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Dishevelled-2 regulates cocaine-induced structural plasticity and Rac1 activity in the nucleus accumbens

Caroline Dias^a, David Dietz^{a,1}, Michelle Mazei-Robison^{a,2}, Haosheng Sun^a, Diane Damez-Werno^a, Deveroux Ferguson^{a,3}, Matthew Wilkinson^a, Jane Magida^a, Virginia Gao^a, Rachael Neve^b, and Eric J. Nestler^{a,*}

^aFishberg Department of Neuroscience and Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, New York, USA

^bDepartment of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

Abstract

Chronic cocaine exposure increases the density of dendritic spines on medium spiny neurons (MSNs), the predominant neuronal cell type of the nucleus accumbens (NAc), a key brain reward region. We recently showed that suppression of Rac1, a small GTPase, is a critical mediator of this structural plasticity, but the upstream determinants of Rac1 activity in this context remain to be elucidated. In this study we examined whether isoforms of Dishevelled, a key hub protein of multiple branches of Wnt signaling, including Rac1, are regulated in the NAc by chronic cocaine, and whether these Dishevelled isoforms control Rac1 activity in this brain region *in vivo*. We found that chronic cocaine administration decreased expression of Dishevelled-2, and several other Wnt signaling components, in the NAc, and that overexpression of Dishevelled-2, but not Dishevelled-1, conversely upregulated Rac1 activity and prevented the cocaine induction of dendritic spines on NAc MSNs. We posit that the cocaine-induced downregulation of Dishevelled-2 in the NAc is an upstream regulator of Rac1 activity and plays an important role in the dynamic structural plasticity of NAc MSNs seen in response to chronic cocaine exposure.

Keywords

Dendritic spines; morphological plasticity; medium spiny neurons; Wnt signaling

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*Corresponding author (eric.nestler@mssm.edu).

¹Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, New York, USA

²Department of Physiology, Michigan State University, East Lansing, Michigan, USA

³Department of Basic Medical Sciences, The University of Arizona College of Medicine, Phoenix, Arizona, USA

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1. Introduction

Although initially studied in the context of development, recent findings have demonstrated an important role for Wnt signaling in the mature vertebrate central nervous system [1]. For example, we recently demonstrated a critical role for this signaling pathway in the nucleus accumbens (NAc), a key brain reward region, in a mouse model of depression [2,3]. Because the NAc is also a brain region critical to the development of addiction pathophysiology, we were interested in studying Wnt signaling in the context of chronic cocaine exposure. Specifically, we focused on a protein that acts as a central hub of Wnt signaling: Dishevelled.

Dishevelled, an intracellular protein named initially based on the *Drosophila* phenotype of unoriented body and wing hairs seen upon knockout of the gene [4], has since been determined to represent a key hub in signal transduction through several different pathways of Wnt signaling [5]. In “canonical” Wnt signaling, activation of Dishevelled through a transmembrane Wnt-receptor complex eventually leads to stabilization of β -catenin, allowing β -catenin to translocate to the nucleus where it acts as a transcriptional activator at Wnt target genes. In the NAc, it is this branch of Wnt signaling that our laboratory has found to be critical in mediating resilience to stress [3]. However, signaling through Dishevelled also regulates distinct, “non-canonical” pathways [6,7]. For example, it can affect calcium signaling and Ca^{2+} /calmodulin-dependent protein kinase 4. Notably, mouse Dishevelled-1 and -2 can in addition activate certain small GTPases, including Rac1 [8]. Humans and mice express three Dishevelled homologs: 1, 2, and 3. Although there is functional redundancy between these three isoforms, an individual knockout approach has demonstrated distinct phenotypes of each protein. For example, Dishevelled-1 knockout mice, but not mice lacking other Dishevelled isoforms, have social interaction abnormalities [9,10]. In contrast, Dishevelled-2 or -3 knockout mice display greater perinatal mortality due to cardiac malformations [6].

