.

Surgery and intracranial injections

The mice (weighing 18-20 g at the time of surgery) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments). Lentiviral-AUTS2-shRNA (knockdown) or lentiviral-AUTS2 (overexpression) (approximately 1.0×10^6 infectious units of virus per μl) was bilaterally injected into the NAc (from the bregma: AP, +1.6 mm; ML, ± 1.5 mm; DV, -4.4 mm) using a Hamilton syringe (needle gauge 31) at a speed of 0.1 $\mu\text{l}/\text{min}$ to a total volume of 0.5 μl . The needle remained in place for an additional 5 min. The behavioral tests were performed 7 days after the viral injection, and the infected NAc areas were collected after sacrifice for western blotting.

Tissue sample preparation and western blot assays

Tissue sample preparation and western blotting were performed as described previously (Wang et al., 2015, Zhao et al., 2015). Briefly, the NAc and CPu were isolated on ice using a 26-gauge needle under a dissecting microscope. The isolated tissue was stored at -80°C until further processing for western blotting. The proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were incubated with blocking solution containing 5% dried non-fat milk in TBST (0.1% Tween-20 in TBS buffer) for 1 h. The membranes were incubated in primary antibody diluted in blocking solution overnight at 4°C . The membranes were rinsed in TBST buffer (3 x

20 min) and subsequently incubated in secondary antibody for 1 h at room temperature. The following primary antibodies were used: rabbit anti-AUTS2 (1:1000, Abcam, ab96326) and mouse anti- β -actin (1:10000, Sigma-Aldrich). The blots were developed with enhanced chemiluminescence (ECL) reagent (Amersham), and the autoradiogram bands were analyzed using Quantity One software (Bio-Rad). All western blot samples were normalized to β -actin.

RNA quantification

Total RNA was extracted from stored NAc or CPu using TRIzol reagent (Invitrogen, Carlsbad, CA). Real-time quantitative PCR (RT-qPCR) was performed on an iQ5 system (Bio-Rad Laboratories, Hercules, CA) using SYBR Premix Ex Taq II (TaKaRa). The data were normalized to β -actin. The following primer pair sequences were used: AUTS2 (NM_177047.3) forward primer 5'-AATGACCGCAATCTCTGCCA -3' and reverse primer 5'-GCTGGAAGCCCTTGGATTCT -3'; β -actin (NM_013540) forward primer 5'-TAGGCGGACTGTTACTGAGC-3' and reverse primer 5'-TGCTCCAACCAACTGCTGTC-3'.

Data analysis

The data are expressed as the means $\pm$ SEM and were analyzed using analysis of variance (ANOVA) with Tukey's post hoc test as appropriate. A $p < 0.05$ was considered statistically significant.

RESULTS

Repeated heroin exposure causes reduced AUTS2 expression in the NAc but not the CPu

To investigate the involvement of AUTS2 expression in heroin dependence, we first examined the mRNA and protein levels of AUTS2 after acute and repeated heroin injection using qRT-PCR and western blotting. As shown in Fig. 1A, the mRNA levels of AUTS2 were unaltered after the acute heroin injection (1 mg/kg, s.c.) in both the NAc and CPu (NAc: SAL 1.00 ± 0.08 vs. HRI 1.02 ± 0.11 ; CPu: SAL 1.00 ± 0.10 vs. HRI 1.02 ± 0.13 , both $p > 0.05$). However, reduced AUTS2 expression was detected after repeated heroin treatment (1 mg/kg, s.c. twice a day for 5 days) in the NAc (SAL 1.00 ± 0.09 vs. HRI 0.45 ± 0.06 , $p < 0.001$) but not the CPu (SAL 1.00 ± 0.07 vs. HRI 0.84 ± 0.05 , $p > 0.05$). Subsequently, we assessed the protein levels after acute and repeated heroin treatment. Fig. 1C shows the protein expression of AUTS2 in the NAc or CPu after the acute heroin injection. One-way ANOVA revealed that acute heroin exposure had no effect on AUTS2 expression in the NAc or CPu (NAc: SAL 1.00 ± 0.16 vs. HRI 1.02 ± 0.18 ; CPu: SAL 1.00 ± 0.12 vs. HRI 0.98 ± 0.05 , both $p > 0.05$). As shown in Fig. 1D, five days after chronic heroin administration, significantly decreased AUTS2 expression was observed in the NAc (SAL 1.00 ± 0.14 vs. HRI 0.56 ± 0.19 , $p < 0.01$) but not in the CPu (SAL 1.00 ± 0.05 vs. HRI 1.03 ± 0.05 , $p > 0.05$).

