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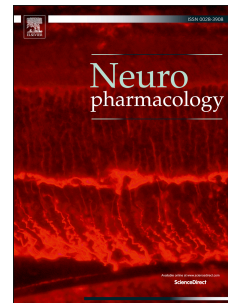
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Title:

BNC210, a negative allosteric modulator of the alpha 7 nicotinic acetylcholine receptor, demonstrates anxiolytic- and antidepressant- like effects in rodents.

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Abstract: This work describes the characterization of BNC210 (6-[(2,3-dihydro-1*H*-inden-2-yl)amino]-1-ethyl-3-(4-morpholinylcarbonyl)-1,8-naphthyridin-4(1*H*)-one), a selective, small molecule, negative allosteric modulator (NAM) of $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChR). With the aim to discover a non-sedating, anxiolytic compound BNC210 was identified during phenotypic screening of a focused medicinal chemistry library using the mouse Light Dark (LD) box to evaluate anxiolytic-like activity and the mouse Open Field (OF) (dark) test to detect sedative and/or motor effects. BNC210 exhibited anxiolytic-like activity with no measurable sedative or motor effects. Electrophysiology showed that BNC210 did not induce $\alpha 7$ nAChR currents by itself but inhibited EC₈₀ agonist-evoked currents in recombinant GH4C1 cell lines stably expressing the rat or human $\alpha 7$ nAChR. BNC210 was not active when tested on cell lines expressing other members of the cys-loop ligand-gated ion channel family. Screening over 400 other targets did not reveal any activity for BNC210 confirming its selectivity for $\alpha 7$ nAChR. Oral administration of BNC210 to male mice and rats in several tests of behavior related to anxiety- and stress- related disorders, demonstrated significant reduction of these behaviors over a broad therapeutic range up to 500 times the minimum effective dose. Further testing for potential adverse effects in suitable rat and mouse tests showed that BNC210 did not produce sedation, memory and motor impairment or physical dependence, symptoms associated with current anxiolytic therapeutics. These data suggest that allosteric inhibition of $\alpha 7$ nAChR function may represent a differentiated approach to treating anxiety and stress-related disorders with an improved safety profile compared to current treatments.

1. Introduction

Anxiety and stress-related disorders are the most common forms of mental illness, which in the US, affect around 19% of the adult population every year and over 30% of adolescents (<https://adaa.org/understanding-anxiety/facts-statistics>). Examples of these disorders include panic disorder (PD), generalized anxiety disorder (GAD), social phobia (SP), depression and post-traumatic stress disorder (PTSD), all chronic illnesses that cause patients to experience symptoms that impact their daily functioning and ability to manage a working life.

The FDA-approved drugs for anxiety and stress-related disorders are certain selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs). These newer generation antidepressants offer an improved therapeutic option and around 25% of responders achieve recovery or remission (Bystritsky, 2006; Berger et al., 2009). However, SSRIs and SNRIs also have safety, tolerability and side effect concerns and a slow onset of action, taking up to 4 weeks or more before the therapeutic benefit is felt. Issues with tolerability include gastrointestinal side effects (diarrhea, nausea), central nervous system side effects (agitation, anxiety, insomnia, dizziness, drowsiness, and suicidal ideation), sexual dysfunction (ejaculatory disorder, erectile dysfunction, decreased libido, and delayed ejaculation) and sweating. Both classes of antidepressant carry a black box warning from the FDA to indicate safety concerns (Jick et al., 2004). Some of the side-effects (in particular, suicidal ideation) are of concern with more vulnerable patients e.g., adolescents and children, while others affect compliance.

The shortcomings of therapeutics for anxiety and stress-related disorders highlight an unmet need for medicines with greater clinical benefit, fewer side effects, and more rapid onset of action. Considerable effort has been invested in the discovery and development of new drugs for anxiety and stress-related disorders and recent approaches have included targeting the GABA_A receptor in new ways (Atack, 2011; Witkin et al.), evaluating new drug targets for anxiety (Davis et al., 2002) and for depression (with a rapid onset of action) (Yavi et al., 2022). With this goal in mind, we initiated a medicinal chemistry program based on the physicochemical properties of a quinolone lead class of GABA_A receptor modulators, which had the unique profile of being anxiolytic-like but non-sedating (Johnstone et al., 2002). An *in vivo* phenotypic screening approach was used, with the mouse Light/Dark (LD) box measuring anti-anxiety-like behavior (Bourin and Hascöet, 2003) and the Open Field-dark (OF) evaluating sedation (Prut and Belzung, 2003). Compounds were evaluated at 3, 10 and 30

mg/kg and several had the desired phenotype, including BNC210 (Fig. 1) which had a minimum effective dose of 3 mg/kg in the LD box and did not cause sedation in the OF test at any dose tested. However, the molecules identified in the screen did not bind to the GABA_A receptor. We next determined if the compounds were active at other members of the GABA_A receptor family i.e., the cys-loop, ligand gated ion channel family which includes GABA_A, all nicotinic acetylcholine receptors (nAChRs), serotonin 5-HT₃ receptor, and the strychnine-sensitive glycine receptor (Alexander et al., 2009). Given the sequence homology between these family members (Connelly et al., 2004), compounds targeting one of these ion channels often display activity at one or more other channels in the family (Prickaerts et al., 2012; Wallace et al., 2011; Jensen et al., 2005; Ng et al., 2007). Several of the compounds in our library with the anxiolytic-like and non-sedating phenotype, were active at 2 or more of these receptors, but BNC210 was the only one selective for α 7 nAChR (Table 3).

BNC210 was tested in an extensive battery of animal studies to further explore its potential for anti-anxiety, anti-stress and anti-depressant-like properties, effects on fear extinction and a differentiated side effect profile from current therapeutics e.g., lack of sedation, memory/motor impairment, or physical dependence (Table 4).

2. Materials and Methods

2.1 Study Design

BNC210 was characterized *in vitro* using both electrophysiology and pharmacology screens (receptor binding and functional) to investigate target and off-target effects, followed by evaluation in a broad range of animal tests relevant for assessing anxiety and stress-related behaviors, and common side effects. Electrophysiology analyses of BNC210 were performed on cells (n=3-5) patch-clamped in whole cell mode in the recording chamber of a 16-channel re-usable Dynaflo ReSolve[®] chip (Celletrion, Sweden) using an EPC10 USB amplifier (HEKA Elektronik, Germany). For the *in vivo* studies, male rats or mice (n=10-12 per group) were randomized in all experiments and investigators were blinded regarding the treatments and allocation sequence. Drug samples were prepared by an independent investigator to maintain the blind. Many of the animal tests of anxiety have been validated by clinically effective benzodiazepine anxiolytics including diazepam which was used as an internal control in several studies (Markou et al., 2009). Apart from the Conditioned Fear Extinction and Non-Precipitated Withdrawal tests, BNC210 was evaluated in animal tests of anxiety, stress, and depression several times, using a variety of dose ranges, usually in log or half log increments,

to establish the most effective doses for each test. The figures in this paper start with a non-effective dose of BNC210 and progress through to doses where the efficacy plateaus. BNC210 was evaluated in side effect tests at doses higher than the most effective doses in behavioral tests. To calculate outliers in the animal data sets, Grubbs' test (GraphPad) was used. If there was only one outlier, it was removed. The figures in this paper do not contain pooled data.

2.2 Statistical Analyses and Calculations

When performing dose responses using electrophysiology, increasing concentrations of BNC210 (0.3, 1, 3, 7, 15 and 30 μ M) were applied and responses were normalized to EC80 concentrations of each nicotinic agonist to construct concentration-response curves.

For in vivo experiments, data were expressed as Mean \pm SEM. The T-maze results were expressed as a percentage of alternation over 14 free choice arm visits and the novel object recognition (NOR) data were expressed as Recognition Index (RI) using the time taken to explore Object A (tA) and Object B (tB) during the retention trial, in the equation: $RI = tB/(tA + tB) \times 100$. In the non-precipitated withdrawal tests, the data were plotted as treatment versus time and were analyzed using two-way ANOVA to determine whether there was a treatment \times time interaction. Other statistical analyses were performed using a simple one-way ANOVA followed by Dunnett's test for multiple comparisons. Statistical analyses were performed using GraphPad 10.0.3 unless otherwise stated. $p \leq 0.05$ was deemed significant. This version of Graph pad (10.0.3) includes the capability to perform normality and lognormality tests on the data prior to analysis using two tests determine the variance of the data; Bartlett's and Brown-Forsythe. Homogeneity of variance is an assumption underlying both t tests and F tests (analyses of variance, ANOVAs) and F test results have been included in the legend of each study.

2.3 Cell Culture

Rat or human $\alpha 7$ nAChR/pCEP4 constructs were stably transfected into GH4C1 cells (CCL-82.2, American Type Culture Collection, Manassas, VA) (rat $\alpha 7$ nAChR reference sequence NM_012832; human reference sequence NM_000746) using Lipofectamine 2000 (Gibco/Life Technologies, USA) according to manufacturer's instructions. The $\alpha 7$ nAChR/GH4C1 cell lines were maintained in complete F10 Ham growth medium supplemented with 15% horse serum, 2.5% foetal calf serum, 2 mM penicillin-streptomycin-glutamine, 10 mM HEPES at 37°C in 5% CO₂. Expression pressure was maintained with hygromycin B (200 μ g/ml). For

electrophysiology, cells were seeded at 2×10^6 cells/ml in complete F10 Ham growth media (without hygromycin B) with 0.5 mM sodium butyrate and incubated at 33°C in 5% CO₂ for 2 days prior to assaying.

2.4 Drugs

All cell culture reagents (Gibco) were purchased from Life Technologies (Carlsbad, CA). acetylcholine (ACh), nicotine, 5HT, epibatidine, DMSO, GABA, CCK-4, diazepam and imipramine were purchased from Sigma-Aldrich (St Louis, MO). For the electrophysiology experiments, BNC210 was dissolved in DMSO (0.1% final concentration) and diluted in extracellular solution. For all *in vivo* studies, if not stated otherwise, BNC210 was prepared in an aqueous vehicle containing 0.5% w/v hydroxypropylmethyl cellulose, 0.5% v/v benzyl alcohol and 0.4% v/v Tween 80, a formulation which gives ~70% bioavailability in rats when administered orally. CCK-4, diazepam and imipramine were prepared in 0.9% saline. In the Contextual Fear Conditioned Extinction study, diazepam was prepared in an aqueous solution containing 5% PEG. BNC210 and diazepam were administered PO, 1 h prior to testing. CCK-4, scopolamine and imipramine were administered IP. Doses selected for the control compounds had previously been established as the most effective for purpose e.g., diazepam (1 mg/kg, 60 min prior) reliably produced effective anti-anxiety behavior in rat and mouse tests without being sedative, scopolamine (1 mg/kg for mice, 20 min prior; 0.6 mg/kg for rats, 30 min prior) produced ~50% inhibition of memory in the mouse T-maze and rat NOR respectively. CCK-4 (0.2 mg/kg, 30 min prior) induced highly anxious, but still measurable behavior in the Elevated Plus Maze (EPM) and imipramine (30 mg/kg, 30 min prior) caused significant decrease in immobility time in the rat Forced Swim Test (FST).

2.5 Electrophysiology

Rat or human $\alpha 7$ /GH4C1 cells were patch-clamped in whole cell mode in the recording chamber of a 16-channel re-usable Dynaflo ReSolve[®] chip (Celletrion, Sweden) using an EPC10 USB amplifier (HEKA Elektronik, Germany). The cells were perfused with extracellular solution that contained NaCl (137 mM), KCl (5 mM), CaCl₂ (2.5 mM), MgCl₂ (1 mM), HEPES (10 mM), D-Glucose (10 mM), pH 7.4. Recording electrodes of 1.5-2.0 (or 2-4) M Ω resistance were fabricated from thin wall borosilicate glass with filament (G150TF-4, Warner Instruments, CT) using a Sutter P-2000 programmable laser pipette puller (Sutter Instrument, CA) and then filled with intracellular solution of K-gluconate (120 mM), KCl (5

mM), HEPES (10 mM), EGTA (10 mM), MgCl₂ (1 mM), ATP (2 mM), pH 7.2. Cells were voltage-clamped at -70 mV and those with series resistance below 15 MΩ were kept, and 40% or 70% resistance compensation was utilized routinely. Those cells that did not maintain a seal resistance of > 500 MΩ were excluded from analysis. For each experiment, ACh or nicotine control applications at EC₈₀ concentration, were followed by increasing concentrations of BNC210 using the fast perfusion system of the *Dynaflow*[®] (Celletricon). Compound application was as follows – extracellular solution or BNC210 alone was pre-incubated for 1.5 minutes followed by 250 ms application of EC₈₀ agonist (ACh or nicotine) or BNC210 plus agonist (EC₈₀ concentration), with a 20 sec or 40 sec washout intervals between episodes. Two control agonist applications were initiated after a final washout of 60 sec or 120 sec following the last compound application. Peak current amplitudes were measured using HEKA analysis and the effect of BNC210 on current peak was calculated as the percentage of inhibition relative to the control response using the following formula: Effect (%) = $((I_{\text{compound}}/I_{\text{control}})) \times 100$. Concentration-response curves and EC₅₀ values were plotted and calculated using GraphPad Prism (GraphPad Software, CA). Non-linear regression using $Y=(100/(1+10^{-(\text{LogIC}_{50}-X) \cdot \text{HillSlope}}))$ equation was used to determine concentration-response curves.

