



Evidence of D₂ receptor expression in the nucleus incertus of the rat



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HIGHLIGHTS

- The nucleus incertus (NI) is a brainstem nucleus projecting widely across the brain.
- RT-PCR, western blotting and immunofluorescence showed expression of D₂ receptors in the NI.
- Infusion of quinpirole (D₂/D₃ receptor agonist) into the NI induced hypolocomotion

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ABSTRACT

The nucleus incertus (NI), located in the caudal brainstem, mainly consists of GABAergic neurons with widespread projections across the brain. It is the chief source of relaxin-3 in the mammalian brain and densely expresses corticotropin-releasing factor type 1 (CRF₁) receptors. Several other neurotransmitters, peptides and receptors are reportedly expressed in the NI. In the present investigation, we show the expression of dopamine type-2 (D₂) receptors in the NI by reverse transcriptase-polymerase chain reaction (RT-PCR), western blotting (WB) and immunofluorescence (IF). RT-PCR did not show expression of D₃ receptors. D₂ receptor short isoform (D_{2S})-like, relaxin-3, CRF_{1/2} receptor and NeuN immunoreactivity were co-expressed in the cells of the NI. Behavioural effects of D₂ receptor activation by intra-NI infusion of quinpirole (a D₂/D₃ agonist) were evaluated. Hypolocomotion was observed in home cage monitoring system (LABORAS) and novel environment-induced suppression of feeding behavioural paradigms. Thus the D₂ receptors expressed in the NI are likely to play a role in locomotion. Based on its strong bidirectional connections to the median raphe and interpeduncular nuclei, the NI was predicted to play a role in modulating behavioural activity and the present results lend support to this hypothesis. This is the first evidence of expression of a catecholamine receptor, D₂-like immunoreactivity, in the NI.

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1. Introduction

The nucleus incertus (NI) is a highly conserved brainstem structure present in invertebrates, rodents, non-human primates and humans. It is located at the base of the fourth ventricle and consists of long GABAergic projection neurons with widespread connections throughout the rat brain [21,45]. Strong inputs from the prefrontal

cortex, motor cortex, lateral habenula, oculomotor control regions and medial septum to the NI indicate a role in integrating information to modulate behavioural activity possibly exerted through its major efferent connections to the median raphe, interpeduncular nucleus, and hippocampal system [21,52]. The NI is the main neuronal source of the neuropeptide relaxin-3 that is known to be involved in various behaviours associated with anxiety, fear, arousal, stress response, reward, addiction, cognition, memory and feeding (for review see: [52,55,59]). For a small group of neurons, the NI expresses a variety of receptors: CRF₁ receptors at high density, GABA receptors, 5-HT receptors, cholinergic receptors and orexin receptors [4,22,29,40,48,61]. However, dopamine receptors have not previously been discovered in the NI.

D₂ receptors bind to Gα_{i/o} and inhibit adenylyl cyclase, in turn reducing cAMP levels [2]. Alternative splicing of a 29 amino acid region

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from the 3rd intracellular loop results in two isoforms of the D₂ receptor, long (D_{2L}) and short (D_{2S}) [10,20,26,27,41]. This region has been found to be important in G-protein coupling thus resulting in distinct functional roles of the two isoforms ([17]; for review see: [11]). D₂ receptors are highly expressed in the striatum, nucleus accumbens and olfactory tubule; and moderately expressed in the substantia nigra, ventral tegmental area, prefrontal cortex, amygdala, hippocampus and hypothalamus of rodents [11,64]. In the brainstem, certain nuclei strongly express D₂ receptors, including the locus coeruleus, trigeminal nucleus and the nucleus of the solitary tract [64].

Our previously published work showed that both typical and atypical antipsychotics induced significant c-Fos expression in the NI [50]. Since the main underlying mechanism of action of antipsychotics is D₂ receptor antagonism-mediated, these findings prompted us to investigate the presence of D₂ receptors in the NI. In the present manuscript, reverse transcription polymerase chain reaction (RT-PCR), western blotting and immunofluorescence labelling techniques have been employed to show the expression of D₂ receptors in the NI. Concurrent immunofluorescent labelling of NeuN, CRF₁ and relaxin-3 was carried out to validate the location of the NI and study receptor co-expression patterns. To further confirm the presence of D₂ receptors, quinpirole (a D₂/D₃ agonist) was microinfused into the NI and the rats were exposed to the Laboratory Animal Behaviour Observation Registration and Analysis System (LABORAS) home cage monitoring system and a novel environment-induced suppression of feeding paradigm.

2. Materials and methods

2.1. Animals

Forty-six male Sprague–Dawley rats (280–350 g), obtained from the Laboratory Animals Centre, National University of Singapore, were utilised in this investigation. The procedures conducted were in compliance with the guidelines of the National Institutes of Health Guide for Care and Use of Animals, and with approval from the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore. Rats were housed in pairs in individually ventilated cages that were maintained in a temperature controlled room (22 °C–24 °C) with a 12 hour light–dark cycle. The animals were given ad libitum access to food and water and were acclimatised to the housing conditions for at least 5 days.

2.2. Drugs

Quinpirole hydrochloride (Tocris, UK), ketamine (Parnell Manufacturing Pty Ltd.; Alexandria, NSW, Australia), xylazine (Ilium Xylazil, Troy Laboratories Pty Ltd.; Glendenning, NSW, Australia), enrofloxacin (Baytril 5%, Bayer Health Care; Seoul, Korea) and carprofen (Carprieve, Norbrook Laboratories (GB), Ltd.; Carlisle, UK) were freshly prepared in sterile isotonic saline (B. Braun, Germany) before use. Pentobarbital (Valbarb) was purchased from Jurox Pty Ltd., Australia.

2.3. Fresh tissue harvest

Rats were anaesthetised with a pentobarbital overdose and decapitated. The brains were removed immediately and 500 µm sections were collected with a rat brain matrix (Roboz Surgical, USA). The position of the NI (Fig. 1A) was verified by light microscopy and punched out using a micropunch (0.75 mm, Sigma-Aldrich, USA) as described previously [30]. NI tissue punches and microdissected regions of the caudate putamen were homogenised for RT-PCR amplification and western blot analysis.