Decreased expression of AUTS2 in the NAc is accompanied by heroin-induced behavioral sensitization

As shown in Fig. 2A, the mice were injected with heroin (1 mg/kg, s.c.) or saline twice a day for five days to generate heroin-induced locomotor sensitization. Two-way ANOVA revealed a significant main effect of treatment on the horizontal distance traveled [$F(1, 26) = 282.7, p < 0.0001$] and SAL or HRI challenge [$F(1, 26) = 67.62, p < 0.0001$] and a significant interaction [$F(1, 26) = 53.45, p < 0.0001$]. Tukey's multiple comparison test revealed that the HRI-HRI group exhibited greater locomotor activity (32864.3 ± 3994.6 cm) than the SAL-SAL group ($3012.4 \pm 1213.5, p < 0.0001$) or the SAL-HRI group ($14341.6 \pm 4333.4, p < 0.0001$). The mice were sacrificed 1 h after the behavioral test, and the NAc was collected for qRT-PCR and western blot analysis. As shown in Fig. 2B, the mRNA and protein expression of AUTS2 in the NAc was negatively correlated with locomotor activity. Specifically, consistent with the results described above, the acute heroin treatment (SAL-HRI challenge group, 1.0872 ± 0.0938) did not affect AUTS2 expression (two-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$ compared with the SAL-SAL challenge group, 0.9999 ± 0.1209), while a significant decrease in AUTS2 mRNA expression was observed after chronic heroin treatment (HRI-SAL challenge group, 0.6278 ± 0.0612) compared with the SAL-SAL group ($p < 0.05$). Importantly, the heroin-sensitized mice (HRI-HRI challenge group, 0.2132 ± 0.0201) not only showed a significant decrease in AUTS2 expression compared with the SAL-SAL group ($p < 0.001$), but they also showed decreased AUTS2 expression compared with the HRI-SAL group ($p < 0.05$). As shown in Fig. 2C, the protein levels of AUTS2 exhibited similar alterations; specifically, the heroin-sensitized mice (HRI-HRI,

0.4304 $\pm$ 0.1278) showed reduced AUTS2 protein levels compared with the SAL-SAL (1.0000 $\pm$ 0.1242) and HRI-SAL (0.6210 $\pm$ 0.2912) groups.

AUTS2 knockdown in the NAc facilitates heroin-induced sensitization

To test the effects of AUTS2 knockdown on heroin-induced locomotor sensitization, lentiviral-AUTS2-shRNA or lentiviral-control-shRNA was microinjected bilaterally into the NAc 1 week before behavioral training (Fig. 3A shows the expression of lentivirus in the NAc 1 week post-injection). As shown in Fig. 3B, the downregulation of AUTS2 in the NAc escalated the increase in heroin-induced locomotor sensitization. A two-way ANOVA of the locomotor activity included the between-subjects factors AUTS2-shRNA and heroin and the within-subjects factor test day, and it revealed a significant main effect of treatment ($F(5, 282) = 206.1, p < 0.001$), a significant main effect of test day ($F(7, 282) = 433.5, p < 0.001$), and a treatment X test day interaction ($F(35, 282) = 80.57, p < 0.001$). Tukey's multiple comparison test revealed that the HRI-HRI group that received the NAc-shRNA exhibited greater locomotor activity (56211.2 ± 2923.2) than the HRI-HRI group with the NAc control virus ($40985.5 \pm 4001.3, p < 0.001$, Fig. 3B and C) after the heroin challenge on day 8. These results suggest that heroin administration progressively increased locomotor activity, likely primed through AUTS2 inhibition in the NAc. The baseline locomotor activity in the saline group was not affected by AUTS2 inhibition in the NAc.

