Brain-Wide Mapping of Afferent Inputs to Accumbens Nucleus Core Subdomains and Accumbens Nucleus Subnuclei

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The nucleus accumbens (NAc) is the ventral part of the striatum and the interface between cognition, emotion, and action. It is composed of three major subnuclei: i.e., NAc core (NAcC), lateral shell (NAcLS), and medial shell (NAcMS), which exhibit functional heterogeneity. Thus, determining the synaptic inputs of the subregions of the NAc is important for understanding the circuit mechanisms involved in regulating different functions. Here, we simultaneously labeled subregions of the NAc with cholera toxin subunit B conjugated with multicolor Alexa Fluor, then imaged serial sections of the whole brain with a fully automated slide scanning system. Using the interactive WholeBrain framework, we characterized brain-wide inputs to the NAcC subdomains, including the rostral, caudal, dorsal, and ventral subdomains (i.e., rNAcC, cNAcC, dNAcC, and vNAcC, respectively) and the NAc subnuclei. We found diverse brain regions, distributed from the cerebrum to brain stem, projecting to the NAc. Of the 57 brain regions projecting to the NAcC, the anterior olfactory nucleus (AON) exhibited the greatest inputs. The input neurons of rNAcC and cNAcC are two distinct populations but share similar distribution over the same upstream brain regions, whereas the input neurons of dNAcC and vNAcC exhibit slightly different distributions over the same upstream regions. Of the 55 brain regions projecting to the NAcLS, the piriform area contributed most of the inputs. Of the 72 brain regions projecting to the NAcMS, the lateral septal nucleus contributed most of the inputs. The input neurons of rNAcC and cNAcC are two distinct populations but share similar distribution over the same upstream brain regions, whereas the input neurons of dNAcC and vNAcC exhibit slightly different distributions over the same upstream regions. Of the 55 brain regions projecting to the NAcLS, the piriform area contributed most of the inputs. Of the 72 brain regions projecting to the NAcMS, the lateral septal nucleus contributed most of the inputs. The input neurons of NAcC and NAcLS share similar distributions, whereas the NAcMS exhibited brain-wide distinct distribution. Thus, the NAcC subdomains appeared to share the same upstream brain regions, although with distinct input neuron populations and slight differences in the input proportions, whereas the NAcMS subnuclei received distinct inputs from multiple upstream brain regions. These results lay an anatomical foundation for understanding the different functions of NAcC subdomains and NAc subnuclei.

Keywords: nucleus accumbens, brain-wide mapping, afferent input, retrograde tracer, tract-tracing, neuroanatomy
INTRODUCTION

The nucleus accumbens (NAc) is a basal forebrain structure located ventromedially to the caudoputamen (CP) and ventrolaterally to the septal nuclei (Groenewegen et al., 1999). It is composed of core (NAcC) and shell (NAcS) regions, with the shell regions further subdivided into medial shell (NAcMS) and lateral shell segments (NAcLS; Záborszky et al., 1985; Heimer et al., 1997; Zahm, 1999, 2000; Yang et al., 2018). The NAc is important in many functions (Floresco, 2015), such as learning and memory (Li et al., 2018), reward processing (Carlezon and Thomas, 2009), addiction behavior, locomotor activity, stress-related aversion, liking (Castro et al., 2016), motivation (Castro and Bruchas, 2019), and sexual motivation (Everitt, 1990; Beny-Shefer et al., 2017). In addition, NAc dysfunction is associated with many mental disorders, including schizophrenia (Cotter et al., 2001), Huntington’s disease (Albin et al., 1989), alcohol addiction and drug abuse (Volkow et al., 2007; Lobo et al., 2010; Pirkulashvili et al., 2017; Morales et al., 2019), Alzheimer’s disease (Schliebs and Arendt, 2011; Nie et al., 2017), and depression (Nestler and Carlezon, 2006).

The NAcS also exhibits functional heterogeneity. The NAcMS plays key roles in facilitating the reinforcement of drug abuse, mediating goal-directed behavior, and suppressing unwrangling