2.4. RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA was extracted from the rats' NI or striatum and purified with the Purelink Mini Total RNA Purification kit (Invitrogen, USA) according to the manufacturer's guidelines. The amount of RNA was quantified and approximately 1 µg of RNA was reverse transcribed with oligo (dT) primers using ImProm-II™ Reverse Transcription system. The PCR reaction for the dopamine receptor subtypes [63] and β-actin was carried out with the following sets of primers: D₂ forward primer 5'-GCA GTC GAG CTT TCA GAG CCA ACC TG-3', reverse primer 5'-AGA ACT TGG CAA TCC TGG GAT TGA C-3' (255 bp expected band size for short isoform and 342 bp expected band size for long isoform); D₃ forward primer 5'-GTG ACT GTC CTG GTC TAT GCC AG-3', reverse primer 5'-GCA GAT GCT GTA GTA GCG CTT CAG-3' (312 bp expected band size) and β-actin forward primer 5'-ATC CTG AAA GAC CTC TAT GC-3', reverse primer 5'-AAC GCA GCT CAG TAA CAG TC-3' (287 bp expected band size). The PCR cycling reactions were carried out at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C–65 °C for 1 min, 72 °C for 1 min followed by 10 min at 72 °C. The amplified products were separated in 1.5% agarose gel, and then visualized under UV irradiation.

2.5. Western blotting

Fresh NI tissue was dissected and lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0). The total protein concentration was determined using a Pierce bicinchoninic acid assay (BCA) kit (Pierce Biotechnology, USA). 50 µg of protein was separated by 10% or 12% sodium dodecyl sulphate SDS-PAGE for about 1.5 h and then electroblotted onto a low immunofluorescence PVDF membrane (Biorad, USA) at 100 mA for 2 h. The membranes were subsequently blocked with 5% skim milk, 5% bovine serum albumin (BSA) or 2% normal goat serum (NGS) in Tris-buffered saline/Tween 20 (TBST) for an hour in room temperature followed by incubation with primary antibodies: anti-D₂ (1:1000, Ab32349, Abcam, Hong Kong); anti-D_{2S} (1:1000; 324,396, Merck Millipore, USA); anti-CRF RI/II (1:1000, Santa Cruz, USA), anti-relaxin-3 (1:1000); or anti-β-actin (1:10,000, A-5441, Sigma-Aldrich, USA) overnight at 4 °C. The mouse monoclonal anti-relaxin-3 antibody was produced from hybridoma cells as previously described [30]. Following primary antibody incubation, the blot was then washed and incubated with a peroxidase-conjugated anti-goat (1:5000; Pierce Biotechnology, USA), anti-mouse (1:5000; ThermoScientific, USA) or anti-rabbit IgG (1:5000; Pierce Biotechnology, USA) for an hour at room temperature with agitation. Protein bands were detected by incubating the membranes with peroxidase substrate Luminata Forte (Merck Millipore, USA) and imaging was conducted with a luminescent image analyzer (Image Quant LAS 4000, GE Healthcare, UK). The relaxin-3 blot was incubated with goat anti-mouse AlexaFluor488 (1:5000) for an hour at room temperature with agitation and the fluorescence signal was detected with the Image Quant LAS 4000.

2.6. Immunofluorescence

Rats were anaesthetized with a pentobarbital overdose. Transcardial perfusion was carried out with isotonic saline followed by 2% paraformaldehyde in phosphate buffer (0.1 M). The brains were subsequently post-fixed in 2% paraformaldehyde at 4 °C for 2 h and soaked in 15% sucrose followed by 30% sucrose at 4 °C for 2 days each. Sections (30 µm) were collected serially in sterile PBS in a cryostat (CM 3050, Leica Biosystems, Germany). The ventral half of the brain sections containing the NI was stored in cryoprotectant solution (1% PVP-10, 30% ethylene glycol, 30% sucrose, 50% 0.1 M phosphate buffer, pH 7.4) at –20 °C until immunofluorescence labelling was conducted [24]. Sections were hydrated by washing in phosphate buffered saline (PBS) and permeabilized by incubation

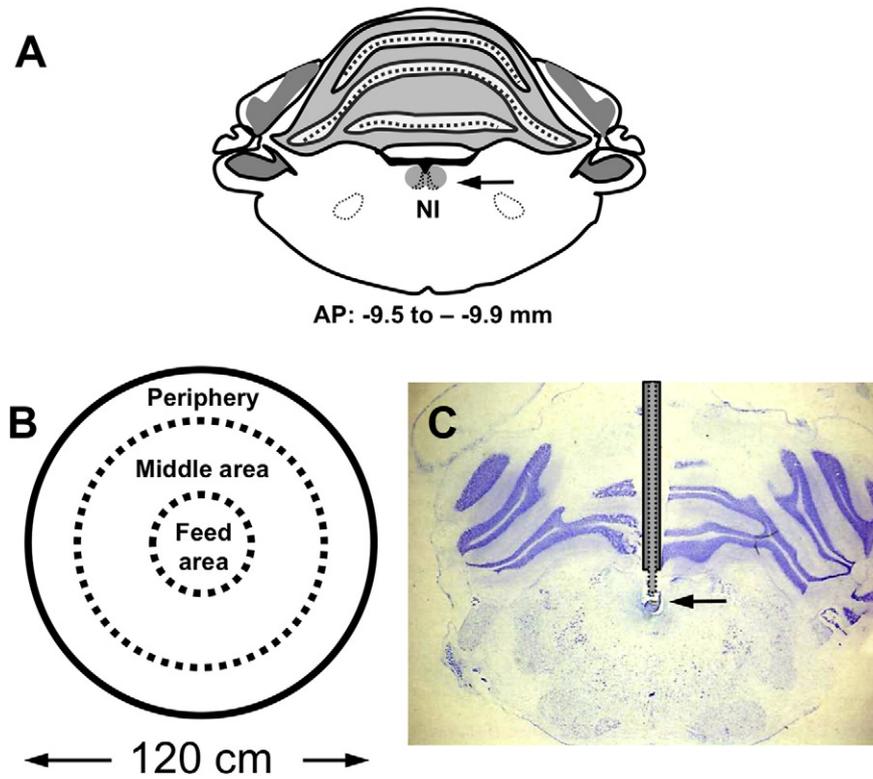


Fig. 1. (A) Schematic representation of a brainstem section in which the arrow points to two grey circles indicating the areas of tissue punched out for RT-PCR or western blot analysis. (B) Schematic diagram of the novel environment-induced suppression of feeding arena. (C) Representative image showing the cannula position in the nucleus incertus. Arrow points to the pontamine sky blue dye spot.