AUTS2 overexpression in the NAc attenuates heroin-induced sensitization

Next, we examined whether the overexpression of AUTS2 in the NAc could block the

observed heroin-induced sensitization. The mice were trained for heroin-induced sensitization 1 week after the injection with a virus expressing AUTS2, as described above. As shown in Fig. 4A, AUTS2 overexpression in the NAc significantly decreased the locomotor activity on Day 8 compared with that of the control group. Two-way ANOVA revealed a significant main effect of treatment ($F(1, 78) = 32.07, p < 0.001$), a significant main effect of test day ($F(5, 78) = 103.4, p < 0.001$), and a treatment X test day interaction ($F(5, 78) = 4.524, p < 0.001$). Tukey's multiple comparison test indicated that the HRI-HRI-AUTS2 overexpression group demonstrated significantly decreased locomotor activity (33409.2 ± 4801.4) compared with the HRI-HRI-control group ($46098.3 \pm 3892.1, p < 0.001$, Fig. 3B and C) following the heroin challenge on day 8. After the behavioral testing, the NAc was removed for western blotting. As shown in Fig. 2B, the AUTS2 overexpression group showed a significant increase in AUTS2 expression. Furthermore, the activation of Rac1 (a small GTPase downstream of AUTS2, associated with repeated cocaine-induced structural plasticity) increased after the heroin challenge. These data suggest that AUTS2 overexpression attenuated heroin-induced sensitization, likely associated with the upregulation of the AUTS2-Rac1 signaling pathway.

DISCUSSION

In the present study, we examined the role of striatal AUTS2 expression in the acute, chronic, and sensitized stimulant effects of heroin. We observed that AUTS2 was down-regulated in the NAc, but not in the CPu, after repeated heroin injections in mice, accompanied by heroin-induced sensitization of locomotor behavior. Moreover, AUTS2 knockdown in the NAc was enhanced, whereas overexpression attenuated heroin-induced locomotor sensitization. To our knowledge, this study is the first to demonstrate a role for AUTS2 in heroin addiction in an animal model. These findings indicate that AUTS2 expression in the NAc, but not the CPu, mediates heroin-induced behavioral adaptations, suggesting that AUTS2 may be a potential target for the treatment of heroin dependence.

AUTS2 was initially identified as an autism susceptibility candidate gene in a pair of monozygotic twins with autism who had an identical balanced chromosomal translocation at t(7;20) (q11.2; p11.2), which disrupted the AUTS2 gene (Sultana et al., 2002). A previous study revealed that AUTS2 physiologically functions in the cytoplasm in neuronal migration and neuritogenesis through the activation of Rac1 (Hori et al., 2014), indicating a potential contribution to early brain development and cognitive processes (Oksenberg and Ahituv, 2013). Notably, the high levels of AUTS2 expression in the adult striatum and genetic analyses of the AUTS2 gene have indicated that the AUTS2 gene may be associated with alcoholism and heroin dependence (Schumann et al., 2011, Zuo et al., 2012, Chen et al., 2013). Although previous animal studies have indicated that the reduced expression of AUTS2 is

associated with high alcohol preference in mice, few studies have examined the molecular and cellular mechanism of AUTS2 involved in addiction. The findings in the present study indicated that AUTS2 expression in the NAc, but not the CPu, was negatively correlated with chronic heroin treatment and heroin-induced sensitization in mice. The striatum comprises the CPu and NAc, which play distinct roles in addictive behaviors (Koob, 1992, Hyman and Malenka, 2001, Nestler, 2001). For example, in action selection, the CPu is involved in salience, while the NAc is involved in the reward value (Threlfell et al., 2010). These results suggested that AUTS2 in the NAc may play a critical role in heroin-induced psychomotor activity. Indeed, we observed a further decrease in AUTS2 expression in the NAc after heroin-induced locomotor sensitization, likely representing the basis for drug cravings (Robinson and Berridge, 1993, 2000).

We next used viral-mediated gene transfer to locally manipulate AUTS2 expression within the NAc of mice subjected to heroin-induced behavioral sensitization using RNAi to knockdown AUTS2 expression. This technique significantly decreased the expression of AUTS2 in the developing cortex (Hori et al., 2014). The data in the present study showed that the intra-NAc injection of lentivirus vectors expressing AUTS2 shRNA increased heroin-induced locomotor sensitization, which is a pro-craving effect. Moreover, the overexpression of AUTS2 in the NAc attenuated the behavioral actions of heroin. These findings are consistent with a previous study describing a role for Rac1 in the NAc in cocaine-induced behavioral adaptation (Dietz et al., 2012). Rac1 activity was enhanced through the overexpression of AUTS2 in the

NAc, consistent with previous studies (Hori et al., 2014). The blockage of the action of repeated heroin treatment could reflect the overactivation of the AUTS2-Rac1 signaling pathway.

