Catecholaminergic innervation and D2-like dopamine receptor-mediated modulation of brainstem nucleus incertus neurons in the rat

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ABSTRACT

Nucleus incertus (NI) is a brainstem structure involved in the control of arousal, stress responses and locomotor activity. It was reported recently that NI neurons express the dopamine type 2 (D2) receptor that belongs to the D2-like receptor (D2R) family, and that D2R activation in the NI decreased locomotor activity. In this study, using multiplex in situ hybridization, we observed that GABAergic and glutamatergic NI neurons express D2 receptor mRNA, and that D2 receptor mRNA-positive neurons belong to partially overlapping relaxin-3- and cholecystokinin-positive NI neuronal populations. Our immunohistochemical and viral-based retrograde tract-tracing studies revealed a dense innervation of the NI area by fibers containing the catecholaminergic biosynthesis enzymes, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), and indicated the major sources of the catecholaminergic innervation of the NI as the Darkschewitsch, raphe and hypothalamic A13 nuclei. Furthermore, using whole-cell patch clamp recordings, we demonstrated that D2R activation by quinpirole produced excitatory and inhibitory influences on neuronal activity in the NI, and that both effects were postsynaptic in nature. Moreover, the observed effects were cell-type specific, as type I NI neurons were either excited or inhibited, whereas type II NI neurons were mainly excited by D2R activation. Our results reveal that rat NI receives a strong catecholaminergic innervation and suggest that catecholamines acting within the NI are involved in the control of diverse processes, including locomotor activity, social interaction and nociceptive signaling. Our data also strengthen the hypothesis that the NI acts as a hub integrating arousal-related neuronal information.

1. Introduction

Maintaining an appropriate level of arousal and locomotor activity is essential for successful functioning within a complex and changing environment, and adequate responding to external stimuli. One of the brain areas involved in the control of these processes is the nucleus incertus (NI), a brainstem structure localized bilaterally beneath the fourth cerebral ventricle in the human (Streeter, 1903), macaque (Ma et al., 2009), rat (Goto et al., 2001; Olucha-Bordonau et al., 2003) and mouse (Smith et al., 2010); and also described in fish (Donizetti et al., 2008).

Abbreviations: ACSF, artificial cerebrospinal fluid; D2, dopamine type 2 receptor; D2R, D2-like receptor; ePSCs, excitatory postsynaptic currents; iPSCs, inhibitory postsynaptic currents; NI, nucleus incertus; NIC, nucleus incertus pars compacta; NId, nucleus incertus pars dissipata; RLN3, relaxin-3; sACSF, standard artificial cerebrospinal fluid; TH, tyrosine hydroxylase.

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glutamatergic NI neuronal populations strongly innervate the medial septum, an area tightly associated with theta rhythmogenesis and related processes, which include arousal and stress responses (Cervera-Ferri et al., 2012; Lu et al., 2020; Oluocha-Bordonau et al., 2012; Szlaga et al., 2022). In this regard, it has been shown that NI neurons express a range of arousal and stress response-associated G-protein-coupled receptors, such as the type 1 corticotropin-releasing hormone receptor (CRHR1), orexin/hypocretin receptors, the melanin-concentrating hormone (MCH) receptor 1, and the D2-like receptor (D2R) (Biasiak et al., 2015; Kastman et al., 2016; Kumar et al., 2015; Ma et al., 2013; Sabethgadom et al., 2018).

Indeed, functional studies indicate that NI neuronal populations control arousal, locomotion, anxiety, the stress response and food intake (Biasiak et al., 2017; Lu et al., 2020; Ma et al., 2013, 2017a; Szlaga et al., 2015; Kastman et al., 2016; Kumar et al., 2015; Ma et al., 2013; Sabethgadom et al., 2018).

Specifically, chemo- and opto-genetic activation of NI neurons promotes locomotor activity, arousal levels, and hippocampal theta power (Lu et al., 2020; Ma et al., 2017a; Szlaga et al., 2019), whereas optogenetic inhibition of NI NMB neurons lead to deceleration of locomotion speed and a decrease in arousal (Lu et al., 2020). In female rats, depletion of RLN3 levels in the NI caused increased anxiety-like behavior in the open field, along with a decrease in body weight and an imbalance in food intake (de Avila et al., 2020), and infusion of an antagonist of the cognate relaxin-3 receptor, RXFP3, into the paraventricular hypothalamic nucleus prevented stress-induced, binge-eating behavior (Kania et al., 2020).

NI-controlled functions are closely related to dopaminergic signaling, which is critically involved in the control of locomotor activity, anxiety, motivation and arousal levels (Klein et al., 2019). Dopamine is known to exert its actions through two G-protein-coupled receptor families: D1-like receptors (D1R), comprising D1 and D5 receptors, and D2-like receptors (D2R), comprising D2, D3 and D4 receptors (Beaulieu et al., 2015). Notably, dopamine is not the only endogenous ligand for these receptors, as the catecholamines, noradrenaline and adrenaline, act as D2 receptor agonists (Lanau et al., 1997; Sánchez-Soto et al., 2016). A recent study reported that D2, but not D3 receptors, are present in the NI in RLN3- and CRHR1-expressing neurons, and D2 receptor activation in the NI leads to hypo-locomotion (Kumar et al., 2015). However, the source of the catecholaminergic innervation of NI remains unknown.

The most common effect of D2R stimulation observed is inhibition of neuronal activity resulting from an increase in potassium conductance and subsequent membrane hyperpolarization (Missale et al., 1998), but D2R stimulation can also exert an excitatory effect, via activation of nonselective cation currents (Aman et al., 2007; Haj-Dahmane, 2001). However, it remains unclear, what influence D2R activation has on NI neuronal activity.

In these studies, we performed multiplex fluorescent in situ hybridization (RNAscope™) to verify the neurochemical profile of NI neurons expressing D2 receptor mRNA. With the use of viral vector-based, retrograde neural tract-tracing combined with immunofluorescent staining, we identified the source of the catecholaminergic innervation of the NI in multiple regions, including hypothalamic, midbrain and brainstem structures. Finally, we performed single-cell, patch-clamp recordings of NI neurons to determine the physiological effect of D2R activation on their activity.

2. Materials and methods

2.1. Ethical approval and animals

Experiments were conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes of January 15, 2015 and approved by the 2nd Local Institutional Animal Care and Use Committee (Krakow, Poland). All efforts were made to minimize stress prior to experimentation and the number of rats used.