Abbreviations: aca, Anterior commissure, anterior part; AAA, Anterior amygdalar area; ACA, Anterior cingulate area; AHN, Anterior hypothalamic nucleus; AI, Agranular insular area; AON, Anterior olfactory nucleus; ARH, Arcuate hypothalamic nucleus; ATN, Anterior group of the dorsal thalamus; AUD, Auditory areas; BLA, Basolateral amygdalar nucleus; BMA, Basomedial amygdalar nucleus; BST, Bed nuclei of the stria terminalis; CA1, Hippocampal field CA1; CA2, Hippocampal field CA2; CA3, Hippocampal field CA3; CEA, Central amygdalar nucleus; CLA, Claustrum; CM, Central medial nucleus of the thalamus; COA, Cortical amygdalar nucleus; CP, Caudoputamen; CS, Superior central nucleus raphe; CTXsp, Cortical subplate; DG, Dentate gyrus; DMH, Dorsomedial nucleus of the hypothalamus; DP, Dorsal peduncular area; ECT, Ectorhinal area; ENTI, Lateral entorhinal area; ENTm, Medial entorhinal area; EP, Endopiriform nucleus; EPI, Epithalamus; fx, Columns of the fornix/fox; GU, Gustatory areas; HY, Hypothalamus; HPF, Hippocampal formation; IA, Interulated amygdalar nucleus; ILA, Intralimbic area; IMD, Intermediodorsal nucleus of the thalamus; LA, Lateral amygdalar nucleus; LHA, Lateral hypothalamic area; LPO, Lateral preoptic area; LS, Lateral septal nucleus; mPFC, Medial prefrontal cortex; MB, Midbrain; MBO, Mammillary body; MD, Mediodorsal nucleus of thalamus; MEA, Medial amygdalar nucleus; MO, Somatomotor area; MOB, Main olfactory bulb; MPO, Medial preoptic area; MPN, Medial preoptic nucleus; MRN, Midbrain reticular nucleus; MSC, Medial septal complex; NAc, Nucleus accumbens; NAcC, Nucleus accumbens core; nNAcC, Rostral nucleus accumbens core; eNAcC, Caudal nucleus accumbens core; dNAcC, Dorsal nucleus accumbens core; vNAcC, Ventral nucleus accumbens core; NAcS, Nucleus accumbens shell; NAcLS, Lateral nucleus accumbens shell; NAcMS, Medial nucleus accumbens shell; NLOT, Nucleus of the lateral olfactory tract; NST, Solitary tract nucleus; OLF, Olfactory; ORB, Orbital area; OT, Olfactory tubercle; PA, Posterior amygdalar nucleus; PAA, Piriform-amygdalar area; PAG, Periaquaductal gray; PAL, Pallidum; PERI, Perirhinal area; PF, Parafascicular nucleus; PH, Posterior hypothalamic nucleus; PIR, Piriform area; PL, Prelimbic area; PMd, Dorsal premammillary nucleus; PMv, Ventral premammillary nucleus; PT, Parataenial nucleus; PVT, Paraventricular nucleus of the thalamus; RAmh, Medbrain raphe nuclei; RE, Nucleus of reuniens; RH, Rhomboid nucleus; RN, Red nucleus; SI, Substantia innominata; SMT, Submedial nucleus of the thalamus; SS, Somatosensory areas; STR, Striatum; SUB, Subiculum; TEa, Temporal association areas; TH, Thalamus; TP, Posterior transition area; TT, Taenia tecta; TU, Tuberal nucleus; VENT, Ventral group of the dorsal thalamus; VL, Lateral ventricle; VMH, Ventromedial hypothalamic nucleus; VISC, Visceral area; VTA, Ventral tegmental area; ZI, Zona incerta.

MATERIALS AND METHODS

Animals

Twenty 6-week-old wild type C57BL/6J mice were purchased from the Beijing Vital River Laboratory Animal Technology Company Limited (China). The animals were housed 3–5 mice/cage (30 cm × 18 cm × 13 cm) under a 12 h:12 h light-dark cycle (light on at 8:00 am), with ad libitum access to rodent food and water in an environmentally controlled room at a consistent ambient temperature (23 ± 2°C) and humidity (50% ± 5%). The mice used in the study were adult (8–10 weeks) male mice.

Ethics Approval

This study was carried out in accordance with the guidelines issued by the Institutional Animal Care and Use Committee (IACUC) at Huazhong University of Science and Technology, Wuhan, China. All protocols were approved by the IACUC and every effort was made to ensure the mice used were treated humanely and any discomfort was kept to a minimum.
Microinjection and Stereotactic Surgery