2.6 Animals

All animal care and experimental procedures (except the Contextual Fear Conditioning Extinction) were performed in accordance with institutional guidelines and were conducted in compliance with French Animal Health Regulation, at Neurofit SAS (Illkirch, France). Male CD-1 mice, 4–5 weeks old, and male Wistar rats (200-215 g) were obtained from Janvier (Le Genest St Isle, France), and group-housed 3-10 per cage in a temperature-controlled room (21–22°C) with a reversed light-dark cycle (12 h/12 h; lights on: 17:30–05:30; lights off: 05:30–17:30) with food and water available *ad libitum*. They were allowed at least one week to acclimatize to the animal facility environment. The Contextual Fear Conditioning Extinction study was performed at Brains Online LLC (San Francisco, CA) with male C57B6 mice (Harlan, USA), 9-11 weeks old, and housed in individual plastic cages with access to food and water *ad libitum*. Animals were kept on a 12/12h light/dark cycle and acclimated to the facility for at least 7 days prior to experimentation. Experiments were conducted in accordance with the declarations of Helsinki and were approved by the Institutional Animal Care and Use Committee of Brains On-Line. All experimental procedures for pharmacokinetic analyses were approved and performed in accordance with the guidelines of the Institutional Animal

Experimentation Ethics Committee (Monash University Ethics approval numbers VCPA.2007.10 and VCPA.2008.2).

2.7 Pharmacokinetic Studies

The pharmacokinetic profile of BNC210 was studied in overnight-fasted male Sprague Dawley rats weighing 263 - 313 g. BNC210 was administered orally at 0.5, 5 or 100 mg/kg (n = 3 rats at each dose level) (Table 1).

On the day prior to dosing, a commercially available BASi Culex[®] cannula (for use with a Culex[®] automated blood sampling device) was inserted into the left carotid artery of all rats under isoflurane anesthesia (2%). Cannulas were exteriorized by tunneling subcutaneously to emerge above the scapulae. Immediately following surgery and through to the end of the experiment, rats were housed in Ratur[®] metabolic cages in the Culex[®] automated blood sampler (ABS). All rats returned to normal grooming, drinking and sleeping behavior within an hour of surgery. Animals were given a small amount of food just after they awoke from the anaesthetic and were then fasted for 16-18 hours prior to drug administration. Animals had free access to water. Food was re-instated 4 hours following drug administration. For oral administration, BNC210 (free base) was formulated in aqueous vehicle containing 0.5% w/v hydroxypropylmethyl cellulose, 0.5% benzyl alcohol and 0.4% v/v Tween 80. The target concentrations of BNC210 in the oral formulations were 0.15, 1.5 and 30 mg/mL to provide doses of 0.5, 5 and 100 mg/kg, respectively, in a 1.0 mL dose volume. At the 0.5 mg/kg dose, the formulation was a solution, whereas at the 5 and 100 mg/kg doses, formulations were fine yellow suspensions. Rats were lightly anaesthetized with isoflurane to facilitate insertion of the oral gavage tube. The formulation was taken up into a 1.0 mL syringe, attached to the gavage tube, and the dose administered into the stomach. After the dose was administered, the tube that previously contained the dosing formulation was rinsed with 1 mL of Milli-Q water to collect any remaining dosing formulation, and this volume was also administered to the animal. Any material remaining in the tube and syringe after dosing was retained, dissolved in a known volume of acetonitrile, and assayed by LCMS to determine the exact dose administered to each animal.

Samples of arterial blood and total urine were collected up to 48 hours after oral dosing. Arterial blood was collected directly into borosilicate vials (at 4°C) containing heparin, Complete[®] (a protease inhibitor cocktail), potassium fluoride, and EDTA to minimize the potential for *ex vivo* degradation of BNC210 in blood/plasma samples. Once collected, blood samples were

centrifuged, supernatant plasma was removed, and plasma was stored at -20°C prior to analysis by LCMS. Plasma concentrations of BNC210 were determined by LCMS. The lower limit of quantitation (LLQ) of the LCMS assay was assessed using the standard curve for each analytical run.

Pharmacokinetic parameter estimation was conducted using the noncompartmental analysis module of the WinNonlin software (version 4.0.1, Pharsight Corporation, Mountain View, CA). The terminal elimination half-life ($t_{1/2}$), total plasma and blood clearance (plasma CL_{total} and blood CL_{total}) and volume of distribution at steady state (V_{ss}) after IV administration were estimated. Bioavailability at each dose level was calculated relative to the IV dose for which comparable plasma concentrations were observed. The apparent bioavailability (BA) after oral administration was estimated using the following equation:

$$BA (\%) = \frac{AUC_{extravasc} * Dose_{IV}}{AUC_{IV} * Dose_{extravasc}}$$

2.8 Behavioral Tests (Summary Table 4)

2.8.1 Light Dark Test (LD) (Bourin and Hascöet, 2003)

In this task mice were given a choice between exploring a brightly lit or a dark chamber as a measure of anxiety. The apparatus consisted of two PVC (polyvinylchloride) boxes (19 × 19 × 15 cm) covered with Plexiglas. One of these boxes was darkened. The other box was illuminated by a 100 W desk lamp placed 15 cm above and providing an illumination of about 4400 Lux. An opaque plastic tunnel (5 × 7 × 10 cm) separated the dark box from the illuminated one. Animals were placed individually in the lit box, with their heads directed towards the tunnel. The time spent in the lit box and the number of transitions between the two boxes was recorded over a 5 min period after the first entry of the animal in the dark box. The total walked distance in the lit box was also recorded. Animals scored without entry into the lit box were excluded from the analysis. The apparatus was cleaned between each animal using 70% alcohol.

2.8.2 Marble Burying Test (MB) (Deacon, 2006)

The apparatus consisted of transparent polycarbonate cages (30 cm x 18 cm x 19 cm) containing a 5 cm layer of fine sawdust bedding and 20 glass marbles (diameter: 1.5 cm) spaced evenly along the walls of the cage. Each animal was placed individually in the cage where it remained for a 20 min test session. On termination of the test session the animals were removed

from the cage and the number of marbles with at least two-thirds buried in the sawdust was recorded.

2.8.3 Contextual Fear Conditioning Extinction

The effect of BNC210 on fear extinction was examined in a contextual fear-conditioning extinction paradigm (Brains On-Line Study QM-5-896-A-01, Brains Online LLC, CNS Contract Research). Fear conditioning was achieved by pairing a conditioned stimulus (CS - tone) with an aversive unconditioned stimulus (US), in this case a mild foot shock. In response to the tone-shock CS-US pairing, the animals exhibited specific fearful behaviors, to the point where the CS alone elicited a freezing behavior response in subsequent behavioral sessions. Following a cue-shock (CS-US) conditioning session, the effect of BNC210 on freezing behavior in response to the CS was examined in subsequent CS-only extinction sessions. Extinction was initiated 24 hours after the acquisition of the fear response. Prior to treatment with compounds, a conditioned fear response (freezing) was acquired during the conditioned stimulus (CS; tone) and unconditioned stimulus (US; shock) pairing session. *CS-US pairing session:* Animals were placed into a 7 x 7 x 12-inch enclosure with an electrified grid bottom (Med Associates, St Albans, VT). Animals were allowed to explore the chamber for 180 sec. Next, a tonal cue was played (CS; 30 sec, 3 kHz, 75 dB) which was concluded by a mild foot shock (US; 1 sec, 0.5 mA). After a 120 sec inter-trial interval (ITI), animals were presented with a second tonal cue-shock pairing (CS-US) followed by an additional 60 sec in the enclosure. Animals were promptly removed and returned to their home cage. *CS only extinction session:* In response to the tone-shock CS-US pairing, animals exhibit specific fearful behaviors, such as freezing, in response to the CS alone in subsequent behavioral sessions. Mice were dosed daily with either vehicle, BNC210 (10, 30, or 100 mg/kg, PO), or diazepam (0.5 mg/kg, IP) 1 hour prior to each extinction session. 24 hours after the initial CS-US pairing, mice were exposed to the enclosure for 60 sec followed by 10 CS only presentations with 60 sec of no tone after each presentation. Mice were exposed to 10 CS only presentations on extinction days 1-3 and 2 CS only presentations on extinction days 4-7. Freezing behavior was measured during this period and used as an endpoint for memory and/or the strength of the CS-US association. After each extinction session, mice were immediately returned to their home cage. Extinction sessions occurred for 7 consecutive days after the initial CS-US pairing session. Time spent freezing during the CS-US pairing and subsequent extinction sessions for each group were scored automatically via the Med Associates Software. Comparisons of all treatment groups were conducted using a one-way ANOVA followed by a

Bonferroni's post hoc test for multiple comparisons using GraphPad Prism (GraphPad Prism 7 Software, CA).

2.8.4 *Elevated Plus Maze (EPM) (Pellow et al., 1985)*

2.8.4.1 *Basal conditions*

The apparatus was made of polyvinylchloride materials and consisted of four equal exploratory arms (45×10 cm) which were all interconnected by a small platform (10×10 cm). Two arms were open, and two others were closed with walls (30 cm high). The apparatus was placed 66 cm above the floor. A video tracking system was used to record the test (ViewPoint, France) with a camera placed 2.50 m above the equipment and connected to the computer via a video capture card (Pinnacle Systems, France). A trial consisted of placing an animal on the central platform facing a closed arm. The number of entries into and the duration spent in open arms were automatically recorded by a video track system during a 5-minute period. The apparatus was cleaned between each animal testing using 70% alcohol.

2.8.4.2 *Stress-induced conditions: Forced Swim Session (Micale et al., 2008)*

Rats were exposed to 90 sec of swim stress 5 minutes prior to being placed on the EPM. The FST apparatus consisted of a glass cylinder (height 39 cm; diameter 26 cm) filled with water (depth 30 cm; temperature 25 ± 1 °C).

2.8.4.3 *Stress-induced conditions: Cholecystokinin tetrapeptide (CCK-4) injection (Rex et al., 1994)*

CCK-4 has a high affinity for CCK_B receptors which are distributed widely throughout the brain. The major biological actions of CCK-4 are the induction of anxiety-related behavior and reduction of food intake (Bradwejn, 1993; Fink et al., 1998; Harro and Vasar, 1991). Other researchers have shown that CCK-4 has an anxiogenic profile in rats by decreasing the number of entries into and the time spent on open arms of the EPM. Rats were administered CCK-4 (0.2 mg/kg; IP), 30 min prior to EPM testing.

2.8.5 *Forced Swim Test (Porsolt et al., 1977)*

The FST apparatus consisted of a glass cylinder (height 35 cm; diameter 24 cm) filled with water to a depth of 25 cm, (temperature: 25 ± 1 °C). The rats were individually placed in the water for 6 min of forced swimming which consisted of an acclimation period of 2 min

followed by a 4-min observation period during which the time of immobility was recorded using a video tracking system (Videotrack-FST, Viewpoint, France). The rat was scored as immobile when it was passively floating in the water only making small movements to keep its head above the surface. Total immobility time is considered to reflect the level of despair-like behavior which can be reduced by effective antidepressants such as imipramine.

2.9 In Vivo Safety Studies (Summary Table 4)

2.9.1 Open Field (OF) (Prut and Belzung, 2003)

The apparatus was an open plexiglass cage (52 × 52 cm) with 40 cm walls. The animal's movements were tracked by a computerized video tracking system, consisting of an overhead camera, diode sensors placed underneath the floor of the cage, computer and video analyzer software (ViewPoint, France). The video camera was placed at 2.50 m above the cage and connected to the computer via a video capture card (Pinnacle Systems, France). The video tracking system was set in a way that the floor of the OF was divided into nine equal squares. The total number of crossed squares and the total walked distance were recorded. Each animal was singly placed in a corner of the apparatus and locomotor activity was automatically recorded over a period of 20 min. The apparatus was cleaned between each animal testing with 70% alcohol.

