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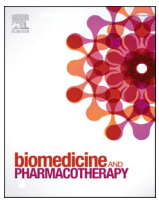

Rescue of respiratory and cognitive impairments in Rett Syndrome mice using NLX-101, a selective 5-HT$_{1A}$ receptor biased agonist

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ABSTRACT

Rett Syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked gene encoding the *methyl-CpG-binding protein 2 (MECP2)*. Impaired function of this transcriptional regulator leads to profound neurological defects, among which respiratory distress, motor function and cognitive disorders are prominent. Despite great advances in understanding RTT neurobiology, therapies that can meaningfully improve patients' symptoms are still needed. Here, we focused on 5-HT$_{1A}$ receptor-mediated serotonergic signaling as a potential therapeutical route for RTT. We report the effects of a drug candidate, NLX-101, a highly selective, biased agonist of 5-HT$_{1A}$ post-synaptic receptors at brainstem and cortical regions, on key phenotypes of RTT. Unrestrained whole-body plethysmography studies confirmed and extended the previous observation that single i.p. administration of NLX-101 dose-dependently reduced the occurrence and length of apneic events in Mecp2$^{tm1.1Bird}$ heterozygous female mice and largely corrected respiratory irregularity. Although no preservation of motor function was observed, early onset chronic administration of NLX-101 entirely prevented the cognitive deficits of the Mecp2$^{tm1.1Bird}$ mice both in the short and the long-term memory paradigms of the Novel Object Recognition upon 10 weeks of treatment, an effect that was maintained throughout animals' age. Similar effects were observed in the Fear Conditioning paradigm, with treated Rett mice performing as well as wild-type controls, highlighting the procognitive properties of NLX-101. This work provides compelling evidence of the therapeutic potential of targeting post-synaptic 5-HT$_{1A}$ receptors to improve cognitive function in patients with RTT while supporting its respiratory-rescue properties.

1. Introduction

Mutations in the X-linked *methyl-CpG binding protein 2* gene, a key regulator of neuronal transcriptomic profile through epigenetic mechanisms [1], are the leading cause of Rett Syndrome (OMIM312750), a severe neurodevelopmental disorder that affects approximately 1 in 10000 girls worldwide [2]. After a seemingly normal development, patients enter a period of rapid developmental regression, characterized by loss of already acquired speech and motor skills, development of stereotypic hand movements, muscle tone and movement abnormalities, as well as seizures and intellectual disabilities, typically with severe cognitive impairments [3,4]. Among RTT core symptoms, breathing abnormalities, characterized by irregular respiratory patterns during wakefulness, breath holding and apnea [5], represent a life-threatening condition. Additionally, because they are associated with significant decreases in blood oxygen saturation, they may further contribute to the aggravation of the remaining neurological symptoms, including the occurrence of seizures, motor deterioration and cognitive decline.

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Current therapies are mostly limited to supportive care and management of individual symptoms [6] and present only a moderate effect, highlighting the importance of seeking novel therapeutic targets and approaches that offer a higher magnitude of response. In this perspective, serotonergic transmission has emerged as a promising target, as it appears to be deficient both in RTT patients [7–9] and in mouse models [10,11]. Among serotonergic receptor subtypes, 5-HT_{1A} receptors play fundamental roles in several physiological functions affected in RTT, including mood, cognition and neuroplasticity [12,13], and are key in breathing termination processes (thought to underlie the breathing defects in RTT), thus rendering 5-HT_{1A} receptors a promising target for Rett Syndrome. However, from the therapeutic point of view, it is vital to identify compounds that specifically activate relevant 5-HT_{1A}R subpopulations, as indistinct activation of pre- and post-synaptic receptors or stimulation of different receptor subpopulations in different brain regions may drive diverging effects, thus dampening the potential therapeutic efficacy of 5-HT_{1A}R activation. Biased agonism has emerged as a promising therapeutic option, as it allows preferential activation of specific receptor subpopulations in certain brain regions that mediate therapeutic actions relevant for the targeted disease [14]. One of these biased agonists, NLX-101 (a.k.a. F15599), is a highly selective agonist of post-synaptic 5-HT_{1A}R [15,16], that preferentially activates serotonergic receptors located in cortical and brainstem areas [17], with marked therapeutic efficacy in multiple animal models of mood and cognition [18–21]. Importantly, administration of NLX-101 has been previously demonstrated to ameliorate breathing deficits in a mouse model of RTT ($Mecp2^{tm1.1Bird}$ mice) [22]. Here, we considerably expanded these studies, by testing a wide range of doses of NLX-101 to evaluate its capacity to achieve more meaningful rescue of breathing dysrhythmias, while assessing its potential to improve key RTT behavioral phenotypes, in light of the potent beneficial effects of NLX-101 in several models of neuropsychiatric and neurological disorders. We used $Mecp2^{tm1.1Bird}$ heterozygous female mice, bearing a large deletion of the *Mecp2* gene [23], whose phenotype mimics several clinical presentations observed in patients. Our data confirmed that single administration of NLX-101 ameliorates respiratory deficits, reducing apnea frequency and length, while correcting the respiratory irregularity pattern. Importantly, the effects of NLX-101 on breathing abnormalities were dose-dependent, with the higher dose almost completely abolishing these defects. This strong effect was accompanied by a complete prevention of the cognitive deficits in this model upon chronic, but not sub-chronic, administration of NLX-101 and with no impact on motor or social memory phenotypes. These results strongly support the therapeutic potential of biased agonism at post-synaptic 5-HT_{1A} receptors for the treatment of RTT symptoms.

2. Materials and methods

2.1. Animal experimentation ethical statement

All the procedures applied to the animals were in accordance with European (Directive 2010/63/EU revising Directive 86/609/EEC on the protection of the vertebrate animals used for scientific purposes) and Portuguese National laws and according to the UK Home Office's Animals (Scientific Procedures) Act (1986). Animal facilities and staff involved in animal experiments were certified by the Portuguese regulatory entity (Direção Geral de Alimentação e Veterinária – DGAV). All protocols were approved by the Animals Ethics Committee of the Life and Health Sciences Research Institute, University of Minho (EM. ICVS-I3Bs.3Bs_012–2021) and by the DGAV (reference 70579) and by the University of Bristol' Animal Welfare and Ethical Review Body. The pharmacokinetic profile and whole-body plethysmography experiments were conducted at the University of Bristol, while behavioral evaluation at the University of Minho.

2.2. Pharmacokinetic profile of NLX-101

2.2.1. Animals

Heterozygous female B6.129P2(C)-$Mecp2^{tm1.1Bird}$/J mice (Jackson Laboratories, USA, strain #003890) (15.0 ± 4.0 months, n = 4 mice per timepoint) (mean ± S.E.M) were used to evaluate the pharmacokinetic profile of NLX-101 after a single administration.

Animals were maintained in a Specific Pathogen-Free (SPF) facility throughout the procedures and underwent regular health screens. Animals were single housed during the study under standard conditions: temperature 20–26ºC, relative humidity 50–70 %, 12 h light: dark cycle with *ad libitum* access to standard rodent diet and water. Water was ultrafiltered before being provided to the animals.

2.2.2. NLX-101 formulation and administration

NLX-101 fumarate (supplied by Neurolixis) was dissolved in saline at a dose of 0.16 mg/kg (all doses are expressed as weight of free base) and administered intraperitoneally (i.p.). Each animal received a single administration of the compound; different mice were used for each of the time points evaluated.