CTB-conjugated Alexa Fluor 488 (CTB-488) and Alexa Fluor 555 (CTB-555) were purchased from Thermo Fisher Scientific, Waltham, MA, USA. The tracer was dissolved in neutral phosphate-buffered saline (PBS) at a concentration of 1 µg/µl, aliquoted at 5 µl each and stored at −20°C until usage. Dexamethasone (30 nl, 2 mg/ml, intraperitoneal injection) was given to the mice half an hour before surgery. Then, they were anesthetized with 5% chloral hydrate (0.1 ml/10 g) before the CTB injection, with a simultaneous intraperitoneal injection of 30 µl of atropine (0.1 µg/µl) and scalp infiltration anesthesia of lidocaine at a concentration of 5 µg/ml. Supplementary doses of chloral hydrate were given throughout the procedure as needed. After the mice were completely anesthetized, they were fixed on a stereotactic stent (68030, RWD Life Science, China). Before adjusting their skulls in parallel to the reference panel, their eyes were covered with eye lube. A 0.5-mm diameter drill bit was used to make a small hole in the skull above the target area. To label upstream inputs to the NAcC, CTB-488 and CTB-555 were stereotactically injected into the right rNAcC (coordinates: AP: +1.8 mm, ML: −1.1 mm, DV: −3.75 ± 0.15 mm) and cNAcC (coordinates: AP: +0.9 mm, ML: −1 mm, DV: −3.9 ± 0.15 mm), respectively, using a glass pipette connected to a pneumatic pump (PV820, pneumatic pico-pump, World Precision Instruments Inc., Sarasota, FL, USA). To label upstream input to the NAcS, CTB-488 and CTB-555 were stereotactically injected into the right NAcMS (coordinates: AP: +1.3 mm, ML: −0.55 mm, DV: −4.2 mm) and NAcLS (coordinates: AP: +1.3 mm, ML: −1.7 mm, DV: −4.15 mm), respectively. 30 nl of CTB solution was slowly injected (6 nl/min) into each injection site with an impulse injection (20 psi at 5–10 Hz with a pulse duration of 10–15 ms). A low positive “holding” pressure was maintained in the injecting pipette between injection pulses to prevent fluid uptake through capillary action. After the last pulse was given, the glass electrode was held at the injection site for 10 min and then slowly retracted. After the injection, the surgical site was rinsed with saline, sutured and disinfected with iodophor. The operated mice were then placed on a heating pad until fully awake. The mice were given 0.03 ml of ketorolac tromethamine analgesic (1 µg/µl) and 0.03 ml of anti-inflammatory drug enrofloxacin (0.5%, Baytril, Bayer Bitterfeld GmbH, Germany) daily in the next 3 days.

Tissue Processing

Two weeks after CTB injection, the mice were deeply anesthetized by an intraperitoneal overdose injection of chloral hydrate, followed by transcardial perfusion with 100 ml of 0.1 M PB and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Mouse brains were carefully removed and then post-fixed with 4% PFA in 0.1 M PB overnight at 4°C. The brains were placed in a 20% sucrose-0.1 M PB solution at 4°C until they sank, then moved to a 30% sucrose-0.1 M PB solution at 4°C until they sank. The brains were sectioned coronally (30-µm thickness) with a freezing microtome (Leica Microsystems, Wetzlar, Germany). One out of every four sections was collected and kept in 0.01 M PBS in a 48-well plate and then mounted on a glass slide. These sections were imaged for all subsequent analyses with a fully automated slice scanning microscope (10× objective, NA 0.4, Olympus VS120, Japan) at a resolution of 0.67 µm. All images were saved as 16 bit grayscale in non-compressed “.tif” format.

Cell Counting and Input Brain Region Identification

We used 30-µm sections from 10 brains to perform cell counting with ImageJ software. For cell counting in each area, we loaded the image into ImageJ and used its Cell Counter multi-point tool to mark the soma. We counted all long-range upstream brain regions in the ipsilateral hemisphere of the injection sites. The pixel position of each marked cell in each brain section was exported as a .csv file.

The interactive WholeBrain1 framework is an R-language based open-source software developed by Fürth et al. (2018). We transform the .csv raw data into R data for further analysis in the interactive WholeBrain framework. After images with cell counting information were loaded into the interactive WholeBrain framework, it automatically loaded the corresponding Allen Brain Atlas for registration. Mostly, after auto-registration, the actual image and Atlas did not match well (Figure 2Bi). However, the framework provides an interface to allow manual adjustment of the atlas to match the image by overlaying their landmarks lateral ventricle (VL), anterior commissure, anterior part (aca) to the image (Figure 2Bii).

Only the regions containing a significant number of labeled cells (i.e., more than 10) were considered as input regions for further analysis (Luo et al., 2019). The input from each upstream region was normalized by dividing the number of labeled neurons found in that region by the total number of labeled neurons from each injection site in each brain, which was then called the proportion of total inputs. When performing a correlation analysis between the co-labeled neurons and the total labeled neurons projecting to the NAcC subdomains and the NAcS subnuclei, proportions of co-labeled neurons were calculated by dividing the number of co-labeled neurons found in that major area by the total labeled neurons from one brain. The total labeled neurons of one major area came from CTB-488 labeled neurons plus CTB-555 labeled neurons then minus the co-labeled neurons in that area.