Experiments were conducted using male, Sprague-Dawley rats (Institute of Zoology and Biomedical Research, Jagiellonian University, Krakow, Poland), which were kept in plastic cages lined with wood-shaving bedding, under constant temperature conditions (21 ± 2 °C), maintained on a 12:12 light-dark cycle with ad libitum access to fresh water and standard laboratory rodent chow. Rats were separated from dams at four weeks old and kept in same-sex cages (up to 7 rats per cage), until use in experiments. For in situ hybridization, immunostaining and neural tract-tracing studies, 10–12 week-old rats were used, and for patch-clamp experiments, 4–6 week-old rats were used. Overall, 124 rats were used in these studies.

2.2. Multiplex fluorescent in situ hybridization (RNAscope™)

In situ hybridization using the RNAscope™ HiPlex Assay (Advanced Cell Diagnostics (ACD), Hayward, CA, USA) with the RNAscope™ HiPlex Alternate Display Module (ACD; for AF488, Atto550 and Atto647 detection) was performed to determine the neurochemical nature, percentage and location of NI neurons expressing D2 receptor mRNAs. All procedures were conducted using fresh frozen 16 µm brain sections from three rats (3 NI-containing sections per brain), according to the manufacturer’s instructions and as described (Szlaga et al., 2022). During the initial hybridization step, the following probes were applied: RLN3 (Rn-Rln3-T3, cat. no. 1037211-T1, ACD; assigned to AF488), CCK (Rn-Cck-T2, cat. no. 532851-T2, ACD; assigned to Atto550), vGAT1 (Rn-Slc32a1-T3, cat. no. 424541-T3, ACD; assigned to Atto647), vGlut2 (Rn-Slcl7a6-T5, cat. no. 31701-T5, ACD; assigned to Atto550) and D2 (Rn-Drd2-T6, cat. no. 315641-T6, ACD; assigned to Atto647).

Images for rounds 1 and 2 were acquired and processed using an Axio Imager M2 fluorescence microscope (Zeiss, Gottingen, Germany) with an automatic z-stage and Axiocam 503 mono camera (Zeiss), and subsequently with the following software: Zen (3.1 blue edition and 3.0 SR black edition, Zeiss), CorelDraw 2020 (Corel Corporation, Ottawa, Canada), ImageJ (Schneider et al., 2012) and HiPlex Image Registration Software v.1.0 (ACD). The borders of the NI were delineated on the basis of the presence of RLN3 mRNA-expressing neurons and a rat brain atlas (Paxinos and Watson, 2007). All T1-T6 mRNA-expressing cells in the NI area from one brain hemisphere per slice (right and left interchangeably), were counted semi-automatically with an ImageJ Cell Counter plugin. Neurons were identified by the presence of an explicit cell-like distribution of fluorescent mRNA dots and/or a nucleus stained with DAPI. A cell was considered as expressing a specific mRNA when at least two unambiguous dots of specific fluorescence were present within its boundary. All counted neurons were categorized by type, depending on mRNA species co-expression (juxtaposition within a cell).

2.3. Immunostaining of TH and DBH fibers in the nucleus incertus

Rats (n = 3) were anesthetized with an intraperitoneal injection of pentobarbital (240 mg/kg) and sacrificed by transcardial perfusion with 250 ml of PBS followed by 250 ml of 4% formaldehyde solution (freshly made from paraformaldehyde powder).Brains were then fixed in 4% formaldehyde at 4 °C overnight, and cut into 50 µm coronal sections, using a Leica VT 1000S vibrating microtome (Leica Instruments, Heidelberg, Germany). Every fourth section containing NI underwent an immunostaining procedure: blocking and permeabilization for 1 h at room temperature (10% normal donkey serum (NDS), 0.3% Triton X-100 in PBS), incubation with primary antibodies for 72 h at 4 °C (one set of sections using rabbit anti-TH (1:100) and another set using rabbit anti-dopamine β-hydroxylase (DBH) (1:1000), 2% NDS, 0.3% Triton X-100 in PBS), followed by incubation with secondary antibodies overnight at 4 °C (donkey anti-rabbit Cy3 (1:400), 2% NDS in PBS). Incubation with primary and secondary antibodies was followed by several washes in PBS. Fluoroshield™ with DAPI was used for section mounting. TH- and DBH-immunoreactive fibers in the NI were imaged using a
2.4. Retrograde neural-tract tracing with viral vectors

Rats (n = 4) were anesthetized with an intraperitoneal injection of ketamine and xylazine (100 mg/kg ketamine + 10 mg/kg xylazine) and with additional doses of ketamine as required (33 mg/kg), and placed in a stereotaxic apparatus (SAS-4100; ASI Instruments, Warren, USA). Retro-AAV2-hSyn-mCherry viral vector was injected into the NI bilaterally (stereotaxic coordinates [mm]: AP 3.7 from lambda, ML ± 0.15, DV 7.2, with the rat’s head angled forward by 15°, 100 nl in each hemisphere), using glass microinjection pipettes (50 μm tip diameter), pulled from borosilicate glass capillaries on a vertical puller (Narishige, Tokyo, Japan) and connected to a 1 μl Hamilton syringe (Hamilton, Bonaduz, Switzerland). In order to improve subsequent immunostaining, after 7 days of recovery, rats were anesthetized as described and a colchicine injection was made into the lateral ventricle ([mm]: AP -0.7 from bregma, ML 1.8, DV -4; 5 μl of 20 mg/ml solution into one hemisphere). After 24 h, rats were anesthetized with an intraperitoneal injection of pentobarbital (240 mg/kg) and sacrificed by transcardial perfusion as described. After post-fixation, brains were cut into 50 μm coronal sections using a Leica VT 1000S vibrating microtome (Leica Instruments). Sections containing NI were mounted onto glass slides, coveredslipped with Fluoroshield™ and injection sites were imaged using an Axio Imager M2 fluorescence microscope with an A-Plan 10 × /0.25 objective (Zeiss). Reconstructions of the injection sites were made using CorelDRAW software (Corel Corporation), according to the rat brain atlas (Paxinos and Watson, 2007). Every fourth section, beginning at the coronal level of the medial septum through to the NI (i.e., from bregma +1.3 mm to -10.3 mm) underwent immunostaining. Free-floating sections were blocked and permeabilized for 1 h at room temperature (10% NDS, 0.3% Triton X-100 in PBS, respectively), incubated with primary antibodies solution for 72 h at 4 °C (mouse anti-T (1:250), rabbit anti-mCherry (1:1000), 2% NDS, 0.3% Triton X-100 in PBS) and subsequently with secondary antibody solutions overnight at 4 °C (donkey anti-mouse Alexa 647-conjugated antibody (1:400), donkey anti-rabbit Cy3-conjugated antibody (1:400), 2% NDS in PBS). Incubations with primary and secondary antibodies were followed by several washes in PBS. After mounting with Fluoroshield™, sections were examined and imaged using an Axio Imager M2 fluorescence microscope with an A-Plan 10 × /0.25 objective (Zeiss). Tyrosine hydroxylase (TH) and mCherry immunoreactive (-ir) neurons were counted using ZEN 2.1 software (Zeiss) and multiplied by 4 to estimate the number of TH-ir neurons innervating the NI.