We demonstrated previously that downregulation of Rac1 activity, without a change in its total expression levels, is a critical mediator of cocaine-induced structural plasticity of medium spiny neurons (MSNs) in the NAc [11]. Given these findings, and our lack of knowledge of what drives decreased Rac1 activity in this brain region, we hypothesized that Dishevelled signaling might be an upstream regulator of Rac1 and contribute to structural changes of NAc MSNs in the context of chronic cocaine exposure. We thus investigated the effects of acute and chronic cocaine on expression levels of Dishevelled isoforms in the NAc. We also investigated the relationship between Dishevelled isoforms and Rac1 *in vivo* in this brain region. Finally, we determined the structural consequences of Dishevelled-2 signaling on cocaine-dependent structural plasticity in the NAc.

2. Materials and methods

2.1. Animals

For all experiments, 7–9 week old male c57bl/6 mice were used; all mice were group housed on a 12-hour light/dark cycle with ad libitum access to food and water. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Icahn

School of Medicine at Mount Sinai. The following number of mice were used for each study: for the mRNA studies, 38 mice total were used; for the protein studies, 73 mice total were used; for the active Rac1 study, 23 mice total were used; for the spine studies, 14 mice total were used.

2.2. Cocaine administration for tissue collection

Mice were given 20 mg/kg cocaine hydrochloride or saline via intraperitoneal (IP), experimenter-administered injections daily for 1 or 7 days as described. The latter is a standard treatment regimen known to induce reduced Rac1 activity in the NAc [11].

2.3. Real-time PCR

PCR was performed as in [3]. Briefly, bilateral 14 gauge punches of NAc were taken; RNA was extracted using Trizol/chloroform in conjunction with the Qiagen RNeasy Micro kit. Purity and concentration were measured on a Nanodrop spectrophotometer. RNA was then reverse transcribed into cDNA with the iScript DNA synthesis kit (BioRad). We used GAPDH, which is not regulated by cocaine [11], to normalize quantification.

2.4. Immunoblotting

Immunoblotting was performed as in [3]. NAc punches were sonicated in a standard buffer with a desktop sonicator and centrifuged. Laemelli buffer was added to quantified protein lysates and equal amounts of protein were loaded onto SDS-PAGE gels. Samples were transferred to membranes, blocked, and incubated in primary antibody overnight at 4°C. After washing, secondary incubation was conducted for one hour at room temperature. Antibodies directed against dishevelled isoforms or GAPDH were used from the following companies: GAPDH Cell Signaling #2118, Dishevelled-1 Santa Cruz # 8025, Dishevelled-2 Cell Signaling #3216, Dishevelled-3 Cell Signaling #3218).

2.5. Active Rac1 assay

We stereotactically injected (see below) Herpes simplex virus (HSV)-Dishevelled-1, HSV-Dishevelled-2, or HSV-GFP into the NAc and killed animals 4 days later. We obtained bilateral 15 gauge NAc punches under a fluorescent microscope, punching only the fluorescent area. We used the NewEast Biosciences Active Rac1 kit with the provided protocol to assay active Rac1 levels [11].

2.6. Stereotactic surgery

Stereotactic surgery was performed on mice under ketamine/xylazine anesthesia. Vectors were infused bilaterally into the NAc with the following coordinates: +1.6 mm A/P, +1.5 mm M/L, -4.4 mm D/V from bregma. The HSV-Dishevelled vectors used in this study have been validated previously to ensure overexpression and functional activity in this brain region [2].

