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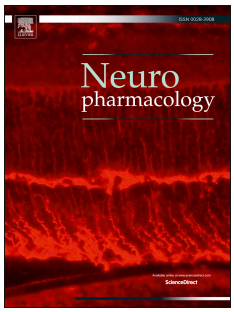

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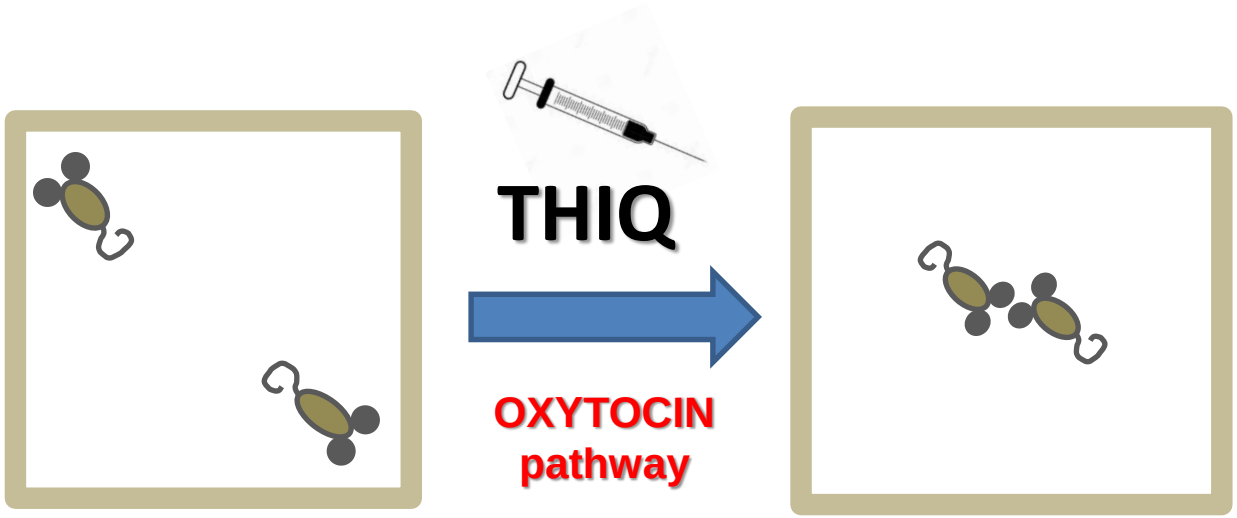
THIQ
OXYTOCIN
pathway

ACCEPTED MANUSCRIPT

Melanocortin 4 receptor stimulation improves social deficits in mice through oxytocin pathway

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ABSTRACT

Several studies on humans and mice support oxytocin's role in improving social behaviour, but its use in pharmacotherapy presents some important limiting factors. To date, it is emerging a pharmacological potential for melanocortin 4 receptor (MC4R) agonism in social deficits treatment. Recently, we demonstrated that the deletion of the *NFKB1* gene, which encodes the p50 NF-κB subunit, causes impairment in social behaviours, with reductions in social interactions in mice. In this work, we tested the acute effects of THIQ, a selective melanocortin 4 receptor (MC4R) agonist. THIQ treatment increased social interactions both in wild type and $p50^{-/-}$ mice. In particular, after treatment with THIQ, $p50^{-/-}$ mice showed a prosocial behaviour analogous to that of basal WT mice. Moreover, intranasal treatment with an oxytocin antagonist blocked social interactions induced by THIQ, demonstrating that its prosocial effects are mediated by the oxytocin pathway. The data obtained reinforce using MC4R agonists to ameliorate social impairment in NDDs.

KEYWORDS: THIQ, melanocortin 4 receptor, oxytocin, p50 KO mouse, social behaviour

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1. Introduction

Social interaction is a primary and adaptive behavioural component of mammalian species. For social animals, the interaction is important for the organization and stability of societies to define social hierarchy and for mate choice (Berry and Bronson, 1992). Several neurodevelopmental disorders (NDDs) such as schizophrenia and autism are characterized by social behavioural deficits. NDDs include developmental brain dysfunction characterized by neuropsychiatric deficits generating impairments in motor function, learning, verbal or non-verbal communication, social, or occupational functioning. All these phenotypic alterations have neuroanatomical basis. Indeed, increased cortical layers thickness, an abnormal columnar organization, cortex misconnections are often associated with NDDs (Innocenti et al., 2003; Budday et al., 2015). Recently, we observed specific neurodevelopmental alterations in a genetic mouse model, namely the NF-κB p50 knock out (p50 KO) mouse (Bonini et al., 2016). p50 KO mice were generated by targeted deletion of the *NFKB1* gene that encodes for the precursor of the p50 NF-κB subunit. NF-κB is involved in many physiological functions such as immune and inflammatory responses, cell survival and death (Grilli and Memo, 1999; Kucharczak et al., 2003), in cell plasticity and morphology remodelling (Mattson, 2005; Gutierrez and Davies, 2011; Bonini et al., 2011). This mouse model has been widely used in studies on inflammation, immunity, metabolic diseases and cancer (de Valle et al., 2016; Minegishi et al., 2015; Southern et al., 2012). Moreover, we found that adult p50 KO mice display abnormal columnar organization in the somatosensory cortex and altered neurite orientation, associated with hyperactivity and impairment in social behaviours with a reduction in social interactions (Bonini et al., 2016). All these cortical and behavioural alterations have also been reported in different NDD models, including models of autism spectrum disorders (ASD) and schizophrenia (Casanova et al., 2006; Casanova et al., 2008; Beasley et al., 2009; Stoner et al., 2014, Gaudissard et al., 2017).

