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Evidence of D_2 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 D_2 receptors in the NI.
- Infusion of quinpirole (D_2/D_3 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 (CRF_1) 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 (D_2) 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 D_3 receptors. D_2 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 D_2 receptor activation by intra-NI infusion of quinpirole (a D_2/D_3 agonist) were evaluated. Hypolocomotion was observed in home cage monitoring system (LABORAS) and novel environment-induced suppression of feeding behavioural paradigms. Thus the D_2 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, D_2-like immunoreactivity, in the NI.

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: CRF_1 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.

D_2 receptors bind to $G\alpha_{i/o}$ 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 D_2 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]). D_2 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 D_2 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 D_2 receptor antagonism-mediated, these findings prompted us to investigate the presence of D_2 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 D_2 receptors in the NI. Concurrent immunofluorescent labelling of NeuN, CRF_1 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 D_2 receptors, quinpirole (a D_2/D_3 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 (Valabarb) 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: D_2 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); D_3 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-D_2 (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