Rac1 is a member of the Rho family of small GTPases and is a critical mediator of structural plasticity in the NAc after chronic cocaine exposure (Dietz et al., 2012, Dias et al., 2015). However, a recent report showed that Rac1 displayed an opposite pattern of activity (increased) in the CPu after repeated cocaine treatment compared with the NAc (decreased). The inhibition of Rac1 activity using specific inhibitors, overexpression of a dominant-negative mutant of Rac1, and local Rac1 knockout attenuated the cocaine-induced increase in dendritic spine density in the CPu and behavioral adaptations (locomotor sensitization and conditioned place preference), whereas mice with constitutively active Rac1 exhibited blunted morphological and behavioral changes after repeated cocaine exposure. These findings suggest a critical role for Rac1 activity in the CPu in regulating cocaine-related structural and behavioral plasticity (Li et al., 2015b). We did not observe a significant difference in AUTS2 expression, one of the upstream determinants of Rac1 activity, in the CPu after repeated heroin exposure. In addition, the loss-of-function experiment using shRNA showed that AUTS2 knockdown mice exhibited facilitated locomotor sensitization after heroin treatment, whereas the overexpression of AUTS2 in the NAc attenuated behavioral sensitization to heroin. Interestingly, the role of AUTS2 in heroin-induced long-lasting adaptations in the NAc was associated with the AUTS2-Rac1 signaling pathway because the activation of Rac1 was correlated with

blunted heroin sensitization through the overexpression of AUTS2 in the NAc. These findings support the hypothesis that AUTS2 in the NAc, but not the CPu, may contribute to Rac1 activity, which directly mediates dendritic morphogenesis and behavioral output.

Accumulating evidence highlights a crucial role for the suppression of Rac1 activity in structural plasticity and behavioral adaptations following chronic cocaine administration; however, the upstream molecular machinery regulating Rac1 activation in the NAc remains largely unknown. Rac1 can be activated through several parallel pathways, and the active form of this protein is typically located in high concentrations in the plasma membrane (Matsuo et al., 2003, Yoshizawa et al., 2005, Stankiewicz and Linseman, 2014). In the context of drug addiction, the cocaine-induced downregulation of Dishevelled-2 in the NAc acts as an upstream regulator of Rac1 activity and plays a critical role in the dynamic structural plasticity of NAc neurons (Dias et al., 2015). The AUTS2-Rac1 signaling pathway is important in cytoskeletal regulation during neuronal migration and neuritogenesis in the developing brain (Hori et al., 2014), and neuronal plasticity in adult neurons requires proteins involved in cytoskeletal remodeling (Hall, 1994, Sebeo et al., 2009). The results of the present study provide the first evidence that the downregulation of AUTS2 in the NAc could be another upstream regulator of Rac1 activity, which is implicated in heroin-induced behavioral sensitization. Additionally, psychostimulant-induced hyperactivity is largely dependent on the enhancement of dopaminergic transmission from midbrain dopamine neurons to the striatum (Berke

and Hyman, 2000). The manner in which AUTS2-Rac1 signaling in the NAc is regulated in response to increased dopamine tone remains largely unknown. Using cultures of primary PFC neurons, a recent report showed that the effects of Rac1 on dendritic morphogenesis are largely dependent on the activation of dopamine (D1) receptor-mediated signaling (Li et al., 2015a). It will be interesting to investigate whether AUTS2-Rac1 signaling is regulated through dopamine (D1) receptors in NAc neurons following heroin treatment.

Moreover, it is interesting to consider AUTS2 as one of the nodal points in the cross-talk between autism spectrum disorder (ASD) and drug addiction, as AUTS2 is susceptible to both ASD and heroin addiction. Several studies have focused on the similarities and differences between ASD and addiction (Kalat, 1978, Johnson et al., 2014, Casartelli and Chiamulera, 2016, Rothwell, 2016), revealing a strong overlap in the dopaminergic dysregulation of the limbic cortico-striatal circuits in individuals with addiction, obsessive-compulsive disorder, and ASD in both animal and human studies (Langen et al., 2011a, Langen et al., 2011b). It is necessary to further study the role of AUTS2 in the regulation of limbic cortico-striatal circuits in both ASD-like and addiction-like behaviors.

CONCLUSION

The present study reveals a novel role for AUTS2 signaling in mediating heroin-induced behavioral and neurochemical adaptations in the NAc, potentially mediated through the control of Rac1 activity. We observed the robust downregulation of AUTS2 in the NAc, but not the CPu, of mice exhibiting heroin-induced behavioral sensitization. In addition, AUTS2 knockdown potentiated the behavioral output of heroin and overexpression of AUTS2, blunting heroin-dependent hyperactivity. Thus, these findings suggest that AUTS2 is an important *in vivo* modulator of the sensitized stimulant effects of heroin in the NAc.

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Contributors

YS. Z and B. X conceived and designed the study and drafted the paper. W. D designed the study, examined behavioral sensitization, and interpreted the results. YY. J constructed the plasmids and prepared the viral stocks. P. Y conducted the surgery and intracranial injections. YX. L and XM. Q prepared the tissue samples and performed the western blot assays. JH. L conceived and designed the study.