Statistical Analysis

All values were presented as Mean ± SEM, with *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Unpaired two-tailed Student’s t-test was performed when comparing inputs between the two groups. One-way analysis of variance (ANOVA) with Dunnett’s post hoc test for single factors was performed when comparing inputs among three or more groups, whereas two-way ANOVA followed by multiple comparisons with Dunnett’s post hoc test was used for double factor experiments. To

1http://wholebrainsoftware.org/
RESULTS

Tracing Whole-Brain Inputs to NAcC Subdomains and NAcS Subnuclei

We stereotaxically injected the retrograde tracer CTB into the NAc subnuclei to map the brain-wide distribution patterns of input neurons. Both CTB-488 and CTB-555 (30 nl/injection site) were stereotaxically microinjected into the rNAcC and cNAcC or NAcLS and NAcMS, respectively (Figure 1A and Supplementary Figures S1, S2). We injected at two depths to label NAcC input neurons, simultaneously allowing us to analyze the input neurons of the vNAcC and dNAcC too. CTB was taken up by the axonal terminals at the injection site and then retrogradely transported to the somata. The neuronal somata across the brain projecting to the rNAcC were labeled with CTB-488 (green), whereas the cNAcC-projecting neurons were labeled with CTB-555 (red). Two weeks after injection, the mice were transcardially perfused, and their brains were fixed and coronally sectioned at a thickness of 30 µm. The CTB-labeled neurons were concentrated in the ipsilateral site, with sparsely labeled neurons also observed in the contralateral hemisphere (data not shown).

To generate overall brain-wide distribution of the CTB-labeled somata, we imaged every fourth brain section with an automated slice scanning system (Figure 1B). The brain slice images were manually aligned along the rostral-caudal axis (Figure 1B). The CTB-labeled neurons of each slice were manually marked using ImageJ, with the results exported and converted to R format for subsequent analysis (Figure 1C). The Allen Brain Atlas at the corresponding rostral-caudal position was registered to each image in the aligned brain-wide stack using the interactive WholeBrain framework (Fürth et al., 2018; Figures 1D, 2). This framework allowed manual tweaking of the atlas to match the images according to the cytoarchitectural landmarks in the brain (Figure 2B). The improvement in cell body segmentation after manual correction is shown in Figures 1D, 2C.

We calculated the number of CTB-labeled neurons in each brain region. Brain regions with more than 10 labeled cells, which equated to 0.1% of all labeled neurons across the brain, were included for quantitative analysis. The median number of whole-brain labeled neurons to the NAc subnuclei was 9,518 (6,878, 7,566, 5,057, 5,654, and 10,121 to the rNAcC; 7,493, 3,661, 16,728, 19,508, and 19,617 to the cNAcC; 10,988, 11,275, 9,450, 7,192, and 10,383 to the NAcLS; and, 9,585, 9,264, 8,439, 14,997, and 17,530 to the NAcMS). The median number of co-labeled neurons to the NAcC subdomains was 175 (558, 175, 58, 44 and 414), and the median number of co-labeled neurons to the NAcS was 11 (11, 14, 6, 17 and 0; Figures 3E, 6E). To minimize the influence of experimental variation on the total number of labeled neurons, the input from each region was normalized by dividing the number of labeled neurons found in that region by the total number of labeled neurons in each injection site to obtain the proportion of total inputs. In total, 75 input regions were compared. Among them, 57 brain regions projecting to the NAcC, 55 to the NAcLS, and 72 to the NAcMS. The 75 brain regions could be grouped into nine major brain areas, including the isocortex, olfactory areas (OLF), hippocampal formation (HPF), cortical subplate (CTXsp), striatum (STR), pallidum (PAL), thalamus (TH), hypothalamus (HY), and midbrain (MB). Thus, these results indicate that the NAc (including the NAcC...
and NAcS) neurons integrated inputs from diverse brain regions, ranging from the cerebrum to the brain stem.

**Global Distributions of Input Neurons to NAcC Subdomains (rNAcC vs. cNAcC and dNAcC vs. vNAcC) Are Similar**

The distributions of input neurons projecting to the rNAcC and cNAcC across the nine major brain areas were similar (two-way ANOVA; Brain areas × Subdomainr-c, $F_{(8,72)} = 1.30, P = 0.26$; Brain areas, $F_{(8,72)} = 19.34, P < 0.0001$; Subdomainr-c, $F_{(1,72)} = 5.02 	imes 10^{-6}, P > 0.99$; **Figure 3A**), whereas the distributions of neurons projecting to the dNAcC and vNAcC were slightly different (two-way ANOVA; Brain areas × Subdomaind-v, $F_{(8,54)} = 10.61, P < 0.0001$; Brain areas, $F_{(8,54)} = 44.54, P < 0.0001$; Subdomaind-v, $F_{(1,54)} = 1.67 	imes 10^{-6}, P > 0.99$; **Figure 3C**). We also found a larger proportion of OLF neurons projecting to the vNAcC (46.93% ± 4.29%) than to the dNAcC (16.30% ± 4.89%), whereas the neurons projecting from the CTXsp and TH to the dNAcC (15.56% ± 2.03% and 12.02% ± 0.46%, respectively) were more than that to the vNAcC (8.25% ± 0.94% and 8.44% ± 0.43%, respectively). We analyzed the correlation of inputs distributions to the NAcC subdomains (i.e., rNAcC vs. cNAcC and dNAcC vs. vNAcC). The squared Pearson’s correlation coefficient ($R^2$) for inputs between rNAcC and cNAcC was 0.81 ($P = 0.001$; **Figure 3B**), and that between
dNAcC and vNAcC was 0.71 ($P = 0.0043$; Figure 3D, the source regions with differential input portions are highlighted in orange circles).

