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Nucleus incertus contributes to an anxiogenic effect of buspirone in rats: Involvement of 5-HT$_{1A}$ receptors

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ABSTRACT

The nucleus incertus (NI), a brainstem structure with diverse anatomical connections, is implicated in anxiety, arousal, hippocampal theta modulation, and stress responses. It expresses a variety of neurotransmitters, neuropeptides and receptors such as 5-HT$_{1A}$, D$_2$ and CRF$_1$ receptors. We hypothesized that the NI may play a role in the neuropharmacology of buspirone, a clinical anxiolytic which is a 5-HT$_{1A}$ receptor partial agonist and a D$_2$ receptor antagonist. Several preclinical studies have reported a biphasic anxiety-modulating effect of buspirone but the precise mechanism and structures underlying this effect are not well-understood. The present study implicates the NI in the anxiogenic effects of a high dose of buspirone. Systemic buspirone (3 mg/kg) induced anxiogenic effects in elevated plus maze, light-dark box and open field exploration paradigms in rats and strongly activated the NI, as reflected by c-Fos expression. This anxiogenic effect was reproduced by direct infusion of buspirone (5 μg) into the NI, but was abolished in NI-CRF-saporin-lesioned rats, indicating that the NI is present in neural circuits driving anxiogenic behaviour. Pharmacological studies with NAD 299, a selective 5-HT$_{1A}$ antagonist, or quinpirole, a D$_2$/D$_3$ agonist, were conducted to examine the receptor system in the NI involved in this anxiogenic effect. Opposing the 5-HT$_{1A}$ agonism but not the D$_2$ antagonism of buspirone in the NI attenuated the anxiogenic effects of systemic buspirone. In conclusion, 5-HT$_{1A}$ receptors in the NI contribute to the anxiogenic effect of an acute high dose of buspirone in rats and may be functionally relevant to physiological anxiety.

1. Introduction

Buspirone is an atypical anxiolytic highly prescribed for generalized anxiety disorder (Apter and Allen, 1999; Flint, 2005; Gale and Millichamp, 2016; Goa and Ward, 1986). It is a 5-HT$_{1A}$ receptor partial agonist and a D$_2$-like receptor antagonist (Loane and Politis, 2012; Newman-Tancredi et al., 1998; Tunnicliff, 1991). Unlike the benzodiazepines, the precise anxiolytic mechanism of buspirone remains unclear with inconsistencies in anxiolytic effects observed in the clinic and animal models of anxiety (Chessick et al., 2006). It is noted from numerous reports that acute treatment of buspirone in rodents causes an anxiolytic effect at a narrow, low dose range that is highly variable across species and anxiety paradigms, while an anxiogenic effect is evident at a wide, high dose range (Bradley et al., 2011; Collinson and Dawson, 1997; de Oliveira Cito Mdo et al., 2012; File and Andrews, 1991; Handley and McBlane, 1993; Hestermann et al., 2014; Inagaki et al., 2010; Lim et al., 2008; Moser, 1989; Paine et al., 2002; Shimada et al., 1995; Soderpalm et al., 1989; Thompson et al., 2015; Varty et al., 2002). The anxiogenic effect of buspirone is a clinical concern as it manifests in the initial acute phase of chronic buspirone treatment regimens (Chignon and Lepine, 1989; Liegghio et al., 1988; Newton et al., 1986; Sinclair et al., 2009).

The complex receptor affinity profile of buspirone may underlie its biphasic pharmacological effects (Tunnicliff, 1991). Buspirone acts as a full agonist at the somatodendritic 5-HT$_{1A}$ autoreceptors

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located at the raphe nuclei reducing firing in the 5-HT neurons and consequentially decreasing the serotonin released in target structures and thus increasing firing. This is thought to be at least partly responsible for its anxiolytic effects (Carli et al., 1989; Hutson et al., 1989; Sharp et al., 1989a, 1989b; Sprouse and Aghajanian, 1987; Tunnicliff, 1991). However, buspirone also acts as an agonist at the post-synaptic 5-HT_{1A} receptors thus reducing neuronal firing. These opposing effects could be responsible for the biphasic anxiety-modulating effects buspirone displays (Andrews et al., 1994; File et al., 1996; Hodges et al., 1987). Some pharmacological interaction studies conducted with WAY 100635, a 5-HT_{1A} antagonist, indicated that the anxiogenic effects and anxiolytic effects of buspirone are mediated by its interaction with the D_2-like and 5-HT_{1A} receptor systems, respectively (Collinson and Dawson, 1997). The above conclusions ignore the considerable affinity and efficacy of WAY-100635 at D_2-like receptors (Chemel et al., 2006; Johansson et al., 1997). Buspirone was recently found to antagonize D_3 and D_4 receptors with higher affinity than D_2 receptors (Bergman et al., 2013). Therefore, we chose to use NAD 299, a high affinity 5-HT_{1A} antagonist that is more selective than WAY-100635 (Johansson et al., 1997) but displays similar potency (Martin et al., 1999), at a dose based on previous central administration studies (Kehr et al., 2010; Ross et al., 1999). The complex receptor affinity profile and the robust reproducible anxiogenic effects of buspirone at an acute high dose level, merit investigation.

The nucleus incertus (NI), a cluster of GABAergic neurons in the brainstem located at the floor of the fourth ventricle, is the principal source of neuropeptide relaxin-3 in mammalian brain (Tanaka et al., 2005) and richly coexpresses CRF_1 (Ma et al., 2013; Tanaka et al., 2005), 5-HT_{1A} (Miyamoto et al., 2008) and D_2 receptors (Kumar et al., 2015). The NI is highly conserved in zebrafish, lower mammals such as rodents and cats, as well as in primates such as monkeys and humans, illustrating its physiological relevance (Ma et al., 2009). The NI/relaxin-3 system is reported to be involved in several functions such as stress responses, arousal, anxiety, fear, memory, cognition, feeding, reward and sleep (for reviews see: Ryan et al., 2011; Smith et al., 2011). The NI is proximally located to the raphe nuclei with robust bidirectional connections to the median raphe in particular, which could explain negative regulation of relaxin-3 expression in the NI by 5-HT (Miyamoto et al., 2008). Exposure to aversive environments and to anxiogenic compounds such as FG 7142, activates relaxin-3 positive NI neurons and dorsal raphe serotonergic neurons simultaneously, suggesting that these interact to modulate physiological anxiety (Lawther et al., 2015). The cognate relaxin-3 receptor, RXFP3, is highly expressed in areas involved in anxiety, thus central administration of an agonist causes anxiolytic effects in the light-dark paradigm and elevated plus maze (Ryan et al., 2013). The NI strongly responds to stressful conditions such as swim stress, exposure to elevated platform, foot shock stress by way of increasing Fos expression as well as relaxin-3 mRNA levels (Li and Sawchenko, 1998; Rajkumar et al., 2016; Tanaka et al., 2005). Interestingly, the NI is more strongly activated by escapable forms of stress that require high behavioural activity rather than those that induce freezing (Goto et al., 2001; Ryan et al., 2011; Timofeeva et al., 2003).The robust bidirectional connections and common target regions of the NI, median raphe and interpeduncular nucleus indicate that they may function as an ascending control of behavioural activation (Goto et al., 2001).

It is generally well-accepted that at low doses, buspirone preferentially acts on the raphe nuclei due to their large (pre-synaptic) 5-HT_{1A} receptor reserve (Carli et al., 1989; Newman-Tancredi et al., 1998). Thus in order to study the role of NI, which expresses post-synaptic 5-HT_{1A} receptors, in the neuropharmacology of buspirone, a high dose is suitable. Buspirone is an anxio-selective drug, and hence it is likely that the structures it modulates are part of the physiological anxiety circuit (Eison and Temple, 1986). Anxiety is characterized by increased arousal and behavioural inhibition, both of which the NI/relaxin-3 system are involved in. The notable expression of receptors and peptides in the NI, that are putative targets for anxiety disorders (especially, 5-HT_{1A}, D_2, CRF and relaxin-3), extensive and strategic connections of this small neuron group to other brain structures that are known to modulate anxiety, arousal and theta activity (especially, median raphe, amygdala, prefrontal cortex, bed nucleus of stria terminalis, medial septum and hippocampus) lead us to hypothesize that the NI may be involved in the anxiogenic effect of a high dose of buspirone.

A high dose of 3 mg/kg intraperitoneal (i.p.) dose of buspirone with a pretreament time of 30 min was selected based on literature (Collinson and Dawson, 1997; Lim et al., 2008) and preliminary studies where 3 doses (0.125 mg/kg, 1 mg/kg and 3 mg/kg) were tested on 3 anxiety paradigms, namely the elevated plus maze, the light-dark box and the open field. Only the highest dose produced a consistent anxiogenic response and was thus selected for further study in context of the NI. In order to determine if this anxiogenic dose activated the NI, c-Fos expression in the NI was quantified. To further examine if the NI played a role in the anxiogenic effect of the high dose of buspirone, the effect of NI lesioning on the drug response was tested. To study the direct anxiety-modulating effects of buspirone on the NI, intra-NI infusion of the drug was conducted. Pharmacological interaction studies with a D_2 agonist (quinpirole)/5-HT_{1A} antagonist (NAD 299) and buspirone were subsequently carried out to determine the receptor system mediating the effects.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (280–350 g), obtained from InVivos Pte Ltd, 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 of the National University of Singapore. Rats were housed in individually ventilated cages, in a temperature controlled room (22–24 °C) with a 12 h light-dark cycle and given *ad libitum* access to food and water.