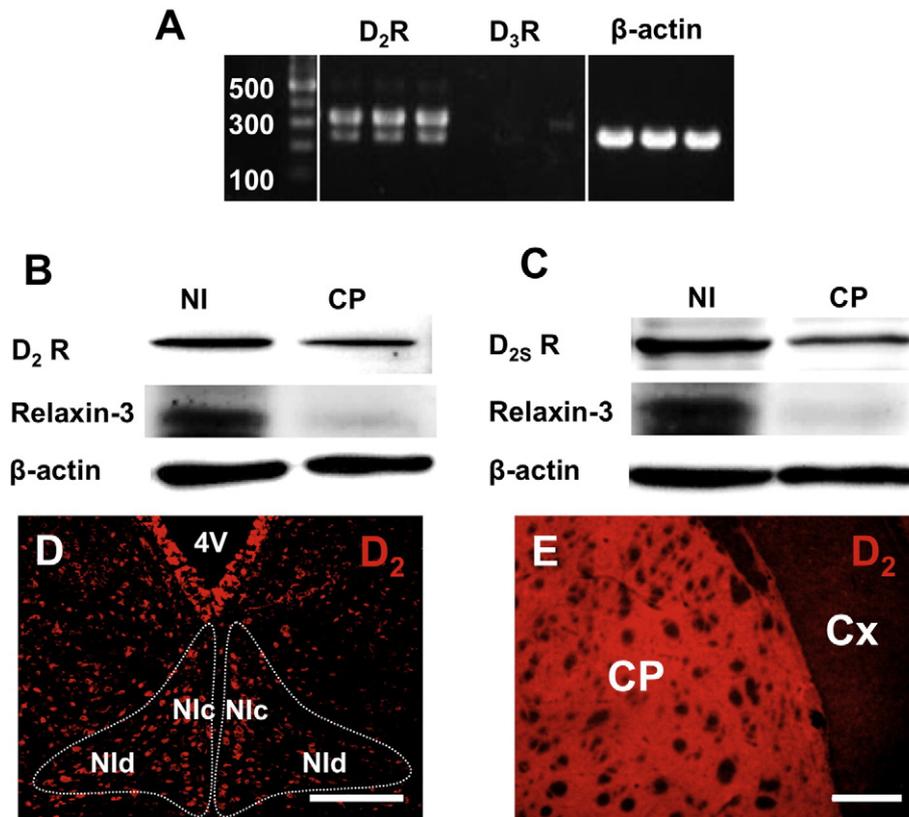


Fig. 2. (A) Agarose gel electrophoresis of PCR products of dopamine receptor D_2 and D_3 subtypes in the nucleus incertus. RT-PCR was conducted on total RNA extracted from the nucleus incertus tissue of rats. Representative western blots showing the evidence of (B) D_2 R and relaxin-3 expression, (C) D_{2s} R and relaxin-3 expression in the nucleus incertus (NI) and caudate putamen (CP). Representative fluorescence microscopy images showing D_2 receptor-like immunoreactivity in the nucleus incertus (D) and caudate putamen (E). 4 V: fourth ventricle; Cx: cortex; Nlc: nucleus incertus pars compacta; Nld: nucleus incertus pars dissipata. Scale bars: 100 μ m.

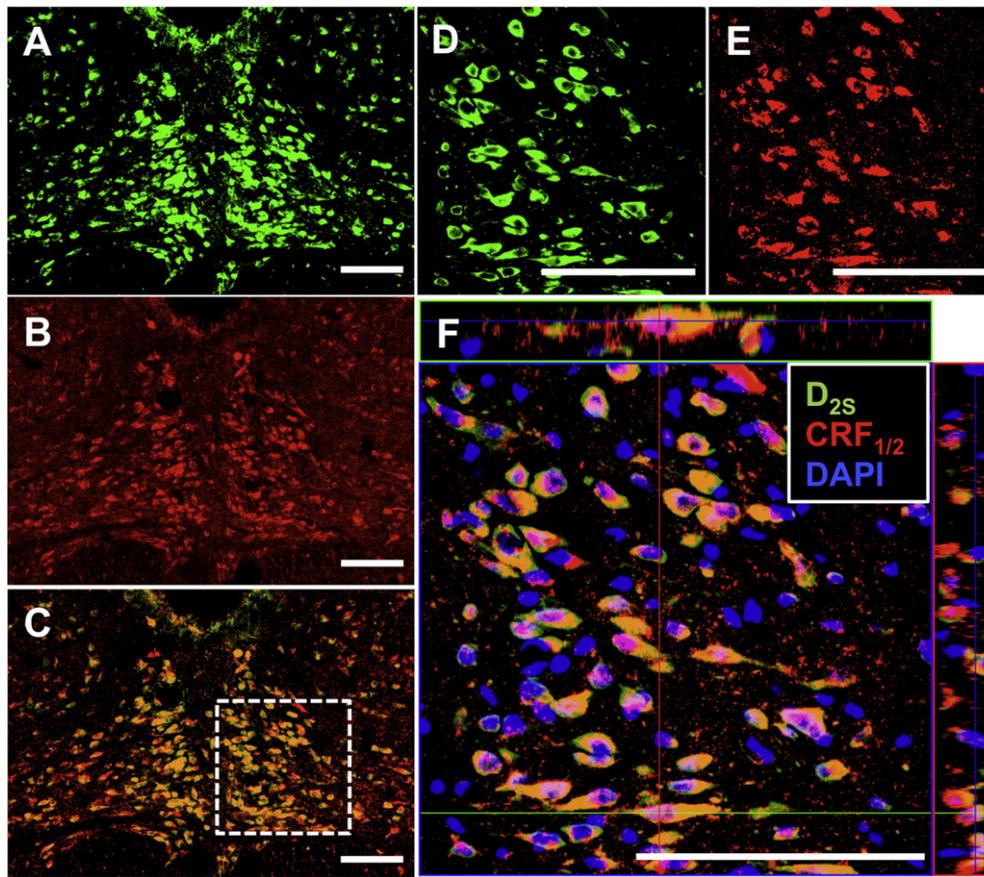


Fig. 3. Representative confocal images showing that D₂₅ receptor-like immunoreactivity in the nucleus incertus is co-expressed with CRF_{1/2} receptors. Figures A–F are confocal images from the same tissue section. (A) and (D) show the expression of D₂₅ receptor-like immunoreactivity (green) at lower and higher magnifications (region boxed with dashed line in C), respectively. (B) and (E) show the expression of CRF_{1/2} (red) at lower and higher magnifications (region boxed with dashed line in C), respectively. (C) represents the merge of (A) and (B). (F) Z-stack confocal image showing the co-localisation of D₂₅ receptor-like immunoreactivity, CRF_{1/2} and DAPI (blue) in the nucleus incertus. Scale bars: 100 μ m.