rNAcC and cNAcC shared similar input patterns but very few co-projecting input neurons. Figure 4 shows representative coronal images of the CTB retrogradely labeled neurons.
in the upstream brain regions. The CTB-488- and CTB-555-labeled neurons indicated populations projecting to the rNAcC and cNAcC, respectively. Notably, in the same brain region, most neurons that projecting to the rNAcC were not the same population that projecting to the cNAcC. Of all neurons projecting to the rNAcC and cNAcC, the proportion of co-labeled neurons was only 1.49% ± 0.70%.

Specifically, correlation analysis showed that the proportions of total labeled neurons and the proportions of co-labeled neurons projecting to the NAcC of corresponding brain regions were closely related \((R = 0.8316, P = 0.0006; \text{Figure 3F})\). Thus, the proportions of co-labeled neurons were not brain-region selective but appeared to be related to total inputs to the NAcC. These results suggest that the rNAcC and cNAcC share common upstream regions but receive input from relatively distinct neuronal populations of each upstream brain region.

Comparison of Inputs to rNAcC vs. cNAcC and vNAcC vs. dNAcC Among 57 Upstream Brain Regions

We further divided the nine major brain areas into finer segmented brain regions and found that input neurons of the NAcC were observed in 57 of them. The distribution of input neurons projecting to the rNAcC differed \((P < 0.0001, F_{(56,228)} = 30.95, \text{one-way ANOVA; \text{Figure 5, left}})\), with the anterior olfactory nucleus (AON) contributing most of the inputs \((34.05% ± 4.68%\), followed by the piriform area (PIR), \(9.59% ± 2.74%\) and orbital area (ORB, \(7.45% ± 1.79%\)). The neurons projecting to the cNAcC also differed \((P = 0.0007, F_{(56,228)} = 1.876, \text{one-way ANOVA})\), with the AON contributing most of the inputs \((19.31% ± 15.23%)\), followed by the infralimbic area (ILA, \(6.76% ± 2.12%\)) and ORB \((6.42% ± 3.03%)\). Most regions showed no statistical differences in their contribution to input neurons projecting to the rNAcC and cNAcC (Student’s \(t\)-test), except the medial amygdalar nucleus (MEA, \(P = 0.04\)), basomedial amygdalar nucleus (BMA, \(P = 0.03\)), and cortical amygdalar area (COA; \(P = 0.01\)).

Among the 57 upstream regions projecting to the NAcC, the distribution of input neurons projecting to the dNAcC differed \((P < 0.0001, F_{(56,171)} = 5.74, \text{one-way ANOVA; \text{Figure 5, right}})\), with the ILA contributing most of the inputs \((8.41% ± 1.71%)\), followed by the ORB \((7.97% ± 2.61%)\) and prelimbic area (PL), \(7.13% ± 2.61%%\). The distribution of input neurons projecting to the vNAcC also differed \((P < 0.0001, F_{(56,171)} = 24.61, \text{one-way ANOVA})\), with the AON contributing most of the input source \((31.38% ± 4.97%)\), followed by the PIR \((11.48% ± 2.56%)\) and ORB \((6.38% ± 1.86%)\). Comparing the upstream regions to the dNAcC and vNAcC, only 9 out of 57 regions showed statistical differences in the input proportion (Student’s \(t\)-test), including the auditory area (AUD; \(P = 0.03\)), ILA (\(P = 0.03\)), AON (\(P = 0.0045\)), dorsal peduncular area (DP, \(P = 0.04\)), COA (\(P = 0.04\)), basolateral amygdalar nucleus (BLA, \(P = 0.009\)), BMA (\(P = 0.02\)), MEA (\(P = 0.049\)), and rhomboid nucleus (RH, \(P = 0.004\)). Overall, the distribution of input neurons to the NAcC subdomains was very similar; therefore, we considered the NAcC, as a whole, to compare to NAcS subnuclei.