2.5. Ex vivo electrophysiology

Whole-cell, patch-clamp electrophysiological recordings were performed as described (Szlaga et al., 2022). Male Sprague-Dawley rats were deeply anesthetized with isoflurane and decapitated. Brains were collected in ice-cold, low-sodium, high-magnesium ACSF, containing (in mM): 185 sucrose, 25 NaHCO3, 3 KCl, 1.2 NaH2PO4, 2 CaCl2, 10 MgSO4 and 10 glucose, pH 7.4; osmolality 290–300 mOsmol/kg) and cut into 250 μm coronal slices on a Leica VT 1000S vibrating microtome (Leica Instruments). Slices containing the NI were transferred to an incubation chamber containing carbonated, warm (32 °C) ACSF, containing (in mM): 118 NaCl, 25 NaHCO3, 3 KCl, 1.2 NaH2PO4, 2 CaCl2, 1.3 MgSO4 and 10 glucose, pH 7.4; osmolality 290–300 mOsmol/kg). After the transfer of a first slice, bath heating was turned off. After recovery (90–120 min), slices were placed in a recording chamber, where the tissue was perfused (2 ml/min) with carbonated, warm (32 °C) ACSF of the same composition.

Recording micropipettes were fabricated from borosilicate glass capillaries (7–9 MΩ; Sutter Instruments, Novato, CA, USA) using a horizontal puller (Sutter Instruments) and filled with a solution containing (in mM): 145 potassium gluconate, 2 MgCl2, 4 Na2ATP, 0.4 Na3GTP, 5 EGTA, 10 HEPES (pH 7.3; osmolality 290–300 mOsmol/kg) and biocytin (0.05%, for subsequent identification of recorded neurons). The calculated liquid junction potential was +15 mV, and that value was subtracted from the data during analysis.

NI neurons were localized and approached using an Examiner D1 microscope (Zeiss) equipped with video-enhanced infrared differential interference contrast. Cell-attached and whole-cell configurations were obtained using a negative pressure delivered by mouth suction. SEC 05XL amplifiers (NPI, Tamm, Germany), Micro 1401 mk II converters (CED, Cambridge Electronic Design, Cambridge, UK) and Signal and Spike2 software (CED) were used for signal recording and data acquisition. Recorded signal was low-pass filtered at 3 kHz and digitized at 20 kHz. All drugs were applied via a perfusion system. The activity of one neuron per slice was recorded.

Current-clamp recordings (zero holding current) were performed in standard ACSF (sACSF) followed by ACSF containing voltage-sensitive, sodium channel blocker, tetrodotoxin citrate (TTX, 0.5 μM) and antagonists of ionotropic glutamate and GABA receptors (10 μM CNQX, 50 μM DL-AP5, and 20 μM bicineulline, respectively). Voltage-clamp recordings were performed in sACSF followed by ACSF containing TTX (0.5 μM) at −50 mV holding potential. Since for the solutions used in the present study, the calculated reversal potential for Cl− currents was −90.51 mV, outward events represented inhibitory (iPSCs), while inward events represented excitatory postsynaptic currents (ePSCs).

2.6. Electrophysiological data analysis

Only neurons with stable input resistance throughout the recording, assessed on the basis of the voltage or current responses to hyperpolarizing current or voltage steps (applied every 60 s), were included in the final analysis. The change in the recorded whole-cell current or voltage in response to the drug application was considered significant if it differed from the baseline by more than three standard deviations (SD). Electrophysiological data were analyzed using custom Spike2 and MATLAB (MathWorks Inc., Natick, MA, USA) scripts. To verify the effect of quinpirole on recorded synaptic activity, 200 s epochs of the baseline recording and 200 s epochs after quinpirole application, were analyzed using Mini Analysis software (Synaptosoft Inc., Fort Lee, NJ, USA). Events were manually detected to measure frequency, amplitude and rise time of postsynaptic currents and the decay time constant of averaged current trace. Analysis of the influence of quinpirole on synaptic activity was performed on all recorded neurons, regardless of whole-cell current change in response to D2R agonist. Statistical analysis was performed using GraphPad Prism v6.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA). All data underwent a test for normality of distribution (Shapiro-Wilk normality test) and outlier detection (ROUT method, Q = 1%) and outliers were eliminated from the analysis. Differences were considered statistically significant at p < 0.05. All tests were two-tailed, and tests used (paired and unpaired t-tests, Mann–Whitney test and Wilcoxon test) are stated in the Results and Figures. Values are provided as mean ± SD, where data was normally distributed or as median ± interquartile ranges, if otherwise.

2.7. Post-recording immunostaining

After recordings, slices underwent immunofluorescent staining in order to verify the neurochemical content of examined neurons. Slices were fixed overnight with 4% formaldehyde at 4 °C. Fixed, free-floating sections were blocked and permeabilized with 10% NDS and 0.6% Triton X-100 in PBS, respectively, at 4 °C overnight or for 3 h at room temperature. Subsequently, after washing in PBS, slices were incubated with mouse anti-RLN3 (1:15), ExtrAvidin-Cy3 (1:200), 2% NDS and 0.3% Triton X-100 in PBS for 48–72 h at 4 °C and, after several washing steps (in PBS), with secondary antibody solution: anti-mouse Alexa 647 (1:400) and 2% NDS in PBS at 4 °C overnight. Slices were mounted onto
glass slides, coverslipped with Fluoroshield™ and imaged using a fluo-
rescence microscope (Axio Imager M2, Zeiss, with an A-Plan 10 × /0.25
objective or EC-Plan-NeoFluar 20 × /0.25 objective) to assess the pres-
ence of RLN3 immunoreactivity within recorded cells. However, a lack
of RLN3 immunoreactivity was not used as a prerequisite for assigning
an NI neuron as non-RLN3, due to the possible dilution of antigen by the
intraperitoneal solution during patch-clamp recording.

2.8. Reagents

Reagents for phosphate-buffered saline (PBS), artificial cerebrospinal
fluid (ACSF) and the intraperitoneal solution were purchased from
Sigma-Aldrich (Darmstadt, Germany), apart from biocytin (Cat No.
3349), which was purchased from Tocris Bioscience (Bristol, UK).