2.7. Dendritic spine studies

Mice were stereotactically injected with HSV-GFP or HSV-Dishevelled-2 into the NAc and allowed 24 hours to recover. Mice were then given 5 IP injections of 10 mg/kg cocaine or

saline over the course of 3 days, a protocol identical to that in [11] with the exception of the dose of cocaine used. This regimen is known to induce dendritic spine density of NAc MSNs within the timeframe of HSV transgene expression [11]. We used a lower (submaximal) dose of cocaine in the present study to facilitate the ability to observe either increases or decreases in thin spine density as compared to saline or cocaine conditions; importantly, the lower dose used here still significantly increases thin spine density of NAc MSNs (see Results and Discussion). 4 hours after the last treatment, mice were anaesthetized and underwent trans-cardial perfusion of PBS, followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were removed and post-fixed overnight in PFA. 100 μ m sections were taken on a vibratome and stained for GFP to enhance the signal. Dendrites that were at least >50 μ m from the soma, sampled from both the shell and core sub-regions, were identified and z-stacks taken on a Zeiss LSM 710 confocal microscope with identical settings in a 64x oil immersion lens. 5–8 dendrites from 4–8 neurons were imaged per animal from the NAc and values averaged to get the mean value per animal that was used for subsequent statistical analysis. 3–4 animals/group were used. Images were deconvoluted with the AutoQuant deconvolution software and semi-automated spine analysis was conducted using Neuron Studio. The number of spines was analyzed and quantified per unit length (<http://research.mssm.edu/cnic/tools-ns.html>). The spine images presented in this paper were enhanced for contrast; this was performed for maximally visualizing 2D images for publication and did not affect spine analysis quantification which was carried out in 3D.

2.8. Statistical analyses

For the active Rac assay, a one-way ANOVA with Tukey post hoc tests were used. For the dendritic spine study, a two-way ANOVA with Bonferroni post-hoc tests were used. For all other analyses, student's two-tailed t-tests were used. Results are expressed as the mean $\pm$ SEM.

3. Results and Discussion

24 hours after 7 days of daily 20 mg/kg cocaine (or saline) IP injections, there was widespread downregulation of Wnt signaling molecules in the NAc (Figure 1A). This regulation was specific to chronic cocaine administration since 24 hours after a single 20 mg/kg cocaine injection no regulation was observed (Figure 1B). Interestingly, when looking at Wnt expression, the secreted signaling proteins transmitting Wnt signaling across cells, following chronic cocaine in the NAc, this pattern of regulation was observed for Wnt1 only with other Wnt isoforms showing no such regulation (Figure 1A). This pattern is notable given that Wnts presumably represent signaling to neurons (or other cell types) in brain regions distinct from the NAc, as opposed to the level of Wnt signaling that occurs within the NAc itself. Additionally, when we examined the protein levels of β -catenin after chronic cocaine, we also saw no regulation, pointing away from canonical signaling playing a critical role in this context (Figure 1C). In contrast, when we examined the protein levels of Dishevelled-2, both 15 minutes and 24 hours after the last chronic dose of cocaine, we observed decreased protein expression in the NAc of cocaine treated animals (Figure 1D). Downregulation of Dishevelled-2 was specific for this Dishevelled homolog, since we found

no cocaine regulation of Dishevelled-1 or Dishevelled-3 protein levels 24 hours following the last chronic dose of cocaine (Figure 1E). Thus, despite the down-regulation of all three homologs at the mRNA level, we thus focused the rest of our studies on Dishevelled-2 which is uniquely downregulated at the protein level in the NAc after chronic cocaine administration.

Based on reports that Dishevelled-1 and -2, but not Dishevelled-3, can activate Rac1 in other systems [8,12], and our data that Rac1 suppression in NAc contributes to cocaine-mediated dendritic plasticity [11], we examined the effects of Dishevelled-1 or -2 overexpression in this brain region on active Rac1 levels. We omitted analysis of Dishevelled-3 not only because it does not activate Rac1 in other systems, but also because it is expressed at much lower levels than Dishevelled-1 in NAc as per the Allen Brain Atlas (<http://mouse.brain-map.org/>). We found that viral-mediated Dishevelled-2 overexpression in NAc increases levels of active Rac1, an effect not seen for Dishevelled-1 (Figure 2). To our knowledge, this is the first demonstration that Dishevelled-2 activation of Rac1 occurs in the fully differentiated adult brain, suggesting a conserved functional mechanism from the earliest stages of embryonic development to maturity. How might this activation occur? Recent work has shown that, within dopaminergic neurons, Dishevelled-2 can interact with Tiam1, a Rac1 guanine nucleotide exchange factor, and is required for Dishevelled-induced activation of Rac1 [13]. Future work will be necessary to conclude whether a similar mechanism of activation is occurring in the context of cocaine action.