Global Distribution of Input Neurons to NAcLS Is Similar to NAcC But Different From NAcMS

Neurons projecting to the NAcC, NAcLS, and NAcMS exhibited distinct distributions across the nine major brain areas (two-way ANOVA; Brain areas × Subnuclei_{C-L-M}, \(F_{(16,153)} = 10.24, P < 0.0001\); Brain areas, \(F_{(8,153)} = 23.41, P < 0.0001\); Subnuclei_{C-L-M}, \(F_{(2,153)} = 6.44 \times 10^{-4}, P > 0.99\)). Among them, the OLF contributed most of the afferent inputs \((39.18% ± 7.66%)\) to the NAcC, followed by the isocortex \((28.67% ± 5.06%)\) and CTXsp \((10.56% ± 1.65%)\). For the NAcLS, the isocortex contributed most of the afferent inputs \((46.69% ± 5.58%)\), followed by the OLF \((20.90% ± 2.80%)\).
FIGURE 5 | Input neurons of NAcC subdomains share similar distribution patterns across the brain. The proportions of total inputs contributed by each brain area to each NAcC subnuclei, including rNAcC (purple), cNAcC (red), dNAcC (yellow) and vNAcC (green). The proportions of input neurons in midbrain (MB), thalamus (TH), and hypothalamus (HY; superior) and TH, pallidum (PAL), and striatum nuclei (inferior) are shown with finer-scale as an inset on the upper right corner (Student's t-test, \( n = 5 \) mice for rNAcC and cNAcC, \( n = 4 \) mice for dNAcC and vNAcC). *\( P < 0.05 \), **\( P < 0.01 \).
FIGURE 6 | Overview of whole-brain inputs to NAcMS, NAcLS, and NAcC. (A) Distribution of input neurons of NAcC, NAcLS, and NAcMS across nine major brain areas (two-way ANOVA, n = 5 mice each). (B–D) Correlation of the distributions of inputs neurons of NAcLS, NAcMS, and NAcC. The brain areas with significantly different contributions to the input neuron of two subnuclei were highlighted by orange circles. (E) The number of total and co-labeled input neurons of NAcLS and NAcMS. (F) Correlation of fraction of co-labeled input neuron in each area and the proportion of input neurons contributed by such brain area to the total inputs to NAcS. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

CTXsp (10.75% ± 1.52%). For the NAcMS, the HPF contributed most of the inputs (27.22% ± 4.63%), followed by the STR (22.63% ± 3.92%) and CTXsp (16.95% ± 3.70%). We found that the NAcC and NAcLS both received more inputs from the isocortex and OLF, whereas the NAcMS received more inputs from the HPF, CTXsp, and STR. Both the PAL and HY sent preferential innervation to the NAcMS (2.88% ± 0.82%, 9.43% ± 1.92%, respectively).

We quantified the correlations among inputs to the NAcC and NAcS. We pairwise compared inputs to the NAcC, NAcLS, and NAcMS in the nine major brain areas, with each circle in the scatter plot representing one input brain area.
Comparison of Inputs to NAcC, NAcLS, and NAcMS Among 75 Upstream Brain Regions

We found in total 75 regions projecting to the NAcC, NAcLS, and NAcMS (57, 55 and 72 brain regions respectively). The distribution of input neurons across those brain regions to NAcC, NAcLS and NAcMS differed from each other (one-way ANOVA; \( P < 0.0001 \) \( F(56,513) = 10.54 \), \( P < 0.0001 \) \( F(74,300) = 24.82 \) and \( P < 0.0001 \) \( F(74,300) = 12.11 \), respectively; Figure 8). The AON contributed the most input neurons projecting to NAcC (26.68% ± 7.90%), followed by the PIR (7.31% ± 1.73%) and ORB (6.94% ± 1.67%). The PIR contributed most of the inputs (18.55% ± 2.64%) to NAcLS, followed by the agranular insular area (AI, 15.11% ± 1.81%) and lateral entorhinal area (ENTl, 6.60% ± 0.35%). The lateral septal nucleus (LS) contributed most of the inputs (14.97% ± 4.37%) to NAcMS, followed by the subiculum (SUB, 14.79% ± 2.37%), BMA (7.80% ± 1.98%) and BLA (5.48% ± 1.43%). 53 out of 75 upstream brain regions contributed different proportions of input neurons to the NAcC, NAcMS, and NAcLS (one-way ANOVA, Supplementary Tables S1, S2). In each major area, the preference for innervating NAc subnuclei was different. For example, the isocortex is preferentially sent axons to the NAcLS, with very little innervation to the NAcMS, except the medial prefrontal cortex (mPFC), including the anterior cingulate area (ACA), PL, ILA, and ORB). The OLF provided almost over 20% of the input neurons to NAcLS and NAcC but only 10% of those to the NAcMS (Figure 6A). Notably, the distribution of input neurons within OLF was not even, with AON and PIR took the most portion of inputs to NAcC and NAcLS (26.68% ± 7.90% and 18.55% ± 2.64% respectively; Figure 8). The HPF and STR provided the largest portions of inputs neurons to NAcMS but only a modest contribution to NAcLS and NAcC (Figure 6A). The SUB, CA1 and ENTm (medial entorhinal area) in the HPF provided much more inputs to NAcM than to NAcLS and NAcC, with the SUB took the largest portion all over the brain (14.79% ± 2.37%). The LS, CEA, and MEA in the STR contained much larger portions of NAcMS projecting neurons (14.97% ± 4.37%, 2.68% ± 1.43% and 3.11% ± 0.45%, respectively), whereas the CP contributed more inputs to NAcLS. All regions in the HY and PAL showed preferential innervation to the NAcMS, especially the HY, in which all regions demonstrated exclusive innervation to the NAcMS. All regions in the TH showed similar distribution patterns of input neurons projecting to the NAcMS, NAcLS and NAcC, with each region containing a relatively smaller portion of...
neurons projecting to NAcMS, as confirmed by correlation analysis (NAcMS vs. NAcLS, $R = 0.64$, $P = 0.0018$; NAcMS vs. NAcC, $R = 0.73$, $P = 0.0004$; NAcC vs. NAcLS, $R = 0.90$, $P < 0.0001$). For the MB, each region in this area contained a similarly small proportion of projecting neurons to each NAc subnucleus.
FIGURE 9 | Schemes of brain-wide input patterns of NAcC and NAcS subdomain. (A) Brain-wide input patterns of NAcC subdomains. Each cross indicates inputs to NAcC subdomains from corresponding major brain areas and the arm length indicates the relative amount of input neurons in the corresponding major brain area. The horizontal arm shows the relative input amount of rNAcC (purple) and cNAcC (orange), and the vertical arm shows the relative amount of input neurons of dNAcC (yellow) and vNAcC (green). (B) Brain-wide input patterns of NAcLS, NAcMS, and NAcC. Every pie indicates the relative amount of input neurons of NAc subnuclei from the corresponding major brain area.