Reagents (suppliers) used in the electrophysiological experiments
were as follows: tetrodotoxin citrate (TTX), Abcam, Cat No. ab120055 or
Tocris Bioscience, Cat No. 1069; 2-amino-5-phosphopentanoic acid (DL-
AP5) Tocris Bioscience, Cat No. 0105; 6-cyano-7-nitroquinoxaline-2,3-
dione disodium salt (CNQX) Tocris Bioscience, Cat No. 1045; bicucul-
line methiodide, Sigma-Aldrich, Cat No. 14343; quinpirole hydrochlo-
rride, Sigma-Aldrich, Cat No. Q102. Reagents were dissolved in deio-
nized water, aliquoted and stored at
-20 °C.

Immunostaining reagents and suppliers were as follows: para-
formaldehyde, Sigma-Aldrich, Cat No. 441244; Triton X-100, Sigma-
Aldrich, Cat No X100; normal donkey serum (NDS), Abcam, Cat No.
Ab7475 or Jackson ImmunoResearch (West Grove, PA, USA), Cat
No. 017-000-121; avidin-Cy3 conjugate ExtrAvidin®-Cy3™, Sigma-Aldrich,
Cat No. E4142. Primary antibodies: mouse anti-tyrosine hydroxylase,
Santa Cruz Biotechnology, Cat No. sc-25269; rabbit anti-tyrosine hy-
droxylase, Sigma-Aldrich, Cat No. T8700; rabbit anti-dopamine β-hy-
droxylase, ImmunoStar (Hudson, WI, USA), Cat No. 22806; rabbit anti-
mCherry, Abcam, Cat no. ab167453. The mouse anti-RLN3 antibody
was prepared in-house (The Florey Institute of Neuroscience and Mental
Health) using a monoclonal cell line originally supplied by the Inter-
national Patent Organism Depository (IPOD) National Institute of
Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki,
Japan. Secondary antibodies: anti-mouse Alexa Fluor 647-conjugated
antibody, Jackson ImmunoResearch, Cat No. 711-606-150; anti-rabbit
Cy3-conjugated antibody, Jackson ImmunoResearch, Cat No. 711-165-
152. NDS, rabbit anti-TH and anti-DBH antibodies were aliquoted and
stored at −20 °C; mouse anti-TH and anti-RLN3 antibodies were stored at
4 °C; secondary antibodies were aliquoted and stored at −80 °C. Fluoroshield™ with DAPI, Sigma-Aldrich, Cat No. F6057 was used for section/slice mounting.

Suppliers of the tract-tracing reagents were as follows: colchicine,
Sigma-Aldrich retro-AAV2-hSyn-mCherry, UNC Vector Core. Viral vec-
tor was aliquoted and stored at −80 °C. Anesthetics used for anatomical
and electrophysiological studies were pentobarbital (Morbital, Biowet,
Warsaw, Poland).

3. Results

3.1. Different neurochemical populations of nucleus incertus neurons
express D2 receptor mRNA

Brain sections containing NI were subjected to RNAscope™ HiPlex in
situ hybridization with probes for RLN3, CCK, and vesicular GABA
(vGAT1) and glutamate (vGlut2) transporter mRNA, as well as D2 re-
ceptor mRNA, to investigate the expression and distribution of different
mRNA species in NI neurons, with a focus on D2 receptor mRNA-
containing populations.

The mean number of NI cells per section, expressing at least one of
the mRNA species tested was 1178, of which 422 (36%) were D2 re-
ceptor mRNA-positive. Cell counting established that 59% of counted NI
neurons expressed vGAT1 mRNA and 48% vGlut2 mRNA. Among them,
a small group exhibited a ‘vGAT1 + vGlut2 mRNA’ phenotype (7% of all
cells). Similar numbers of vGAT1 and vGlut2 mRNA-expressing cells, as
well as vGAT1/vGlut2 mRNA-expressing cells, were also D2 receptor
mRNA-positive (52%, 56% and 8% of total D2 receptor mRNA-
expressing cells, respectively).

vGAT1- and vGlut2 mRNA-expressing neurons were found to express
RLN3 and CCK mRNA, with 21% of all counted cells vGAT1/RLN3
mRNA-positive; including a small group of vGAT1/vGlut2/RLN3
mRNA-expressing cells. RLN3 mRNA-expressing neurons always co-
expressed vGAT1 mRNA, and vGlut2/RLN3 only mRNA-positive neu-
rons were not identified. CCK mRNA-positive cells accounted for 11% of
total counted cells and comprised: vGAT1/CCK, vGAT1/vGlut2/CCK
and very rarely CCK only mRNA-expressing neurons. Interestingly, a
small group (4%) of the total NI neurons counted expressed RLN3 and
CCK mRNA, with vGAT1/RLN3/CCK mRNA and rare vGAT1/vGlut2/
RLN3/CCK mRNA-positive cells observed. Importantly, all identified
cell types included subpopulations that expressed D2 receptor mRNA,
except the CCK mRNA only and vGAT1/vGlut2/RLN3/CCK mRNA-
positive cells, in frequencies roughly proportional to the general abun-
dance of each cell type within the NI. Scarcely D2 receptor mRNA only
expressing neurons were also detected. For a detailed summary see
Tables 1 and 2, and Fig. 1.

Notably, different neurochemical NI cell types were differentially
distributed throughout the structure, as reported (Szlaga et al., 2022).
vGlut2 mRNA-expressing neurons were mostly located laterally, while
RLN3 and CCK mRNA-positive cells were more medial, and vGAT1 and
D2 receptor mRNA-expressing neurons were evenly distributed.

3.2. Catecholaminergic fibers densely innervate the nucleus incertus

Immunostaining for TH, the rate-limiting enzyme in catecholamine
biosynthesis, and DBH, the synthetic enzyme for noradrenaline, revealed
the presence of dense TH-ir and DBH-ir fibers within the NI region
(Fig. 2A). Both TH-ir and DBH-ir fibers were observed in close appo-
sition to RLN3-ir neurons, suggesting the presence of synaptic connections between them (Fig. 2B).