2.2.3. Sample collection

Drug exposure in blood plasma and brain was evaluated over 24 hours. Samples were collected at 0.25-, 0.5-, 1-, 2-, 3-, 4-, 8- and 24-hours post drug administration. Blood samples were collected via cardiac puncture under deep anesthesia (5 % isoflurane; 100 % O2) into pre-chilled BD Microtainer tubes with Microgard closure (BD #365975), spray-coated with K2 EDTA solution (0.8 mg) and placed on ice. After blood collection, brains were harvested and immediately placed on ice. Plasma and brain homogenates processing was performed within 30 minutes from sample collection.

2.2.4. Sample processing

Blood samples were processed for plasma by centrifugation at 4ºC, 4000 rpm for 20 min. The obtained plasma was snap frozen over dry ice and kept at −70 ± 10ºC. Brain samples were homogenized with 3-fold cold 10 mM PBS (w/v) using a Pro Scientific Bio-Gen PRO200 Homogenizer for 2 min on full power and then stored at −70 ± 10ºC.

NLX-101 concentration in biological samples was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at WuXi AppTec facilities (Xangai, China).

2.2.5. Liquid chromatography-tandem mass spectrometry

For plasma samples, a 40 μL aliquot of the sample was protein precipitated with 160 μL of isopropanol and centrifuged at 4000 rpm for 20 min (20–25ºC). A 60 μL aliquot of the supernatant was then mixed with 120 μL of water and centrifuged at 4000 rpm at 4ºC; 7 μL of the sample were injected in the LC-MS/MS (API 4000) instrument for NLX-101 concentration analysis. For brain samples, a 50 μL aliquot of the homogenate was protein precipitated with 200 μL of isopropanol and centrifuged at 4000 rpm for 20 min (20–25ºC). A 60 μL aliquot of the supernatant was then mixed with 120 μL of water and centrifuged for 4000 rpm at 4ºC: 7 μL of the sample were injected in the LC-MS/MS system.

2.3. Breathing analysis

2.3.1. Animals

Female B6.129P2(C)-$Mecp2^{tm1.1Bird}$/J aged 11.0 ± 5.0 months were used in this study. Animals were maintained in a SPF facility throughout the procedures and underwent regular health screens. Animals were single housed during the study under the standard conditions reported above. Water was ultrafiltered before being provided to the animals. Humane endpoints were defined prior to experiment initiation: 20 % body weight reduction, inability to reach food or water, presence of wounds in the body and signs of dehydration. These criteria were never

met during the experiment.

2.3.2. NLX-101 administration

NLX-101 or saline (vehicle) were administered by intraperitoneal injection, at increasing doses of 0.04, 0.16, 0.63 and 2.5 mg/kg. Each dose of NLX-101 was administered with a 48-h minimum washout period (n = 16 animals receiving vehicle and n = 15 animals receiving NLX-101). Animals were randomly assigned to NLX-101 or saline with no cross-over, and the experimenter remained blinded to the treatment conditions. NLX-101 and saline groups were age- and body weight-matched; the mean age and body weight of mice for each tested NLX-101 dose can be found in Table 1.

2.3.3. Plethysmography analysis

Unrestrained whole-body plethysmography (EMKA Technologies, Paris, France) with a bias flow of 0.5 mL/min and continuous chamber temperature and humidity compensation was used to evaluate breathing parameters (apnea frequency, length, successive breaths variation, breathing frequency and tidal volume). All experiments were performed during the lights-on phase. Pairs of animals receiving either saline or NLX-101 were recorded simultaneously. The volume of saline administered was adapted to the volume of NLX-101 solution administered to each animal's counterpart. Briefly, the animals were placed in the plethysmography chamber and allowed to adapt for 20 minutes. Baseline respiratory activity was recorded during the following hour. Animals were removed from the recording chamber for administration of either saline or NLX-101, returned to the chamber and allowed to rest for an additional 20 minutes. Post-treatment respiratory activity was recorded for the following hour.

Breathing parameters of NLX-101 treated animals were normalized to the mean of the vehicle group (post-vehicle administration respiratory activity). Data analysis was automatically performed using the IOX v2.9 software (EMKA Technologies, France) and a custom written script in Spike 2 (CED, Cambridge, UK – script available at [24]) . Breaths were considered valid if the difference between inspired and expired volumes was 30 % or less. The time of expiration (TE) was calculated for all valid breaths (in seconds). The analysis period was then divided into 1-min blocks and an average TE was calculated for each 1-min block. Apneas were defined as breaths with TE longer than 4-times the local average TE (4xTE). Analysis of apneas longer than 1 second was also performed. All identified apneas underwent manual inspection, during which behavioral artifacts such as sniffing were eliminated from the dataset. Successive breaths variation (SBV) was calculated as the root mean square of the differences between the length of successive breaths, as follows:

$$SBV = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n-1}(x_{i+1} - x_i)^2}$$

where n indicates the number of breaths in an epoch, and xi indicates the length of individual consecutive breaths in seconds.

2.4. Effects of NLX-101 on behavior

2.4.1. Animals

Female B6.129P2(C)-Mecp2$^{tm1.1Bird}$/J and sex- and age-matched WT (Jackson Laboratories, USA, strain #00664) animals were used to assess the therapeutic potential of NLX-101 on behavioral performance. Animals were maintained under standard laboratory environmental conditions: 12 h light/dark cycle (lights on from 8 a.m. to 8 p.m.), room temperature 21 ± 1ºC, relative humidity of 50–60 % and water and food *ad libitum*. Humane endpoints were defined before the beginning of the experiment as previously described above; these criteria were never met.

Table 1
Average age and body weight for each group of mice at each of the tested NLX-101 dose.

Dose	0.04 mg/kg		0.16 mg/kg		0.63 mg/kg		2.5 mg/kg	
NLX−101	-	+	-	+	-	+	-	+
Age (weeks)	36.2	34.9	36.9	35.7	37.5	36.3	38.3	37.1
Weight (g)	26.5	27.8	26.6	28	26.5	28.2	26.5	27.7

This study used three distinct groups of animals for the experiments, as outlined below:

(i) To evaluate the effects of chronic NLX-101 administration, mice were randomly assigned to four groups as follows: wild-type (n = 18), Mecp2$^{tm1.1Bird}$ vehicle (n = 20) and Mecp2$^{tm1.1Bird}$ receiving NLX-101 at 2.5 mg/kg (n = 20) and at 5 mg/kg (n = 20); **(ii)** To assess the effects of sub-chronic administration of NLX-101 on cognitive performance, an additional subset of B6.129P2(C)-Mecp2$^{tm1.1Bird}$/J mice (n = 6), along with age- and sex-matched WT controls (n = 15) was used; **(iii)** Finally, a set of WT mice was used to assess the effects of chronic NLX-101 administration on behavioral performance (WT vehicle, n = 10; WT NLX-101 2.5 mg/kg, n = 8).

At the conclusion of the study, all animals were humanely euthanized using the proper method to ensure the subsequent experiments, either by decapitation or exsanguination. In the later, saline infusion was followed by PFA 4 % fixative solution perfusion. The animals were under deep anaesthesia (a mixture of ketamine hydrochloride, 150 mg/kg, and medetomidine, 0.3 mg/kg) during the procedures. Brain samples were harvested and properly stored until further processing (either in fixative solution or frozen at −80ºC).

2.4.2. NLX-101 treatment

Chronic treatment with NLX-101 was initiated at 7 weeks of age and lasted until the animals reached 27 weeks of age. NLX-101 was administered daily via i.p. at two doses: 2.5 mg/kg and 5 mg/kg, diluted in vehicle (saline, NaCl 0.9 %). The subset of wild type animals used to evaluate the effects of chronic administration started treatment with NLX-101 at 13 ± 7 weeks of age. NLX-101 was administered via daily i.p. at 2.5 mg/kg, for 28 days. For the sub-chronic NLX-101 treatment, animals aged 4 ± 2 months were used. NLX-101 was administered via daily i.p. at 2.5 mg/kg, for 7 days. Animals were weighed every week and NLX-101 was prepared weekly adjusting to animals' weight. WT and Mecp2$^{tm1.1Bird}$ vehicle-treated animals received a daily i.p. of saline solution (0.1 mL).