Drugs currently used for NDDs are mainly symptomatic and often are ineffective for social deficits (Ji and Findling, 2016). Therapeutic intervention commonly aims to reduce psychotic symptoms and relies on atypical antipsychotics, especially risperidone, aripiprazole and olanzapine (Lamberti et al., 2016; Mastinu et al., 2012; Lazzari et al., 2017). No current medical treatments exist for prevention of NDDs and for amelioration of social deficits. Indeed, risperidone treatment in p50 KO mice resulted in decreased hyperactivity but had no effect on social deficits (Bonini et al., 2016). A promising compound to rescue social impairments in NDDs is represented by oxytocin (Woolley et al., 2015; Gordon et al., 2013; Kirsch 2015). Oxytocin is synthesized in the neurons of the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus and released in the brain or in the blood (Ludwig and Leng, 2006). Oxytocin exerts several neuroendocrine functions when released in the peripheral bloodstream and modulates social behaviour when released in the brain. In particular, oxytocin has been reported to increase social stimuli, to promote parental nurturing and social bonds in mouse models of ASD (Peñagarikano et al., 2015; Teng et al., 2016). Furthermore, intranasal oxytocin improved social deficits both in patients with schizophrenia/schizoaffective disorder (Shilling and Feifel, 2016) and in ASD patients (Andari et al., 2010; Guastella et al., 2010). Studies in humans support the pharmacological role of oxytocin in stress, anxiety disorders, social phobia, postpartum depression, and bipolar disorder, with a positive action on reward in social behaviour and communication (MacDonald et al., 2013; Singer et al., 2008). Lastly, oxytocin is involved also in peripheral inflammatory responses mediated by NF-κB, as previously reported (Soloff et al., 2006; Kim et al., 2015). In particular, Soloff and

colleagues hypothesize that the NF-κB p65 subunit interacts with the promoter of the oxytocin receptor and down regulates its expression. At present the specific mechanisms of p65 interaction with the oxytocin receptor promoter remains considerably complex.

Despite these encouraging results both in animal models and in humans, the use of oxytocin in pharmacotherapy presents some relevant limitations. First of all, oxytocin is a peptide therefore it cannot be used by oral administration. Another adverse factor is represented by oxytocin's short half-life. Indeed, oxytocin degrades very rapidly by intravenous administration, resulting in short-lasting effects (Modi et al., 2016). Furthermore, its brain penetrance is uncertain; indeed, some studies report that only a small fraction can pass the blood brain barrier and enter the brain (Mens et al., 1983; Guastella et al., 2013). On the contrary, Lee and colleagues demonstrated cerebrospinal fluid penetrance of exogenous oxytocin administered peripherally, by intranasal or intravenous route (Lee et al., 2017). Intranasal delivery resulted to be the more efficient pathway for oxytocin compared to intravenous administration, but unfortunately it presented wide variability of both cerebrospinal fluid and plasma concentrations between different treated animals (Lee et al., 2017). Hence, it is not a manageable treatment.

To circumvent these problems, compounds able to enhance endogenous oxytocin release can be used. Sabatier et al. (2003) showed that α melanocyte-stimulating hormone (αMSH) has a selective modulatory action on SON oxytocin neurons via melanocortin 4 receptor (MC4R) agonism. Moreover, MC4R agonist treatment increases oxytocin release in the brain (Sabatier et al., 2003) and promotes partner preference in voles (Modi et al., 2015). THIQ is a selective and potent MC4R agonist; it is an orally active small non-peptide MC4R mimetic compound (Grunewald et al., 1999). In addition, THIQ can penetrate the blood-brain barrier (BBB), as previously reported by an in vitro BBB permeability study (Grunewald et al., 1999). Moreover, it shows chemical properties of log P 5.65 ± 0.93, calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02. THIQ has already been reported to induce MC4R-mediated erectogenic effects in both mice and rats with sexual dysfunctions (Martin et al., 2002).