3.3. Multiple sources of the descending catecholaminergic innervation of the nucleus incertus

In studies to determine which brain structures provide the cate-
cholaminergic innervation of the NI, the brains from four rats that
received an intra-NI injection of a retrograde viral vector (retro-AAV2-
hSyn-mCherry) were examined for colocalization of mCherry and TH
immunoreactivity throughout the brain (+1.3 mm to −10.3 mm from
bregma). In all four brains (cases A-D, Fig. 3A and B), TH-ir and
mCherry-ir cells were consistently observed in the periaqueductal gray
Darkschewitsch nucleus (Fig. 3C), raphe nuclei (including the para-
median raphe nucleus, dorsal raphe nucleus (dorsal, lateral and caudal
parts), caudal linear nucleus of the raphe, rostral linear nucleus of the
raphe) (Fig. 3D) and in the A13 dopaminergic group (Fig. 3E). In three

| mRNA combination | Mean ± SD (%) | + D2 mRNA (%) |
|-------------------|--------------|--------------|
| vGAT1 only        | 250 ± 26 (21) | ≤ 0.3 (1)    |
| vGAT1/RLN3        | 94 ± 48 (8)   | ≤ 0.3 (1)    |
| vGAT1/CCK         | 58 ± 16 (5)   | ≤ 0.3 (1)    |
| vGAT1/RLN3/CCK    | 30 ± 6 (3)    | ≤ 0.3 (1)    |
| vGAT1/vGlut2      | 35 ± 10 (5)   | ≤ 0.3 (1)    |
| vGAT1/vGlut2/RLN3 | 7 ± 5 (1)     | 1 ± 0.3 (1)  |
| vGAT1/vGlut2/CCK  | 4 ± 1 (0.3)   | ≤ 0.3 (1)    |
| vGAT1/vGlut2/RLN3/CCK | 2 ± 0.2 (0.2) | ≤ 0.3 (1)    |
| vGlut2 only       | 270 ± 31 (23) | 194 ± 78 (16)|
| vGlut2/CCK        | 7 ± 3 (1)     | 9 ± 2 (1)    |
| D2 only           | 2 ± 0.2 (0.1) | ≤ 0.3 (1)    |
| CCK only          | 1 ± 0.1 (0.1) | ≤ 0.3 (1)    |
dopaminergic cell group. Single catecholaminergic neurons immunoreactive for mCherry and TH were observed in the lateral and pleoglial periaqueductal gray and the A11 dopaminergic cell group. Single catecholaminergic neurons immunoreactive for mCherry and TH were not observed in more than two brains studied (Table 3).

3.4. D2-like receptor activation in nucleus incertus has excitatory and inhibitory effects

Recorded NI neurons were divided into two groups based on their unique electrophysiological properties. Type I neurons were identified by the presence of a delay before the first action potential after a hyperpolarizing current pulse, underlined by a robust A-type potassium current; and type II neurons were characterized by a lack of a delay and/or the presence of rebound depolarization after the hyperpolarizing current pulse, underlined by calcium current passing through voltage-dependent channels (Blasiak et al., 2015; Szlaga et al., 2022).

Whole-cell, zero current clamp recordings revealed that 64.7% (11 of 17) of the recorded neurons were responsive to bath application of the D2R agonist, quinpirole (20 μM) in sACSF. Within this group, six neurons (56%) were excited, and the remaining five neurons were inhibited by D2R activation. Notably, type I neurons were inhibited (n = 5) and type II neurons were excited (n = 2) by quinpirole application, whereas type II neurons were exclusively excited (n = 4; Fig. 4A and B).

In studies to test for the possible pre-vs post-synaptic localization of D2R in the NI, five neurons that were responsive to quinpirole in sACSF, were re-treated with the agonist under conditions of pharmacological isolation, i.e., in the presence of TTX, and ionotropic GABAergic and glutamatergic receptors antagonists. All tested neurons remained responsive to quinpirole under pharmacological isolation; four neurons (type I) were hyperpolarized (−2.44 ± 1.07 mV, mean voltage change) and one neuron (type II) was depolarized by 4.31 mV (Fig. 4A and B).

In voltage-clamp recordings (holding potential −50 mV), 52% (34 of 66) of recorded cells were sensitive to quinpirole (20 μM) in sACSF, and within this group, in 74% of neurons (n = 25, 13 type I, 12 type II) an increase in inward current in response to quinpirole was observed (14.67 ± 9.42 pA, mean current change). In the remaining 26% (n = 9, 7 type I, 2 type II), an increase in outward current was recorded (13.6 ± 6.53 pA, mean current change; Fig. 4C and D). The mean change in quinpirole-induced inward current was significantly different between NI neuron types (Mann–Whitney unpaired test p = 0.0001, Fig. 4E), with type I neurons displaying significantly greater excitatory responses to quinpirole application.

Similar to current-clamp recordings, in voltage-clamp experiments, the majority (n = 17, 80.95%) of neurons responsive to quinpirole in sACSF, exhibited a change in whole-cell current after quinpirole application in TTX-enriched ACSF; 82.35% (n = 14) of responsive neurons exhibited an increase in inward current (8 type I, 6 type II, mean amplitude of the increase of inward current: 14.29 ± 8.19 pA, Fig. 4F, H), and in 17.65% (n = 3), an increase in outward current was observed (2 type I, 1 type II, mean amplitude of increase of outward current: 8.07 ± 1.86 pA, Fig. 4H). In TTX-enriched ACSF, type I neurons displayed a tendency for a higher amplitude of D2R activation-induced inward current (Mann–Whitney test, p = 0.081), suggesting the larger increase in inward current is due to postsynaptic actions of D2R (Fig. 4F).

Both RLN3-immunopositive and RLN3-immunonegative neurons were responsive to D2R activation (Fig. 4I). Of nine RLN3-ir neurons (7 type I, 2 type II), six were inhibited and the remaining three were excited by D2R activation in sACSF.

3.5. D2R activation influences synaptic currents in the nucleus incertus in a neuron type-specific manner

In order to assess possible effects of D2R activation on spontaneous postsynaptic currents in type I (n = 21) and type II (n = 15) NI neurons, quinpirole (20 μM) was applied during voltage-clamp recordings performed in sACSF. Subsequent analysis revealed that D2R activation caused a drop in frequency of IPSCs by 0.12 ± 0.18 Hz (paired t-test, p = 0.024) in type II neurons (Fig. 5F), and a decrease in ePSC rise time by 0.19 ± 0.32 ms (paired t-test, p = 0.011) in type I neurons (Fig. 5L). The remaining parameters associated with the recorded synaptic currents were not affected by quinpirole application (Table 4).

4. Discussion

In these studies, we characterized the catecholaminergic innervation of the NI at the molecular, circuit and electrophysiological level. Using multiplex in situ hybridization histochemistry, we demonstrated that both GABAergic and glutamatergic NI neurons express D2 receptor mRNA, and that transcripts for these dopaminergic receptors were present in one-third (36%) of all NI cells counted. Our immunohistochemical data and viral-based, retrograde tract-tracing studies revealed an abundance of TH- and DBH-ir fibers within the NI area, and identified the major descending sources of the NI catecholaminergic innervation in the periaqueductal gray, raphe nuclei and hypothalamic A13 nuclei. Finally, using whole-cell, patch-clamp recordings, we demonstrated that a majority of NI neurons is sensitive to D2R activation by quinpirole, in a cell type-dependent manner; and that D2R activation had a direct excitatory or inhibitory influence on NI neuronal activity. Our results indicate that the rat NI is under a strong catecholaminergic influence, that can be both dopaminergic and noradrenergic in nature, and provide insights into the possible involvement of this innervation in the regulation of diverse functions including locomotor activity, social interactions and nociceptive signaling. Moreover, our findings support the hypothesis that the NI acts as an integration hub for arousal-related neural information.