2.4.3. Behavioral phenotype

The effect of chronic NLX-101 treatment on motor, cognitive and social performance was evaluated at two timepoints. Motor evaluation comprised Open field test at 13 weeks of age, motor swimming test and balancebeam walk test at 15 and 24 weeks of age, and rotarod test at age 24 weeks. Cognitive performance was assessed with Novel object recognition at 17 and 22 weeks of age, and with Fear conditioning, at 25 weeks of age. Performance in the 3-chamber sociability test at 14 and 23 weeks of age was used as an indicator of social memory. The behavioral testing protocol is summarized in Fig. 2. The effect of chronic NLX-101 on wild type animals' behavior was evaluated using the Novel object recognition and Open Ffield paradigms following 28 days of NLX-101 treatment.

For the sub-chronic NLX-101 treatment, effects on cognitive performance were assessed using the Novel object recognition which was firstly performed to obtain a baseline measure of cognitive performance and repeated on the 4th and 5th days following treatment.

2.4.4. Open field (OF)

Mice were placed in an open arena (32x32cm) and allowed to freely explore for 10 min. Locomotor activity was automatically tracked using ANY-maze software. Total distance traveled was taken as a measure of spontaneous locomotor activity.

2.4.5. Motor swimming test (MST)

The test was performed as previously described [25]. The animals were trained for two consecutive days (three trials per animal) to transverse a clear Perspex water tank (60 cm long; water temperature at 23ºC) towards a safe platform located at one end of the pool. Mice were tested over the next three days (two trials/animal), and the latency to cross the tank was recorded.

2.4.6. Balance beam walk test (BBT)

The test was conducted as previously described [25]. Mice were trained for three consecutive days to transverse a 12 mm squared beam (two trials/day). On day four, the time taken to traverse the training beam (square beam) was recorded (two trials/animal) and any time the animal stopped was discounted. Animals were allowed to fail twice during the test paradigm, either by falling or turning around on the beam.

2.4.7. Rotarod

Mice were tested in a rotarod apparatus on an accelerating rod (4–40 rpm, 5 min) during three consecutive days (three trials per day with a 15-min rest between trials); the latency to fall from the rod was evaluated (protocol adapted from [26]).

2.4.8. Novel object recognition (NOR)

Mice were first acclimatized to a testing arena × 30 cm) for 3 days during 20 minutes under dim light. Following habituation, mice were presented with two identical objects and allowed to freely explore for 10 minutes. Twenty-four hours later, the animals returned to the arena, where one of the previously presented objects was replaced by a novel one (different in shape, color and texture), and allowed to explore for 10 minutes (long-term memory test). On the next day, animals were again presented with two additional objects, equal between them but differing from the ones previously presented. One hour later, the animals returned to the arena for 10 minutes, with one of the objects replaced by a new one (short-term memory test). Animal's exploratory behavior towards the objects was scored using the Kinoscope software [27]. The discrimination index was calculated based on the following formula [time spent in (novel-familiar)/(novel+familiar) x 100] [28].

2.4.9. Fear conditioning (FC)

Standard operant chambers (20 ×16 x 20.5 cm; Med Associates Inc, Virginia, USA) with a stainless-steel shock grid and a light bulb (CM1820) mounted directly above the chamber were used to conduct the first two trials of the test. On the first day (fear acquisition) animals received three pairs of light and a co-terminating foot shock (2 sec, 0.5 mA) with a 5-min interval between them, following a 3 minutes habituation period to the chamber. Animals were allowed to stay in the chamber for 30 seconds before returning to their home-cage. Twenty-four hours after receiving the shock (context probe – Context A) animals returned to the same cage in the absence of light-shock pairings. Two hours later, the animals were introduced to a modified version of the chamber (Context B), coated with a colored cover and a different scent. The room, experimenters' lab coat and gloves were different from Context A to ensure both contexts had distinct spatial, visual and odor cues. Twenty-four hours later, mice were returned to the same chamber and exposed to the light previously paired with the shock (context cue – Cue). Freezing behavior was defined as the absence of motion for longer than 1 sec and manually scored during 3 minutes for Context A and B and 1 min for the Cue (% freezing Cue = % freezing Cue - % freezing Context B) presentation using the Kinoscope software [27].

2.4.10. 3-chamber sociability and preference for social novelty test (3-CST)

The test was adapted from a previously described protocol [29]. The apparatus consisted of a transparent rectangular arena (56 cm long x 50 cm wide x 39 cm high) divided into three equal chambers with openings (10 cm wide) to allow free arena exploration. The test consisted of four 10-minute trials conducted under red light during the animals' active period (from 8 pm to 8 am). After each 10-minute block, the access to the side chambers was restricted with two doors to confine the test subject to the centre arena and allow for trial condition modifications. In the first trial, the test subject was placed in the centre chamber and allowed to freely explore. In the subsequent trial, two empty round wire cups (9.8 cm high with an 8 cm diameter and several horizontal opened bars spaced 3.7 × 0.5 cm to allow visual, auditory, olfactory and nose contact between animals) were placed in each of the side chambers and the mouse was allowed to freely explore. A weight was placed on top of the wire cups to prevent the mice from climbing on top of the cups. In the third trial block, an unfamiliar mouse (age and sex-matched – novel subject) with no prior contact with the test mouse was placed inside one of the cups. In the last trial of the test, the previous mouse remained enclosed in the cup (now-familiar subject) and a second strange mouse was placed in the previously empty cup (now-novel mouse) to evaluate the social novelty component of the task. The location of the novel and familiar subject was always alternated between testing animals. The amount of time spent interacting with each one of the cups in the last trial of the test was manually scored using the Kinoscope software and used as a measure for social novelty discrimination.

2.5. Western blot analysis

Pre-frontal cortex (PFC) and dorsal hippocampus (dHipp) were homogenized in cold RIPA buffer (NaCl 150 mM, Triton X-100 0.1 %, Sodium deoxycholate 0.1 %, SDS 0.5 %, Tris HCl 50 mM, EDTA 5 mM, EGTA 1 mM) supplemented with protease (Complete; Roche, Switzerland) and phosphatase inhibitors (Cocktail II and III, Sigma-Aldrich, Missouri, USA), followed by 4 cycles of sonication for 14 seconds. The Pierce BCA protein kit (Thermofisher, Massachusetts, USA) was used to determine protein concentration of each sample. The samples were heated at 70°C for 10 minutes and centrifuged for 10 seconds before loading. For each sample, 10 or 20 μg of protein were loaded into SDS-PAGE gels followed by wet transference to PVDF (IPFL0010, Millipore, Germany) membranes. Membranes were incubated with the respective primary antibodies: rabbit $5HT_{1A}R$ (1:1500, ABCAM, ab85615), mouse PSD-95 (1:1500, Invitrogen, MA1–045) and rabbit synaptophysin (1:3000, ABCAM, ab32127). Subsequently, membranes were probed with the respective secondary antibodies: goat anti-mouse (1:10000, LI-COR, 926–68070) or anti-rabbit (1:10000, LI-COR, 926–32211). Antibody affinity was detected by fluorescence. Band quantification was performed using AzureSpot, according to manufacturer's instructions. For total protein level normalization, proteins were detected using a fluorescent dye (TotalStain Q, Azure biosystems, California, USA).