in 0.3% Triton-X 100 in PBS (PBST) for 10 to 30 min. The sections were then incubated in 5% fetal bovine, goat or donkey serum for 1 h at room temperature followed by incubation in the following primary antibody solutions for 24–72 h at 4 °C: anti-D₂ (1:100, Ab32349, Abcam, Hong Kong); anti-D₂₅ (1:150; 324,396, Merck Millipore, USA); anti-CRF RI/II (1:600, Santa Cruz, USA); anti-relaxin-3 (1:100); and anti-NeuN (1:200, MAB 377, Merck Millipore, USA). The mouse monoclonal anti-relaxin-3 antibody was produced from hybridoma cells as previously described [30]. For the co-staining with anti-relaxin-3 (raised in mouse) and anti-CRF RI/II (raised in goat), anti-D₂₅ (raised in rabbit) was used instead of anti-D₂ (raised in goat) due to compatibility of the antibody source. All sections were rinsed in PBS prior to incubation in solutions containing appropriate Alexa Fluor secondary antibodies (Invitrogen, Life Technologies, CA, USA) for an hour at room temperature in the dark. All sections were rinsed 4 \times 5 min each in PBS and mounted on glass slides. The sections were gently shaken during all incubation periods including the rinses. The sections were dried, covered with Prolong Antifade Gold reagent with DAPI (Invitrogen, Life Technologies, CA, USA) and coverslipped. The sections were viewed and photographed with a confocal Microscope (Zeiss LSM 510 Meta, Zeiss, Germany) or a fluorescence microscope (BX-51, Olympus, Japan).

2.7. Intra-NI administration of quinpirole and behaviour

2.7.1. Surgery

Rats were anaesthetised with a cocktail of ketamine (75 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally and mounted on to a

stereotaxic frame. The scalp was shaved and a midline sagittal incision exposed the bare skull. A burr hole was drilled at AP: -9.7 mm, ML: -0.1 mm from the bregma to target the nucleus incertus [46]. A guide cannula (Plastics One, USA) 8 mm in length was carefully inserted to a depth of 7.4 mm from the skull surface and was held in place with dental cement and anchoring screws fitted to the skull. The guide cannula was closed using a cap with a stylette. The rats were then allowed a rehabilitation period of 1 week, with analgesic (carprofen) and antibiotic (enrofloxacin) treatments injected subcutaneously for the first 5 days.

2.7.2. General considerations for behavioural assessments

All behavioural assessments were conducted during the light phase of the 12 h light–dark cycle. The NI cannula-implanted rats had free access to water but food was restricted for 18 h prior to some of the experiments as specified below. They were brought to the experimental room at least 1 h before the commencement of behavioural assessments. They were gently handled and quinpirole (6 μ g/0.1 μ l) or vehicle (equivalent volume of sterile isotonic saline) was infused into the NI over a period of 30 s into the brain. The administration was done using an infusion cannula that had a 1 mm projection beyond the guide cannula. The infusion cannula was left in place for 1 min before re-capping and placing the rat in the exploration arenas. Behavioural assessment began immediately. Behavioural apparatus was cleaned with 70% ethanol between the trials. All the behavioural observations were video recorded and analysed using a video tracking system (Ethovision, version 8.2, Noldus, Netherlands).

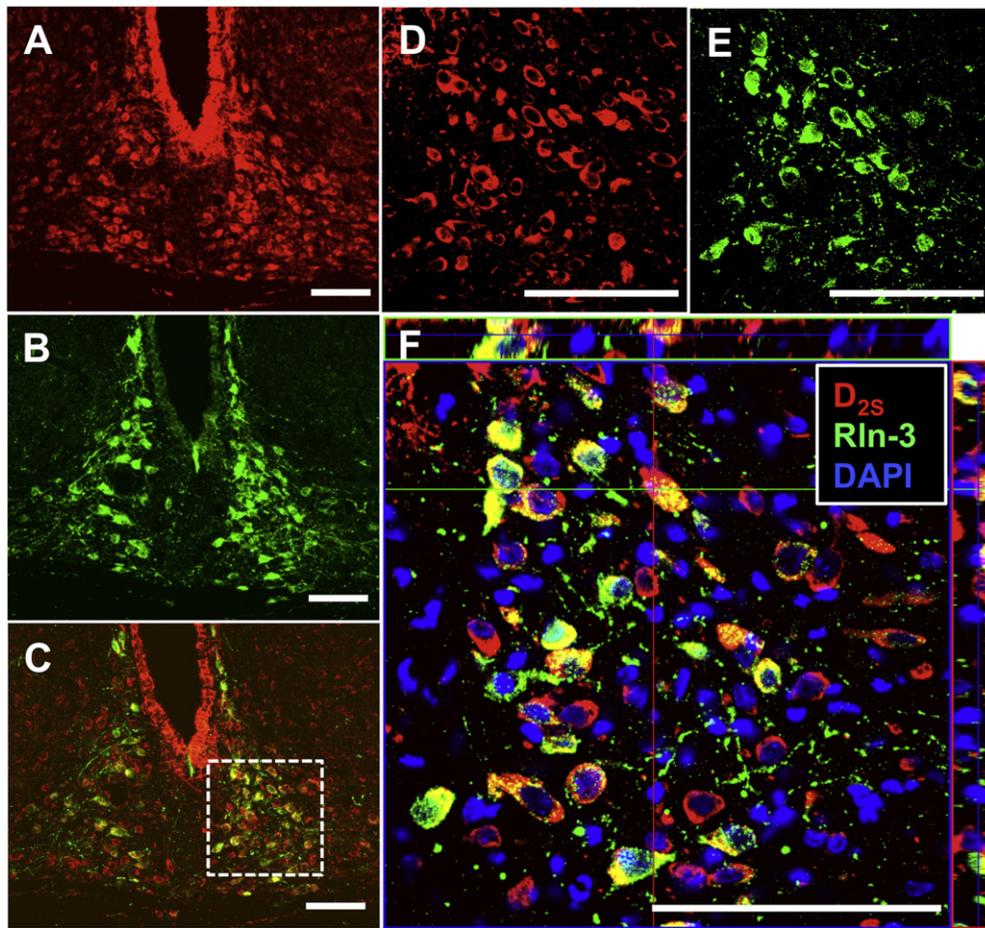


Fig. 4. Representative confocal images showing that D_{25} receptor-like immunoreactivity in the nucleus incertus is co-expressed with relaxin-3. Figures A–F are confocal images from the same tissue section. (A) and (D) show the expression of D_{25} receptor-like immunoreactivity (red) at lower and higher magnifications (region boxed with dashed line in C), respectively. (B) and (E) show the expression of relaxin-3 (green) at lower and higher magnifications (region boxed with dashed line in C), respectively. (C) represents the merge of (A) and (B). (F) Z-stack confocal image showing the co-localisation of D_{25} receptor-like immunoreactivity, relaxin-3 and DAPI (blue) in the nucleus incertus. Scale bars: 100 μ m.

