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TARGET-DEPENDENT EXPRESSION OF THE NETRIN-1 RECEPTOR, UNC5C, IN PROJECTION NEURONS OF THE VENTRAL TEGMENTAL AREA

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Abstract—We have shown previously that the netrin-1 receptor, *unc*-5 homologue C (UNC5C), is expressed by ventral tegmental area (VTA) dopamine (DA) neurons of rodents, but only from adolescence onwards (Manitt et al., 2010; Auger et al., 2013). The goal of this study was to characterize the expression of UNC5C by these neurons. Specifically, we assessed whether UNC5C expression is selective to DA neurons that project to the medial prefrontal cortex (mPFC), which undergo significant maturation during the adolescent period. To this end, we injected fluorescent retrograde tracer beads into the mPFC, nucleus accumbens (NAcc) core, or NAcc lateral shell of adult male wild-type C57Bl/6J mice and processed their brains for tyrosine hydroxylase (TH) and UNC5C immunofluorescence 2–3 weeks later. VTA neurons with any combination of these immunolabels were visualized and counted using optical fractionator stereology. Our analysis revealed two main findings: (1) there are no differences in the proportions of UNC5C-positive DA neurons projecting to the mPFC, NAcc core, or NAcc lateral shell, and (2) the proportion of non-DA UNC5C-positive neurons targeting the mPFC is greater than the proportions of non-DA UNC5C-positive neurons targeting the NAcc core or lateral shell. These findings show that, contrary to our hypothesis, DA neurons projecting to the mPFC do not express UNC5C selectively. However, UNC5C expression by non-DA VTA neurons is predominantly found in those projecting to the mPFC and, as such, may play a role in their function.

Key words: dopamine, prefrontal cortex, nucleus accumbens, UNC5C, retrograde tracer, stereology.

INTRODUCTION

Netrin-1 is a bi-functional guidance cue critically implicated in the formation of neural networks during development (Manitt and Kennedy, 2002; Manitt et al., 2010, 2011). Our group has demonstrated a role for the netrin-1 receptor, DCC (deleted in colorectal cancer), in the selective development and organization of mesocortical dopamine (DA) circuitry (Flores et al., 2005; Grant et al., 2007; Yetnikoff et al., 2007, 2010; Flores, 2011; Manitt et al., 2011). Briefly, we have found that mice with a heterozygous loss-of-function mutation in the *dcc* gene ($dcc^{+/-}$) display elevated baseline levels of extracellular DA in the medial prefrontal cortex (mPFC; "mesocortical"), but not in the nucleus accumbens (NAcc; "mesolimbic"), when compared to wild-type controls (Grant et al., 2007). Furthermore, adult $dcc^{+/-}$ mice have a significant increase in the span of DA synapses within the cingulate 1 and prelimbic subregions of the mPFC, but not in the NAcc, relative to wild-type controls (Manitt et al., 2011). These changes are associated with reduced spine density and dendritic arbor length of mPFC layer V pyramidal neurons selectively, which receive robust DA innervation (Benes et al., 2000; Grant et al., 2007; Manitt et al., 2011). However, no structural modifications are observed in NAcc medium spiny neurons.

The phenotypic traits of $dcc^{+/-}$ mice only become evident after adolescence (Grant et al., 2009; Manitt et al., 2011; Yetnikoff et al., 2011). This finding is intriguing because the DA innervation of the mPFC, which continues to develop until early adulthood, undergoes dramatic refinement during adolescence (Kalsbeek et al., 1988; Voorn et al., 1988; Rosenberg and Lewis, 1995; Benes et al., 2000; O'Donnell, 2010). In contrast, the DA innervation of the NAcc achieves an adult level of density soon after birth (Voorn et al., 1988).

Recent findings from our group suggest that the UNC5 homologue netrin-1 receptors, may also contribute to the adolescent development of ventral tegmental area (VTA) projections to the mPFC (Manitt et al., 2010; Auger et al., 2013). Both DCC and UNC5 receptors are highly expressed in the VTA by DA and non-DA neurons in rodents. However, whereas DCC is highly expressed in this region throughout life, UNC5 expression increases dramatically during adolescence (Manitt et al., 2010). In fact, VTA DA neurons only begin to express UNC5 receptors from adolescence onwards (Manitt et al., 2010). We now have demonstrated that DA neurons express the *unc*-5 homologue C (UNC5C) homologue specifically (Manitt et al., 2010; Auger et al., 2013). Importantly, we have shown that (a) single VTA DA neurons co-express DCC and UNC5C in adulthood (Manitt et al., 2010); (b) $dcc^{+/-}$ and $unc5c^{+/-}$ mice exhibit almost identical behavioral responsiveness to

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Abbreviations: ANOVA, analysis of variance; DA, dopamine; DCC, deleted in colorectal cancer; mPFC, medial prefrontal cortex; NAcc, nucleus accumbens; PBS, phosphate-buffered saline; SSC, saline-sodium citrate; TH, tyrosine hydroxylase; UNC5C, *unc*-5 homologue C; VTA, ventral tegmental area.