2.6. Statistical analysis

All statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA), and a significance level of $p < 0.05$ was used. The G*Power 3.1.9.2 software was used to calculate sample size, based on a power of 0.8. For continuous variables, normality assumption was verified by qualitative analysis of Q-Q plots and frequency distribution, the z-score of skewness and kurtosis, as well as by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Homogeneity of variances assumptions were assessed by Levene's test. Due to the nature of the model used, and our expectation for great heterogeneity between subjects due to somatic mosaicism of X-inactivation, no outliers were removed. Animals were excluded from the analysis only when the test criteria were not met (a minimum of 20 sec object/animal exploration or absence of overall exploration). Two-tailed unpaired Student's *t*-test or Mann-Whitney *U* test (when data met the normality assumption or not, respectively) and paired Student's *t*-test or Wilcoxon test (when data met the normality assumption or not, respectively) were used for

comparisons of means between two groups (breathing parameters). All other mean comparisons with more than two groups were carried out using a one-way ANOVA followed by Tukey's HSD post-hoc test, or a Kruskal-Wallis test (when data were normally or non-normally distributed, respectively) (NOR, CFC, MST, BBT) or a two-way ANOVA (3-CST). Concerning non-normally distributed data and/or for the comparison of medians of discrete variables across time-points, a Friedman's ANOVA was carried out, with pairwise comparisons through the Kruskal–Wallis statistic test. Statistical analysis is reported in Table S2. Graphical representation was carried out using Graphpad Prism 9, with measures of location and distribution indicated in the figure legends.

3. Results

3.1. Administration of NLX-101 restores breathing patterns in Mecp2 female mice

Breathing abnormalities are among the most distressing features of RTT [5], and are accurately mimicked in mouse models of the disease [30]. Building on previous reports demonstrating that selective 5-HT_{1A}R agonism improved breathing in mouse models of RTT [22], we firstly confirmed the effects of acute NLX-101 administration on reversing the breathing dysrhythmias in Mecp2$^{tm1.1Bird}$ mice and assessed whether these effects were dose-dependent, with increasing doses yielding more pronounced effects.

Time course and exposure of NLX-101 in brain and plasma were first determined in separate pharmacokinetic experiments. Single i.p. administration of NLX-101, at one of the lowest doses used in the follow-up studies (0.16 mg/kg), resulted in similar initial concentrations of the drug in both brain and plasma (0.25 h post-injection), although overall exposure levels in the brain were about 2.8-fold higher than plasma. NLX-101 availability was prolonged in the brain, with the drug exhibiting a half-life three times greater than in plasma (Figure S1, Table S1). These levels correspond to total drug, not corrected for plasma or brain binding.

Acute administration of NLX-101 greatly improved breathing parameters in Mecp2$^{tm1.1Bird}$ mice. We demonstrated that NLX-101 dose-dependently reduced the occurrence of apneic events (breaths with a time of expiration superior to 4-times the local average time of expiration) in Mecp2$^{tm1.1Bird}$ females with an MED of 0.63 mg/kg in comparison to vehicle-treated animals (Fig. 1a) or compared to their own baseline (Figure S2a, Figure S1g). This was accompanied by a significant decrease in the length of the remaining apneas (Fig. 1b, Figure S2b), starting at 0.16 mg/kg. This potent effect was sustained even when examining longer apneas (time of expiration greater than 1 second) (Fig. 1c,e, Figure S2c,e). Importantly, treatment with the higher dose of NLX-101 led to a complete abolishment of these longer apneas in five of the recorded mice. Additionally, NLX-101 administration led to a near complete reversal of the variation in duration of successive breaths (Fig. 1e and Figure S2e). NLX-101 administration at the lower and higher dose led to a decrease in breathing frequency (Figure S4a), while no major impact was found regarding tidal volume, although treatment with the lower dose elicited a decrease on this parameter (Figure S4b). No correlation was found between apnea frequency and animals' age (Figure S3a, b) or body weight (Figure S3c, d).

3.2. Early symptomatic treatment with NLX-101 restores cognitive performance of Mecp2$^{tm1.1Bird}$ mice

In light of the normalizing effects of NLX-101 on breathing parameters, together with the implication of serotonergic dysfunction for RTT pathology [8,10], we next evaluated whether activation of 5-HT_{1A} receptors could ameliorate the behavioral deficits present in these mice. We tested whether treatment with NLX-101 (Fig. 2a), at the dose which most efficaciously alleviated respiratory distress (2.5 mg/kg i.p.) or higher (5 mg/kg i.p.) was able to prevent motor, cognitive and social performance impairments when administered to mice starting at 7 weeks of age.

Mecp2$^{tm1.1Bird}$-vehicle treated female mice exhibited severe short- and long-term memory deficits both at 17 and 22 weeks of age, as demonstrated by a decrease in the discrimination index during Novel object recognition paradigm performance. Early symptomatic NLX-101 administration prevented the cognitive impairments of Mecp2$^{tm1.1Bird}$ females in this task, as demonstrated by an increase in the discrimination index between the novel and familiar objects while performing both the short- and the long-term paradigms (Fig. 2b), in comparison to the vehicle-treated animals. Improvement of the memory deficits was observed after 10 weeks of treatment (i.e., at age 17 weeks) and was sustained over time at the next measure (i.e. at age 22 weeks). Both doses of NLX-101 achieved significant improvement of the discrimination index. Notably, for long-term memory, at the latter timepoint tested, NLX-101 returned the discrimination index to levels that were not significantly different to those of WT mice. Importantly, chronic administration of NLX-101 had no impact on wild-type cognitive performance, highlighting that the observed beneficial effects are specific to Rett mice (Figure S6).

During Fear conditioning test performance, heterozygous vehicle-treated females exhibited a decreased freezing response both when re-exposed to the context previously paired with a noxious stimulus (Fig. 2c – Context A) or to the light cue paired with the foot shock (Fig. 2c – Cue) in comparison to WT animals, indicative of cognitive impairments. NLX-101 treatment restored the context-induced freezing behavior to WT levels and partially rescued the cue-induced freezing behavior of Mecp2$^{tm1.1Bird}$ mice, suggesting preservation of cognitive function.

These beneficial effects of NLX-101 on cognitive performance occur independently of any impact on the levels of 5-HT_{1A} receptor and synaptic proteins – PSD-95 and synaptophysin - in key memory-related brain areas, as the dorsal hippocampus (Figure S7a-d) and the prefrontal cortex (Figure S7e-h).

3.3. Treatment with NLX-101 did not impact Mecp2$^{tm1.1Bird}$ mice motor and social impairments

NLX-101 did not improve motor phenotypes of Mecp2$^{tm1.1Bird}$ mice, as observed by a similar performance to the vehicle-treated mice in the motor swimming test (Fig. 3a), in the balance beam test (Fig. 3b) and in the rotarod task (Fig. 3c). Similar findings were observed regarding social memory preference, where treatment with NLX-101 was unable to improve 3-chamber sociability test performance (Fig. 3d), with both vehicle- and NLX-101-treated animals being unable to distinguish between a familiar and a novel conspecific mouse.

In summary, acute 5-HT_{1A}R selective agonism strongly ameliorated breathing dysrhythmias of Mecp2$^{tm1.1Bird}$ females, while chronic administration of NLX-101 largely prevented the emergence of cognitive deficits in this model, confirming its potential for Rett Syndrome treatment.