Summary of Distribution of Input Neurons to Subregions of NAcC and NAcS

Overall, we compared the brain-wide input patterns of the NAcC subdomains (Figure 9A), most of which received inputs from different neuronal populations in the same upstream brain regions and with a little difference in the proportion of projecting. Comparing the brain-wide input patterns of the NAcC and NAcS subnuclei (Figure 9B), we found that: (1) the brain-wide input patterns of the NAcC and NAcLS were similar, with the main difference being the proportion of input neurons from the same upstream brain region. As shown in Figure 8, most upstream regions projecting to the NAcLS also contain neurons sending inputs to the NAcC, and most often, in the same upstream brain region, the proportion projecting to the NAcLS was greater than that to the NAcC, except for the AON, TT, and ATN; and (2) the NAcMS had a distinct distribution of upstream neurons across the brain compared with the NAcC and NAcLS. In the cerebrum, the isocortex and OLF neurons preferred to send innervation to the NAcLS and NAcC, whereas the HPF, CTXsp, STR, and PAL contained more neurons projecting to the NAcMS. The brain stem TH demonstrated preferential innervation to the NAcC and NAcLS, whereas the HY showed preferential innervation to the NAcMS.

DISCUSSION

We mapped the organization of input neurons projecting to different NAcC (rNAcC, cNAcC, dNAcC, and vNAcC) and NAc shell subnuclei (NAcLS and NAcMS) using retrograde tracing strategy combined with the interactive WholeBrain framework. We found that NAc neurons integrated inputs from diverse brain regions, from the cerebrum to the brain stem. For the NAcC subdomains, input neurons projecting to the rNAcC and cNAcC showed similar distributions across the same upstream brain regions but from almost non-overlapping populations, whereas those projecting to the dNAcC and vNAcC had relatively different distribution patterns over the same input regions (Figure 9A). The NAc shell subnuclei showed more diverse input patterns from numerous brain areas. The input neurons of NAcMS exhibited a very distinct distribution pattern, mainly concentrating on the HPF, CTXsp, and STR (Figure 9B). The PAL and HY neurons also send innervation to NAcLS but seldom to NAcC. Input regions of NAcLS and NAcC were similar, but the distributions of input neurons across those regions were different. Both received inputs from a large portion of neurons in the isocortex and OLF. The similarities and differences in their input distribution observed in our study may provide new insights into the diverse functions of the NAc.

Using the WholeBrain framework for brain-wide maps (Fürth et al., 2018), we can quantify the brain-wide distribution of input neurons of different brains and injection sites. We analyzed the input patterns of different subregions of NAcC and shell subdomains in each upstream brain region. Compared with Brog et al. (1993), we found that the NAcMS had distinct upstream brain regions from the NAcC as well as the NAcLS, whereas the NAcC and NAcLS had similar upstream brain regions but a different distribution of input proportions across those regions.