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Figure legends

Fig. 1 AUTS2 expression in the NAc and CPu of mice exposed to heroin (HRI) or saline (SAL). The mice were acutely treated with heroin (1 mg/kg, s.c.) for 1 h (n = 8 mice) or twice a day for five consecutive days (7 d, 1 mg/kg, s.c.; n = 8) or with saline (0 h, n = 8). Protein lysates were isolated from the NAc and CPu, and Western blotting was performed to measure AUTS2 expression. The results are displayed as the mean $\pm$ SEM relative band intensity for AUTS2 expression in the NAc and CPu relative to saline controls. (A) No difference in AUTS2 mRNA expression was observed in the NAc and CPu after acute heroin treatment. (B) Decreased AUTS2 mRNA expression was observed in the NAc, but not in the CPu, in mice after repeated heroin exposure. *** $p < 0.001$ compared with the SAL controls. (C) No difference in AUTS2 protein expression was observed in the NAc and CPu after acute heroin treatment. (D) A reduced protein level of AUTS2 was detected in the NAc, but not in the CPu, of the mice with repeated heroin treatment. * $p < 0.05$ compared with the SAL controls.

Fig. 2 AUTS2 expression in the NAc is negatively correlated with the sensitized stimulant effects of heroin. The mice were treated with heroin (HRI; 1 mg/kg) or saline twice a day for five consecutive days. The mice were challenged with heroin (0.5 mg/kg) 3 days after the last injection, and locomotor activity was recorded. A daily saline (SAL) treatment group and a saline challenge group were included as controls. (A) The HRI-HRI group exhibited increased distance travelled compared

with the SAL-SAL and SAL-HRI groups, indicating locomotor sensitization induced through HRI. The values are presented as the mean $\pm$ SEM. *** $p < 0.001$ compared with the SAL-SAL group, # $p < 0.05$ compared with the SAL-HRI group. (B) A reduced level of AUTS2 mRNA was detected in the HRI-SAL and HRI-HRI groups. Post-hoc analysis further indicated decreased AUTS2 mRNA levels in the HRI-HRI group compared with the HRI-SAL group. One-way ANOVA followed by Tukey's test. * $p < 0.05$, *** $p < 0.001$ compared with the SAL-SAL group, ## $p < 0.01$ compared with the HRI-SAL group. (C) Western blotting for AUTS2 expression in the NAc of the mice after the locomotor test. The representative blots (left) and summary graph (right) indicate a significant decrease in AUTS2 expression in the HRI-SAL and HRI-HRI groups. Post-hoc analysis indicated lower levels of AUTS2 expression in the HRI-HRI group compared with the HRI-SAL group. One-way ANOVA followed by Tukey's test. * $p < 0.05$, *** $p < 0.001$ compared with the SAL-SAL group, # $p < 0.05$ compared with the HRI-SAL group.

Fig. 3 AUTS2 inhibition through RNAi in the NAc resulted in enhanced expression of heroin-induced locomotor sensitization. The mice were injected in the NAc with lentivirus-shRNA one week prior to repeated exposure to heroin (1 mg/kg, two times per day for five days). After 3 days of withdrawal, the mice were challenged with 0.5 mg/kg heroin. Locomotor activity was measured after the heroin (HRI) or saline (SAL) challenge. A saline vehicle group was used as a control. (A) An example of lentivirus-AUTS2 shRNA expression (in Green) in the NAc. The scale bar represents

50 μ m. (B and C) AUTS2 knockdown in the NAc did not affect the locomotor activity during the initiation of sensitization (first 5 days of heroin treatment) but enhanced the distance travelled after the heroin challenge on Day 8. Two-way repeated measures ANOVA followed by Tukey's multiple comparison test, *** $p < 0.001$.

Fig. 4 Overexpression of AUTS2 in the NAc attenuated the heroin-induced sensitization. (A) Overexpression of AUTS2 in the NAc through viral-mediated gene transfer did not affect the initiation of heroin-induced sensitization (day 1 to day 5), but it significantly attenuated the hyperactivity after a heroin challenge on day 8. Two-way repeated measures ANOVA followed by Tukey's post hoc multiple comparison test, *** $p < 0.001$. (B) The representative Western blot (left) and summarized graph demonstrate an increased expression of AUTS2 and overactivation of AUTS2 in the NAc after the locomotor test. ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

A

B

C

A

B

C

A

B