2.2. Drugs and chemicals

Buspirone hydrochloride (Chemical name: 8-[4-[4-(2-Pyrimidinyl)-1-pipirazinyl]butyl]-8-azaspiro[4,5]decane-7,9-dione hydrochloride; Purity: >99%; Tocris Bioscience, Bristol UK), NAD 299 hydrochloride (Chemical Name: (3R)-3-(Dicyclobutylamino)-8-fluoro-3,4-dihydro-2H-1-benzopyran-5-carboxamide hydrochloride; Purity: >98%; Tocris Bioscience), quinpirole hydrochloride (Chemical Name: (4aR-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline hydrochloride; Purity: >98%; Tocris Bioscience), ketamine (Parnell Manufacturing Pty Ltd., Sydney, Australia), xylazine (Ilium Xylazil, Troy Laboratories Pty Ltd., New South Wales, Australia), enrofloxacin (Baytril 5%, Bayer HealthCare, Leverkusen, Germany), and carprofen (Carprieve, Norbrook Laboratories, Newry, Northern Ireland) were freshly prepared in sterile isotonic (0.9%) saline (B. Braun, Melsungen, Germany) before use. Pentobarbital (Valabarb) was purchased from Jurox Pty Ltd., New South Wales, Australia.

2.3. Surgery

The rats were anaesthetised with a cocktail of ketamine and xylazine and mounted onto a stereotaxic frame. The scalp was

depilated and a midline sagittal incision exposed the bare skull. A burr hole was drilled at AP: -9.7 mm, ML: 0 mm from the bregma to target the NI (Paxinos and Watson, 2007). These coordinates have been used to successfully target the NI in previous studies conducted at our laboratory (Farooq et al., 2013, 2016; Kumar et al., 2015; Lee et al., 2014; Rajkumar et al., 2016).

NI-CRF-saporin lesioning was recently established by our laboratory and we showed that the CRF-saporin lesioning selectively ablates CRF expressing cells resulting in significantly lowered expression of CRF_1 receptors, relaxin-3, GAD65 and 5-HT_{1A} receptors (Lee et al., 2014). Serotonin expression in the neighboring dorsal raphe and CRF_1 expression in the adjacent dorsal tegmentum and locus coeruleus remained intact showing that the lesioning technique only targets CRF_1 expressing cells at the NI coordinates (Lee et al., 2014). The surgery was conducted as described (Lee et al., 2014). Briefly, bilateral injections of 0.2 μl/site, made 7.5 mm ventral to the surface of the skull, delivered 86 ng/site of CRF-saporin or blank saporin (Advanced targeting Systems, San Diego, California, USA) over 5 min to cause NI or sham-lesioning, respectively. The needle was left in place for 5 more minutes before withdrawal. The scalp was sutured, and postoperative care with subcutaneous injections of antibiotic (Baytril) and analgesic (Carprofen) was conducted daily for the first 5 days. The rats were allowed a total rehabilitation period of 14 days before any experiments were carried out.

Cannula implantation surgery targeting the NI was carried out as previously described (Farooq et al., 2013; Kumar et al., 2015; Rajkumar et al., 2016). Briefly, a guide cannula (PlasticsOne, Roanoke, Virginia, 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 rats were allowed to rehabilitate for 1 week with the same postoperative care schedule as described above.

2.4. Behavioural testing

Rats were acclimatized to the behaviour room for at least 1 h before the experiments. For systemic buspirone experiments, the rats were weighed and intraperitoneally injected with buspirone (0.125 mg/kg, 1 mg/kg or 3 mg/kg dose) or vehicle (isotonic sterile saline) in a volume of 1.0 ml/kg. Behavioural assessment began after the pretreatment time of 30 min.

For the intra-NI experiments, buspirone (2.5 μg or 5 μg); NAD 299 (0.5 μg); quinpirole (1 μg) or vehicle (sterile isotonic saline) were infused in a 0.1 μl volume. The doses of NAD 299 (Kehr et al., 2010) and quinpirole are based on previous studies conducted where micro-infusion of these drugs produced an experimental effect indicating an efficacious dose (Amato et al., 2012; Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Wan and Swerdlow, 1993). The drug administration was conducted by gently restraining the rat and inserting an infusion cannula (PlasticsOne, USA), which had a 1 mm projection from the guide cannula connected to a plastic tubing. A 0.1 μl volume of the solution was infused using a syringe (SGE Analytical Science, Trajan Scientific Pty Ltd, Melbourne, Australia) connected to the plastic tubing over a period of 30 s, after which the infusion cannula was left in place for 1 min before re-capping and placing the rat in the exploration arenas. Behavioural testing began immediately.

Behavioural apparatus (as depicted in Fig. 1B–E) was cleaned with 70% ethanol between the trials. All the behavioural observations were video recorded and analyzed using a video tracking system (Ethovision, version XT10, Noldus, Wageningen, Netherlands) except for the home-cage monitoring which was monitored through the laboratory animal behaviour observation registration and analysis system (LABORAS, Metris B.V., Hoofddorp, Netherlands). Elevated plus maze and light-dark box exploration lasted for 5 min while the open field paradigm was a 10 min trial. The elevated plus maze and open field test were conducted in dim room lighting while in the light-dark box the light area was illuminated at 440 lux. The LABORAS home-cage monitoring was conducted in normal lighting and behavioural parameters were continuously assessed for 2 h by the LABORAS software. For the elevated plus maze, light-dark exploration test and open field paradigm, the rat was placed in the central square facing an open arm, against the wall in the light area and against the wall in the periphery of the maze, respectively.

Motor coordination and sedation were assessed using an accelerating rotarod (4–40 rpm over 5 min). The rats were given 2 training sessions and the following day they were placed on the rotarod (Ugo Basile S. R. L., Monvalle VA, Italy) prior to dosing and either directly after dosing for the intra-NI infusion experiments, or after a pretreatment time of 30 min for the systemic buspirone injection experiments (Hughes et al., 2013; Walker et al., 2001).

2.5. Post-mortem tissue processing

Cannula implanted rats were anaesthetised 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 with isotonic saline followed by 4% paraformaldehyde in phosphate buffer (0.1 M). The brains were post-fixed in 4% paraformaldehyde at 4 °C followed by 30% sucrose. 30 μm sections were collected on glass slides in a cryostat (CM 3050, Leica Microsystems, Wetzlar, Germany) and allowed to air-dry before being studied under a light microscope (BX-51, Olympus, Tokyo, Japan) to determine if the tracks were in the NI. Rats with incorrect cannula positions were excluded from analysis. Representative sections were Nissl stained and imaged under the light microscope for illustration purposes (Fig. 1A). NI CRF-saporin lesioning was confirmed with post-mortem Western blotting conducted as per Lee et al., 2014.

2.6. c-Fos immunofluorescence and cell counting

Briefly, naive rats housed in pairs were allowed to acclimatize for at least 3 days in the vivarium before being transferred to the experimental room (20–23 °C, normal room lighting). The rats received intraperitoneal injections of either buspirone (3 mg/kg) or vehicle in a volume of 1.0 ml/kg and after a pretreatment time of 2 h, the rats were anesthetized 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. Free-floating sections (30 μm) were collected serially in PBS in a cryostat (CM 3050, Leica Microsystems, Germany), blocked in 5% goat serum in 1xPBS with Triton X; incubated in the primary antibodies [anti-c-Fos (1:600; Calbiochem, Merck Millipore, Billerica, Massachusetts, USA); anti-CRF-RI/II (1:400; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA))] diluted in blocking solution overnight at 4 °C; followed by incubation in solutions containing appropriate Alexa Fluor secondary antibodies (Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for an hour at room temperature in the dark. The sections were rinsed, plated, covered with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and imaged with fluorescence microscope (BX-51, Olympus, Japan). The sections were gently shaken during all incubation periods including the rinses. The immunostaining procedures were conducted at 20–23 °C under normal room lighting.