2.7.3. Home cage monitoring

Cannula-implanted rats were fasted for 18 h and then acclimatised to the experimental room for 1 h. Following intra-NI infusion of saline

or quinpirole (6 μ g/0.1 μ l), the rats were individually placed in the Laboratory Animal Behaviour Observation Registration and Analysis System (LABORAS, Metris, Netherlands) home cages, which were similar

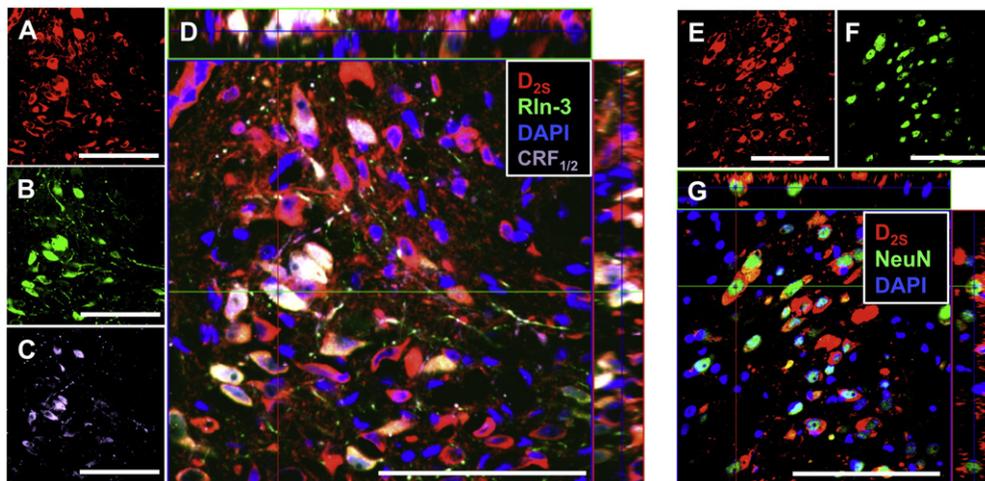


Fig. 5. D_{25} receptor-like immunoreactivity, relaxin-3 and $CRF_{1/2}$ are co-localised in many neurons of the nucleus incertus. Representative confocal images show (A) D_{25} receptor-like immunoreactivity (red), (B) relaxin-3 (green) and (C) $CRF_{1/2}$ (magenta) receptor expression (C). (D) Z-stack confocal image showing the co-localisation of D_{25} receptor-like immunoreactivity, relaxin-3, $CRF_{1/2}$ and DAPI (blue) in the nucleus incertus. D_{25} receptor-like immunoreactivity is also shown to be co-localised with NeuN in the nucleus incertus. (E) D_{25} receptor-like immunoreactivity (red), (F) NeuN expression (green) and (G) Z-stack showing the co-localisation of D_{25} receptor-like and NeuN immunoreactivity in the nucleus incertus. Scale bars: 100 μ m.