2.9.2 Continuous Alternation T-Maze (T-maze) – Working Memory (Gerlai, 1998).

The T-maze apparatus was made of grey Plexiglas with a main stem (55 cm long × 10 cm wide × 20 cm high) and two arms (30 cm long × 10 cm wide × 20 cm high) positioned at a 90-degree angle relative to the main stem. A start box (15 cm long × 10 cm wide) was separated from the main stem by a guillotine door. Horizontal doors were provided to close specific arms during the forced-choice alternation task. The experimental protocol consisted of one single session, which started with one (1) forced-choice trial, followed by 14 free-choice trials. In the first “forced-choice” trial, the animal was confined for 5 sec in the start arm and then released while either the left or right goal arm was locked by horizontal door. The animal negotiated the maze and entered the open goal arm and returned to the start position. Immediately after the return of the animal to the start position, the left or right goal door was opened, and the animal was allowed to choose freely between the left and right goal arm (free-choice trials). The animal was considered to have entered an arm when it placed its four paws in the arm. A session finished and the animal was removed from the maze as soon as 14 free-choice trials had been

performed or 10 min had elapsed, whichever event occurred first. Between each animal test, urine and faeces were removed from the maze and the apparatus was cleaned using 70% alcohol. During the trials, animal handling and the visibility of the operator were minimized as much as possible.

2.9.3 *Novel Object Recognition – Short Term Memory (Ennaceur and Delacour, 1988)*

The apparatus consists of an open acrylic glass cage (101 cm × 101 cm; with 45 cm walls) within which animals could move freely. One of the objects was a metallic ball and the other was a black box. The animal's approaches to the objects were recorded by an observer using a stopwatch. The experiment consisted of four sessions. Habituation: 24 h before the first trial, animals were habituated to the open-field apparatus for 15 min. Acquisition trial: Object A was placed in a particular corner of the central square. Animals were randomly exposed to the experimental situation for 10 min. Their explorative approaches to the object were recorded. Animals that did not display locomotor activity (i.e., total immobility) or did not explore the object were excluded. Retention trial: The test for retention was performed 30 min after the acquisition trial. Objects A and B were placed on two adjacent corners of the central square. Each animal was exposed to the experimental situation for 10 min during which their exploratory approaches to the two objects were recorded. Recognition index: For each animal, the times taken to explore Object A (tA) and Object B (tB) was recorded and the recognition index (RI) determined. $RI = tB / (tA + tB) \times 100$. Both tB and tA were the values collected during the retention trial.

2.9.4 *Non-Precipitated Withdrawal Test of Physical Dependence (Goudie et al., 1993; Porsolt et al., 2006).*

Rats were dosed daily for 14 days with doses of BNC210 or vehicle. From Day 15, body weights and rectal temperatures were recorded to Day 19. Food intake was recorded over four consecutive periods of 24 hours. The effects of abrupt cessation of 14 days treatment on body weight, food intake and rectal temperature, compared to vehicle treated rats were recorded. Statistical analyses were performed using 2-way ANOVA: $p \leq 0.05$ was deemed significant.

3. Results

3.1 *BNC210 is a negative allosteric modulator of the $\alpha 7$ nAChR.*

In electrophysiology studies, BNC210 (Fig. 1) did not induce $\alpha 7$ nAChR currents by itself, indicating no agonist effect. However, in the presence of an EC₈₀ concentration of acetylcholine, BNC210 inhibited $\alpha 7$ nAChR currents. Dose responses were performed with four different agonists by applying increasing concentrations of BNC210 (0.3 to 30 μ M) and normalizing responses to EC₈₀ concentrations of each agonist (Fig. 2A-D; Table 2). The four agonists were acetylcholine (AChR agonist), nicotine (nAChR agonist), and two selective agonists for $\alpha 7$ nAChR; PNU-282987 and choline. IC₅₀ values were determined on rat and/or human $\alpha 7$ receptors and were 1.5 μ M and 3.0 μ M, respectively at human $\alpha 7$, and 1.3 μ M for both nicotine and acetylcholine on rat $\alpha 7$. Representative traces of concentration-dependent inhibition of rat $\alpha 7$ nAChR showed that most of the inhibition was removed following a 2-minute wash period (Fig. 2E). Percentage of inhibition by BNC210 was independent of agonist concentration as determined by electrophysiology (n=5 cells) using 3 μ M BNC210 (the IC₅₀ of BNC210 for human $\alpha 7$) with 60 or 280 μ M of acetylcholine. The percent inhibition was similar for each concentration of acetylcholine; 39.27 ± 2.0 (Mean \pm SD) for 60 μ M, and 33.98 ± 10 (Mean \pm SD) for 280 μ M (data not shown). In a binding assay, BNC210 did not inhibit α -Bungarotoxin (α -BGTX) binding to the orthosteric site of $\alpha 7$ nAChR. These data, in conjunction with BNC210 having no effect on $\alpha 7$ nAChR currents by itself, indicated that it was an allosteric modulator. Direct determination of receptor occupancy for BNC210 was not possible as suitable radioligands are not available to perform binding studies and the allosteric sites on the $\alpha 7$ nAChR have not been precisely identified although they are proposed to lie in the transmembrane domain (Young et al., 2008; Spurny et al., 2015).

3.1.2 BNC210 is selective for $\alpha 7$ nAChR.

The selectivity of BNC210 for $\alpha 7$ nAChR was confirmed by screening against other members of the cys-loop ligand-gated ion channel family, using stable cell lines expressing each of the following: $\alpha 1\beta 1\delta\epsilon$ nAChR, $\alpha 3\beta 4$ nAChR, $\alpha 4\beta 2$ nAChR, GABA_A and 5HT_{3a}. BNC210 (3 and 10 μ M) was evaluated under EC₂₀ agonist and EC₈₀ agonist conditions to detect positive or negative allosteric activity or agonist/antagonist activity. BNC210 did not affect currents on any of these channels in any mode (Table 3). In a broad pharmacology screen, BNC210 (10 μ M) was evaluated at 109 receptors and 35 enzymes and showed >50% inhibition of binding at three targets: human noradrenaline transporter (hNET), human neurokinin 2 receptor (hNK2) and human adenosine A3 receptor (hA3); binding IC₅₀ values were determined to be 6.9, 9.6 and 13 μ M, respectively. BNC210 was determined to have a K_i of 31.9 μ M (95% CI 28-36

μM) in a functional assay of noradrenaline uptake, a concentration unlikely to be reached in the brain. No other off-target effects were identified by further screening which included a panel of 300 kinases and 65 possible anti-anxiety receptor targets tested in both allosteric and orthosteric modes.

3.2 *In vivo* characterization of BNC210 in behavioral tests

3.2.1 In the mouse light dark test, BNC210 reduced anxiety-like behavior.

The light dark paradigm is based on a conflict between the innate aversion of mice to brightly illuminated areas and their spontaneous exploratory behavior. If given a choice between a bright compartment versus a dark compartment, mice spontaneously prefer the dark area. Anxiolytic compounds have been found to increase the number of entries into, distance walked, and total time spent in the bright compartment. Anxiogenic compounds affect these parameters in the opposite way. BNC210 (0.1, 1, 10 and 50 mg/kg), diazepam (1 mg/kg) or vehicle were administered PO, 1 hour prior to testing. BNC210 significantly increased Entries (10-50 mg/kg), Time spent (0.1-50 mg/kg) and Total Distance walked (0.1-50 mg/kg) in the lit area of the box compared to vehicle treated animals. Diazepam (positive control, 1 mg/kg) was also significantly active for all parameters. The behavioral effects of BNC210 peaked at 10 mg/kg and this level of efficacy was maintained up to the highest dose administered (50 mg/kg), demonstrating a plateauing of response (Fig. 3A-C).

3.2.2 BNC210 reduced the number of marbles buried in the Marble burying test.

When mice are placed individually in a cage, they will spontaneously bury glass marbles arranged on top of the cage bedding. Anxiolytic compounds like BZDs reduce this burying behavior compared to vehicle treated mice, at doses which do not impair locomotor activity. The marble burying test has also been validated with drugs used to treat compulsive behaviors e.g., SSRIs. BNC210 (1, 3 and 10 mg/kg), vehicle or diazepam (1 mg/kg) were administered PO, 1 hour prior to testing. All doses of BNC210 and diazepam significantly and dose dependently reduced the number of marbles buried compared to vehicle control animals (Fig. 4).

3.2.3 BNC210 demonstrated enhancement of fear extinction in a model of contextual fear-conditioned extinction in mice.

A failure to extinguish conditioned fear responses is a symptom of anxiety and stress-related disorders and extinction of Pavlovian fear responses provides a model for assessing new drugs

to treat these disorders including PTSD. This protocol uses freezing behavior in mice as a measure of the acquisition and extinction of learned fear. Across extinction sessions, mice reduced their freezing behavior, an effect modulated by treatment. Mice were dosed daily for 6 days with either vehicle, BNC210 (10, 30 and 100 mg/kg) or diazepam (0.5 mg/kg) IP, 1 hour prior to the first extinction session each day. No significant effects were observed with BNC210 at 10 and 30 mg/kg, however mice treated with 100 mg/kg of BNC210 froze significantly less in response to the conditioned stimulus (tone) compared to vehicle treated mice (70.12 ± 11.21 sec versus 119.10 ± 18.24 sec; $p < 0.05$) thus demonstrating enhanced fear extinction. On Day 2 of extinction, this trend continued, although not significant, in the mice treated with 100 mg/kg BNC210. Conversely, diazepam treated mice (0.5 mg/kg) spent more time freezing on Day 1 compared to vehicle and BNC210 treated mice and the increased freezing behavior reached significance on Day 2 demonstrating an inhibitory effect on fear extinction. On Day 3, time spent freezing was similar for all groups and by Day 4 the conditioned response (freezing) was extinguished (Fig. 5).

3.2.4 BNC210-treatment increased entries into and time spent in the open arms of the Elevated Plus Maze.

The EPM is based on the conflict between the innate tendencies of rodents to explore novel environments and to avoid elevated, open and brightly lit areas. Rats prefer to enter the closed arms but will venture out into the open arms providing a means of studying their relative anxiety status. Anxiolytic drugs like diazepam increase the number of entries and time spent on the open arms of the maze. BNC210 (0.1, 1, 10 and 50 mg/kg), diazepam (1 mg/kg) or vehicle were administered PO, 1 hour prior to testing. BNC210 significantly increased Time at all doses and Entries at 1, 10 and 50 mg/kg compared to vehicle treated animals. Diazepam (positive control, 1 mg/kg) was also significantly active for both parameters. The behavioral effects of BNC210 on Time plateaued between 10 to 50 mg/kg and produced a linear dose response for Entries up to the highest dose evaluated (50 mg/kg) (Fig. 6A, 6B). The EPM protocol was also modified to determine whether BNC210 could attenuate the increased levels of anxiety produced by physical (forced swim) or pharmacological stress prior to evaluation on the EPM.

3.2.4.1 BNC210 moderated behavioral changes produced in rats exposed to a period of Forced Swim prior to evaluation on the EPM.

Swim-stress caused an increase in anxiety-like behavior, as shown by stressed control rats making fewer Entries and spending less Time in the open arms of the maze compared to non-stressed control rats. Swim stressed rats were dosed with BNC210 (1, 10 and 100 mg/kg, PO) which resulted in significantly improved performances for Time (10 and 100 mg/kg) and Entries (100 mg/kg). (Fig. 7A, 7B).

3.2.4.2 *BNC210 moderated behavioral changes produced in rats injected with CCK-4 prior to evaluation on the EPM.*

In this experiment, vehicle control rats treated with CCK-4 were significantly more anxious than control rats without CCK-4 treatment. BNC210 was administered at doses of 10, 50 and 100 mg/kg, PO; for Time and Entries, 50 and 100 mg/kg BNC210 significantly reversed the effects of CCK-4 (Fig. 7C, 7D).

3.2.5 *BNC210 was active in the Rat forced Swim Test following single and repeat dosing.*

FST is a widely used paradigm for the evaluation of the potential antidepressant effects of drugs. When rodents are placed in a cylinder containing water, they rapidly become immobile after unsuccessful attempts to escape, a state which is thought to resemble behavioral despair, a feature of depression. Antidepressants decrease the duration of immobility (i.e., increase the length of active periods with escape attempts) and this is used as a measure of antidepressant effect. Single (10, 20, 50 and 100 mg/kg) and repeat (10, 30, 100 mg/kg) doses of BNC210 were evaluated in this test. Following acute dosing, BNC210 (100 mg/kg) significantly decreased immobility time compared to vehicle control (Fig. 8A). The antidepressant Imipramine was used as a comparator and significantly reduced immobility time as expected. When doses of BNC210 were administered daily for 14 consecutive days, both the lower 30 and 100 mg/kg/day doses caused a significant reduction in immobility time compared to vehicle control (Fig. 8B). In summary, acute and repeat dosing with BNC210 at 100 mg/kg significantly reduced total time of relative immobility in rats thus demonstrating an antidepressant-like effect in the FST. However, with repeat dosing, 30 mg/kg also gained significance suggesting that administration for 14 days augmented the effect of BNC210.