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stimulant drugs of abuse; and (c) the behavioral phenotypes of both *dcc*$^{+/-}$ and *unc5c*$^{+/-}$ mice become evident only after adolescence (Flores et al., 2005; Grant et al., 2007; Manitt et al., 2011; Auger et al., 2013). Together, these findings suggest that the emergence of UNC5C receptors by VTA DA neurons during adolescence may be a contributing factor to the effects of DCC on the development of the DA projection to the mPFC. We therefore hypothesized that UNC5C receptors may be expressed predominantly by VTA DA neurons that project to the mPFC. To address this idea, we assessed whether UNC5 receptors in the VTA are expressed differentially by neurons that target the mPFC versus the NAcc. We combined retrograde transport of fluorescent tracer beads injected into the mPFC or NAcc with immunofluorescence against TH and UNC5C in the VTA of adult male wild-type mice.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted according to the guidelines of the Canadian Council of Animal Care and all animal procedures were approved by the McGill University/Douglas Mental Health University Institute Animal Care Committee. Animals were kept on a 12-h light/dark cycle with food access given *ad libitum*. In all studies, we used adult male wild-type C57/Bl6 mice (post-natal day 75 ± 15; average body weight of 30 ± 5 g) bred from our in-house colony.

Primary antibodies

A monoclonal tyrosine hydroxylase (TH) antibody raised in mice (Chemicon International, Temecula, CA, USA; cat # MAB318) was used at a 1:1000 dilution. This primary antibody specifically recognizes an epitope on the regulatory N-terminus of TH (Wolf and Kapatos, 1989; Manitt et al., 2010). It generates a single band of 60 kDa on Western blot, corresponding to the predicted molecular weight for TH. This antibody does not bind to other closely related catecholamine enzymes including DA-β-hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase, dehydropteridine reductase, sepiapterin reductase, and phenylethanolamine-M-methyl-transferase (manufacturer's technical information). As described previously, this antibody stains mesencephalic DA neurons in the adult rodents (Wolf and Kapatos, 1989; Lu et al., 2002; Yetnikoff et al., 2007; Manitt et al., 2010).

A polyclonal UNC5 antiserum was kindly provided by Dr. Pawson (University of Toronto). This antibody was raised against a GST fusion protein expressing a region of UNC5C between the ZU-5 domain and the death domain (residue 605–877, GST-RCMZO-DD, 6th bleed of rabbit #52). This 272-amino-acid peptide antigen shares high amino acid identity with corresponding sequences in other mammalian UNC5 homologues. Specificity of this antibody for UNC5C has been demonstrated by immunoprecipitation, Western blotting, and immunostaining of HEK-293 cells transfected with unc5c cDNA (Tong et al., 2001). Using RT-PCR, our group has shown mRNA expression of *unc5c* and *unc5d*, but not *unc5a* or *unc5b*, in VTA homogenates of adult mouse brains (Manitt et al., 2010). In addition, we have now confirmed that only *unc5c* mRNA is expressed in adult mouse midbrain DA neurons and that, in the VTA region, the UNC5 antiserum detects UNC5C protein exclusively (Auger et al., 2013). Importantly, using this UNC5 antiserum we have recently shown that adult *unc5c* heterozygous mice have a significant reduction in immunoreactivity in the VTA in comparison to wild-type controls (Auger et al., 2013).

Retrograde tracer

We used Red Retrobeads^{TM} IX (Lumafluor Inc., Naples, FL, USA) to perform fluorescent retrograde tract tracing. These latex microspheres are 0.02–0.2 μm in diameter and contain a rhodamine fluorescent dye with peak excitation and emission wavelengths at 530 nm and 590 nm, respectively. Previous studies have shown that when this tracer is injected into the cat visual cortex there is little diffusion observed, no cytotoxicity or phototoxicity, and strong labeling which persists up to 10 weeks (Katz et al., 1984). Indeed, Lammel et al. (2008) have demonstrated strong labeling of DA soma in the VTA 2 and 3 weeks after intracranial microinjection of Retrobeads to the NAcc and mPFC, respectively).

Stereotaxic surgery

Mice were anesthetized with aerosolized isoflurane (5% for induction and 2% for maintenance of anesthesia) and were placed under stereotaxic control. We performed intracranial microinjections of 0.1 μL of red fluorescent retrograde tracers unilaterally (right hemisphere) (Lammel et al., 2008). The tracer was injected into three different target regions: (1) mPFC, aiming at inner layers of cingulate 1 and prelimbic subregions (1.8 mm anterior from bregma, 2.2 mm lateral from the midline, and 1.6 mm below the skull; Manitt et al., 2011), (2) the NAcc core (with the stereotaxic arm at a 30° angle to the sagittal plane, 1.3 mm anterior from bregma, 3 mm lateral from the midline, and 4.8 mm below the skull), and (3) the NAcc lateral shell (with stereotaxic arm in parallel to the sagittal plane, 1.1 mm anterior from bregma, 1.8 mm lateral from the midline, and 5.0 mm below the skull). All coordinates were obtained from the mouse brain atlas (Franklin and Paxinos, 2007). To allow for adequate transport of the retrograde tracer from the injection site to the VTA, we followed the same protocol as Lammel et al. (2008) and kept mice in isolation for 2 or 3 weeks after an injection to the NAcc or mPFC, respectively.

Tracer injection placements were verified by assessing the central point of each injection site. To this end we performed Nissl staining (0.1% Cresyl Violet stain solution) on 30-μm-thick coronal sections of the forebrain corresponding to Plates 6–26 of the mouse brain atlas encompassing both the mPFC and NAcc