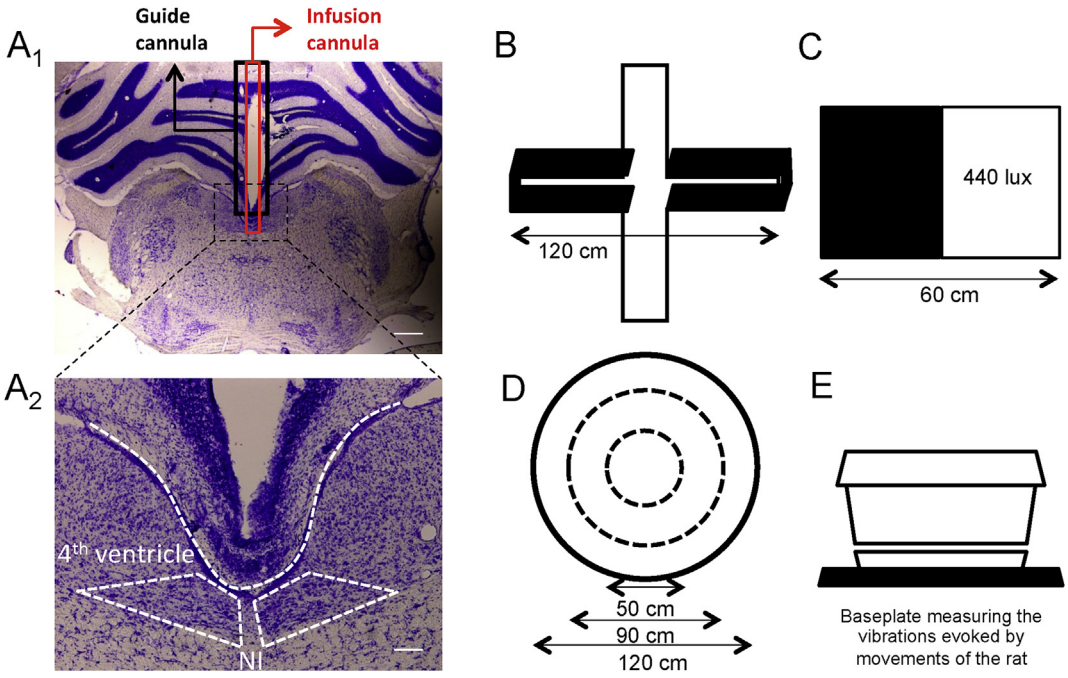

Fig. 1. (A) Representative images showing the cannula position in the nucleus incertus at 2x (A1; scale bar represents 1 mm) and 10x (A2, scale bar represents 200 μm) magnification. Schematic diagram of the behavioural apparatus, the elevated plus maze (B), the light-dark box (C), the open field (D), the LABORAS home-cage monitoring system (E).

For the c-Fos study, the NI was sampled regularly every 60 μm and at least 6 sections were stained per rat. The images were analyzed with ImageJ (NIH, Maryland, USA). Based on the distribution of CRF positive cells, a region of interest was created bilaterally on either side of the fourth ventricle. The c-Fos positive cells within this region were quantified. c-Fos positive cells per unit area were calculated for each rat and averaged for each treatment group. Similar c-Fos immunofluorescence and cell counting techniques were established at our lab and conducted in several studies (Rajkumar et al., 2013, 2016; Verma et al., 2007; Verma et al., 2006).

2.7. Statistical analysis

All statistical analysis conducted, except for the rotarod assay, were between-subjects. If there were more than 2 experimental groups, one-way ANOVA with post hoc Bonferroni's test was conducted. All the behavioural and c-Fos data with 2 experimental groups were analyzed with unpaired Student's *t*-tests except for the rotarod assay, for which a paired *t*-test was conducted. All comparisons were made against the vehicle treatments. 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. GraphPad Prism Version 5.00 (GraphPad Prism, La Jolla, California, USA) was used for the statistical analysis and the level of statistical significance was set at $p < 0.05$. The data are expressed as mean ± SEM.

3. Results

3.1. Effect of systemic buspirone on behavioural paradigms and c-Fos expression

Buspirone (1 mg/kg and 3 mg/kg) induced an anxiogenic effect on the elevated plus maze. One-way ANOVA on the data from the elevated plus maze showed that systemic buspirone significantly influenced percentage time spent in the open arms of the elevated plus maze [$F_{3, 53} = 8.754$, $P < 0.0001$]. *Post hoc* Bonferroni's tests showed that buspirone at 1 mg/kg ($P < 0.05$) and 3 mg/kg ($P < 0.001$) significantly reduced percentage time spent in open arms compared to the vehicle (Fig. 2A). There was no significant difference between treatment groups in the percentage of open arm entries [$F_{3, 53} = 1.851$, $P = 0.149$]. One-way ANOVA showed that systemic buspirone significantly influenced total number of closed arm entries in the elevated plus maze [$F_{3, 53} = 3.624$, $P < 0.05$]. *Post hoc* Bonferroni's tests showed that there was no significant difference between any of the doses of buspirone compared to the vehicle (Fig. 2B) indicating that the buspirone treatment did not affect locomotion.

In the light-dark box test, the highest dose of buspirone induced anxiety. One-way ANOVA showed that systemic buspirone significantly influenced duration in the light area of the light-dark box [$F_{3, 51} = 8.293$, $P < 0.0001$]. *Post hoc* Bonferroni's test showed that buspirone at 3 mg/kg ($P < 0.001$) significantly reduces percentage time spent in the light area compared to the vehicle treatment (Fig. 2C). One-way ANOVA showed that systemic buspirone influenced the number of transitions made in the light-dark box [$F_{3, 51} = 8.427$, $P < 0.0001$]. *Post hoc* Bonferroni's tests showed that there was no significant difference between any of the doses of buspirone compared to the vehicle (Fig. 2D).

The highest dose of buspirone tended to induce an anxiogenic response on the open field test based on the anxiety indices but also reduced distance travelled, indicating a possible suppression of locomotion effect. One-way ANOVA showed that buspirone (3 mg/kg) dose significantly influenced time spent in the centre [$F_{3, 52} = 4.141$, $P < 0.05$], frequencies of entries to centre [$F_{3, 52} = 5.864$, $P = 0.0016$] and distance travelled [$F_{3, 52} = 17.3$, $P < 0.0001$] in the open field (Fig. 2E–G). *Post hoc* Bonferroni's test showed that buspirone (3 mg/kg) significantly ($P < 0.05$) reduced the time spent in the centre (Fig. 2E), buspirone (1 and 3 mg/kg) significantly

reduced the frequency of entries to the centre ($P < 0.05$ and 0.01, respectively, Fig. 2F), reduced the distance travelled ($P < 0.01$ and 0.001, respectively, Fig. 2G) compared to the vehicle treatment.

Based on the anxiety paradigms conducted above, only the highest dose of 3 mg/kg induced a consistent anxiogenic effect. Therefore, this dose was tested on the subsequent experiments.

In the rotarod test, there was no significant ($P = 0.428$, two-tailed paired *t*-test) difference in the pre- and post-buspirone fall latency (Fig. 2H). This shows that buspirone treatment at the highest dose did not affect motor coordination or sedation thus lending credibility to its anxiogenic effects.

The number of c-Fos positive cells per unit area with the total area being demarcated by CRF_1 expressing cells were significantly increased ($P < 0.001$) in buspirone (3 mg/kg) treated rats compared to vehicle-treated rats (Fig. 3C) indicating that the NI is activated and may play a role in the anxiogenic effect induced by this dose of buspirone.

In order to study the effect of buspirone treatment on NI-lesioned rats, the baseline effect of lesioning the NI on anxiety was examined. In the elevated plus maze, the NI-lesioned rats spent a shorter percentage of time in the open arms ($P < 0.001$) and had a lower percentage of open arm entries ($P < 0.05$) compared to the sham-lesioned animals (Fig. 4A–B). There was no significant difference in the total closed arm entries ($P = 0.871$; Fig. 4C). In the study examining the effect of buspirone (3 mg/kg) on sham-lesioned and NI-lesioned rats, the percentage time spent in the open arms of the elevated plus maze ($P < 0.01$) and percentage of open arm entries ($P < 0.05$) were significantly increased in the NI-lesioned rats compared to the sham-lesioned rats (Fig. 4D–E). There was no significant difference in the total closed arm entries ($P = 0.826$; Fig. 4F). Selective lesioning of the NI by CRF-saporin attenuates the anxiogenic effect of buspirone thus drawing attention to the possible causal role of the NI in the robust elevation of anxiety by a high dose of buspirone.

3.2. Effect of intra-NI buspirone on behavioural paradigms

Buspirone was directly infused into the NI at 2 doses, 2.5 μg and 5 μg, and its effect of the anxiety paradigms was measured. Two-tailed unpaired *t*-tests were conducted on all the following data unless otherwise specified. Buspirone (2.5 μg) infused into the NI did not significantly affect the percentage time spent in the open arms ($P = 0.833$) and the percentage of open arm entries ($P = 0.946$; Fig. S1).

Buspirone (5 μg) caused anxiogenic effects in rats when tested on the elevated plus maze and open field exploration paradigms. In the elevated plus maze, the percentage time spent in open arms ($P < 0.01$, Fig. 5A) was significantly reduced in the buspirone-treated group compared to the vehicle-treated group. However, there was no significant difference between treatment groups in the percentage of open arm entries ($P = 0.928$, Fig. 5B) and total closed arm entries ($P = 0.358$, Fig. S2) indicating that the locomotion of the buspirone-treated rats was unaffected. In the open field paradigm, the time spent in the centre ($P < 0.05$, Fig. 5C), frequency to enter the centre ($P < 0.01$, Fig. 5D) and distance travelled ($P < 0.05$, Fig. 5F) were significantly reduced and, the latency to enter the centre ($P < 0.01$, Fig. 5E) and time spent in periphery ($P < 0.05$, Fig. S3A) were significantly increased in the buspirone (5 μg)-treated group compared to the vehicle-treated group. The frequency of entries to middle area was significantly reduced in buspirone (5 μg) treated rats compared to vehicle-treated rats ($P < 0.05$; Fig. S3B).