3.4. Sub-chronic NLX-101 administration had no impact on Mecp2$^{tm1.1Bird}$ mice cognitive performance

Given the striking beneficial effects of chronic NLX-101 treatment on cognitive performance, we next sought to evaluate whether sub-chronic NLX-101 administration for 5 days was able to recapitulate the observed effects in the Novel object recognition task. Female Mecp2$^{tm1.1Bird}$ mice performed significantly worse than their WT counterparts, as observed by a decrease in the discrimination index both in the short- and long-term memory components of the task at baseline period (Fig. 4, left panel of the graphic). Sub-chronic NLX-101 treatment failed to improve animals' cognitive deficits, as observed by a similar discrimination index following treatment to the baseline levels (right panels of the graphic). Sub-chronic administration of NLX-101 did not impact wild type

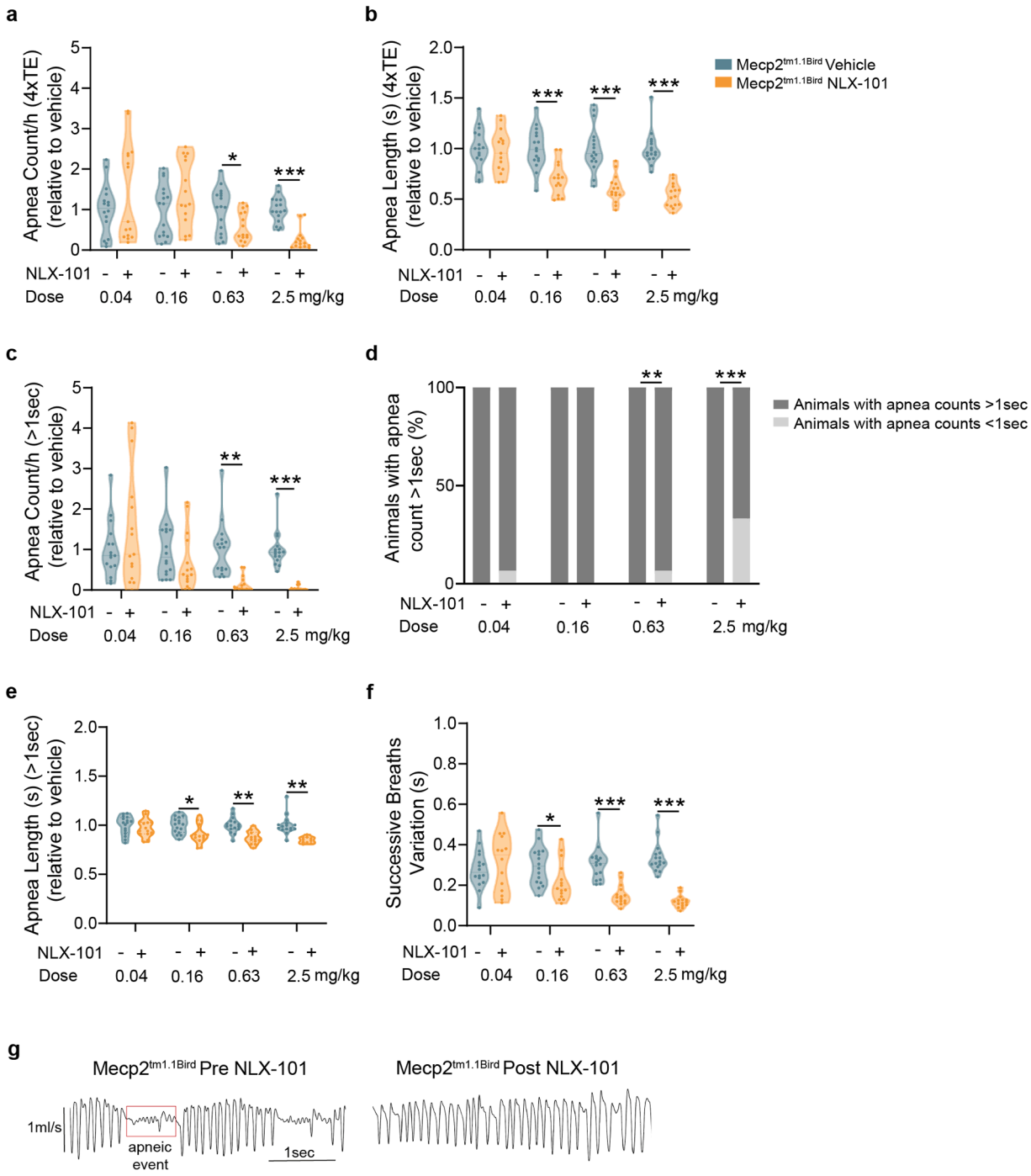

Fig. 1. Administration of NLX-101 via i.p. dosing improves breathing abnormalities in Mecp2$^{tm1.1Bird}$ heterozygous females. (a) An apneic event was defined as a breath with a time of expiration longer than 4-times the local average time of expiration (4xTE). Acute treatment with NLX-101 elicited a decrease in the frequency of apneas at the 0.63 mg/kg dose or higher [0.63 mg/kg: Z: −2.272, $p = 0.023$; 2.5 mg/kg: Z = -3.408, $p < 0.001$]. (b) Length of the remaining apneas was also diminished by treatment with NLX-101 [0.16 mg/kg: t(14) = 4.976, $p < 0.001$; 0.63 mg/kg: t(14)= 5.016, $p < 0.001$; 2.5 mg/kg: Z = -3.408, $p < 0.001$]. (c) Administration of NLX-101 attenuated even the longer apneas (time of expiration longer than 1 second), decreasing their occurrence [0.63 mg/kg: Z = -3.238, p=0.001; 2.5 mg/kg: Z = -3.408, $p < 0.001$] or (d) even abolishing their occurrence in some animals treated with the higher doses [0.63 mg/kg: Z = -3.238, p=0.001; 2.5 mg/kg: Z = -3.408, $p < 0.001$]. (e) Similar effects were observed regarding these apneas length [0.16 mg/kg: t(14) = 2.680, $p = 0.018$; 0.63 mg/kg: t (13) = 3.646, $p = 0.003$; 2.5 mg/kg: Z = -2.701, $p = 0.007$] (f) Breathing irregularity was also partially corrected by the treatment, as represented by a decrease in the variation between successive breaths [0.16 mg/kg: Z = -2.159, $p = 0.031$; 0.63 mg/kg: Z = -3.351, $p < 0.001$; 2.5 mg/kg: Z = -3.409, $p < 0.001$]. (g) Examples of a breathing trace from the same mouse before and after NLX-101 2.5 mg/kg administration; for a clear visualization, original traces were transformed using Adobe Illustrator, with Image Trace Tool, Sketched art preset. Individual data points are overlaid to show the raw data distribution within each category. A total of 16 animals receiving vehicle and 15 animals receiving NLX-101 were used. Data spread and density are depicted through the height and width of the violins, respectively. *p < 0.05, * * p < 0.01, * **p < 0.001.