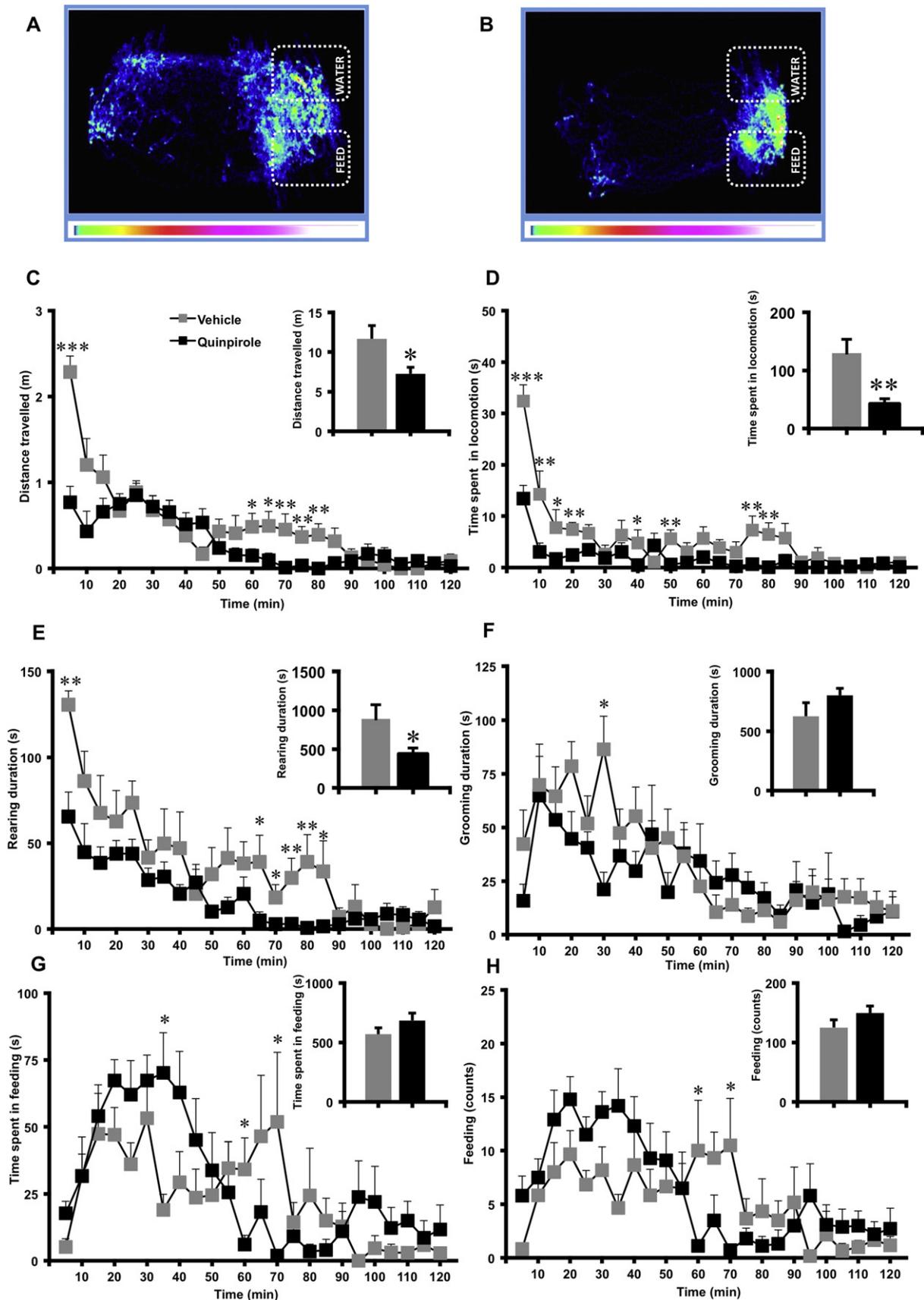


Fig. 6. Intra-NI injection of quinpirole (6 $\mu\text{g}/0.1 \mu\text{l}$) causes locomotor and exploratory deficits in LABORAS home cage activity. Representative images of heat maps of space utilisation showing the behavioural activity of (A) saline and (B) quinpirole treated rats during a 2-hr observation period. Locations of feed and water are schematically represented as rectangular zones (broken lines). Regions with dark colour indicate least visits and those with light colour indicate higher visit frequency. Data points represent the mean of the parameter over the preceding 5 min (C–H). Columns represent mean of values over 2 h and error bars represent SEM (insets in C–H). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to vehicle (sterile isotonic saline) treatment. Repeated measures ANOVA followed by multivariate analysis. $N = 6–10$.

to the regular home cages in which they were housed. Behavioural parameters such as locomotion, velocity, distance travelled, feeding, drinking, rearing and grooming were then continuously assessed for 2 h by the LABORAS software.

2.7.4. Novel environment-induced suppression of feeding

The behavioural assessment was carried out as per previously published reports [7] with certain modifications as mentioned. Cannula-implanted rats, fasted for 18 h, were acclimatised for 1 h in a dimly lit experimental room. Feed pellets were placed in the centre of the circular arena (diameter: 120 cm and height 50 cm, Fig. 1B) with a dark floor and white coloured walls. The observation area was virtually divided into zones, namely feed area, middle area and periphery in the acquisition software (Ethovision Version 8). Rats were individually infused with saline or quinpirole (6 µg/0.1 µl), placed in the periphery of the arena and behaviour was monitored for 10 min. The latency to enter the feed area and the durations of time spent in the feed area and periphery were measured with the Ethovision behavioural tracking and analysis software. The latency to feed was observed manually from the video file by an experimenter blind to the treatment. The quantity of food consumed and the number of faecal pellets left after each trial were also recorded.

2.7.5. Post-mortem analysis

The rats were anaesthetized with an overdose of pentobarbital and 0.1 µl of pontamine sky blue dye was infused into the NI via the guide cannula in the same manner as the drug infusion described above. Subsequently, transcardial perfusion was carried out with isotonic saline followed by 4% paraformaldehyde in phosphate buffer (0.1 M). The brains were subsequently post-fixed in 4% paraformaldehyde at 4 °C overnight and soaked in 30% sucrose at 4 °C for 2 days. Sections (30 µm) were collected serially in PBS with a cryostat (CM 3050, Leica Biosystems, Germany). The sections were then Nissl stained to identify the track of the implanted cannula (Fig. 1C). Rats with incorrect cannula positions were excluded from analysis.

2.7.6. Statistical analysis

All statistical analyses conducted were between-subjects. The behavioural data from home cage monitoring (LABORAS) at various time points was subjected to repeated measures ANOVA across time followed by multivariate analysis to identify the differences at specific time points. The data from other behavioural studies were subjected to unpaired two-tailed Student's *t*-test. IBM SPSS version 20.0 software package was used for the statistical analysis and the level of statistical significance was set at $P < 0.05$. The data are expressed as mean \pm SEM.

3. Results

3.1. Molecular biology

3.1.1. RT-PCR

The expression of D₂ (long and short forms), but not D₃ receptor mRNA was evident in the triplicate NI samples (Fig. 2A).

3.1.2. Western blotting

The nonselective D₂ receptor antibody recognizing the 1–100 amino acids of the human D₂ receptor, produced a band at ~105 kDa present in both the NI sample and the caudate putamen (Fig. 2B). This band is likely to be the D₂ receptor dimer [31]. Using an antibody selective for the short isoform of the D₂ receptor, the strongest band was ~41 kDa, which is the expected weight for the glycosylated D₂₅ receptor. This band is strongly expressed in the NI and caudate putamen (Fig. 2C). The strong relaxin-3 bands (Fig. 2B and C) present in the NI samples validated the position of the NI microdissection.

3.1.3. Immunofluorescence

Immunofluorescence with the non-selective D₂ receptor antibody showed D₂ receptor-like immunoreactivity in the NI (Fig. 2D) and in the caudate putamen region, which was used as the positive control (Fig. 2E). As mentioned earlier, the co-expression studies were conducted with D₂₅ due to the compatibility of the antibodies. Robust D₂₅ receptor-like immunoreactivity was found in the NI. D₂₅ positive neurons of the NI co-expressed CRF_{1/2} receptors (Fig. 3) and relaxin-3 (Fig. 4). Triple immunofluorescence labelling showed the co-expression of D₂₅, CRF_{1/2} and relaxin-3 in several NI neurons (Fig. 5A–D). The D₂₅ positive cells also co-labelled for NeuN (Fig. 5E–G) confirming the expression of D₂₅ receptor-like immunoreactivity in neurons.

3.2. Behaviour

3.2.1. Home cage exploration

Heat maps of space utilisation within the home cage showed hypolocomotion after intra-NI quinpirole treatment (Fig. 6A,B). Repeated measures ANOVA on the data across time showed significant changes in distance travelled ($F_{23,322} = 16.427$, $P < 0.0001$), time spent in locomotion ($F_{23,322} = 21.213$, $P < 0.0001$), time spent in rearing ($F_{23,322} = 14.864$, $P < 0.0001$), time spent in grooming ($F_{23,322} = 4.560$, $P < 0.0001$), time spent in feeding ($F_{23,322} = 4.571$, $P < 0.0001$) and frequency of feeding ($F_{23,322} = 4.216$, $P < 0.0001$). Intra-NI quinpirole (6 µg) significantly reduced distance travelled ($F_{1,14} = 6.962$, $P = 0.019$), duration of time spent in locomotion ($F_{1,14} = 1.286$, $P = 0.001$) and rearing ($F_{1,14} = 6.958$, $P = 0.019$) compared to saline infusion (Fig. 6C–F). Other parameters, grooming ($F_{1,14} = 2.304$, $P = 0.151$, NS), feeding duration ($F_{1,14} = 1.823$, $P = 0.198$, NS), and feeding frequency ($F_{1,14} = 1.829$, $P = 0.198$, NS), were not significantly affected by intra-NI quinpirole administration (Fig. 6C–H).