The NI has attracted considerable research recently, and has been shown to play a role in theta rhythm control, locomotor activity, arousal, and stress responses in rats and mice (Lu et al., 2020; Szlaga et al., 2022; Szőnyi et al., 2019; Trenk et al., 2022). Chemo- and opto-genetic activation of NI neurons increased theta power and locomotor speed and induced arousal (Lu et al., 2020; Ma et al., 2017a). It was also shown that D2R activation in the rat NI by intra-NI infusion of the D2R agonist, quinpirole, induced home cage hypolocomotion and suppression of the velocity and distance travelled in a novel environment (Kumar et al., 2015), suggesting the main effect of D2R activation in the NI is inhibition of neuronal activity. However, the NI consists of heterogeneous neuronal populations that differ in their in vitro electrophysiological properties and their neurochemical phenotype, and distinct NI neuronal populations belong to separate neuronal circuits (Cervera-Ferri et al., 2012; Sutin and Jacobowitz, 1988; Szlaga et al., 2022; Trenk et al., 2022). Our present in situ hybridization data revealed that D2 receptor mRNA is present in both putative GABAergic and glutamatergic NI neurons, and that ~50% of RLN3 and ~25% of CCK NI neurons express D2 receptor transcripts. These data indicate that different NI neuronal populations, possibly involved in distinct neuronal

| mRNA combination | Mean ± SD (%) |
|------------------|---------------|
| D2 only          | 2 ± 2 (0.4)   |
| vGAT1/D2         | 77 ± 30 (18)  |
| vGAT1/RLN3/D2    | 90 ± 43 (21)  |
| vGAT1/CCK/D2     | 8 ± 2 (2)     |
| vGAT1/RLN3/CCK/D2| 10 ± 6 (2)    |
| vGAT1/vGlu2/D2   | 26 ± 16 (6)   |
| vGAT1/vGlu2/RLN3/D2| 5 ± 3 (1)   |
| vGAT1/vGlu2/CCK/D2| 2 ± 0 (0.4)  |
| vGlu2/D2         | 194 ± 78 (46) |
| vGlu2/CCK/D2     | 9 ± 2 (2)     |
Fig. 1. D2 receptor mRNA expression by different types of nucleus incertus neurons. (A) Schematic representation of the NI region (coronal section), –9.6 mm from bregma, based on a rat brain atlas (Paxinos and Watson, 2007), with an area matching the region illustrated in (B) delineated in red. (B) Representative image of the NI area containing RLN3 (green), CCK (pink), vGAT1 (yellow), vGlut2 (light-gray) and D2 (red) mRNA-expressing neurons. Scale bar: 100 μm. (C-F) A series of images illustrating the diversity of D2 receptor mRNA-positive NI cells. DAPI-stained nuclei are shown in blue; (C) a D2/vGAT1/RLN3 mRNA-expressing neuron (green arrowhead); (D) a D2/vGAT1/CCK mRNA-expressing neuron (pink arrowhead); (E) a D2/vGAT1 mRNA-expressing neuron (yellow arrowhead); (F) two D2/vGlut2 mRNA-expressing neurons (white arrowheads). Scale bars: 10 μm. (G, H) Schematics of the proportions of and the relationship between the distinguished types of D2 receptor mRNA-expressing NI cells (G), and the total of counted NI cells (H). The area of each ellipse matches the percentage of each specific cell type. Abbreviations: 4V – 4th ventricle; Cb – cerebellum; CCK – cholecystokinin; D2 – dopamine receptor type 2; LC – locus coeruleus; mlf – medial longitudinal fasciculus; Nlc – nucleus incertus pars compacta; NId – nucleus incertus pars dissipata; PDTg – posterodorsal tegmental nucleus; RLN3 – relaxin-3; vGAT1 – vesicular γ-aminobutyric acid (GABA) transporter; vGlut2 – vesicular glutamate transporter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 2. Catecholaminergic fibers in the nucleus incertus. (A) Confocal projection images of NI sections stained for TH (red) and DBH (yellow) at different coronal levels. Note the abundance of both TH- and DBH-immunoreactivity in the Nlc region. (B) High magnification images of the Nlc area, illustrating the close appositions of TH-ir (left) and DBH-ir (right) fibers with RLN3 neurons (green). Scale bars 50 μm. Abbreviations: 4V – 4th ventricle, DBH – dopamine β-hydroxylase, DR – dorsal raphe nucleus, LDTg – laterodorsal tegmental nucleus, mlf – medial longitudinal fasciculus, Nlc – nucleus incertus pars compacta, NId – nucleus incertus pars dissipata, PDTg – posterodorsal tegmental nucleus, RLN3 – relaxin-3, TH – tyrosine hydroxylase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
with the relatively dense innervation of the NI by catecholaminergic retrogradely-labeled after injection of a retrograde viral vector (retro-AAV2 that the catecholaminergic innervation of the NI may be both dopami circuits, are sensitive to D2R agonists. The potential physiological dance was ~ 0 Relative abundance of catecholaminergic neurons in distinct brain regions – PDR nucleus; Me5 DMTg 4Sh Darkschewitsch nucleus – sphenoid nucleus; VLPAG A7 noradrenergic group – * Medial accessory oculomotor nucleus Locus coeruleus Zona incerta Pre–Pleoglial periaqueductal gray Interstitial nucleus of Cajal Locus coeruleus Retinorecipient nucleus Magnocellular nucleus of posterior commissure Dorsal hypothalamic nucleus, lateral part Subincertal nucleus Subparaventricular hypothalamic nucleus

Table 3

Distribution of catecholaminergic neurons innervating the nucleus incertus.