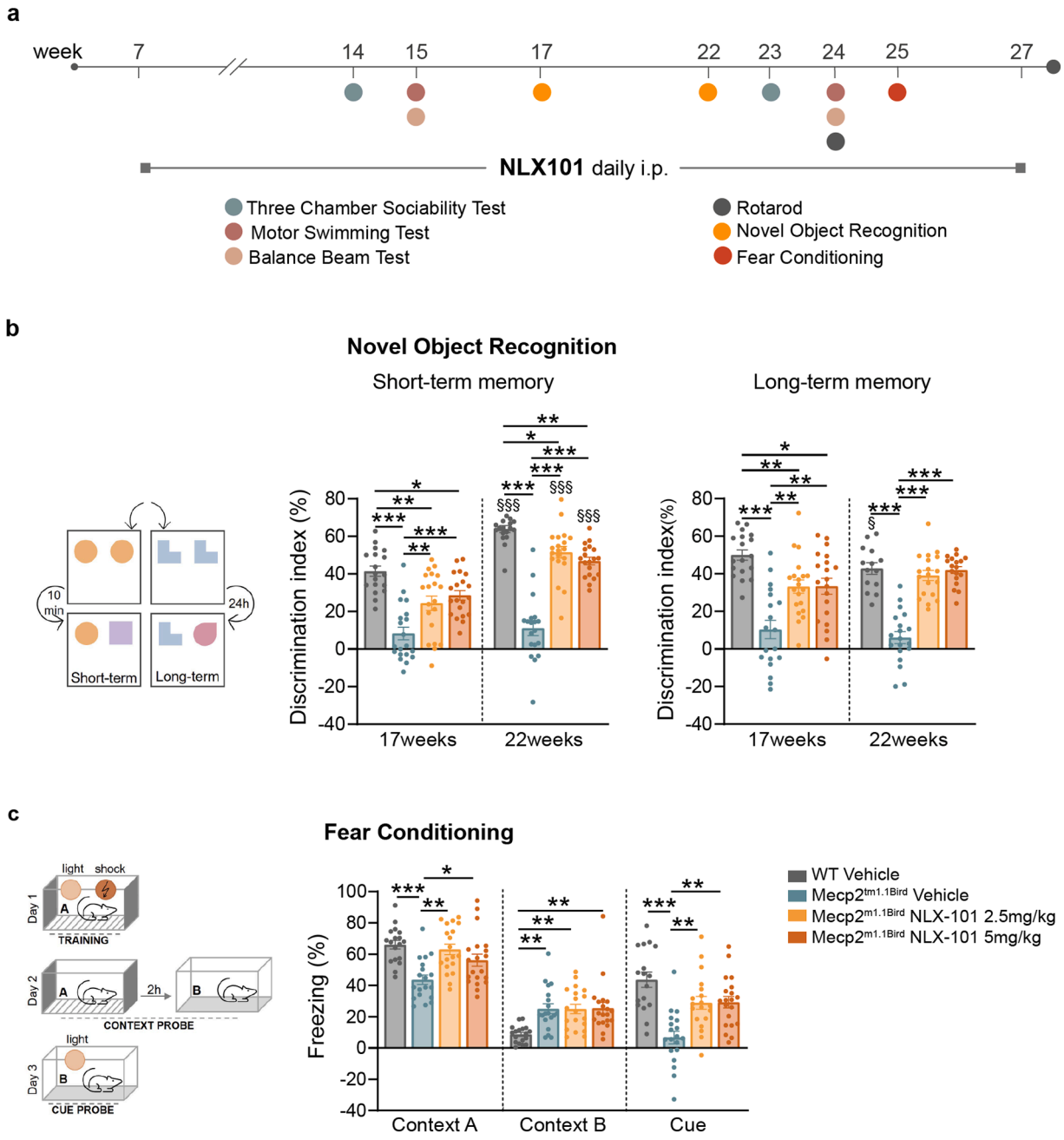

Fig. 2. Treatment with NLX-101 prevents cognitive deficits in Mecp2$^{tm1.1Bird}$ mice. (a) Daily administration of NLX-101 (2.5 and 5 mg/kg i.p.) started at 7 weeks of age and motor, cognitive and social performance were evaluated at two different timepoints. (b) Administration of NLX-101, at both doses tested, prevented the cognitive deficits of Mecp2$^{tm1.1Bird}$ mice, when performing the short- [17 weeks: $F_{(3,74)}= 18.063$, $p < 0.001$; 22 weeks: $F_{(3,72)}= 60.195$, $p < 0.001$] and the long-term [17 weeks: $F_{(3,73)}= 16.252$, $p < 0.001$; 22 weeks: $F_{(3,66)}= 43.680$, $p < 0.001$] component of the Novel object recognition task, as indicated by an increase in the discrimination index. **(c)** Mecp2$^{tm1.1Bird}$ females exhibited a decreased freezing response both in the context and to the cue (Context A and Cue, respectively) associated with the foot shock [Context A: $F_{(3,72)}= 8.964$, $p < 0.001$; Cue: $F_{(3,71)}= 13.012$, $p < 0.001$], that is rescued by NLX-101; all animals exhibited an overall reduction in their freezing behavior in the Context B [$F_{(3,72)}= 7.262$, $p < 0.001$], not associated with any external stimulus. A total of 18–20 animals per experimental condition was used. Data presented as mean±SEM. * represents differences among groups for each tested age whereas differences between the same groups across ages are represented as §. * ,§ p < 0.05; * * p < 0.01; * ** , §§§ p < 0.001.

animals' performance.

4. Discussion

Despite major breakthroughs in the understanding of RTT pathophysiology, currently available treatments fail to offer a substantial improvement of patients' symptoms [31]. The demonstration that restoring the expression of *Mecp2* in adult symptomatic mice reverses their phenotype [32] fostered the prospect that gene therapy and editing strategies could eventually cure RTT. However, this requires complex genetic fine-tuning, as overexpression of Mecp2 also causes neurodevelopmental deficits, and the therapeutic means to achieve such fine tuning are still in their infancy. These strategies will therefore not be available in the near future and will likely be restricted to a small minority of patients [33,34]. Such considerations highlight the importance of finding efficacious pharmacological treatments that can improve patients' quality of life. Although the US FDA recently approved trofinetide (DAYBUE®) for the treatment of RTT, this modified peptide of the