Given the well-described dendritic changes that occur in the NAc with chronic cocaine exposure, we next asked whether Dishevelled-2, based on its activation of Rac1 described above, might contribute to changes in dendritic spine structure following cocaine administration. We used a submaximal cocaine treatment regimen that still induces NAc spine density within the limited timeframe of maximal expression of HSV-mediated transgenes. Indeed, while we observed no change in total spine density following cocaine treatment, there was a significant induction of thin, immature spines, the subtype of spine selectively regulated by cocaine [11], in the GFP condition, with no effect seen for mushroom or stubby spines (Figure 3). Interestingly, Dishevelled-2 overexpression in NAc completely blocked this effect of cocaine, without significantly affecting spine density under control conditions, consistent with Dishevelled-2's induction of Rac1 activity, which opposes the cocaine-induced decrease in Rac1.

Dishevelled-1-Rac1 signaling has previously been demonstrated to increase dendritic growth in cultured hippocampal neurons [8]. Although this finding is opposite to our demonstration that Dishevelled-2-Rac1 signaling opposes cocaine-induced dendritic growth of NAc MSNs, we have shown previously that Rac1's effects on neuronal structural plasticity are dependent on brain region and context [11]. The mechanisms underlying such differences require future investigation. One possible explanation lies in the fact that Rac1 signaling can have subcellular specificity [14], raising the possibility that the subcellular localization of active Rac1 in different neuronal types or contexts may contribute to these differences.

Another question arising from this study is why Dishevelled-2 seems to be specifically important in cocaine action. Although there is high sequence homology among the

Dishevelled isoforms, as well as some functional redundancy, the individual isoforms nonetheless have clearly distinct functions in both development and adulthood [6] (see Introduction). There is also distinct functionality at the molecular level, as the different isoforms have been shown to interact preferentially with different Dishevelled-interacting proteins [15]. Although the exact structural differences which lead to this functional specialization remain to be determined, our findings are consistent with past work in cultured cells, which reported that altered expression of Dishevelled-2 has the least effects on canonical signaling as compared to Dishevelled-1 and Dishevelled-3 [16]. Furthermore, our findings that Dishevelled-2, but not Dishevelled-1, in the NAc increases Rac1 activation could provide a basis for this functional difference with regards to the selective importance of Dishevelled-2 in cocaine action.

It should be noted that there has been previous conflict in the literature over which Dishevelled isoforms lead to activation of Rac1. For example, Habas et al. (2003) showed that Dishevelled-2 but not -3 activated Rac1 [12], while Cajanek et al. (2012) showed that Dishevelled-2 or other isoforms could activate Rac1 [13]. The latter authors concluded that cell-type specific factors could account for the discrepancy. Given the wide array of Dishevelled interacting proteins, which presumably vary in expression in different tissues and cells, we agree that these superficially conflicting findings likely represent region and cell-type specific complexity of Dishevelled-Rac1 signaling.