In the home-cage activity (LABORAS) study, repeated measures ANOVA on the data across time showed significant changes in distance travelled ($F_{23,230} = 16.67$, $P < 0.0001$), time spent in locomotion ($F_{23,230} = 15.63$, $P < 0.0001$), time spent in immobility ($F_{23,\ 230} = 5.75$, $P < 0.0001$), time spent in rearing ($F_{23,230} = 17.02$, $P < 0.0001$), time spent in feeding ($F_{23,230} = 5.75$, $P < 0.0001$) and frequency of feeding ($F_{23,230} = 1.753$, $P < 0.05$) (Fig. 6A–E, H). Time spent in grooming ($F_{23,230} = 1.131$, $P = 0.312$) and drinking ($F_{23,\ 230} = 1.038$, $P = 0.418$) were not significantly altered over time (Fig. 6F–G). Intra-NI buspirone (5 μg) significantly increased the time spent in immobility ($F_{1,10} = 7.497$, $P < 0.05$) and time spent in feeding ($F_{1,10} = 7.497$, $P < 0.05$) compared to saline infusion (Fig. 6C–D). Other parameters, distance travelled ($F_{1,10} = 2.268$, $P = 0.163$), time spent in locomotion ($F_{1,10} = 2.485$, $P = 0.146$), feeding frequency ($F_{1,10} = 1.316$, $P = 0.278$), time spent in drinking ($F_{1,\ 10} = 1.814$, $P = 0.2078$), time spent rearing ($F_{1,10} = 3.722$, $P = 0.0825$) and time spent grooming ($F_{1,10} = 0.8075$, $P = 0.39$) were not significantly affected by intra-NI buspirone administration (Fig. 6A–B,E–H). Two-tailed unpaired *t*-tests conducted on the 2 h data indicated that the time spent in immobility ($P < 0.05$) was significantly increased in buspirone-treated rats (Fig. 6C. Inset graph). All the other parameters: frequency of immobility (counts) ($P = 0.743$), distance travelled ($P = 0.163$), time spent in locomotion ($P = 0.14601$), time spent in rearing ($P = 0.0825$), frequency of rearing ($P = 0.141$), time spent in grooming ($P = 0.390$), frequency of grooming ($P = 0.235$), time spent in feeding ($P = 0.290$), frequency of feeding ($P = 0.359$), time spent in drinking ($P = 0.208$) and frequency of drinking ($P = 0.216$) failed to attain statistical significance (some of these are illustrated in inset graphs of Fig. 6). Despite the reduction in locomotion observed in the open field, in a home-cage enviroment, the time spent in locomotion and distance travelled were unaffected by intra-NI buspirone (5 μg) thus indicating that the latter does not affect locomotion per se.

3.3. Pharmacological interaction studies

The silent dose study for NAD 299 (0.5 μg) revealed that at this dose the anxiety and locomotion parameters were unaffected. Two-tailed unpaired *t*-test were conducted on all the following data. Intra-NI administration of NAD 299 (0.5 μg) did not affect the percentage time spent in the open arms of the elevated plus maze ($P = 0.659$, Fig. S4A) and the percentage of open arm entries ($P = 0.234$; data not shown). There were no significant differences in the time spent ($P = 0.354$) and frequency of entries ($P = 0.279$) in the centre and middle area; and the distance travelled ($P = 0.545$) in the open field (Fig. S4B–D) test. These data confirm that NAD 299 (0.5 μg) did not alter baseline behaviour and therefore could be utilised for the subsequent experiments.

In a cohort of rats treated with systemic buspirone (3 mg/kg), the percentage time spent in open arms was significantly ($P < 0.05$) increased in the intra-NI NAD 299 (0.5 μg)-treated rats compared to the vehicle-treated rats in the elevated plus maze (Fig. 7A). There was no significant difference between the treatment groups in the percentage of open arm entries ($P = 0.191$) (data not shown) and the total closed arm entries ($P = 0.361$) (Fig. 7B). In the cohort of rats treated with systemic buspirone (3 mg/kg), the total time spent in the centre and middle area ($P < 0.05$) and frequency of entries to centre and middle area ($P < 0.05$) were significantly increased in the intra-NI NAD 299 (0.5 μg) treated rats compared to the vehicle-treated rats in the open field test (Fig. 7C–D). There was no significant difference between the treatment groups in the latency to enter centre ($P = 0.984$) (data not shown) and distance travelled ($P = 0.520$) (Fig. 7E).

In the pharmacological interaction study with buspirone (3 mg/kg, i.p.) and quinpirole (1 μg, intra-NI infusion), the percentage time spent in the open arms ($P = 0.344$) and the percentage of open arm entries ($P = 0.676$) in the elevated plus maze were unaffected (Fig. S5A–B). Similarly, there was no significant difference in the