3.3 Safety Studies

3.3.1 *Mice treated with BNC210 did not show signs of sedation or motor changes in the Open Field test.*

The OF task is a simple sensorimotor test used to determine general activity levels, gross locomotor activity, and exploration habits in rodents. This test can provide an early indication of the potential of new compounds for sedative, stimulating or adverse effects. BNC210 was evaluated at doses of 20, 50 or 100 mg/kg. The total walked distance (Fig. 9A) and total number of crossed squares (Fig. 9B) were not significantly different to vehicle treated animals. In contrast, the sedative and motor side effects of diazepam, used at a dose of 3 mg/kg, showed significant difference when compared to vehicle in both measured parameters. These results demonstrated that BNC210 does not affect spontaneous locomotor activity at the doses tested.

3.3.2 BNC210 did not impair working memory in the Mouse T-Maze.

Spontaneous alternation is the innate tendency of mice to alternate free choices in a T-maze over a series of successive runs. This sequential procedure relies on working memory and is sensitive to various pharmacological manipulations affecting memory processes. Treatment with scopolamine (1 mg/kg) reduced spontaneous alternation in mice by ~50% compared to untreated controls. Spontaneous alternation (%) in animals treated with BNC210 at 1, 10 or 100 mg/kg (Fig. 9C) was not different to the performance of vehicle treated animals demonstrating that working memory was not impaired by BNC210 at the doses tested.

3.3.3 Short term memory was not impaired by BNC210 in the rat novel object recognition test.

The NOR test is used to assess short-term memory in rats. The task is based on the natural tendency of rats to preferentially explore a novel versus a familiar object, which requires memory of the familiar object. Scopolamine (0.6 mg/kg) was used to impair rat performance and demonstrated cognitive impairment by decreasing the Recognition Index (RI) by ~50% compared to control animals. Doses of BNC210 (20, 50, 100 or 200 mg/kg) did not significantly differ in the RI compared to controls (Fig. 9D). These results demonstrated that BNC210 did not impair short term memory in this model at the doses tested.

3.3.4 No signs of physical dependence were produced by BNC210 treatment in a non-precipitated withdrawal study.

Identifiable physical signs of withdrawal include changes in body weight, food intake and body temperature following sudden cessation of chronic treatment with drugs which may have addiction potential and induce physical dependence. These measures can be used to evaluate whether novel compounds may have physical dependence/withdrawal liability and the

principle has been validated in rats following chronic treatment with BZDs, antidepressants, and opioids. After repeat dosing with BNC210 for 14 days (0, 10, 30 or 100 mg/kg/day) rats were assessed over the subsequent 5-day period for these physical signs. Abrupt cessation of BNC210 did not produce significant changes in body temperature, body weight or food intake (Fig.10; Table 4) compared to the no-drug treatment group, indicating that 14-days of dosing with BNC210 did not produce any signs of physical dependence.

4.0 DISCUSSION

4.1 Summary

BNC210 is a selective, negative allosteric modulator (NAM) of the $\alpha 7$ nAChR. The focused ion channel chemical library from which it was identified, was designed around a quinolone starting point described as a subunit selective GABA_AR modulator with anxiolytic activity and no sedative or motor side effects (Johnstone et al., 2004). Phenotypic screening of compounds from the focused library identified several leads with non-sedating, anxiolytic-like effects that were devoid of GABA_AR modulatory activity. BNC210 was the most potent in the LD box, non-sedating and unique in its selectivity for the $\alpha 7$ nAChR, which was confirmed by evaluation at over 400 targets in binding and functional assays.

To overcome the imperfect translational power of animal models and tests for neuroscience, multiple tests were used to investigate various behavioral aspects of anxiety and stress-related disorders and to provide convergent validation of the research findings (Markou et al, 2009). Evaluation of BNC210 in a battery of rodent tests demonstrated effective reduction of behaviors associated with anxiety and stress-related disorders such as anxiety, depression, panic, stress, and deficits in fear extinction. Manipulations of the EPM protocol with physical or pharmacological stressors (forced swim or CCK-4 injections) administered prior to testing on the maze, produced additional data in support of the anti-stress and anti-panic potential of BNC210.

BNC210 had antidepressant-like activity in the rat FST following single and repeat dosing for 14 days. Single doses of BNC210 reduced the immobility time of rats, showing significance at 100 mg/kg. After two weeks of daily dosing, significant effects were seen at 30 and 100 mg/kg, suggesting enhancement of the effect of BNC210 over time as seen with SSRIs/SNRIs (Figures 8A, 8B). It has been reported that desensitization and antagonism of nicotinic receptors promotes serotonin release and signaling in the hippocampus (Seth et al., 2002; Kenny et al.,

2000) which may explain the development of the antidepressant effects of BNC210 with repeat dosing.

Fear extinction deficits are characteristic of many anxiety- and stress- related disorders including PTSD (Bierwirth and Stockhorst, 2022). Daily doses of BNC210 at 100 mg/kg/day for 6 days significantly enhanced conditioned fear extinction in the first 2 days of extinction testing compared to vehicle treated mice. In the same experiment, diazepam (1 mg/kg) showed significant impairment of fear extinction. This effect of diazepam has been reported in humans, where attempts to combine exposure-based therapy for PTSD patients with benzodiazepines dampened anxiety but also reduced extinction learning (Singewald et al., 2015) (Fig. 5). A relationship between fear extinction and nicotinic receptors has been described in other publications (Kutlu et al., 2016) including the role of $\alpha 7$ nAChR on fear learning, memory persistence and fear extinction. (Zhang et al., 2021; Miguelez Fernandez et al., 2021).

Given that positive allosteric modulators (Wang et al., 2020), partial agonists (Prickaerts et al., 2012) and agonists of $\alpha 7$ nAChR (like nicotine) (Terry and Decker, 2011) are pro-cognitive, it was important that part of the characterization of BNC210 was to show that $\alpha 7$ nAChR NAMs did not impair cognition. Using rodent tests suitable for evaluating drug effects on working memory and short-term memory such as the mouse T-maze and rat NOR respectively, it was demonstrated that BNC210 did not impair cognition at the doses used (Figures 9C, 9D). Other safety studies showed that BNC210 did not affect spontaneous locomotor activity (OF, dark) (Figures 9A, 9B) or physical dependence (non-precipitated withdrawal) (Figures 10A, 10B, 10C). All safety studies used doses higher than the range required for anti-anxiety and stress-related behaviors.

4.2 Implications of this work

The finding that our molecules were targeting cys-loop ligand gated ion channels other than GABA_A, and that the most efficacious one was a selective, allosteric inhibitor of $\alpha 7$ nAChR, was noteworthy. The association between cholinergic signaling and mood disorders is not new and was first postulated in the 1950s when it was observed that increased levels of acetylcholine, resulting from exposure to acetylcholine esterase inhibitors (AChEIs), caused depression and/or mania in humans (Rowntree et al., 1950). Subsequently, these researchers showed that levels of ACh are elevated in depressed patients (Janowsky et al., 1972). Similarly, nicotine has effects on anxiety and depression in both human and animal studies and activation of nAChRs can modulate many systems associated with stress responses, such as stress

hormone pathways, by stimulating the release of the hypothalamic pituitary adrenal (HPA) hormones ACTH and prolactin (Matta et al., 1998) and by stimulating monoaminergic transmission and release of other neurotransmitters throughout the brain (Picciotto et al., 2002). Mecamylamine is a widely used, non-competitive and non-selective nicotinic antagonist that has been thoroughly investigated in pre-clinical and clinical studies for effects on mood disorders. This compound inhibits most neuronal nAChRs with IC₅₀ values typically in the range 0.1-1 μ M (Wonnacott and Barick, 2007, Wonnacott, 2014) and has been shown to reduce anxiety and stress-induced behavior in animal studies such as the Social Interaction Test, Marble Burying and Elevated Plus Maze (File et al., 1998; Tucci et al., 2003; Roni and Rahman, 2011; Newman et al., 2002), thus implicating inhibition of nicotinic receptors in these behaviors. However, mecamylamine's lack of selectivity produces different results in animal tests compared to the selective modulator BNC210, such as bell shaped and u-shaped dose responses (Newman et al., 2002b). TC-5214, an enantiomer of mecamylamine, was evaluated in clinical trials as an adjunct therapy in patients with major depressive disorder. Despite positive results in Phase 2, the molecule failed in Phase 3, possibly due to its modest selectivity among neuronal nicotinic subtypes and a different mode of inhibition (channel block) (Lippiello et al., 2008).

Allosteric modulators possess several advantages as prospective therapeutics, including a greater chance of being subunit selective due to binding at less conserved sites. Allosteric effects can only be achieved in the presence of endogenous agonists, thus spatiotemporal signaling of natural ligands is maintained. In addition, cooperativity between binding to allosteric (NAM) and orthosteric (agonist) sites imposes a ceiling on the effects of NAM binding in terms of magnitude and direction, thereby restoring homeostasis to the system it modulates rather than overbalancing (Zhang et al., 2020). In animal tests used to evaluate the anti-anxiety-like activity of BNC210 (LD, MB and EPM tests), dosing over a broad range (0.1-50 mg/kg) gave a plateau of effect at the highest doses rather than U-shaped or bell-shaped responses that are often seen for CNS active compounds. These properties persisted in modified protocols of the EPM, where stress was enhanced by forced-swim or CCK-4 injections prior to performing the task. When BNC210 was evaluated in animal tests for side effects, at much higher doses than those used in efficacy studies, it lacked the side effects commonly experienced with current therapies for anxiety- and stress- related disorders such as sedation, potential for physical dependence, motor or memory impairment, and slow onset of action, with the exception of sexual dysfunction which was not evaluated.

$\alpha 7$ nAChRs play a role in the regulation of neuronal excitability due to their position on GABAergic and glutamatergic neurons in brain regions such as the hippocampus, amygdala, and pre-frontal cortex (PFC), or by pre-synaptic modulation of neurotransmitter release (Alkondon and Albuquerque, 2001; Alkondon et al., 1996; Alkondon et al., 1998; Alkondon et al., 2000; Arnais-Cot et al., 2008; Khiroug et al., 2003; Klein and Yakel, 2006), (Barik and Wonnacott, 2009; Dickinson et al., 2008; Livingstone et al., 2009; Quarta et al., 2009). We have observed that the effects of BNC210 on anxiety- and stress- related behaviors in animals are state dependent, as reflected by the requirement for higher doses of BNC210 to effectively reduce the higher excitability produced in some of the animal tests e.g., the CCK-4 challenge, Forced Swim before the EPM, Forced Swim Tests and Conditioned Fear Extinction test which needed higher doses to show significant effects on behavior, in the range of 50-100 mg/kg, whereas in simple ethological tests like the LD box, EPM and Marble Burying, effective doses were in the 0.1-10 mg/kg range.

There is a growing body of evidence to support the idea that negative modulation of excess cholinergic tone via $\alpha 7$ nAChR may have therapeutic potential for anxiety- and stress-related behaviors. Mecamylamine, the non-selective nicotinic antagonist, showed antidepressant-like activity in the forced swim and tail suspension tests in mice. This effect could be blocked by specific antagonists of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs (MLA and Dh β E respectively), implicating these nicotinic receptors in the antidepressant effect. These experiments were then repeated in mice lacking either the $\beta 2$ or $\alpha 7$ nAChR subunits. The antidepressant effects of mecamylamine were lost, suggesting that either receptor could be modulated to produce an antidepressant effect (Rabenstein 2006). The relationship between elevated levels of ACh in the brain with depression and anxiety was also demonstrated by direct manipulations of ACh levels in the hippocampus using the acetylcholine esterase (ACHE) inhibitor physostigmine or by genetic knockdown of ACHE. The resulting increased levels of acetylcholine caused depression and anxiety-like behaviors in mice which could be reversed by an infusion of an AChE transgene (Mineur, 2013). Direct infusions of mecamylamine into the amygdala, or viral mediated knock down of $\beta 2$ or $\alpha 7$ subunits, produced anti-anxiety-like and antidepressant-like behaviors in several mouse models (Mineur, 2015). The effect of $\alpha 7$ nAChR antagonism was further explored in mice following systemic administration of the specific $\alpha 7$ receptor antagonist methyllycaconitine, which had an antidepressant-like effect in the tail suspension and forced swim tests (Mineur, 2018 BJP). Hippocampal knockdown of $\alpha 7$ nAChR showed some antidepressant effects when the depression was induced by a local increase in ACh (by

physostigmine infusions) but did not alter resilience to social stress, another measure of antidepressant effects (Mineur, 2018 BJP).