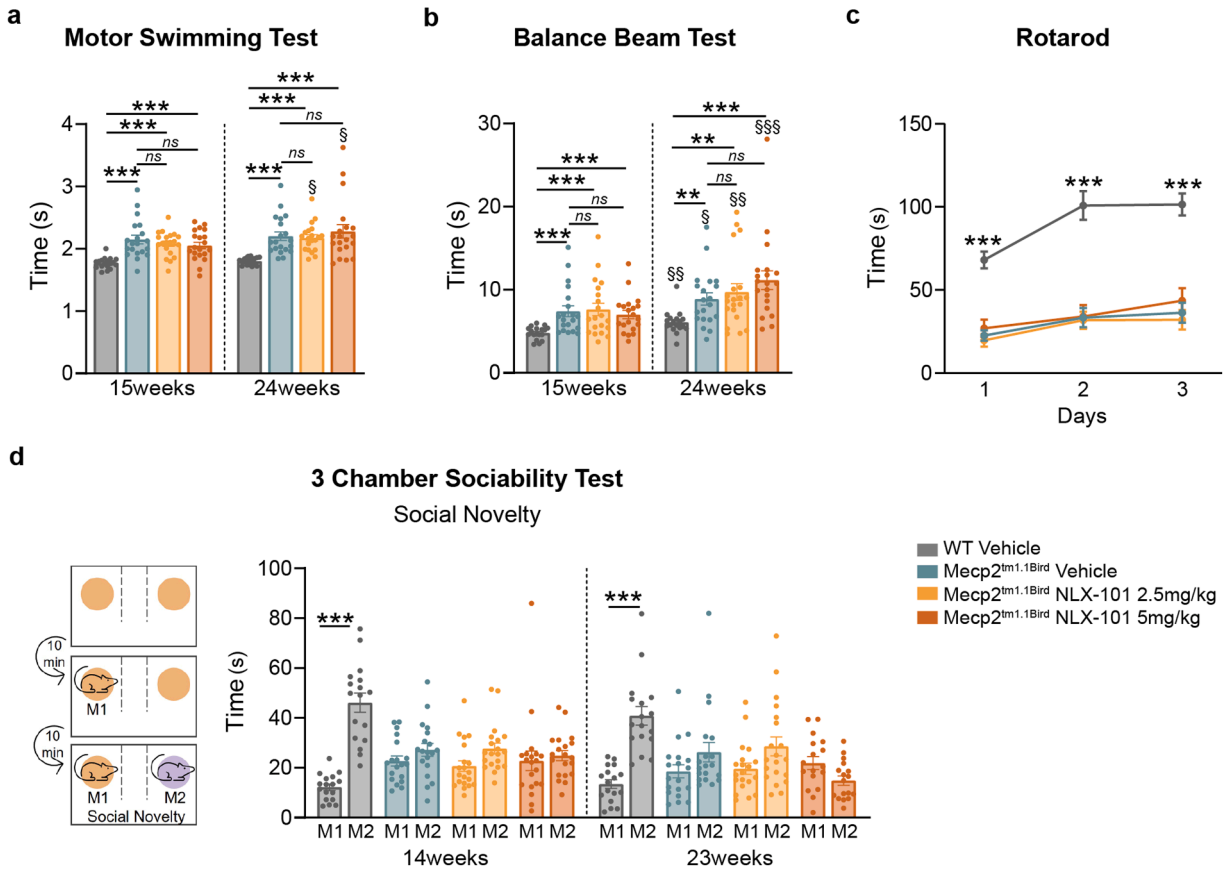

Fig. 3. NLX-101 administration exerted no beneficial effect on animals' motor or social performance. (a) Mecp2$^{tm1.1Bird}$ vehicle-treated females take longer to cross a swimming platform [15 weeks: $F(3,74)= 10.031$, $p < 0.001$; 24 weeks: $\chi^2(3)= 33.377$, $p < 0.001$] and to (b) cross an elevated beam [15 weeks: $\chi^2(3)= 19.074$, $p < 0.001$; 24 weeks: $\chi^2(3)= 20.543$, $p < 0.001$] towards a safe space than WT animals, which is not improved by NLX-101 administration, regardless of tested dose or animals' age. (c) Latency to fall from the rotarod apparatus is also not improved by NLX-101 administration [$\chi^2(2)= 37.026$, $p < 0.001$]. (d) As observed by similar times exploring the novel and the familiar conspecific mice (represented as M2 and M1, respectively), lack of preference for social novelty [14 weeks: $F_{(3144)}= 14.805$, $p < 0.001$; $F_{(3138)}= 4.611$, $p = 0.004$] was not rescued by NLX-101 administration, at any of the tested ages. A total of 18–20 animals per experimental condition was used. Data represented as mean±SEM. *represents differences among groups for each timepoint whereas differences between the same group across the two tested ages are represented as §. § $p < 0.05$; * *,§§ $p < 0.01$; * ** , §§§ $p < 0.001$.

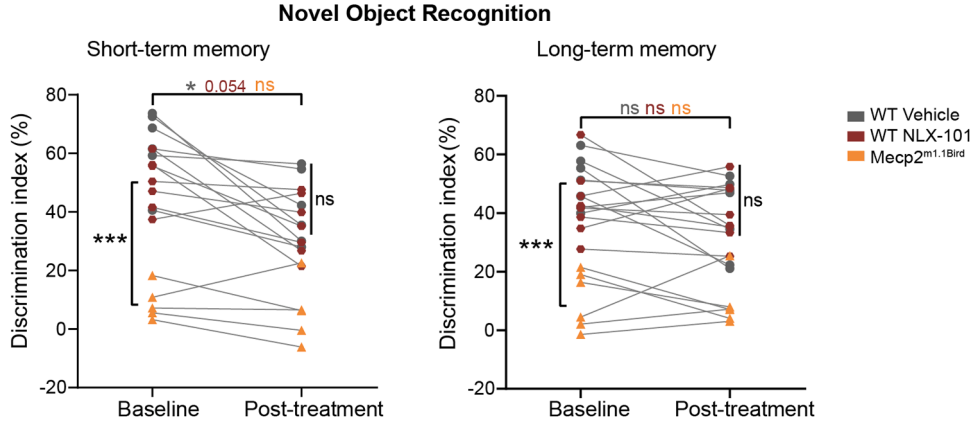

Fig. 4. Sub-chronic NLX-101 administration had no impact on Mecp2$^{tm1.1Bird}$ or wild-type female mice cognitive performance. Mecp2$^{tm1.1Bird}$ mice performed significantly worse than WT vehicle mice in baseline conditions, both in the short-term [$t(16) = 8.255$, $p < 0.001$] and in the long-term memory [$t(10.078) = 7.564$, $p < 0.001$] paradigms of the Novel object recognition test (left panel of the graphic under baseline). NLX-101 administration for 5 days failed to revert these cognitive deficits (right panel of the graphic under post-treatment). Sub-chronic administration of NLX-101 had no impact on the cognitive performance of WT animals (right panel of the graphic under post-treatment). A total of 13–15 wild-type and 5–6 Mecp2$^{tm1.1Bird}$ mice was used. Data represented as mean±SEM. * $p<0.05$; *** $p<0.001$.

N-terminal tripeptide cleavage product of insulin-like growth factor-1 (IGF-1) has only modest therapeutic efficacy [35,36], with 61 % of the patients experiencing no benefit, and it is associated with gastrointestinal side effects in the majority of patients [35,36]. In this context, reports showing that stimulation of serotonergic neurotransmission either with Selective Serotonin Reuptake Inhibitors (SSRIs) or 5-HT receptor agonists ameliorates deficits in *Mecp2* mice [22,37–40] draw attention to the therapeutic potential of targeting the serotonergic system in RTT. Accordingly, some case reports show that SSRIs may be effective in ameliorating psychiatric, behavioral, breathing and motor symptoms in RTT patients [41,42]. Nevertheless, lack of discrimination between pre- and post-synaptic serotonergic receptors by agonists may be a limiting factor in their therapeutic efficacy, and thus, compounds with selective affinity for specific receptors subtypes and locations hold promising potential.

4.1. NLX-101 rescues breathing deficits in RTT mice

Previous studies performed by Levitt et al. [22] demonstrated that acute administration of NLX-101 ameliorated the breathing phenotypes of Mecp2$^{tm1.1Bird}$ mouse models. Here, we confirmed NLX-101's efficacy in ameliorating breathing abnormalities, demonstrating that acute treatment with NLX-101 is sufficient to reduce the apnea frequency and duration as well as inter-breath irregularities in Mecp2$^{tm1.1Bird}$ mice. We demonstrated that these beneficial effects were dose-dependent, with no meaningful effect at the lower doses, but significant and quite striking beneficial effects at the higher doses used, an effect that was maintained even when looking at long-lasting apneas, which are more likely to produce clinically relevant oxygen desaturations. Notably, these beneficial effects occurred independently of changes in mice locomotor activity, strengthening the notion that NLX-101 acts directly on breathing circuitry, rather indirectly improving breathing as a result of improved locomotion. This is supported by reports demonstrating that the breathing defects seen in RTT are linked to difficulties in terminating inspiration, an event dependent on 5-HT$_{1A}$R [43,44]. Additionally, administration of a 5-HT$_{1A}$R antagonist induced apneas in C57BL6/J mice, underscoring the intrinsic involvement of 5-HT$_{1A}$R in maintaining the stability of the breathing rhythm [45]. 5-HT$_{1A/7}$ receptor agonism has also been shown to decrease apneas in Mecp2 females [46], while treatment with other 5-HT$_{1A}$ receptor agonist, sarizotan, reduced apnea and improved breathing regularity in RTT mice [40]. Likewise, case reports support the use of buspirone for breathing dysfunction in RTT patients [47]. However, since SSRIs rely on the availability of serotonin, reported to be reduced in the brain of RTT patients [48], one can speculate that the beneficial effects of these compounds may only reach a modest threshold. Furthermore, previous 5-HT agonists lack target selectivity (sarizotan), or only activate partially 5-HT$_{1A}$ receptors (buspirone), which can limit their potential therapeutic effect. Indeed, a phase III clinical trial failed to demonstrate a beneficial impact of sarizotan in breathing difficulties in RTT patients. Together, this data reinforces the need for a potent and selective full agonist of 5-HT$_{1A}$ receptors, such as NLX-101, to achieve maximal therapeutic benefit.