3.2.2. Novel environment-induced suppression of feeding

Two-tailed unpaired *t*-test on the data revealed that infusion of quinpirole (6 µg/0.1 µl) into the NI suppressed the distance travelled ($P = 0.001$), velocity of the rat movement ($P = 0.001$), frequency of visits to the central feed area ($P = 0.004$), duration of time spent in the feed area ($P = 0.007$), quantity of feed consumed ($P = 0.022$), increased latency to visit feed area ($P = 0.021$), and latency to feed ($P = 0.002$) compared to the saline infusion (Fig. 7A–G). Thigmotaxis (measured by the duration of time spent close to the wall) and defecation were significantly greater in the quinpirole infused rats ($P = 0.0002$ and $P = 0.026$, respectively) than saline infused rats (Fig. 7H and I).

4. Discussion

The presence of D₂ receptors in the NI is demonstrated through RT-PCR, western blotting and immunofluorescence labelling techniques and further confirmed by studying the effect of quinpirole, microinfused into the NI, on behavioural paradigms.

The RT-PCR shows that while the D₂ receptor gene is transcribed into mRNA in the NI, the D₃ receptor gene is not. The D₂ (L + S) receptor antibody produced an ~100 kDa band corresponding to the dimeric form of D₂ receptors [31]. These dimers are unaffected by the reducing and denaturing conditions of the western blot [9]. Notably, similar bands were observed in the caudate putamen, a region highly innervated by dopaminergic projections from the substantia nigra and known to have rich D₂ receptor expression [62,64]. Immunostaining conducted with the same D₂ antibody provides further evidence that D₂ receptor-like immunoreactivity is found specifically at the base of the 4th ventricle corresponding to the NI region. The D₂₅ antibody produced a strong ~41 kDa band in the NI and striatum indicating the presence of the D₂₅ isoform in both of these regions. However,

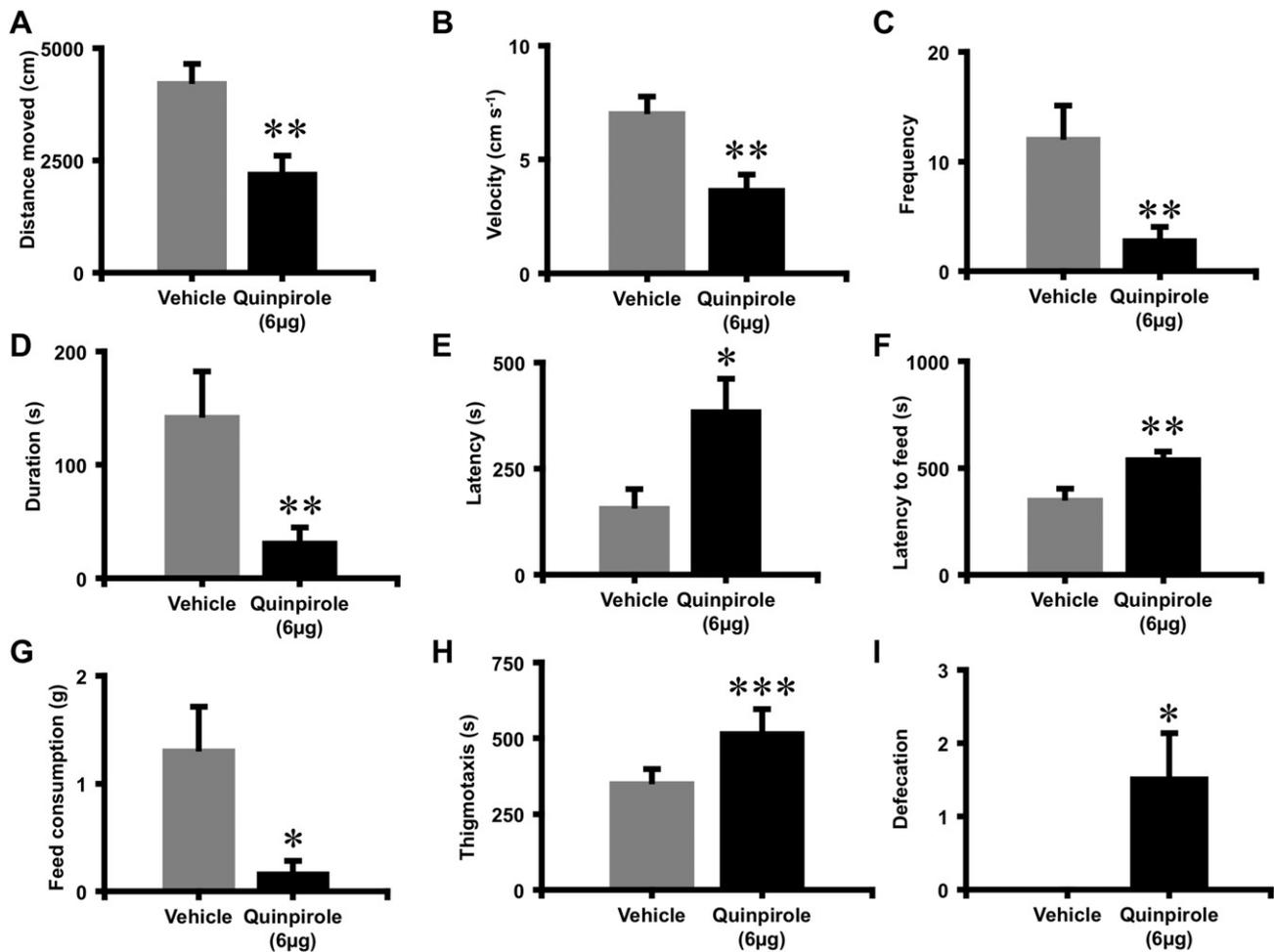


Fig. 7. Intra-NI administration of quinpirole (6 µg/0.1 µl) causes changes in behaviour in the novel environment-induced suppression of feeding paradigm. (A–I) Columns represent mean of values and error bars represent SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to vehicle treatment (sterile isotonic saline). Individual sample two-tailed t -test. $N = 8$ –10.

due to the lack of a specific D_{2L} receptor antibody appropriate for immunostaining/western blotting, the expression of D_{2L} receptors in the NI could not be investigated in the present study.