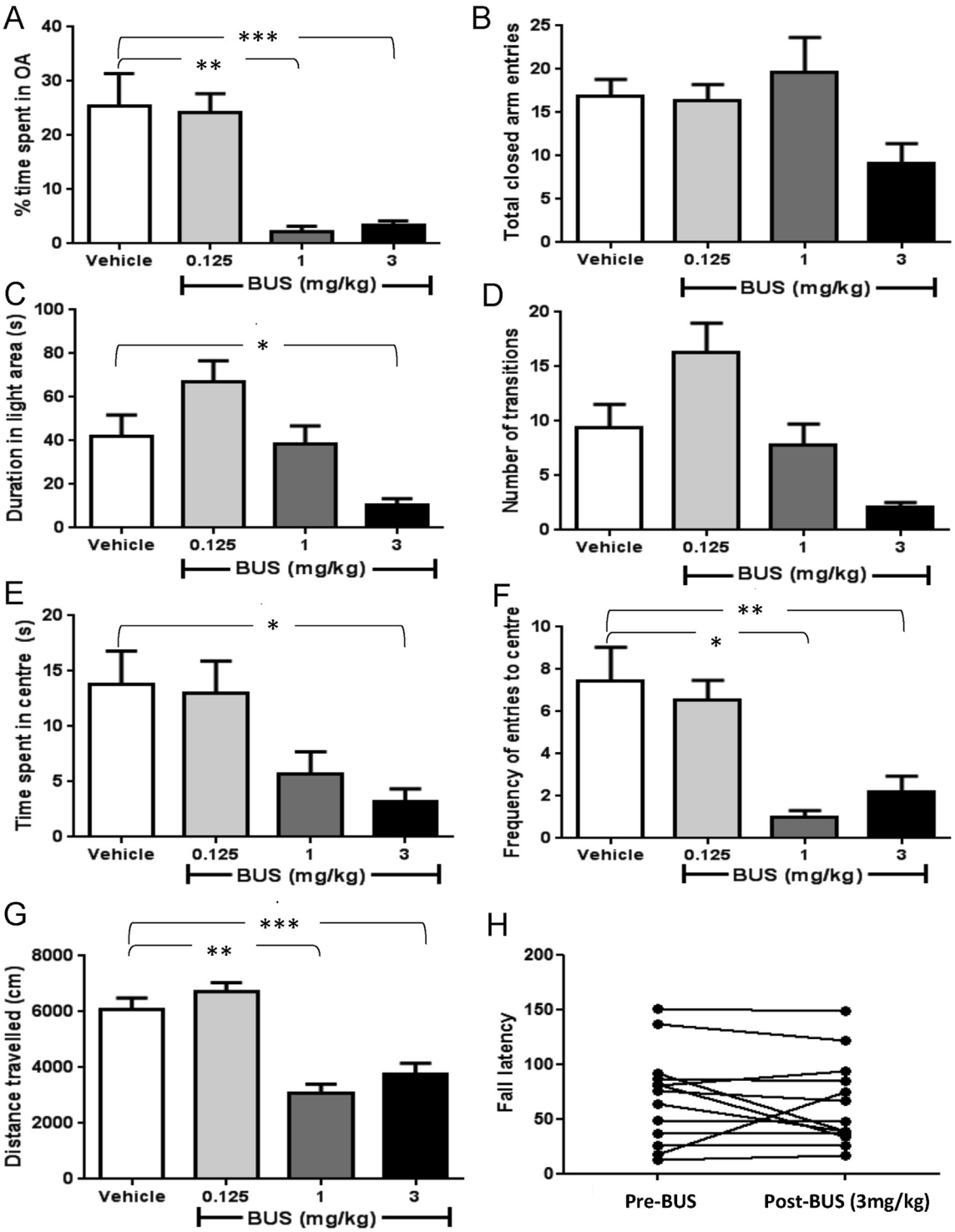

Fig. 2. Effect of systemic buspirone (BUS) on the EPM, LDB, open field and rotarod assay. Different doses (0.125 mg/kg; 1 mg/kg; 3 mg/kg) of buspirone were tested in anxiety paradigms (A–E) while only the 3 mg/kg dose was tested in the rotarod (H). (A) Percentage time spent in the open arms (OA) in the EPM. n = 16 (vehicle) n = 18 (buspirone 0.125 mg/kg) n = 5 (buspirone 1 mg/kg) n = 18 (buspirone 3 mg/kg). (B) Total entries into the closed arms in the EPM. n = 16 (vehicle) n = 18 (buspirone 0.125 mg/kg) n = 5 (buspirone 1 mg/kg) n = 18 (buspirone 3 mg/kg). (C) Duration in the bright area of the light-dark box. n = 17 (vehicle) n = 17 (buspirone 0.125 mg/kg) n = 5 (buspirone 1 mg/kg) n = 16 (buspirone 3 mg/kg). (D) Number of transitions in the light-dark box. n = 17 (vehicle) n = 17 (buspirone 0.125 mg/kg) n = 5 (buspirone 1 mg/kg) n = 16 (buspirone 3 mg/kg). (E–G) Time spent in the centre, the frequency of entries to the centre and the distance travelled in the open field, respectively. n = 16 (vehicle) n = 18 (buspirone 0.125 mg/kg) n = 5 (buspirone 1 mg/kg) n = 17 (buspirone 3 mg/kg). (H) Fall latency before and after buspirone (BUS; 3 mg/kg) treatment in the rotarod assay. n = 13. For (A–G) One-way ANOVA followed by post hoc Bonferroni's test. For (H) paired *t*-test. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$. Data represented as mean ± SEM.

time spent in the centre (P = 0.612), latency to enter the centre (P = 0.580) and frequency of entries in the centre (P = 0.625) and distance travelled (P = 0.187) in the open field between the treatment groups (Fig. S5C–F).

Thus intra-NI infusion of NAD 299 (0.5 μg) but not quinpirole (1 μg), attenuated the anxiogenic effect of buspirone (3 mg/kg) on

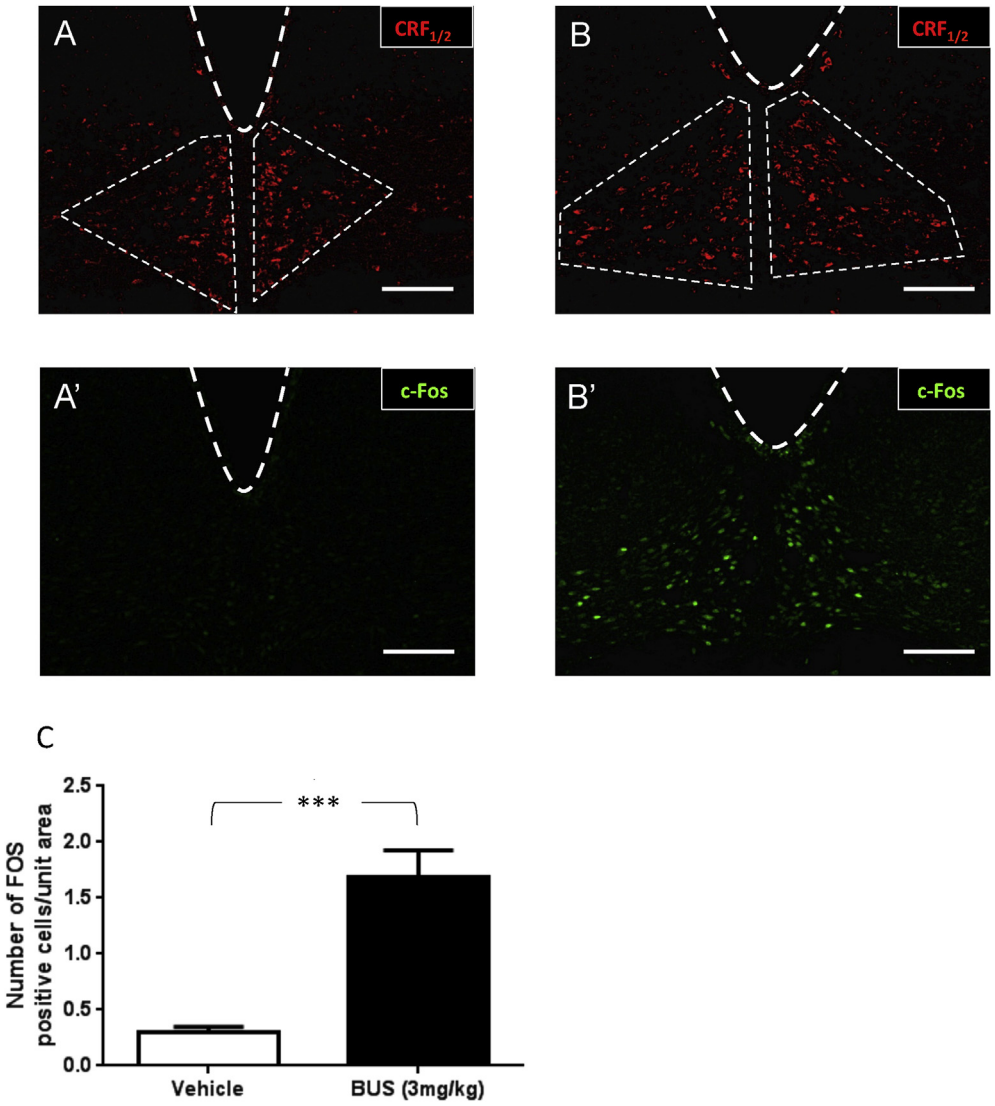

Fig. 3. Effect of buspirone (BUS, 3 mg/kg) on Fos protein levels in the NI cells. (A–B) Representative fluorescence images of vehicle treatment (A) and buspirone treatment (B). The $CRF_{1/2}$ expressing cells demarcate the region of interest (A, B). Buspirone (3 mg/kg) treatment increased the number of cells expressing Fos protein (A′, B′). The dotted lines indicate the fourth ventricle that serves as a landmark. Scale bars: 200 μm. (C) Number of Fos positive cells per unit area in the NI. n = 6 (Vehicle) n = 6 (Buspirone). Students' *t*-test ***p < 0.001. Data represented as mean ± SEM.

the elevated plus maze and open field paradigm.

4. Discussion

In the present study, systemic buspirone (3 mg/kg) produced significant anxiogenic effects on all three anxiety paradigms tested (elevated plus maze, light-dark and open field exploration). This supports a wealth of preclinical literature on buspirone that reveals an anxiogenic effect after an acute high dose (Belzung et al., 2001; Collinson and Dawson, 1997; Dekeyne et al., 2000; Hascoet et al., 2000; Hestermann et al., 2014; Leong et al., 2012; Moser, 1989). Although the rats treated with buspirone (3 mg/kg) travelled a shorter distance in the open field, the locomotor indices in the elevated plus maze (total closed arm entries) and rotarod performance were unaffected. The rotarod assay tests for ataxia, which could result from impairments in gait, posture, motor coordination, motor learning, sedation and intoxication and is frequently used to study the sedative effects of anxiolytics such as benzodiazepines (Jones and Roberts, 1968; Martin et al., 1993; Monville et al., 2006). Further, only a marginal decrease in locomotion was previously reported with 3 mg/kg of buspirone (Leveleki et al., 2006). However, there are other methods that could detect subtle ambulatory changes such as gait analysis which could be used in future studies. The reduction in locomotion in the anxiety paradigms could be a result of fear or anxiety which can induce behavioural inhibition, reducing exploration and inhibiting ongoing activity (Gray and McNaughton, 2003; Weiss et al., 1998).

Based on the aforementioned experiments, only the highest (3 mg/kg) dose of buspirone produced a marked anxiogenic effect consistent across the three paradigms and this dose was therefore selected for the subsequent experiments. Systemic administration of the selected anxiogenic dose of buspirone was found to significantly increase c-Fos expression in the NI neurons within the region of interest based on the CRF_1 receptor expression, a marker frequently used to delineate the cytoarchitecture of the NI (Rajkumar et al., 2013, 2016; Tanaka et al., 2005). Increased c-Fos expression is taken as a measure of NI activation as basal c-Fos expression in the NI is minimal (Cullinan et al., 1995; Herdegen