BNC210 has now been evaluated in several human studies. Three of note include, a CCK-4 challenge in healthy volunteers which showed that BNC210 significantly reduced the number and intensity of panic symptoms on the Panic Symptoms Scale (O'Connor et al., 2011) an fMRI study in GAD subjects, where treatment with BNC210 significantly reduced left and right amygdala hyperactivation and connectivity between the amygdala and the anterior cingulate cortex while performing the Fearful Faces Task (Wise et al., 2020). In the same study, BNC210 had a significant effect on threat avoidance behavior in a human equivalent of the Mouse Defense Test Battery (Perkins et al., 2021; Griebel et al., 1997). In the third human study, BNC210 and lorazepam were compared for effects on attention, psychomotor speed, visual and motor coordination, sedation, addiction liability and mood. The data showed that lorazepam had significant, detrimental effects on all parameters while BNC210 did not affect any of them (O'Connor et al., 2011 Poster). A maximum tolerated dose has not been established for BNC210. On September 29th, 2023, BNC210 reported positive results in a Phase 2 Study in adults with Post-Traumatic Stress Disorder (NCT04951076). <https://ir.bionomics.com.au/news-releases/news-release-details/bionomics-announces-positive-topline-results-phase-2b-attune>

In conclusion, the preclinical data presented in this paper demonstrate that the first reported, selective, $\alpha 7$ nAChR NAM (BNC210) modulates anxiety- and stress- related behaviors, including depression, in animal studies. Anxiety and depression frequently co-occur and may share similar deficits in the processing of emotional stimuli. BNC210 also enhances conditioned fear extinction in mice, which adds to the utility of an $\alpha 7$ nAChR therapeutic approach to anxiety- and stress- related disorders, considering that high anxiety and stress related disorders like PTSD are associated with deficits in fear acquisition and fear extinction. (Dibbets et al., 2015; Maren and Holmes, 2016).

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Methodology: SOC, EA, DP, EBdS (*which animal models to use, drug formulation*)

Investigation: CC, SW, EP, BH (*performing electrophysiology and calcium assays, animal studies*)

Visualization: CC, SOC, SW, EA (graphing and data tables)

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Writing – All authors, TW

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Table 1:

Mean pharmacokinetic parameters following oral administration of BNC210 to male Sprague Dawley rats at doses of 0.5, 5 and 100 mg/kg. Values are presented as the mean \pm SD of n=3 rats at each dose level.

Parameter	0.5 mg/kg	5 mg/kg	100 mg/kg
Measured Dose (mg/kg)	0.55 \pm 0.02	4.8 \pm 0.2	102.4 \pm 7.8
Apparent $t_{1/2}$ (h)	c.n.c.	8.8 \pm 3.5	6.3 \pm 2.3
C_{max} (μ M)	0.2 \pm 0.05	1.1 \pm 0.2	5.7 \pm 0.8
T_{max} (min)	30 \pm 0	30/300/30	150 \pm 90
AUC _{0-t_{last}} (μ M*min)	53.8 \pm 17.6	496.8 \pm 85.6	3055.1 \pm 1054.8
AUC _{0-t_{inf}} (μ M*min)	c.n.c.	497.6 \pm 85.3	3062.9 \pm 1048.8
AUC _{0-t_{inf}} /D (μ M*min*kg/ μ mol) ^a	41.4 \pm 15.1	43.7 \pm 8.7	12.5 \pm 3.7
Apparent BA (%) ^b	66.7 \pm 24.3 ^c	70.3 \pm 14.1	13.2 \pm 3.9 ^d
% Dose in urine ^e	1.6 \pm 0.5	0.7 \pm 0.4	0.2 \pm 0.1

^a Value is the AUC_{0- t_{last}} /D; the profile could not be extrapolated to infinity because the terminal phase was not defined

^b Calculated using truncated AUC (i.e. AUC_{0- t_{last}}) values after oral administration and AUC_{0- t_{inf}} after IV administration. Due to evidence of non-linear clearance after IV administration, bioavailability values should be considered as estimates only

^c Calculated relative to the dose-normalised AUC_{0- t_{inf}} after IV administration at 0.5 mg/kg

^d Calculated relative to the dose-normalised AUC_{0- t_{inf}} after IV administration at 5 mg/kg

^e Unchanged BNC210 present in pooled urine (collected over 0-48 hours post-dose)

c.n.c.: Could not calculate as terminal phase was not defined

Table 2 :

BNC210 inhibits agonist induced currents on rat and human $\alpha 7$ nAChRs. IC_{50} values were determined in whole cell manual patch-clamp electrophysiology using the fast perfusion system of the *Dynaflow*[®] (Cellecetricon). BNC210 inhibited acetylcholine (cholinergic receptor agonist), nicotine (specific nicotinic receptor agonist), choline (an $\alpha 7$ nAChR specific agonist) and PNU-282987 (a small molecule agonist, also specific for $\alpha 7$ nAChRs) at EC_{80} concentration, when evaluated in GH4C1 cell lines stably expressing rat and/or human $\alpha 7$ nAChRs.

Dose response values for inhibition of a selection of nicotinic receptor agonists by BNC210						
	Rat $\alpha 7$ nAChR			Human $\alpha 7$ nAChR		
Agonist	IC_{50} (μM)	n_H	n	IC_{50} (μM)	n_H	n
Acetylcholine	1.5	1.16	5	3	1.0	3
Nicotine	1.3	1.0	5	1.5	0.8	3
Choline	2.4	1.13	4	ND*	ND*	-
PNU-282987	1.2	1.4	4	ND*	ND*	-

*ND: Not determined.

Table 3:

BNC210 is not active at other members of the cys-loop ligand gated ion channel family when tested at 3 and 10 μM for selectivity against the $\alpha 1\beta 1\delta \epsilon$, $\alpha 3\beta 4$, $\alpha 4\beta 2$ nAChRs and the 5HT3a and GABA_A receptors in a Ca⁺⁺ flux fluorescence-based functional assay. % Inhibition values were obtained with EC_{80} ligand concentration. % potentiation values were obtained with EC_{20} ligand concentration. Ligands: Nicotine ($\alpha 7$); Epibatidine ($\alpha 1\beta 1\delta \epsilon$, $\alpha 3\beta 4$, $\alpha 4\beta 2$); Serotonin (5HT3a); γ -Aminobutyric acid (GABA_A).

BNC210 Concentration	Rat $\alpha 7$	h $\alpha 4\beta 2$	h $\alpha 1\beta 1\delta \epsilon$	h $\alpha 3\beta 4$	h5HT3a	hGABA _A
	% Inhibition of Ca ⁺⁺ flux \pm SD					
3 μM	57 \pm 10	3 \pm 8	10 \pm 7	16 \pm 15	0 \pm 0	18 \pm 5
10 μM	90 \pm 3	4 \pm 4	7 \pm 4	17 \pm 8	0 \pm 0	3 \pm 25
	% Potentiation in Ca ⁺⁺ flux \pm SD					
3 μM	0 \pm 0	0 \pm 1	0 \pm 2	0 \pm 1	0 \pm 2	0 \pm 0
10 μM	0 \pm 0	0 \pm 2	0 \pm 1	0 \pm 3	0 \pm 2	0 \pm 1

Table 4: A Summary of the in vivo studies performed with BNC210

Figure	Test	Dose mg/kg; PO (unless specified)	Species
3A, B, C	Light Dark Box	BNC210: 0.1, 1, 10, 50 Diazepam: 1	Mouse
4	Marble Burying	BNC210: 1, 3, 10 Diazepam: 1	Mouse
5	Conditioned Fear Extinction	BNC210: 10, 30, 100/day Diazepam: 0.5 / day	Mouse
6 A, B	Elevated Plus Maze	BNC210: 0.1, 1, 10, 50 Diazepam: 1	Rat
7A, B	Elevated Plus Maze preceded by a period of Forced Swim	BNC210: 1, 10, 100 Swim Stress: 90 sec	Rat
7C, D	Elevated Plus Maze with CCK-4	BNC210: 10, 50, 100 CCK-4: 0.2, IP	Rat
8A	Forced Swim Test	BNC210: 10, 20, 50, 100 Imipramine: 30, IP	Rat
8B	14-day Forced Swim Test	BNC210: 10, 30, 100 Imipramine: 30, IP	Rat
9A, B	Open Field	BNC210: 20, 50, 100 Diazepam: 3	Rat
9C	T-Maze Continuous Alternation Task	BNC210: 1, 10, 100 Scopolamine: 1, IP	Rat
9D	Novel Object Recognition	BNC210: 20, 50, 100, 200 Scopolamine: 0.6, IP	Rat
10	Non-Precipitated Withdrawal Study 14 days	BNC210: 10, 30, 100/day	Rat

Table 5:

BNC210 did not show signs of physical dependence when evaluated in a rat model of non-precipitated withdrawal. The potential consequences of abrupt cessation of BNC210 were assessed in rats following 14 days of treatment at 10, 30 and 100 mg/kg/day. Over the 5 days following drug cessation, changes in (A) body temperature (B) food consumption and (C) body weight were measured and compared to the vehicle control group. BNC210 treated rats did not show any statistical differences compared to vehicle treated animals for weight loss, reduced appetite, or temperature changes, suggesting that BNC210 does not cause physical dependence. Data represent Mean \pm SEM. n=10-12 rats.

A: Rectal Temperature in °Celsius

Dose of BNC210	0		10		30		100	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day Post Dosing								
1	37.91	0.07	37.68	0.06	37.86	0.06	37.66	0.08
2	38.11	0.18	37.71	0.11	37.84	0.10	37.78	0.11
3	38.08	0.13	37.86	0.11	38.01	0.11	38.03	0.16
4	38.03	0.11	37.93	0.11	37.98	0.09	37.93	0.17
5	37.90	0.11	37.78	0.08	38.10	0.07	38.38	0.10

B: % Variation in Body Weight

Dose of BNC210	0		10		30		100	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day Post Dosing								
1	2.92	0.35	2.70	0.27	3.03	0.29	2.55	0.51
2	5.96	0.29	5.59	0.27	5.90	0.35	6.53	0.69
3	9.11	0.30	8.95	0.35	8.59	0.42	8.94	0.76
4	11.45	0.37	11.15	0.39	11.73	0.48	11.32	0.56
5	14.11	0.38	13.11	0.45	13.82	0.42	13.76	0.62

C: Food Intake (grams)

Dose of BNC210	0		10		30		100	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day Post Dosing								
1-2	29.00	0.64	27.00	0.21	28.75	0.81	29.25	1.07
2-3	29.75	0.25	27.25	0.39	29.75	0.69	28.75	0.25
3-4	28.25	0.62	26.75	0.39	28.75	0.94	27.25	0.39
4-5	26.50	0.34	25.25	0.13	27.00	0.37	26.00	0.43

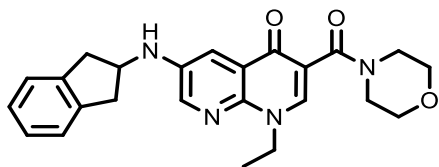
FIGURES:

Fig. 1. Representation of the chemical structure of BNC210, (6-[(2,3-dihydro-1*H*-inden-2-yl)amino]-1-ethyl-3-(4-morpholinylcarbonyl)-1,8-naphthyridin-4(1*H*)-one).