The immunofluorescence co-expression experiments were carried out with the D_{2S} antibody due to the robust staining quality and compatibility with the CRF_{1/2} and relaxin-3 antibodies. Strong D_{2S} receptor-like immunoreactivity was detected in the cell bodies of the NI neurons comparable to previously reported labelling in other brain regions [32,47]. The co-localisation of the D_{2S} receptors with NeuN validates the expression of these receptors in neuronal cells. CRF₁ receptors are densely expressed in the NI and considered a marker to delineate its cytoarchitecture [16,38,51,56] while relaxin-3 expression is a feature relatively unique to NI neurons [6,39]. The strong co-localisation of D_{2S} receptor-like immunoreactivity with CRF₁ and relaxin-3 confirms its position in the NI. A limitation of the current study is that the precise localisation of D_2 receptors in the NI was only demonstrated by immunostaining. This should be confirmed by in situ with which isoform-specific D_2 receptor mRNA could be precisely localised in the NI. While, the present study investigates the D_2 and D_3 receptors, motivated by the *c-fos* inducing effects of antipsychotics in the NI [50], additional studies aimed at other dopamine receptor subtypes will be of interest.

The source of dopaminergic innervation to the NI is unlikely to be from the midbrain dopaminergic structures, namely the ventral tegmental area and substantia nigra, as they do not significantly project to the NI [21]. Despite poor afferents from the VTA, the NI projects strongly to the VTA providing a possible route for dopaminergic feedback regulation [21]. Alternatively, dopamine input to the NI

could arise from the raphe nuclei which possess significant numbers of dopaminergic cells, specifically the median raphe which projects robustly to the NI [21,44,58]. Similarly, the ventral periaqueductal grey, which sends strong inputs to the NI, contains groups of dopaminergic cells [21,25,37]. To ascertain the source of dopamine innervation to the NI, retrograde tracing combined with immunolabelling of the dopamine transporter, tyrosine hydroxylase and dopamine beta-hydroxylase should be conducted.

Based on previous literature, direct administration of ligands into the NI is an apt method to study the functional properties of the nucleus. Inactivation of the NI by direct lidocaine infusion during the Morris water maze task indicated the structure's involvement in spatial and working memory [42]. Similarly, a study conducted in our lab showed that CRF infusion into the NI suppressed long-term potentiation, reflecting the role of the NI in the stress-driven modulation of working memory [16]. To study the functional relevance of the D_2 receptors in the NI, quinpirole was micro-infused into the NI and the behavioural effects were studied. Among the available D_2 receptor agonists, quinpirole (previously reported as LY 171555) has been relatively well investigated in molecular, autoradiography, electrophysiological and preclinical behavioural studies. It is a full agonist at the D_2 receptor and has been used to study effects of D_2 receptor stimulation in vivo in both rodents and primates by many investigators [1,12,14,18,23,28,33–36,53]. Though quinpirole has five-fold stronger affinity to D_2 receptors than D_3 receptors [19,54], we have made an effort to rule out the D_3 effects by using a dose (6 µg) that was recently reported to be reasonably selective for D_2 receptors over D_3 [5]. Furthermore, the RT-PCR conducted shows negligible expression of the D_3 receptor

gene in the NI. Hence the effects of quinpirole observed are most likely mediated by the D₂ receptors in the NI.

In the present study, intra-NI quinpirole infusion significantly suppressed locomotion-related parameters in the behavioural tests conducted (home-cage monitoring and novel environment-induced suppression of feeding). The home cage monitoring was carried out using LABORAS, which is a sensitive behaviour data acquisition system that utilizes a vibration sensitive platform to record and classify the different kinds of movements exhibited by the rat [49]. Intra-NI infusion of quinpirole significantly suppressed distance travelled, duration of time spent in locomotion and rearing duration, measured during the first 5 min and entire 2 h observation period. During the first 5 min, the behavioural effects observed are likely to be primarily due to the effect of quinpirole on the NI D₂ receptors. Therefore we focused on the immediate locomotion and rearing suppression. Rearing is frequently used to reflect both exploration and emotionality [57]. Based on the reduction in rearing behaviour, it is possible that NI D₂ receptors could play a role in behavioural suppression.

Similar to the homecage monitoring, intra-NI quinpirole at the tested dose suppressed locomotion in the novel environment-induced suppression of feeding paradigm. In this paradigm, quinpirole altered thigmotaxis, latency to reach the feed area, frequency of visits to feed area, duration of time spent in feeding, latency to feed, quantity of feed consumed and defecation. Although these results appear to indicate an anxiogenic effect, the locomotor suppression effect of quinpirole might have increased the latency to reach the feeding area and reduced time spent in the feeding area, confounding the hypophagic effects observed in this study. It is interesting to note that feeding was not affected in the low stress home cage environment despite suppression of locomotion. In fact, quinpirole infusion tended to increase feeding duration ($P = 0.0678$) and frequency ($P = 0.0618$) during the first 5 min. However, in the presumably high stress novel environment, intra-NI quinpirole infusion reduced feeding significantly. It is tempting to speculate that the D₂ receptors in the NI modulate feeding differentially in high and low stress environments, particularly since there is high co-expression of CRF₁ and D₂₅ receptor-like immunoreactivity in the NI.

Previous investigations have shown that administration of quinpirole into various structures in dopaminergic circuitry affected locomotion and rearing [3,8,13,15,60]. Similarly, the results of the present study support the idea of D₂ receptors in the NI being involved in locomotion. The behavioural tests in this investigation were conducted immediately after infusion of a small volume (0.1 µl) to limit diffusion to other areas. Although the possibility of the drug diffusing to neighbouring structures cannot be completely ruled out, the usage of a small volume of a specific agonist (quinpirole) at a concentration previously proven to be effective at D₂ receptors suggests that the hypolocomotor effects observed are very likely to be due to action in the NI. The consistent hypolocomotion effect induced by quinpirole infusion to the NI regardless of the paradigm points to a functional role of these receptors in locomotion. Based on its strong bidirectional connections to the median raphe and interpeduncular nuclei, the NI was predicted to play a role in modulating behavioural activity [21] and the present results lend support to this hypothesis. The theta-modulating effects of the NI [43] could be a possible mechanism through which the behavioural activity levels are altered.

5. Conclusion

The current study reports RT-PCR and western blot evidence for the expression of D₂ receptor mRNA and protein, respectively, in micro-punched tissue from the position of the NI. Additionally, D₂ receptor-like immunoreactivity co-expresses with relaxin-3, CRF₁ and NeuN in the NI. Activation of these D₂ receptors by microinfusion of quinpirole into the NI caused hypolocomotion in the behavioural paradigms tested. To the best of our knowledge, this is the first evidence

for the expression of a catecholamine receptor, the D₂ receptor, in the NI.

Statement of interest

None.

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