| Brain Structure                         | Case A | Case B | Case C | Case D | Abundance |
|----------------------------------------|--------|--------|--------|--------|-----------|
| Nucleus of Darkschewitsch             | +      | +      | +      | +      | ***       |
| Raphe nucleus                         | +      | +      | +      | +      | ***       |
| A13 nucleus                            | +      | +      | +      | +      | ***       |
| A11 nucleus                            | +      | +      | +      | +      | ***       |
| Lateral periaqueductal gray            | +      | +      | +      | +      | ***       |
| Pleoglial periaqueductal gray          | +      | +      | +      | +      | ***       |
| Pre–Edinger–Westphal nucleus           | +      | +      | +      | +      | ***       |
| Posterior hypothalamic nucleus         | +      | +      | +      | +      | ***       |
| Zona incerta                           | +      | +      | +      | +      | ***       |
| Edinger–Westphal nucleus               | +      | +      | +      | +      | ***       |
| Locus coeruleus                       | +      | +      | +      | +      | ***       |
| Interstitial nucleus of Cajal          | +      | +      | +      | +      | ***       |
| Medial accessory oculomotor nucleus    | +      | +      | +      | +      | ***       |
| Laterodorsal tegmental nucleus         | +      | +      | +      | +      | ***       |
| A7 noradrenergic group                 | +      | +      | +      | +      | ***       |
| Pararubral nucleus                     | +      | +      | +      | +      | ***       |
| p1 reticular formation                 | +      | +      | +      | +      | ***       |
| Substantia nigra pars compacta         | +      | +      | +      | +      | ***       |
| Retroparafascicular nucleus            | +      | +      | +      | +      | ***       |
| Magnocellular nucleus of posterior commissure | +  | +  | +  | +  | ***       |
| Dorsal hypothalamic nucleus, dorsal    | +      | +      | +      | +      | ***       |
| Subincertal nucleus                    | +      | +      | +      | +      | ***       |
| Subparaventricular hypothalamic nucleus | +      | +      | +      | +      | ***       |

Relative abundance of catecholaminergic neurons in distinct brain regions retrogradely-labeled after injection of a retrograde viral vector (retro-AAV2-hSyn-mCherry) into the NI. Reconstructions of the injection sites are shown in Fig. 3A. The presence of double-stained, TH-ir and m-Cherry-ir neurons in a given structure is indicated by the + sign; and the relative abundance was ~ 0–7 neurons; * 8–23 neurons; ** 24–44 neurons; *** 45–100 neurons; **** 101–400 neurons.

circuits, are signaling to D2R agonists. The potential physiological importance of D2R signaling in the NI is reflected by the fact that D2 receptor mRNA is present in one third of all NI cells, which is consistent with the relatively dense innervation of the NI by catecholaminergic fibers, as shown by the levels of TH and DBH staining. These data further establish the anatomical and molecular basis of the observed behavioral effects of D2R activation in the NI (Kumar et al., 2015), and indicate the need for additional research to investigate the involvement of specific, D2R-expressing NI neuronal populations in defined neuronal circuits. Our immunohistochemical and neural tract-tracing data indicate that the catecholaminergic innervation of the NI may be both dopaminergic and noradrenergic in nature, as TH– (the rate-limiting enzyme of catecholamine synthesis) and DBH– (the enzyme that catalyzes the synthesis of noradrenaline from dopamine) positive fibers were detected within the NI area, and TH-positive neurons from both dopaminergic and noradrenergic brain structures were shown to directly innervate the NI. Brain areas identified as major sources of the catecholaminergic input to the NI were the Darkschewitsch nucleus (Dk), raphe nuclei and A13 dopaminergic cell group. Input from the Dk to the NI in the mouse has been described (Lu et al., 2020), but the neurochemical nature of this projection was unknown until now. We demonstrated that the Dk-NI projection is, at least in part, catecholaminergic, but to the best of our knowledge, it is not established what kind of catecholamine(s) is/are synthetized in the Dk. An early study of intracerebroventricular injections of radioactive noradrenaline, suggested the possible noradrennergic nature of Dk neurons in the rat (Reivich and Glowinski, 1967). However, in situ hybridization data does not detect the presence of DBH mRNA, while confirming the presence of TH mRNA in this brain area in mice (Allen Brain Atlas, 2004).

Surprisingly little is known about the neurophysiological role of Dk neurons, and most information come from studies in cats. Early anatomical data suggested the Dk is involved in oculomotor control (Bianchi and Gioia, 1986), while some later anatomical studies implicated Dk in the control of locomotor activity (Onodera and Hicks, 1995; Rutherford et al., 1989). More recent studies in mice have revealed that neurons in Dk and the neighboring Edinger–Westphal nucleus which also innervates the NI express mRNA for urocortin, a peptide closely related to corticotropin-releasing hormone (CRH), which indicates a possible role of Dk in stress responses (Weninger et al., 2000). Notably, urocortin-positive fibers have been identified in the rat NI (Bittencourt et al., 1999). Taken together, these data on the anatomical relationship between the Dk and NI, suggest this pathway is involved in stress and locomotor activity control.

Among the structures identified in the current studies as sources of a catecholaminergic innervation of the NI were the raphe nuclei, which together with the dopaminergic neurons of the ventrolateral periaqueductal gray (vPAG), can be considered as a dorso-caudal extension of the A10 group (with the majority of dopaminergic cells localized in the ventral tegmental area) (Lager et al., 2016; Li et al., 2014; Stratford and Wirtschafter, 1990). Bidirectional connections between the raphe nuclei/PAG and the NI have been described in rat and mouse (Goto et al., 2001; Lu et al., 2020), but the role of these pathways is currently unclear. It has been reported that dorsal raphe nucleus (DRN) and PAG dopamine neurons are involved in arousal control, and their optogenetic activation promoted wakefulness, whereas their chemogenetic inhibition strongly opposed wakefulness, even in the presence of salient stimuli (Cho et al., 2017; Lu et al., 2006). Therefore, the dorsal raphe/vPAG dopaminergic innervation of the NI, may be an important element of the arousal control circuit, as it has been shown that chemo- and optogenetic activation of NI neurons promotes arousal, cortical desynchronization, hippocampal theta rhythm and locomotor speed (Lu et al., 2020; Ma et al., 2017a).