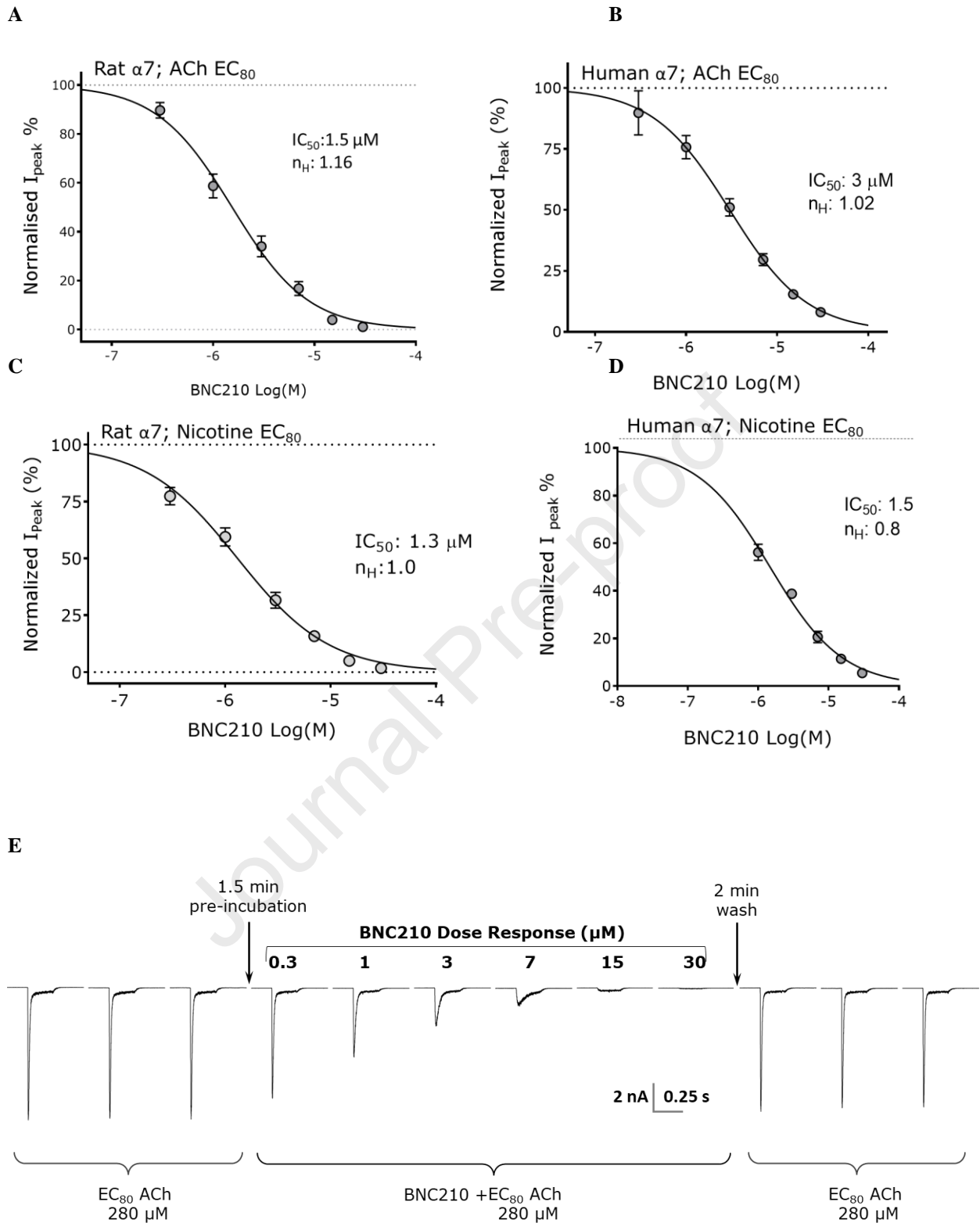
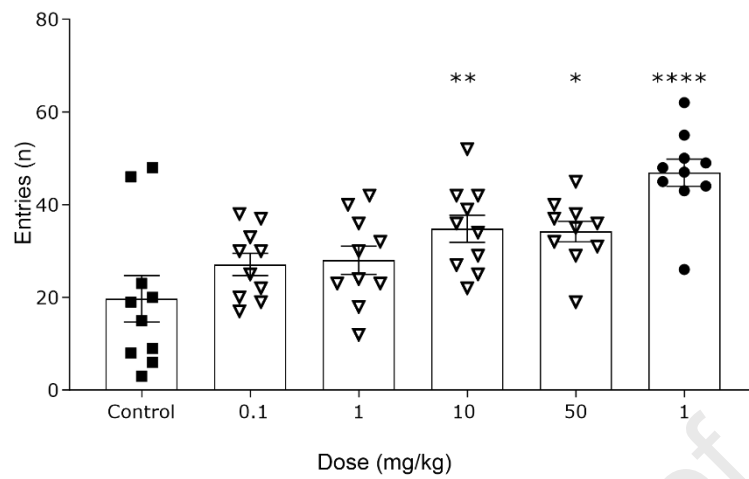


Fig. 2. BNC210 inhibits currents induced by acetylcholine or nicotine in rat and human $\alpha 7$ nAChRs. (A) Concentration-response curve showing inhibition of EC_{80} acetylcholine on rat $\alpha 7$ nAChR by BNC210 (Mean \pm SEM, $n=5$) (B) Concentration-response curve showing inhibition of EC_{80} acetylcholine on human $\alpha 7$ nAChR by BNC210 (Mean \pm SEM, $n=3$) (C) Concentration-response curve showing inhibition of EC_{80} nicotine on rat $\alpha 7$ nAChR by BNC210 (Mean \pm SEM, $n=5$) (D) Concentration-response curve showing inhibition of EC_{80}

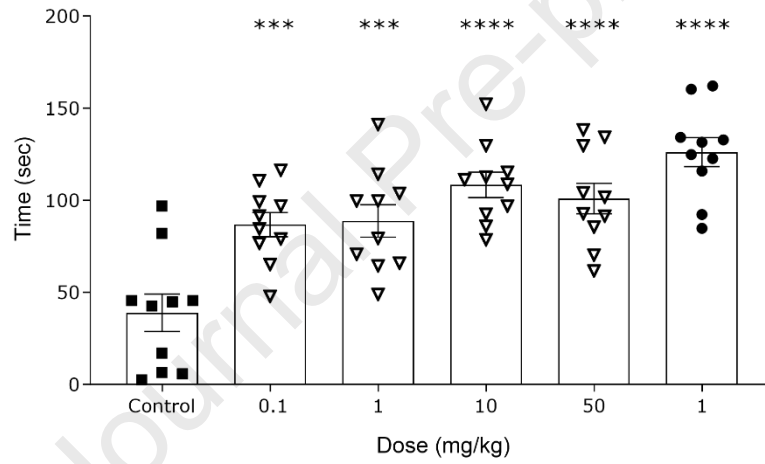
acetylcholine on human $\alpha 7$ nAChR by BNC210 (Mean \pm SEM, n=3) (E) Representative traces showing concentration-dependent inhibition of rat $\alpha 7$ nAChR by BNC210 using EC₈₀ ACh (40 s interval). Increasing concentrations of BNC210 (0.3, 1, 3, 7, 15 and 30 μ M) were applied and normalization of the responses to EC₈₀ concentration of the ligands were used to construct concentration-response curves. Results were calculated using Prism 6.04, non-linear curve fit.

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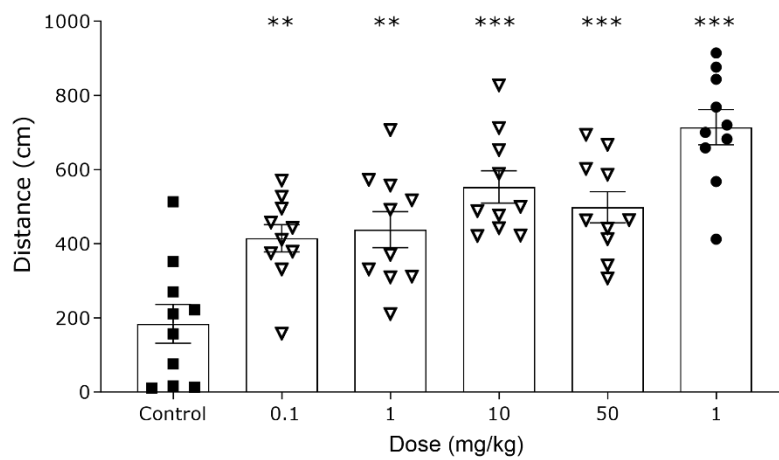
3A



3B



3C



Vehicle ■ BNC210 ▽ Diazepam ●

Fig. 3. BNC210 reduced anxiety-like behavior in the Light Dark Box. **(A) Entries:** BNC210 treated mice showed significantly increased number (n) of entries into the lit box at 10 (35 ± 3.0 ; $p=0.0073$) and 50 (34 ± 2.2 ; $p=0.0107$) mg/kg compared to control mice (20 ± 5.0). Ordinary one-way ANOVA: ($F(5, 54)=8.206$, $p<0.0001$).

(B) Time: BNC210 treated mice showed significantly increased time (sec) spent in the lit box at all doses i.e. 0.1 (87 ± 6.5 ; $p=0.0006$), 1 (89 ± 9.0 ; $p=0.004$), 10 (108 ± 7.0 ; $p<0.0001$) and 50 (101 ± 8.3 ; $p<0.0001$) mg/kg compared to control mice (39 ± 10). Ordinary one-way ANOVA: ($F(5, 54)=13.04$, $p<0.0001$).

(C) Distance: BNC210 treatment significantly increased the total distance travelled (cm) by mice in the lit box at 0.1 (415 ± 37 ; $p=0.0033$), 1 (438 ± 49 ; $p=0.0011$), 10 (553 ± 44 ; $p<0.0001$) and 50 (498.2 ± 42 ; $p<0.0001$) mg/kg compared to control mice (184 ± 52). Diazepam, the positive control, was statistically significant for all three parameters (Entries 46.9 ± 2.9 ; $p<0.0001$; Time 126 ± 7.7 ; $p<0.0001$; Distance 714.4 ± 47.5 ; $p<0.0001$). $n=10$ mice. Ordinary one-way ANOVA: ($F(5, 54)=14.84$, $p<0.0001$).

Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. Significant difference to vehicle control * $p\leq 0.05$; ** $p\leq 0.01$; *** $p\leq 0.001$; **** $p\leq 0.0001$.

4

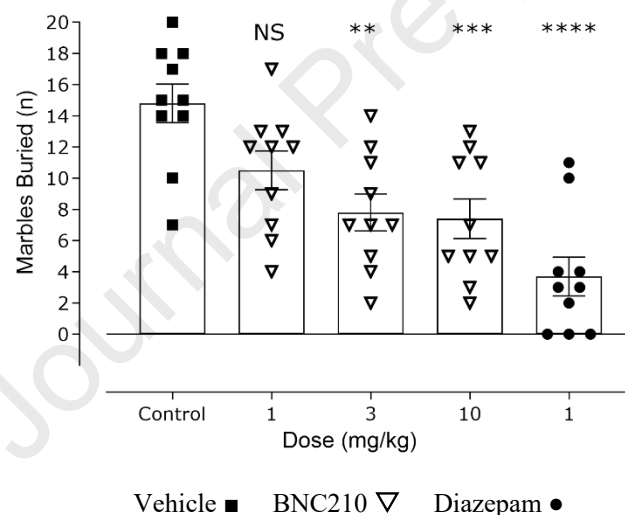


Fig. 4. BNC210 treated mice buried less marbles in the Marble Burying test. Mice treated with BNC210 buried significantly less marbles at 1 ($p=0.000$); 3 (7.8 ± 1.2 ; $p=0.0008$) and 10 (7.4 ± 1.3 ; $p=0.0004$) mg/kg compared to vehicle treated control mice (14.8 ± 1.2). Diazepam treated mice (1 mg/kg) buried the most marbles (3.7 ± 1.2 ; $p<0.0001$) ($n=10$ male mice). Ordinary one-way ANOVA: ($F(4, 45)=11.16$, $p<0.0001$).

Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. Significant difference to vehicle control *** $p\leq 0.001$; **** $p\leq 0.0001$.