4.2. NLX-101 exerts pro-cognitive effects in RTT mice

In addition to restoring breathing defects, chronic administration of NLX-101 completely prevented the cognitive deficits present in the Mecp2$^{tm1.1Bird}$ strain, independently of age. In both paradigms, the two tested doses of NLX-101 were equally effective at preventing the emergence of cognitive deficits in this model. Importantly, as NLX-101 had no effects on motor behavior, we can untangle the beneficial effects on cognition from a potential change in motor behavior. These observations are in line with previous reports indicating that acute administration of NLX-101 improved the performance of healthy rats in the Object pattern separation task, an effect that was maintained upon repeated administration and also observed in aged rats [49,50]. Likewise, NLX-101 attenuated deficits in working, reversal and reference memory induced by phencyclidine in the hole board task, [18,20] or induced by scopolamine in the delayed non-matching to position model of cognition [51]. Moreover, NLX-101 treatment rescued chronic stress-induced impairments in the Novel object recognition task [19], indicating that NLX-101 can ameliorate cognitive deficits through different mechanisms. Importantly, these effects were not shared by the non-biased 5-HT$_{1A}$R agonist 8-OH-DPAT, or by the preferential presynaptic 5-HT$_{1A}$R-biased agonist, F13714 [49], pointing towards a specific role of cortical post-synaptic 5-HT$_{1A}$R in cognitive performance. In contrast to reports demonstrating that a single NLX-101 injection is sufficient to induce pro-cognitive effects in other models, sub-chronic administration of NLX-101 in fully symptomatic RTT mice failed to recapitulate the beneficial effects on cognitive performance upon chronic treatment. This likely translates into an involvement of different pathological mechanisms in the case of Rett Syndrome, and/or suggests that treatment needs to be initiated at younger ages to sustain beneficial effects. Of note, few other compounds tested in RTT mouse models improve their cognitive performance to this extent [52–55], supporting the importance of post-synaptic 5HT$_{1A}$R as drug targets and expanding the therapeutic options for the treatment of cognitive deficits in RTT patients.

NLX-101 chronic treatment did not exert any effects on motor deficits present in *Mecp2* females. While treatment with fluoxetine or citalopram has been shown to improve motor deficits in *Mecp2* mice [37], an effect that was blocked by inhibition of 5-HT synthesis, supporting a role of serotonergic system for this effect, the absence of beneficial effects of NLX-101 likely results from its preferential activity in brain areas that are not as principally relevant for motor control. Accordingly, a related compound, NLX-112, which is also a selective 5-HT$_{1A}$ receptor agonist with biased activity for motor regions, improved motor control in models of Parkinson's disease [56]. NLX-101 administration was also not able to rescue social memory deficits, indicating that probably other circuits are also at play for this aspect of Rett model's phenotype.

4.3. Potential mechanisms of action underlying beneficial effects of NLX-101 on cognitive performance

Although the mechanisms underlying the unique profile of action of NLX-101 remain to be fully elucidated, it is known that its systemic administration results in an increased electrical activity of pyramidal neurons in the pre-frontal cortex (PFC). This likely arises from preferential activation of 5-HT$_{1A}$R expressed by GABAergic interneurons by NLX-101, thus decreasing its activity and, consequently, disinhibiting downstream glutamatergic pyramidal neurons, resulting in a net increase of their electrical tone [17,57].This is consistent with the increased firing rate of PFC pyramidal neurons upon systemic administration of NLX-101 [17], and with previous studies implicating cortical pyramidal activity in procognitive effects [58,59]. This hypothesis aligns with reports of cortical hypofunction in Mecp2-null mice, likely attributed to an inhibitory shift in the balance of synaptic excitation and inhibition, resulting in a reduced net cortical activity [60,61]. This is further supported by the notion that activation of pyramidal neurons in mPFC improves Rett Syndrome mice cognitive performance [61]. The observed effects seem to be solely dependent on receptor activity, as no alterations in 5-HT$_{1A}$ receptor expression were found in memory-related brain regions. Unlike previous reports demonstrating that NLX-101 administration produces long-term effects on neuronal plasticity by regulating synaptic proteins expression [12,62] in addition to the modulation at the functional circuit level, in our case, we found no evidence of such effects. Although this is not surprising, as no defects in the expression of these proteins were present in Mecp2$^{tm1.1Bird}$ mice, it further supports the notion that different mechanisms underly the cognitive deficits present in Rett mice. Several other findings suggest that, in addition to symptomatic improvement of neurological function and modulation of synaptic plasticity, NLX-101 also has the potential to

impact long-term neuronal function by stimulating neurogenesis and neuronal remodeling as well as the expression of neurotrophic factors [12,49,63]. Although not tested in the context of this work, we can speculate that a combination of these effects is likely contributing to the beneficial impact of NLX-101 in Rett Syndrome mice. In summary, the present study confirmed that the 5-HT_{1A} receptor-biased agonist, NLX-101, improved breathing defects in a female mouse model of RTT while demonstrating that it completely prevented the cognitive deficits of this model. Importantly, although current commercialized 5-HT_{1A} agonists (such as buspirone and tandospirone) are characterized by low efficacy, lack demonstrated biased signaling, and possess poor selectivity profile, they are generally well tolerated [64,65], supporting the development of improved 5-HT_{1A} agonists to the clinic.

The present findings provide a rationale for targeting post-synaptic 5-HT_{1A} receptors as a treatment for RTT and, potentially, other intellectual disability syndromes involving serotonergic dysfunction.

Author contributions

Conceptualization: A.P.S.A, M.S.K, A.N.-T, S.D.-S, P.M, D. M.-F; Data curation: A.P.S.A, I.C, D. M.-F; Formal analysis: A.P.S.A, I.C, S.D.-S, D. M.-F; Funding acquisition: A.P.S.A, M.S.K, A.N.-T, P.M; Investigation: D. M.-F, I.C, S.G, D.C.-G, J. P.-S, S.O, S.D.-S; Methodology: A.P.S.A, M.S.K, A.N.-T, S.D.-S, P.M, D. M.-F; Project administration: A.P.S.A, M.S.K, A. N.-T, S.D.-S, P.M; Resources: A.P.S.A, M.S.K, A.N.-T, S.D.-S, P.M, A.T.-C; Software: A.P.S.A; Supervision: S.D.-S; P.M, A.P.S.A; Validation: all authors; Visualization: D. M.-F; Writing – original draft: D. M.-F; Writing – review and editing: A.P.S.A; M.S.K; A.N.-T; M.A.V, S.D.-S; P.M

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CRediT authorship contribution statement

Kleven Mark S.: Writing – review & editing, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Varney Mark A.:** Writing – review & editing, Validation. **Oliveira Stéphanie:** Investigation, Validation. **Pereira-Sousa Joana:** Investigation, Validation. **Cunha-Garcia Daniela:** Validation, Investigation. **Guerreiro Sara:** Validation, Investigation. **Maciel Patricia:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Charles Ian:** Validation, Investigation, Formal analysis, Data curation. **Duarte-Silva Sara:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Monteiro-Fernandes Daniela:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization, Writing original draft, Data Curation, Visualization. **Abdala Ana P. Sheikh:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Newman-Tancredi Adrian:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Teixeira-Castro Andreia:** Resources, Validation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adrian Newman-Tancredi reports a relationship with Neurolixis that includes: employment and equity or stocks. Mark S. Kleven reports a relationship with Neurolixis that includes: employment and equity or stocks. Mark A. Varney reports a relationship with Neurolixis that includes: employment and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.117989.

Data availability

All data is available in the manuscript or supplementary files.

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