In any event, results of the present study demonstrate an important role for Dishevelled-2 in the NAc in response to chronic cocaine and suggest that this role is mediated at least partially through the control of Rac1 signaling. We found robust downregulation of Dishevelled-2 expression in this brain region after chronic cocaine administration at both the mRNA and protein levels, regulation that was not seen with acute cocaine or for other Dishevelled isoforms. As well, we found that Dishevelled-2 overexpression blocked cocaine's induction of thin spines on NAc MSNs. This action could be mediated by Dishevelled-2's ability to increase the levels of active Rac1 in the NAc *in vivo*. Indeed, our laboratory has previously shown that active Rac1 can block cocaine-induced thin spine increases in this brain region [11]. Additionally, our data suggest that the suppression of Dishevelled-2 expression in the NAc by chronic cocaine contributes to the decreased levels of Rac1 activity and increased thin spine density seen under these conditions. The fact that there is not a decrease in thin spine density with Dishevelled-2 overexpression in saline-treated animals emphasizes the interplay between cocaine exposure and regulation of the Dishevelled-2-Rac1 signaling cascade.

In conclusion, we uncovered here a novel role for Dishevelled-2 signaling in mediating cocaine-induced structural plasticity of NAc MSNs, an effect possibly mediated via control of Rac1 activity. We found robust downregulation of Dishevelled-2 in the NAc of mice following chronic cocaine administration, with overexpression of Dishevelled-2 leading to an increase in active Rac1 levels and a blunting of cocaine-dependent thin spine induction in NAc MSNs. We thus identify Dishevelled-2 as a novel upstream regulator of Rac1 activity in the NAc *in vivo* that controls spine dynamics resulting from chronic exposure to cocaine.

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Highlights

- Down-regulation of Dishevelled-2 occurs in the nucleus accumbens with cocaine.
- Dishevelled-2 activates Rac1 in vivo in the mature adult brain.
- Dishevelled-2 is important in controlling spine dynamics in response to cocaine.

Figure 1. Chronic cocaine regulation of Dishevelled-2 (DVL2) expression in NAc

(A) 7 days of experimenter-administered cocaine (20 mg/kg) IP injections leads to widespread downregulation of Wnt signaling molecules as compared to saline 24 hours after the last dose (n=10/group, * $p < 0.05$, ** $p < 0.01$). (B) A single 20 mg/kg cocaine injection leads to no such changes (n=9/group, $p > 0.05$). (C) There is no change in β -catenin protein expression 24 hours after chronic cocaine (n=10/group, $p > 0.05$). (D) There is decreased Dishevelled-2 (DVL2) protein expression both 15 minutes and 24 hours after chronic cocaine (top panel-15 minutes: ** $p < 0.01$, n=9-10/group; bottom panel and blot- 24 hours: ** $p < 0.01$, n=6/group). (E) There is no regulation of Dishevelled-1 (DVL1) or Dishevelled-3 (DVL3) protein expression 24 hours after chronic (7 days) cocaine (n=11/group, $p > 0.05$).

Figure 2. Dishevelled-2 overexpression increases active Rac1 levels in NAc

Overexpression of Dishevelled-2 (DVL2), but not Dishevelled-1 (DVL1), with an HSV vector in NAc leads to significant increases in active Rac levels as compared to GFP overexpression (n=7–8/group, *p < 0.01).

Figure 3. Dishevelled-2 blunts cocaine-induced thin spine increases in NAc MSNs

(A) There was a significant interaction effect between repeated cocaine exposure and HSV treatments when looking at thin spines, which revealed that Dishevelled-2 (DVL2) overexpression blocks the cocaine-induced increase in thin spine density of NAc MSNs (thin spines, two-way ANOVA, $n=3-4$ animals/group, $*p < 0.05$, interaction effect; Bonferroni: $*p < 0.05$, GFP-saline vs. GFP-cocaine, GFP-cocaine vs. DVL2 cocaine). DVL2 overexpression did not significantly affect spine density under control conditions. In contrast, there were no changes in total spine density, stubby spine density, or mushroom spine density under these various conditions. (B) Representative dendrite segments from the different conditions demonstrating increased thin spines in GFP-cocaine conditions but not in DVL2-cocaine conditions. Arrows highlight the increase in thin spine density between GFP-saline and GFP-cocaine conditions that is not seen in the DVL2-cocaine condition.