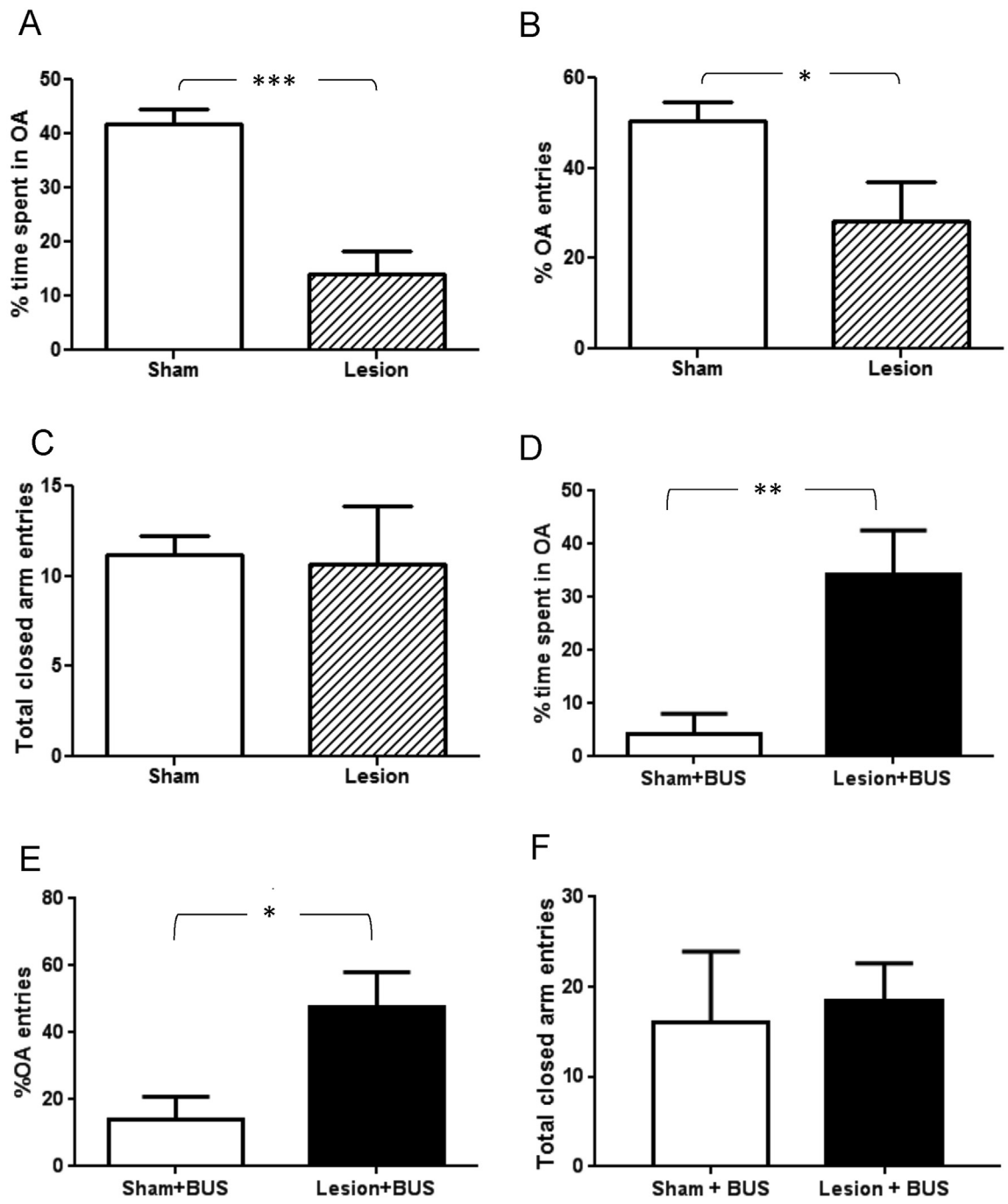

Fig. 4. (A–C) Effect of NI lesioning on rats tested in the EPM. (A) Percentage time spent in the open arms. (B) Percentage of entries into the open arms. (C) Total entries into the closed arms. n = 9 (Sham-lesioned) n = 9 (NI-lesioned). (D–F) Effect of systemic buspirone (BUS; 3 mg/kg) on sham- and NI-lesioned rats tested on the EPM. (D) Percentage time spent in the open arms. (E) Percentage of entries into the open arms. (F) Total entries into the closed arms. n = 10 (Sham + buspirone) n = 6 (NI-Lesion + buspirone). Unpaired *t*-test. $^{*}p < 0.05$ $^{**}p < 0.01$. $^{***}p < 0.0001$ Data represented as mean ± SEM.

et al., 1995; Herrera and Robertson, 1996) as is evident in the vehicle-treated rats in this study. While D_2 receptor activation usually induces Fos production, activation of 5-HT_{1A} receptors differentially induces c-Fos expression depending upon the brain region (Missale et al., 1998; Peroutka, 1988). One study shows that 5-HT_{1A} activation in the frontal cortex, hippocampus and cerebellum increases c-Fos expression albeit at different time points, however, c-Fos expression in the striatum is unaltered, despite all of these regions having post-synaptic 5-HT_{1A} receptors (since they do not express 5-HT) (Tilakaratne and Friedman, 1996). Similarly, several studies show that 5-HT_{1A} receptor activation stimulates adenylate cyclase activity, thus increasing cAMP levels and in turn c-Fos expression particularly in the hippocampus (Buritova et al., 2003; Cadogan et al., 1994; Lucas et al., 1993; Markstein et al., 1986; Sassone-Corsi et al., 1988; Sijbesma et al., 1991). But on the other hand, some studies indicate a negative correlation of activation of 5-HT_{1A} and adenylate cyclase activity (Bockaert et al., 1987; De Vivo and Maayani, 1986; Hoyer and Schoeffter, 1991; Peroutka, 1988; Weiss et al., 1986). Pre-synaptic 5-HT_{1A} receptors in the raphe nuclei when activated by 8-OH-DPAT attenuated the c-Fos

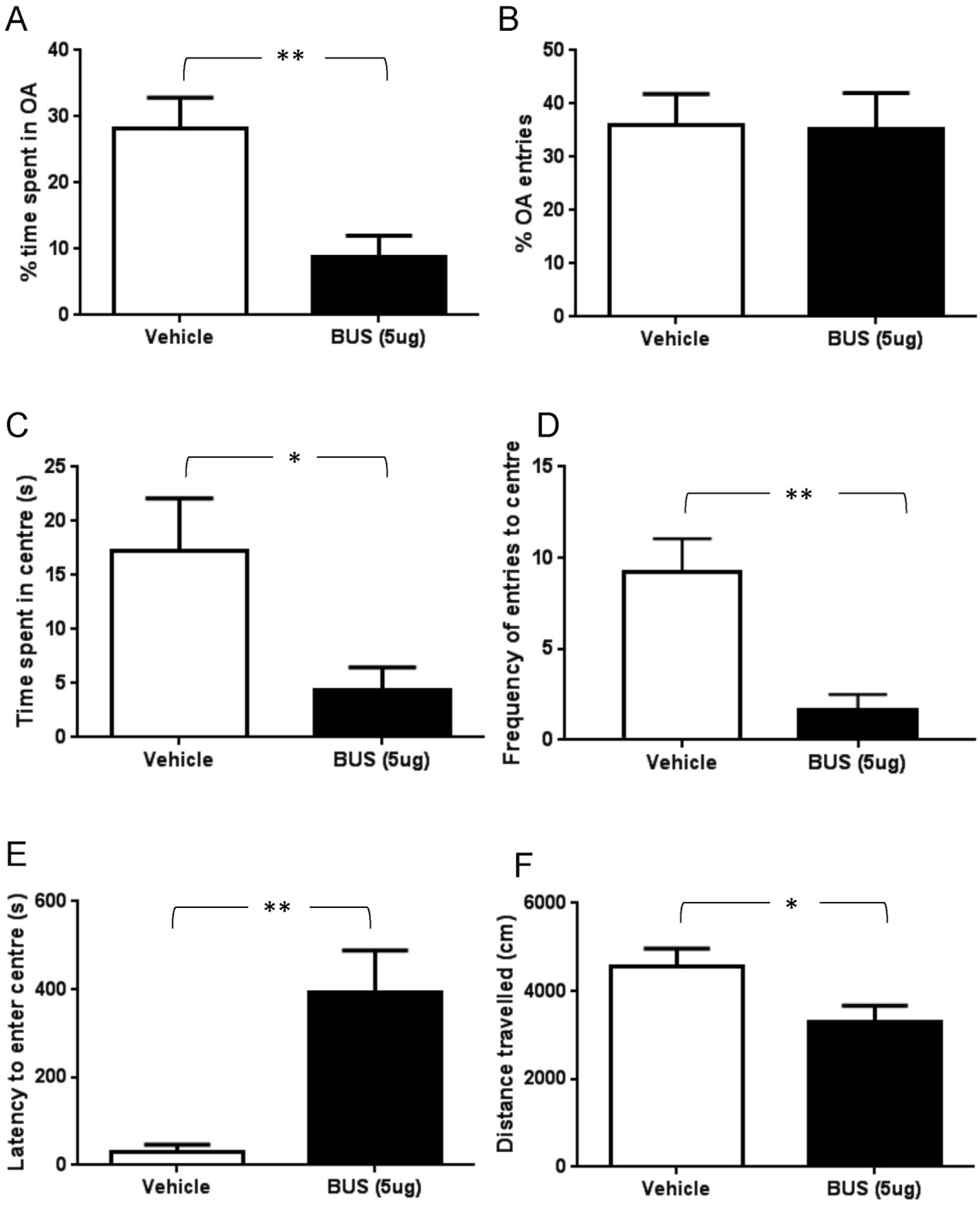

Fig. 5. Effect of intra-NI buspirone (BUS; 5 μg) on the EPM and open field. (A) Percentage time spent in the open arms in the EPM. (B) Percentage of open arm entries in the EPM. n = 6 (Vehicle) n = 7 (Buspirone). (C–F) Duration in the centre, the frequency of entries to the centre, the latency to enter the centre and the distance travelled in the open field, respectively. n = 5 (Vehicle) n = 6 (Buspirone). Unpaired *t*-test *p < 0.05 **p < 0.01. Data represented as mean ± SEM.

increase induced by immobilization stress (Rioja et al., 2006). It may be speculated that the c-Fos induced in the NI by buspirone, a partial agonist at 5-HT_{1A} receptors is via activation of the 5-HT_{1A} receptors in the NI, similar to the hippocampus, where activation of the post-synaptic 5-HT_{1A} receptors increases c-Fos (Tilakaratne and Friedman, 1996).