Previous study has also reported differences in the responses to application of the dopamine agonist in rNAcC and cNAcC (Bowers et al., 2000) and to deep brain stimulation of dNAcC and vNAcC (Rodriguez-Romaguera et al., 2012), which can be a result of three types of the input patterns of NAc subregions. First, the rNAcC and cNAcC receive inputs from different upstream brain regions; second, the rNAcC and cNAcC received inputs from the same upstream brain regions but with different input proportions; and, third, the rNAcC and cNAcC receive inputs from different subpopulations of neurons within the same upstream brain regions with same or different input proportions. Our brain-wide NAcC mapping results demonstrated the third one can be a possible explanation.

Previous studies have indicated that dNAcC has the opposite effect on the extinction of fear memory and drug-seeking behaviors. For example, activation of the dNAcC with deep brain stimulation promoted fear memory extinction (Rodriguez-
and functional terms. Our study found that some brain regions, including the ILA and BLA, preferentially innervate to the dNAcC. Earlier research has confirmed the role of the ILA in fear extinction using lesion, drug infusion, and stimulation approaches (Milad and Quirk, 2012), and showed that the BLA→NAc pathway regulated the reinstatement of alcohol-seeking (Baldi and Bucherelli, 2010; Keisler et al., 2017). We found that dNAcC received much more BLA and ILA inputs than vNAcC (Figure 5), indicating that the ILA→dNAcC pathway may be an important circuit involved in fear extinction, whereas the BLA→dNAcC pathway may be involved in the extinction of drug addiction.

We compared the NAcc, as a whole, with the NAcLS and NAcMS in terms of their brain-wide distribution of input neurons. The main upstream regions containing inputs neurons to the NAcc and NAcS were consistent with those reported in a previous retrograde tracking study, which focused on the D1 dopamine receptor (D1R-) and D2R-expressing medium spiny neurons (MSNs) within the NAcc and NAcS (Li et al., 2018). In that research, Li et al. (2018) systematically identified the brain areas projecting to the D1R- and D2R-MSNs in the NAcc and NAcS, whereas we focused on comparing the similarities and differences in upstream brain regions of different subdomains of the NAcc (rNAcc, cNAcc, dNAcc, and vNAcc) and subdomains of the NAcS (NAcLS and NAcMS). Li et al. (2018) found the distributions of input neurons in all upstream brain regions projecting to the NAcc D1R-MSNs and D2R-MSNs were similar, with only 2 out of 84 brain regions showed different proportions of projecting to the NAcc D1R- and D2R-MSNs. We found that the number of input neurons in the same upstream brain regions (9 out of 57) projecting to the dNAcC and vNAcC was slightly different. They found that D1R-MSNs and D2R-MSNs in both Nac subregions receive similar inputs from diverse sources, but we found that NAcLS and NAcMS have different input patterns, and particularly, HY send innervation almost exclusively to NAcMS. Prior functional studies have indicated that the NAcMS and NAcS have different input patterns, and particularly, HY send innervation almost exclusively to NAcMS. Prior functional studies have indicated that the NAcMS and NAcS have different input patterns, and particularly, HY send innervation almost exclusively to NAcMS. 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Prior functional studies have indicated that the NAcMS and NAcS have different input patterns, and particularly, HY send innervation almost exclusively to NAcMS. Prior func...
attention, and motivation (Yu et al., 2019). In this study, we found that the ENTI and ENTm demonstrated the opposite projecting patterns to the NAcLS, NAcMS, and NAcC. However, the exact role of these different projecting patterns still remains unclear.

CTB retrograde tracing is widely used for elucidating neuronal connectivity; however, it does have several limitations (Köbbert et al., 2000). Although retrograde CTB is useful for marking the identity of cell bodies, as it remains in vesicles and is granular in cells, it cannot provide detailed morphology of neurons. Additionally, although we revealed the input neural circuitries of different NAc subregions, quantification of inputs largely depended on the location of the CTB injection and its diffusion at the injection site. It can be difficult to cover an entire NAc subregion without spilling over to the adjacent areas. To lower the possibility of nonspecific infection, we injected a small volume of CTB and used a very slow injection rate to limit its spread to a small range. We perhaps overlooked some input regions in our experiments because we biased our injections towards smaller volumes and confined regions. In the future, combining new genetic and viral approaches will be necessary to explore the diverse cell subtypes in the NAc with higher specificity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines (IACUC) at Huazhong University of Science and Technology, Wuhan, China. All protocols were approved by the IACUC and every effort was made to ensure the mice were treated humanely and any discomfort was kept to a minimum.

AUTHOR CONTRIBUTIONS

LM conceptualized the project, performed most experiments, analyzed the data, and wrote the manuscript with DY. WC conceptualized the project and performed most experiments. DY wrote and edited the manuscript. YH supervised the research, discussion, and writing of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnsys.2020.00015/full#supplementary-material.

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