Another suggested role of PAG and DRN dopaminergic neurons is in the control of aversion-related responses, as it was shown that these neurons are involved in coping with pain and are a critical site for the antinociceptive actions of opioids (Li et al., 2016; Yu et al., 2021). In this regard, the presence of oxytocin receptors on dopamine dorsal raphe neurons should be further noted, as in addition to the mentioned role in

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**Fig. 3. Catecholaminergic neurons innervating the nucleus incertus.** (A) Reconstruction of injection sites of retro-AAV2-hSyn-mCherry into the NI. (B) Light microscopic images of case B injection site of retro-AAV2-hSyn-mCherry (red); scale bars 200 µm. (C–E) Fluorescence microscopic images of mCherry-expressing (red), retrogradely-labeled TH-ir (yellow) neurons in the Darkschewitsch nucleus, interstitial nucleus of Cajal and medial accessory oculomotor nucleus (C), dorsal raphe nucleus lateral part (D); and A13 dopaminergic cell group (E). Lower rows in C–E are enlargements of boxed outlines from the upper rows. In the case of the Darkschewitsch nucleus (C), the enlargements are counter-clockwise rotated 90°; scale bars 100 µm. Abbreviations: 1C – 1st cerebellar lobe; 3V – 3rd ventricle; 4Sh – trochlear nucleus shell region; 4V – 4th ventricle; Aq – aqueduct; DA – dorsal hypothalamic area, Dk – Darkschewitsch nucleus; DMTg – dorsomedial tegmental area; DRD – dorsal raphe nucleus, dorsal part; DRL – dorsal raphe nucleus, lateral part; DRV – dorsal raphe nucleus, ventral part; INc – interstitial nucleus of Cajal; LC – locus coeruleus; LDTg – laterodorsal tegmental nucleus; LPAG – lateral periaqueductal gray; MA3 – medial accessory oculomotor nucleus; Me5 – mesencephalic trigeminal nucleus; mlf – medial longitudinal fasciculus; Nc – nucleus incertus pars compacta; Nid – nucleus incertus pars dissipata; PDR – posterodorsal raphe nucleus; PDTg – posterodorsal tegmental nucleus; pEW – pre-Edinger–Westphal nucleus; RTg – reticulotegmental nucleus of the pons; Sph – sphenoid nucleus; vLPAG – ventrolateral periaqueductal gray, ZID – zona incerta, dorsal part; ZIV – zona incerta, ventral part. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Type II neurons were almost exclusively excited, while type I NI neurons were equally excited or inhibited upon D2R activation. This effect also occurred under conditions of pharmacological blockade of spiking and synaptic activity (in the presence of TTX and ionotropic glutamate and GABA receptor antagonists), which indicates that the D2R activated receptors were situated postsynaptically on recorded neurons. However, this does not exclude the possibility of presynaptic expression of D2R within the NI.

In accordance with the excitatory effect of D2R activation on type II NI neurons, manifested either as depolarization in the current clamp or an increase in the whole cell inward current in voltage clamp mode, were our observations of the influence of quinpirole on postsynaptic currents (PSCs) in these neurons. The recorded decrease in frequency of excitatory PSCs and concomitant decrease in the rise time of the event indicated that the D2R activated receptors were situated postsynaptically on recorded neurons. However, this does not exclude the possibility of presynaptic expression of D2R within the NI.

The possible association of catecholaminergic signaling within the NI with diverse neuronal populations belonging to different neuronal circuits, is supported not only by the current in situ hybridization and neural tract-tracing data, but also by the different responses of NI neurons to D2R activation. We observed that quinpirole-induced D2R activation could result in both excitation and inhibition of recorded neurons, and that the type of response was dependent on the NI cell type. Type II neurons were almost exclusively excited, while type I NI neurons were equally excited or inhibited upon D2R activation. This effect also occurred under conditions of pharmacological blockade of spiking and synaptic activity (in the presence of TTX and ionotropic glutamate and GABA receptor antagonists), which indicates that the D2R activated receptors were situated postsynaptically on recorded neurons. However, this does not exclude the possibility of presynaptic expression of D2R within the NI.

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intracellular signaling cascades within a given type of neuron.

The heterogeneous nature of neuronal responses to D2R activation has been described earlier, although an inhibitory effect is more commonly observed (Bonci and Hopf, 2005). The inhibitory D2R action is mediated by coupling to $G_{\alpha i/o}$-protein and inhibition of adenylyl cyclase, and a subsequent drop in cAMP production (Missale et al., 1998). This may result in the reduction of Ca$^{2+}$ flow through L- and N-type channels (Hernández-López et al., 2000; Yan et al., 1997), activation of inwardly-rectifying potassium channels (GIRKs) (McCall et al., 2019), and interaction with Kir3 potassium channels (Lavine et al., 2002). The excitatory action of D2R activation has been shown to be associated with G$\beta\gamma$ protein coupling and activation of phospholipase C (PLC) (Valler et al., 1990). PLC can directly modulate inositol 1,4,5-trisphosphate (IP3), diacylglycerol (DAG) and phosphatidylinositol 4, 5-bisphosphate (PIP2) signals, and lead to an increase in calcium levels in the cytoplasm (Putney and Tomita, 2012). For example, a direct excitatory action of D2R activation was observed in parvalbumin interneurons in primary motor cortex (Cousineau et al., 2020), supraoptic nucleus neurons (Yang et al., 1991) and dorsolateral geniculate nucleus interneurons (Munsch et al., 2005). D2R activation was also shown to have an excitatory action on dorsal raphe serotonin neurons, via activation of a nonselective cation current (Aman et al., 2007;
variety of neurochemical profiles of NI neurons expressing D2 receptor Visualization, Writing (Gil-Miravet et al., 2021; Ma et al., 2017b; Olucha-Bordonau et al., hypothesis that the NI is a hub integrating arousal-related information involved with distinct neuronal circuits. This in turn strengthens the opposite effects of D2R activation on neuronal activity, as well as the sources, Writing further studies are needed to map the distribution of TH-retrogradely-labeled structures innervate defined areas of the NI. Therefore, further studies of the effects of catecholamines on NI neuronal activity in which the catecholaminergic origin to the NI is specified. 5. Technical considerations regarding retrograde tracing studies It is important to note that in the current study, some structures were retrogradely labeled in some but not all examined brains, which may be due to differences in the area of NI that was transfected with viral vectors in the different cases. This finding suggests that the described retrogradely-labeled structures innervate defined areas of the NI. Therefore, further studies are needed to map the distribution of TH-positive fibers, originating from defined dopaminergic and noradrenergic brain structures, across the NI area. It is also worth noting that in tracing studies there is the possibility that some of the retrogradely labeled neurons, accumulate viral vectors (or other molecules in the case of conventional tracers) from the near vicinity of the structure targeted for injection. Therefore, the relative abundance of retrogradely-labeled neurons is an important aspect of interpreting such labelling results.

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Declarations of interest
None.

CRediT authorship contribution statement
Agata Szlaga: Investigation, Formal analysis, Visualization, Writing – original draft, Funding acquisition. Patryk Sambak: Investigation, Writing – review & editing. Anna Gugula: Formal analysis, Visualization, Writing – original draft. Aleksandra Trenk: Formal analysis, Visualization, Writing – review & editing. Andrew L. Gundlach: Resources, Writing – review & editing. Anna Blasiak: Conceptualization, Validation, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Data availability
Data will be made available on request.

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