However, the role of D_2 receptors cannot be completely ruled-out since it has been previously demonstrated in our laboratory that systemic treatment with antipsychotics (which are D_2 antagonists) induces c-Fos in the NI (Rajkumar et al., 2013). These effects may also be secondary to buspirone-mediated modulation of other brain areas, such as the raphe nuclei that project strongly to the NI. Indeed other studies have shown that the modulation of the HPA axis by 5-HT_{1A} agonists trans-synaptically altered c-Fos expression in other brain regions (Compaan et al., 1996). It would be of interest to study the effect of an anxiolytic dose of buspirone on c-Fos expression in the NI as it would allow us to examine if the NI plays in both opposing anxiety-modulating responses or just the

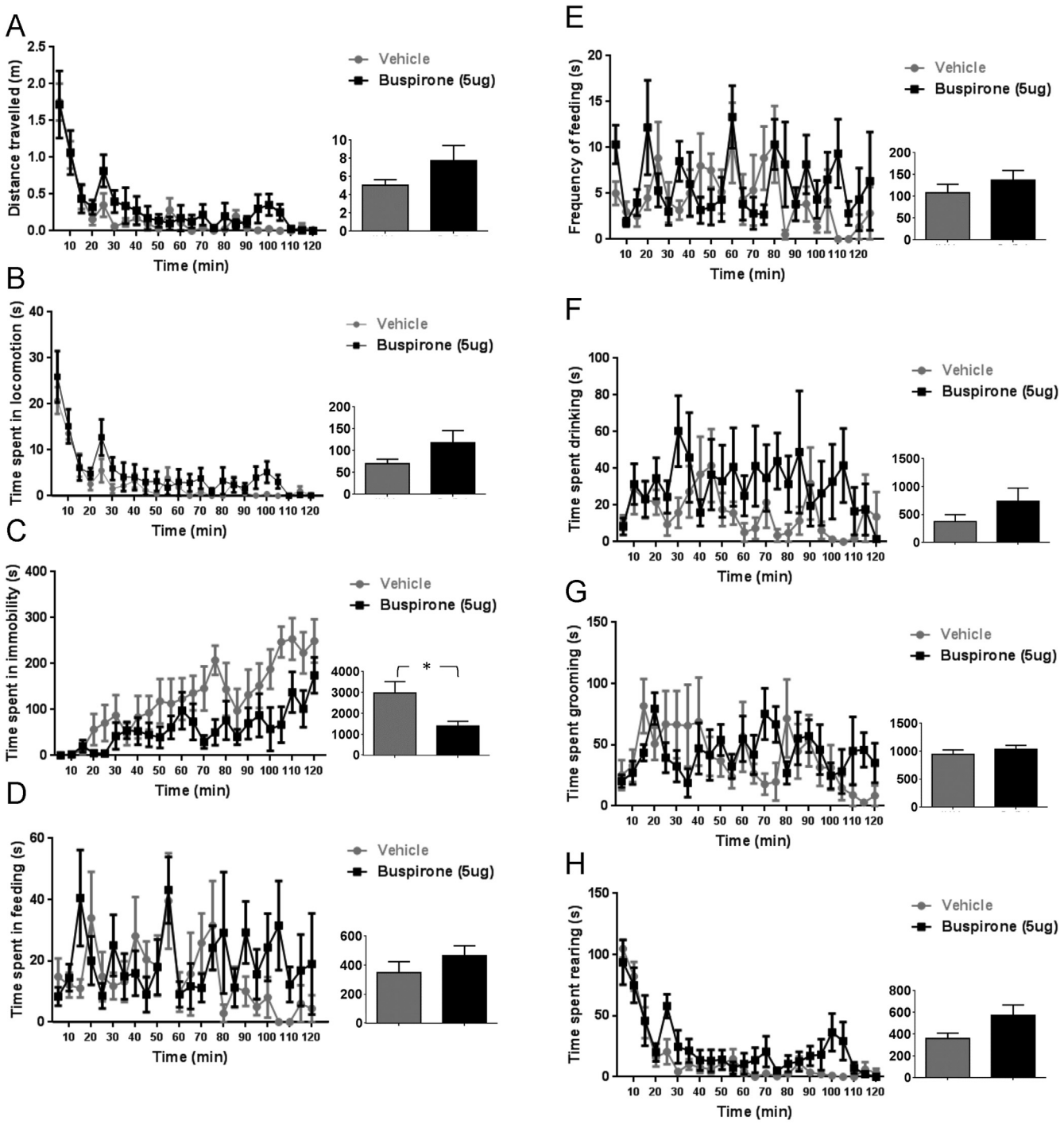

Fig. 6. Effect of intra-NI buspirone (BUS; 5 μg) on the LABORAS home-cage activity. Data points represent the mean of the parameter over the preceding 5 min (A–H). Columns represent mean of values over 2 h and error bars represent SEM (insets in A–H). n = 6 (vehicle) n = 6 (buspirone). Repeated measures ANOVA followed by multivariate analysis. *$p < 0.05$.

anxiogenic response.

Having shown that the anxiogenic dose of buspirone activates NI, we examined whether the NI is necessary for the anxiogenic effect. CRF-saporin lesioning of the NI, specifically ablates the CRF_1 expressing NI neurons significantly reducing CRF_1, relaxin-3, GAD 65 and 5-HT_{1A} expression levels (Lee et al., 2014). Lesioning the NI resulted in an anxiogenic effect illustrated by reduced time spent and entries into the open arms of the elevated plus maze. Buspirone (3 mg/kg) was tested on NI-lesioned rats in the elevated plus maze paradigm. The anxiogenic effect of systemic buspirone was abolished in the NI- lesioned rats but preserved in the sham-lesioned rats with no significant differences in locomotion. The present data suggests that the NI is involved in mediating the anxiogenic response of buspirone.

To examine whether the observed anxiogenic effects were due to a direct action of buspirone on the NI or an indirect action via a secondary structure that modulates the NI, buspirone was infused directly into the NI. A significant anxiogenic response is seen in the