5A

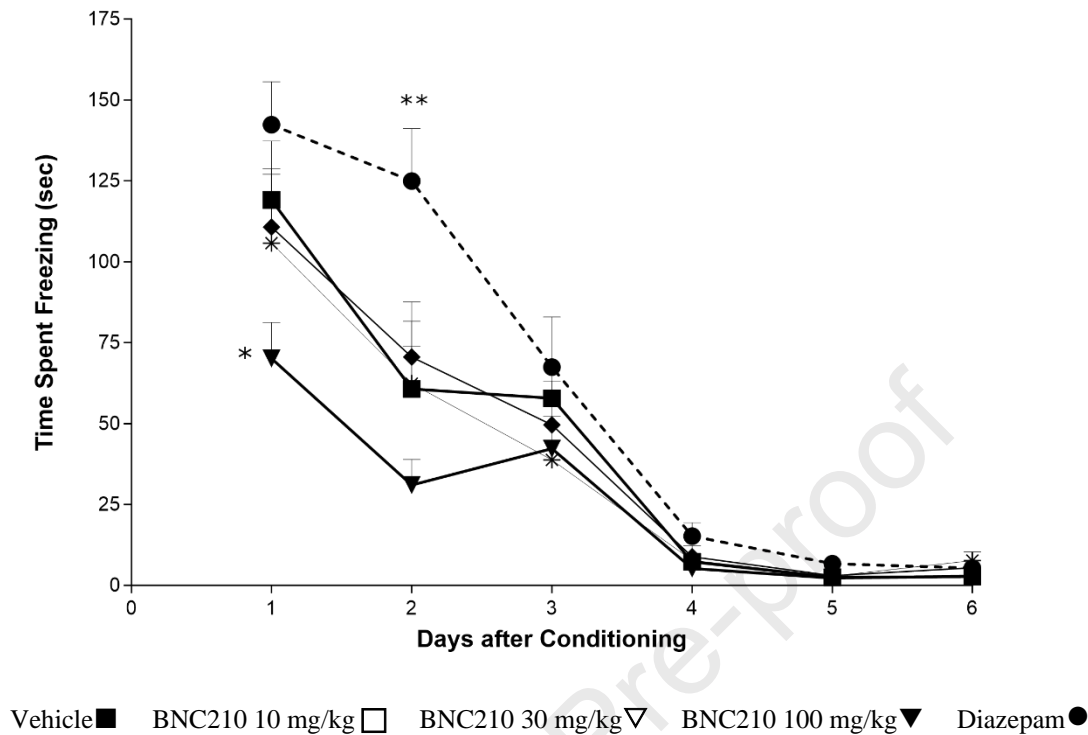
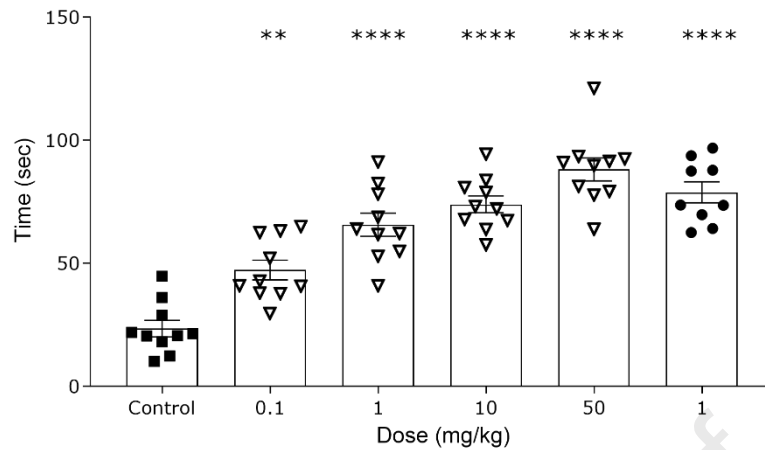


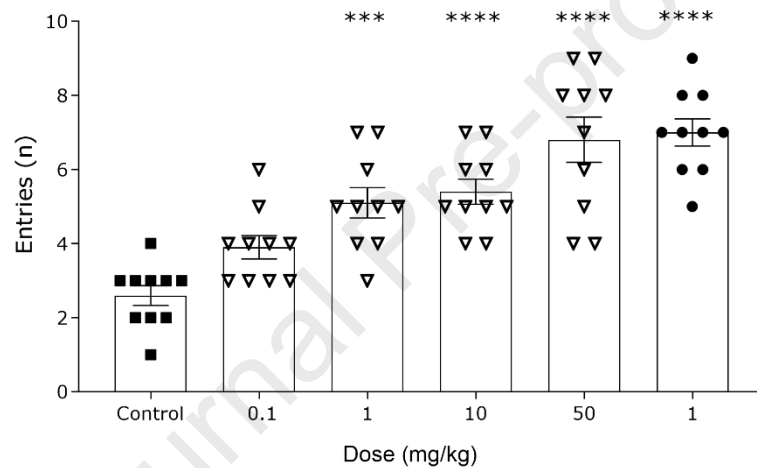
Fig. 5. BNC210 enhanced fear extinction in a conditioned fear extinction paradigm. On Day 1 of the extinction period, mice treated with 100 mg/kg/day of BNC210 showed significant reduction of time spent freezing (70.12 ± 11.21 sec) compared to vehicle control animals (119.10 ± 18.24 sec) (Mean Difference 48.98 sec; 95% CI of difference -95.05 to -2.897; $p \leq 0.05$). This reduction continued as a trend on Day 2 for 100 mg/kg/day of BNC210 (31.0 ± 7.9 sec). Conversely, diazepam treated mice (0.5 mg/kg/day), appeared to experience delayed fear extinction as these animals showed significantly greater freezing behavior on Day 2 compared to control (Day 2: 124.94 ± 16.23 ; Mean Difference 64.22 sec; 95% CI of difference 18.14 to 110.3; $p \leq 0.001$). By Day 4 most of the animals had stopped freezing.

Two-way Repeated Measures ANOVA for CS Freezing Time Across Days: $F=2.64$, $p < 0.0434$ followed by Bonferroni's multiple comparisons test. Data represents Mean \pm SEM. Significant difference to vehicle control * $p \leq 0.05$; ** $p \leq 0.01$.

6A



6B



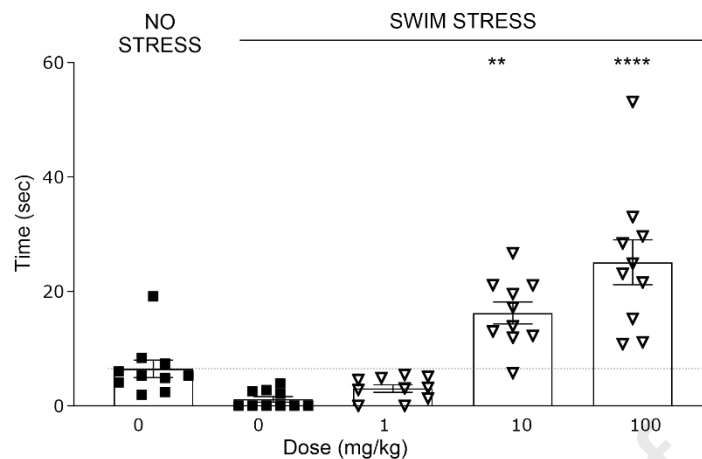
Vehicle ■ BNC210 ▽ Diazepam ●

Fig. 6. BNC210 reduced anxiety-like behavior in the elevated plus maze under basal conditions. (A) Time: Rats treated with BNC210 at 0.1 (47.2 ± 4.0 ; $p=0.0006$), 1 (66 ± 5.0 ; $p<0.0001$), 10 (73 ± 3.4 ; $p<0.0001$) and 50 (88 ± 4.7 ; $p<0.0001$) mg/kg spent significantly more time (sec) on the open arms of the elevated plus maze compared to controls (23 ± 3.3). Ordinary one-way ANOVA: ($F(5, 53) = 33.79$, $p<0.0001$).

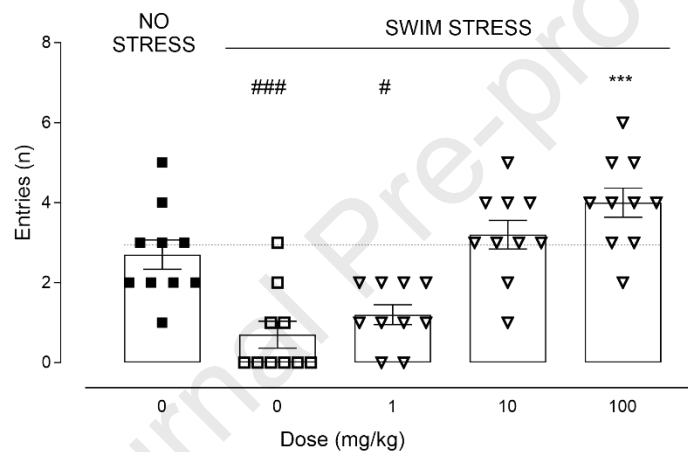
(B) Entries: Rats treated with BNC210 at 1 (5.1 ± 0.4 ; $p=0.0002$), 10 (5.4 ± 0.3 ; $p<0.0001$) and 50 (6.8 ± 0.6 ; $p<0.0001$) mg/kg made significantly more entries into the open arms compared to control animals (2.6 ± 0.3). Diazepam (1 mg/kg) also significantly increased Time (78.8 ± 4.2 ; $p<0.0001$) and Entries (7 ± 0.4 ; $p<0.0001$) compared to controls (\pm). Data represent Mean \pm SEM. $n=9-10$ rats per group. Ordinary one-way ANOVA: ($F(5, 54) = 17.88$, $p<0.0001$).

Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. Significant difference to unstressed vehicle control ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

7A



7B



Non-Stressed Controls ■ Swim-Stressed Controls □ BNC210 + swim stress ▽

Fig. 7. (A, B). The anxiolytic-like activity of BNC210 was maintained in rats exposed to swim stress prior to the EPM. Rats exposed to swim stress showed increased levels of anxious behavior by spending significantly less Time (sec) (1.1 ± 0.5) and making significantly less Entries (n) (0.7 ± 0.3) into the open arms of the EPM compared to unstressed control animals (2.7 ± 0.4).

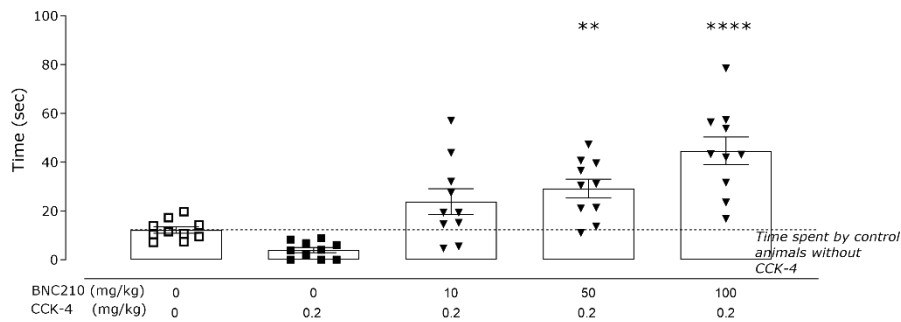
(A) Time: BNC210 at 1 mg/kg (3 ± 0.6) did not reduce the effects of swim stress. However, rats treated with BNC210 at 10 (16.2 ± 2.0 ; $p = 0.0009$) and 100 (25 ± 4 ; $p = 0.0003$) mg/kg, spent significantly more time in the open arms compared to non-stressed controls, demonstrating relief of the increased anxious behavior induced by swim-stress. Ordinary one-way ANOVA: ($F(4,45) = 22.90$, $p < 0.0001$).

(B) Entries: BNC210 at 1 mg/kg (1.2 ± 0.2) did not reduce the effects of swim stress on Entries into the open arms of the maze. However, the dose of 10 mg/kg demonstrated a reversal of the swim stress-induced anxiety by restoring behavior to the level of the unstressed controls (3.2 ± 0.4 , $p = 0.3428$), and 100 mg/kg (4 ± 0.4 ; 0.03) produced a significant reversal of the swim stress behavior. Ordinary one-way ANOVA: ($F(4,45) = 16.66$, $p < 0.0001$).

Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. n=10 rats. Significant difference to unstressed vehicle control *** $p \leq 0.001$; Significant difference between control animals, ## $p \leq 0.01$; ### $p \leq 0.001$. Significant difference to swim-stressed control animals: ## $p \leq 0.01$; ### $p \leq 0.001$.

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7C



7D

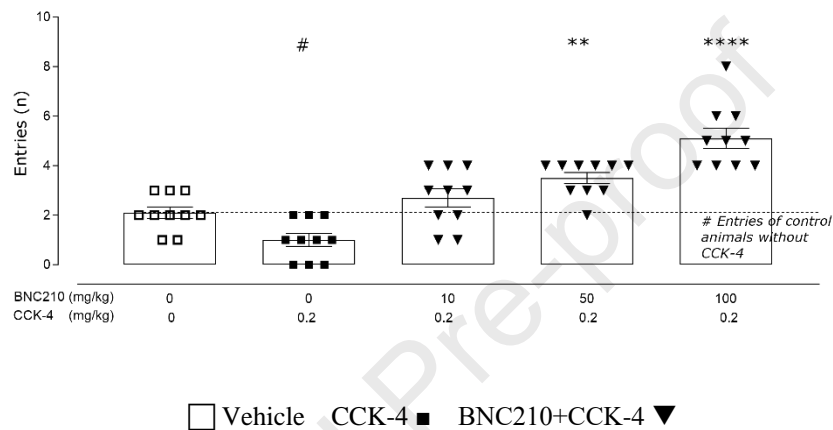


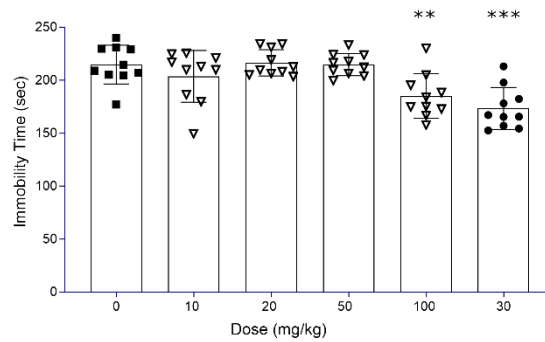
Fig. 7. (C, D). BNC210 produced dose-dependent reductions of CCK-4-induced behavioral effects in rats.