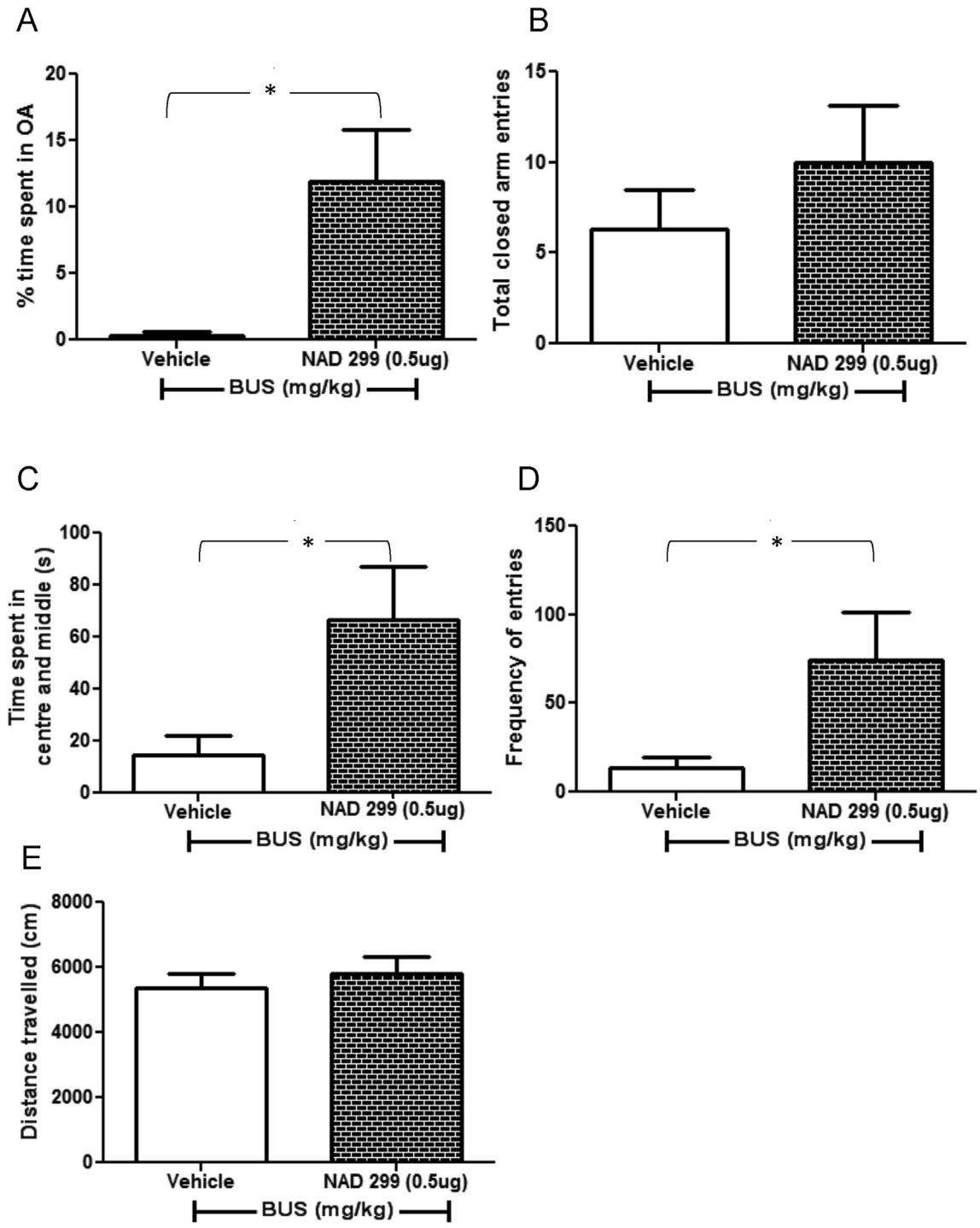

Fig. 7. Effect of intra-NI NAD 299 (0.5 μg) in rats treated with systemic buspirone (3 mg/kg) on the EPM and open field. (A–B) Percentage time spent in the open arms of the EPM and total entries into the closed arm respectively. n = 6 (Vehicle) n = 6 (NAD 299). (B–D) Total time spent in the centre and middle, total frequency of entries to the centre and middle and distance travelled in the open field, respectively. n = 6 (Vehicle) n = 5 (NAD 299). Students' *t*-test *p < 0.05. Data represented as mean ± SEM.

elevated plus maze and open field exploration paradigms. This points to the idea that a direct action of buspirone on the NI contributes to the anxiogenic effects. No significant differences were detected in the anxiety parameters of the light-dark box, indicating that the anxiogenic effect was mild. Preliminary studies conducted with a 2.5 μg dose administered to the NI did not show an anxiogenic response indicating that the effect may be dose-dependent.

Intra-NI infusion of buspirone significantly reduced the distance travelled in the open field. However, the locomotor indices in the elevated plus maze, namely the total closed arm entries were not altered by buspirone. To determine if intra-NI buspirone altered locomotion per se, the buspirone infused rats were assessed in the LABORAS home-cage monitoring system. The anxiogenic dose of intra-NI buspirone did not affect distance travelled, the time spent in locomotion, the frequency of feeding, time spent drinking, time spent grooming and time spent rearing in LABORAS. The time spent

in immobility was lower in the buspirone-treated rats over the entire 2 h period. However, the repeated measures ANOVA indicated that the difference between treatments was only observed at the 105 min bin, a time point at which a significant increase in the feeding was observed, which could explain the reduction in immobility. No significant differences were found in any of the parameters during the first 10 min, the time period representing the immediate action of infused buspirone in the NI. Further, this observation indicates that intra-NI buspirone treatment may not affect the behaviour in a low stress home-cage environment. On the other hand, the higher-stress paradigms, namely elevated plus maze and open field exploration, involve risk analysis (that might involve NI) and thus buspirone induces behavioural inhibition (Goto et al., 2001; McNaughton and Gray, 2000; Ryan et al., 2011; Walf and Frye, 2007). As mentioned earlier, the NI has been shown to be strongly activated by stressors while being minimally active under basal conditions (Cullinan et al., 1995; Herdegen et al., 1995; Herrera and Robertson, 1996). The reduction in the distance travelled observed in the open field is likely to be due to the increase in anxiety level rather than motor impairment or sedation as the distance travelled in the LABORAS home-cage system was unaffected (Gray and McNaughton, 2003; Weiss et al., 1998).

While the aforementioned observations indicate that the NI is a target of buspirone, pharmacological studies were conducted to determine if 5-HT_{1A} or D_2 receptors were involved. Opposing the D_2 receptor antagonism by buspirone, with quinpirole, a D_2 agonist, resulted in no significant difference in the elevated plus maze, light-dark box and open field exploration. These results indicate that the D_2 receptors in the NI may not be directly involved in the anxiogenic effects of buspirone. To examine the effects of 5-HT_{1A} receptors, intra-NI infusion of NAD 299, a selective antagonist of 5-HT_{1A} receptors was carried out and the behavioural changes in elevated plus maze and open field paradigms were studied. A study was conducted initially to ensure that intra-NI NAD 299 at 0.5 μg administered alone, did not alter any anxiety or locomotor parameters. Since NAD 299 is an antagonist, it is important to measure if there is a 5-HT basal tone in the NI which would induce any behavioural effect when blocked. At the 0.5 μg dose, the relevant parameters were unaffected.

In the systemic buspirone and intra-NI NAD 299 pharmacological interaction study, compared to the intra-NI saline treated controls, intra-NI NAD 299 treated rats spent significantly more time in the open arms of the elevated plus maze. Likewise, rats treated with NAD 299, spent more time in and had a larger number of entries to the centre and middle of the open field with no effect on locomotor indices such as total closed arm entries in the elevated plus maze and distance travelled in the open field. These results demonstrate that 5-HT_{1A} inhibition, attenuated the anxiogenic response induced by systemic buspirone (3 mg/kg). Overall, the observations from this behavioural study, indicate that 5-HT_{1A} receptors expressed in the NI are centrally involved in mediating the anxiogenic response of a high dose of buspirone. Since buspirone is an anxio-selective drug, it is likely that structures involved in its mechanism of action are part of the physiological anxiety circuit (Riblet et al., 1983; Temple et al., 1982). Therefore, the $5HT_{1A}$ receptors in the NI may be important in the expression and modulation of anxiety. It has been shown that these receptors are co-expressed with relaxin-3 and may be instrumental in the negative regulation of relaxin-3 expression by serotonin signalling (Miyamoto et al., 2008). The nature and pattern of these 5-HT_{1A} receptors should be studied in greater detail. The role of 5-HT_{1A} receptors in anxiety has been widely studied due to the biphasic effects of 5-HT_{1A} ligands and conflicting results in different tests of anxiety (Celada et al., 2013; De Vry, 1995). It has been convincingly shown that the anxiolytic effect of buspirone is mediated via the 5-HT_{1A} autoreceptors in the raphe nuclei (Carli et al., 1989; File et al., 1996). To date most studies have considered post-synaptic 5-HT_{1A} in the hippocampus as the main target due to its high receptor density (Pazos et al., 1987). Infusion of buspirone into the hippocampus, produced an anxiolytic effect making it an unlikely target for the anxiogenic effect observed at high doses (Kostowski et al., 1989).

It is interesting to note a contrasting effect between the anxiogenic effect of NI lesioning and buspirone treatment. Lesioning of the NI produces an anxiogenic effect indicating that the NI reduces anxiety physiologically, however buspirone's action on the NI increases anxiety. CRF-saporin lesioning of the NI is a chronic procedure that ablates the NI cells over a period of 14 days and has been shown to reduce relaxin-3 expression in the target regions such as the medial septum (Lee et al., 2014). Studies involving central infusion of RXFP3 selective ligands have indicated that global relaxin-3 signalling is anxiolytic (Nakazawa et al., 2013; Ryan et al., 2013). Thus lesioning the NI likely reduces relaxin-3 signalling in target regions increasing baseline anxiety. Buspirone (3 mg/kg) treatment on the other hand, is an acute procedure that, based on the present study, stimulates the 5-HT_{1A} receptors in the NI producing an anxiogenic effect. When the NI is lesioned and these receptors are reduced, the anxiogenic effect of buspirone is attenuated. The NI releases GABA, relaxin-3 and glutamate and responds to a variety of neurotransmitters and thus is likely to modulate anxiety levels differentially based on its level of activation and the type of neurotransmitter signalling.

Although there is a lack of detailed clinical literature on the direct anxiogenic effects associated with acute buspirone treatment, several reports show that patients treated with buspirone initially experience akathisia (a syndrome characterized by motor restlessness, feeling of muscular quivering and inability to sit still), jitteriness and panic (Brody et al., 1990; Chignon and Lepine, 1989; Liegghio et al., 1988; Patterson, 1988; Pohl et al., 1989; Sinclair et al., 2009). In order to understand the neuropharmacology of buspirone, it is important to establish the target structures buspirone acts on and the nature of this interaction. Actions in the NI could play a role in the delayed efficacy and side effects of buspirone. Future studies should examine the role of the NI/relaxin-3 system in a chronic buspirone regimen in rodents, while on the clinical side, more information should be obtained regarding the acute effects of buspirone during the initial phase of the treatment.

Statement of interest

None.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.07.019.

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