CCK-4 treatment induced anxious behavior as shown by comparing maze performance of control rats administered CCK-4, with control rats receiving vehicle only). **(C) Time (sec):** CCK-4-only treated rats spent less time in the open arms of the maze (4 ± 1) compared to the vehicle control animals (12.2 ± 1.3). Rats treated with CCK-4 and BNC210 at 10 (23.9 ± 5.2), 50 (29.2 ± 3.8) or 100 (44.6 ± 5.7) mg/kg spent increasingly more time (sec) on the open arms of the elevated plus maze compared to the no-CCK-4 control animals (12.2 ± 1.3); 10 mg/kg restored Time to the level of the no-CCK-4 controls, while 50 ($p=0.0133$) and 100 ($p<0.0001$) mg/kg were significantly different. Ordinary one-way ANOVA ($F(4, 45) = 15.83$, $p<0.0001$).

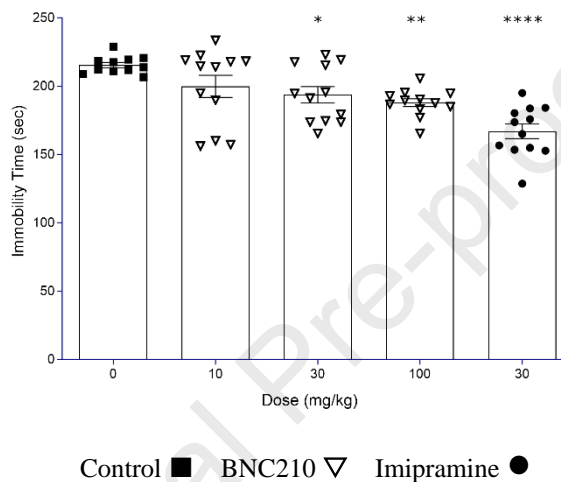
(D) Entries (n): CCK-4-only treated rats made significantly less entries into the open arms (1 ± 0.3) compared to the vehicle control animals (2.1 ± 0.2 ; $p=0.0497$). Rats treated with CCK-4 and BNC210 made dose dependent increases in number of Entries into the open arms at 10, 50 (3.5 ± 0.2) or 100 (5.1 ± 0.4) mg/kg. The 10 mg/kg (2.7 ± 0.4) dose restored Entries to the level of the CCK-4-only control animals (2.1 ± 0.2) while 50 ($p=0.0085$) and 100 mg/kg ($p<0.0001$) significantly increased Entries into the open arms compared to the CCK-4-only control animals (2.1 ± 0.2). Data represent Mean \pm SEM. $n=10$ rats. Ordinary one-way ANOVA ($F(4, 45) = 25.17$, $p<0.0001$).

Dunnett's Multiple Comparisons test. Data represent Mean \pm SEM. $n=10$ rats. Significant difference to vehicle control * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$. Significant difference between CCK-4 treated and vehicle treated control rats; # $p \leq 0.0497$.

8A



8B



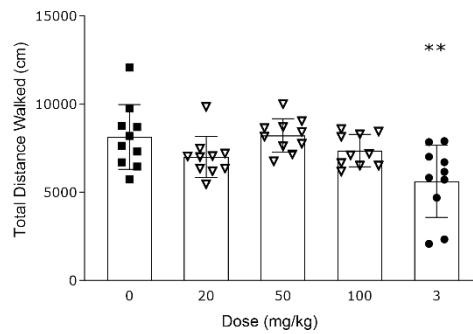
Control ■ BNC210 ▽ Imipramine ●

Fig. 8. (A, B) BNC210 demonstrated antidepressant effects in the forced swim test following single and repeat dosing regimens. (A) Acute dosing: BNC210 was administered to rats at 10, 20, 50 and 100 mg/kg and the positive control was imipramine (30 mg/kg). The highest dose of BNC210 (100 mg/kg) gave a statistically significant reduction in immobility time (184.9 ± 6.6 sec; $p=0.0029$) compared to control rats (214.7 ± 5.8 sec), demonstrating an antidepressant-like effect. Imipramine (30 mg/kg) also significantly reduced immobility time (173.3 ± 6.2 sec; $p<0.0001$). Ordinary one-way ANOVA ($F(5, 54) = 9.728$, $p<0.0001$).

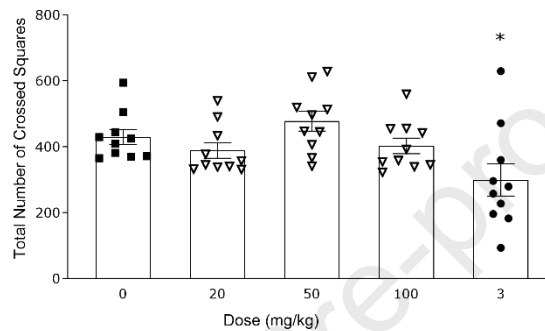
(B) 14-day repeat dosing: BNC210 was administered to rats at 10, 30 and 100 mg/kg/day for 14 days. On day 14, 1 hour after drugs were administered, the animals were tested on the FST. Chronic administration produced some augmentation of the antidepressant effect of BNC210 as demonstrated by a trend towards significant reduction by 30 mg/kg/day (193.7 ± 5.9 sec; $p=0.060$) with 100 mg/kg/day (187.9 ± 2.9 sec; $p=0.009$) producing significant reduction of immobility time compared to control rats (212.5 ± 3.4). The effect of imipramine (167.5 ± 5.3 sec; $p<0.0001$) was also statistically significant compared to controls following repeat dosing. Ordinary one-way ANOVA ($F(4, 55) = 10.54$, $p<0.0001$).

Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. $n=10$ rats (single doses), $n=12$ rats (repeat dosing). Significant difference to vehicle control * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

9A



9B



Control ■ BNC210 ▽ Diazepam ●

Fig. 9. (A, B). BNC210 does not demonstrate sedative or motor-impairing side effects in the Open Field. (A)

Total Walked Distance: Mice treated with BNC210 at 20 (6994±368 cm), 50 (8222±301 cm) and 100 (7361±290 cm) mg/kg did not show significant alterations in total walked distance for any dose compared to vehicle control (8130 ±582 cm) suggesting that BNC210 was not sedating. Diazepam was used as a ‘sedating’ control at 3 mg/kg and demonstrated significant reduction of distance walked (5624±649 cm; p=0.0015). Ordinary one-way ANOVA (F (4, 45) =5.183 p<0.0016).

(B) The number of crossed squares (n) parameter measures the spontaneity of mouse behavior in the open field and may also reflect anxious behavior, particularly when walked distance is not affected. BNC210 at 20 (388±23), 50 (477±30), and 100 (402±23) mg/kg did not reduce the number of squares crossed by mice in the open field compared to control (429±23) which indicated no effects on spontaneous activity, whereas diazepam treated mice crossed significantly less squares (299±49; p=0.0188). Ordinary one-way ANOVA (F (4, 45) =4.341, p=0.005). Dunnett’s Multiple Comparisons test. Data represents Mean ± SEM. n=10 mice. Significant difference to vehicle control * p≤0.05

9C

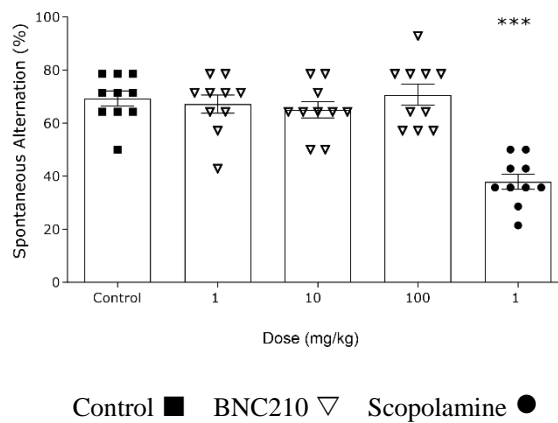


Fig. 9C. BNC210 did not impair working memory in the T-maze Continuous Alternation Task in mice. BNC210 was evaluated in the T-maze at 1, 10 and 100 mg/kg. Positive control mice were treated with scopolamine at 1 mg/kg, a dose previously shown to cause around 50% inhibition of memory (37.9 ± 2.8 ; $p < 0.0001$). The percentage of spontaneous alternation was not significantly different at 1 (67.1 ± 3.4), 10 (65 ± 3.1) or 100 (70.1 ± 3.9) mg/kg doses compared to vehicle controls (69.3 ± 2.8). Ordinary one-way ANOVA ($F(4, 45) = 17.84$, $p < 0.0001$). Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. $n = 10$ mice. Significant difference to vehicle control. **** $p \leq 0.0001$.

9D

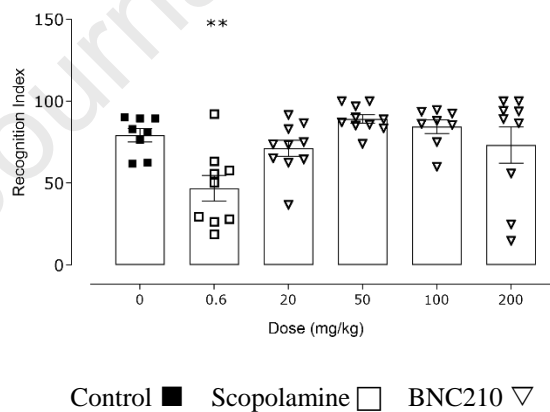
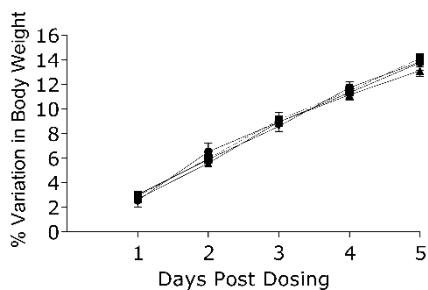


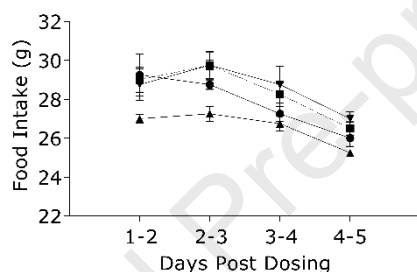
Fig. 9D. BNC210 did not impair short term memory in the rat NOR. The effect of BNC210 on short-term memory was assessed in the rat NOR at doses of 20, 50, 100 and 200 mg/kg PO. Results are expressed as a percentage of control. Positive control rats were treated with scopolamine at 0.6 mg/kg (46.8 ± 7.8), a dose previously shown to cause approximately 50% inhibition of memory in rats. The effects of BNC210 on Recognition Index at 20 (71.2 ± 4.9), 50 (89.1 ± 2.5), 100 (84.4 ± 4.1) and 200 mg/kg (73.2 ± 11.0) were not significantly different to control animals (79.23 ± 4.1) indicating that BNC210 did not impair short term memory in this model, whereas scopolamine caused significant memory impairment (46.9 ± 7.8 ; $p = 0.0051$). Ordinary one-way ANOVA ($F() = 9.404$, $p < 0.0005$).

Dunnett's Multiple Comparisons test. Data represents Mean \pm SEM. n=8-10 rats. Significant difference to vehicle control ** $p \leq 0.0051$.

10A



10B



10C

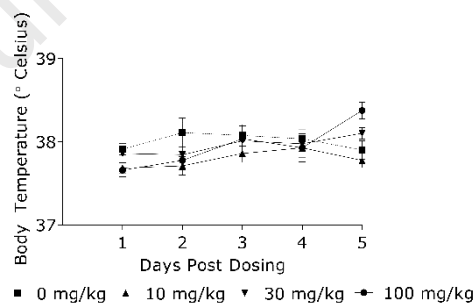


Fig. 10. BNC210 does not produce symptoms of physical dependence in rats. After repeat dosing with BNC210 for 14 days (0, 10, 30 or 100 mg/kg/day) rats were assessed for physical signs of dependence (body temperature, body weight or food intake) over the subsequent 5-day period. Graphs of these data plot treatment versus time and were analysed using two-way ANOVA. BNC210 treated rats did not show any statistical differences compared to vehicle treated animals for weight loss, reduced appetite, or temperature changes, suggesting that BNC210 does not cause physical dependence. Abrupt cessation of BNC210 did not produce any significant changes between the groups for any parameter. Data represents Mean \pm SEM. n=12 rats.

BNC210 - an $\alpha 7$ nAChR negative allosteric modulator identified from a focused library built on a GABA_A active compound:

- is selective for $\alpha 7$ nAChR and demonstrated anxiolytic-like behavior in rodent tests
- has antidepressant-like activity in rodent tests
- enhances fear extinction in mouse model of conditioned fear
- A Phase 2B PTSD trial with BNC210 has recently reported positive results

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

All authors were employed by Bionomics Ltd at the time the work was done, either as full-time employees or under contract.

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