To identify a correlation between social deficit and the oxytocin pathway in p50 KO mice, we initially measured the oxytocinergic tone. Then, we evaluated the THIQ effect on social deficits and whether this effect was mediated by oxytocin pathway.

2. Materials and Methods

2.1. Animals

Experiments were conducted in conformity with the European Communities Council Directive of 1986 (86/609/EEC), approved by the Italian Ministry of Health, and the Animal Care and Use Committee of the University of Brescia. Animals were housed two-to-three per cage in a 12 hours light/dark cycle (light phase from 8:00 a.m. to 8:00 p.m.) with food and water available ad libitum. The cage size was 15 cm wide x 35 cm long x 12 cm deep. Temperature (22 °C) and humidity (50% ± 10) in the cages were automatically regulated by the Sealsafe Aero System by individually ventilated cages with EPA filters (Tecniplast Group, Italy). NF-κB $p50^{-/-}$ mice (B6;129P2-Nfkb 1tm 1 Bal/J) and wild-type mice (B6;129PF2) were purchased from The Jackson Laboratories (Bar

Harbor, ME, USA). Stable mating couples were maintained to amplify the colony and the progeny was used for the experiments. In particular, wild-type (WT) and $p50^{-/-}$ (p50 KO) age-matched mice (4-6-month-old mice; weight = 25-35 g) were used. All the experiments were performed on male mice.

2.2. Drugs and pharmacological treatment

For pharmacological treatment, melanocortin 4 receptor agonist THIQ (*N*-[(1*R*)-1-[(4-Chlorophenyl)methyl]-2-[4-cyclohexyl-4-(1*H*-1,2,4-trazol-1-ylmethyl)-1-piperidinyl]-2-oxoethyl]-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide, Tocris Bioscience, Bristol, UK) was administered acutely via intraperitoneal injection 30 min before the behavioural tests, according to previously reported data (Peñagarikano et al., 2015). THIQ doses were 0.5, 1, and 2 mg/kg in a 10 ml/kg volume, 250 µl for an average 25 g mouse and were chosen on the basis of previous data reporting K_i, IC_{50} and MC4R activation (Martin et al., 2002; Sebhat et al., 2002). Oxytocin receptor antagonist, L-371,257 (Tocris Bioscience, Bristol, UK) was intranasally administered at a dose of 300 µg/kg; a drop of a 2.5 µL solution was placed in the animal nostril with a P10 pipette to induce the mouse to aspirate the drop into its nasal cavity. The mice received L-371,257 15 min before THIQ administration (Peñagarikano et al., 2015). All drugs, suspended in 0.9% saline solution, were prepared daily, sonicated, and administered.

2.3. Radioimmunoassay

To correlate oxytocin levels with THIQ treatment, we measured hypothalamic oxytocin levels 30 min after intraperitoneal drug administration as reported previously (Peñagarikano et al., 2015). Mice (6 for WT and 5 for the p50 KO mice group) were sacrificed and hypothalamus was isolated, weighted and homogenized with acetic acid (Sigma Aldrich, Milano, Italy) 50% v/v. Then, the tissue homogenate was boiled for 10 minutes at 100 °C and centrifuged at 12000 rpm for 30 minutes at 4 °C. One mL of supernatant was combined with 1 mL of Buffer A (Phoenix Pharmaceuticals, Burlingame, CA, USA) and centrifuged at 12000 x g for 20 minutes, and we collected the supernatant. After, the samples were extracted onto the C-18 sep column (Phoenix Pharmaceuticals, Burlingame, CA, USA). The eluted sample was frozen at least three hours before being placed in the lyophilizer. The lyophilized sample was reconstituted with one x assay buffer for RIA detection (Phoenix Pharmaceuticals, Burlingame, CA, USA). Briefly, the standard peptide, the antibody, the positive control, and unknown samples were reconstituted, and RIA buffer was diluted with 150 mL of distilled water. Dilutions of the standard peptide and samples were prepared and incubated at 4 °C for 16 or 24 hours. Subsequently, the ^{125}I-peptide was added and all tubes were incubated for another 16 or 24 hours at 4 °C. After the addition of antibody, all tubes were incubated at room temperature, centrifuged and a γ-counter was used to count the cpm (counts per minute) of the pellet.

2.4. qRT PCR

Total RNA was isolated from hypothalamic tissue and dissected from WT and p50 KO mice brain. To purify RNA, the RNeasy kit (Qiagen, Milano, IT) was used and RNA was digested with the RNase Free DNase set (Qiagen, Milano, IT), according to the manufacturer's protocol. RNA quality of samples was tested by RNA electrophoresis to ensure RNA integrity. RNA was quantified by means of mySpec spectrophotometer (VWR, Milano, IT). One micrograms of total RNA from WT and KO hypothalamic tissue was transcribed into cDNA using murine leukemia virus reverse transcriptase (Promega, Milano, IT) and oligo(dT) 15–18 as a primer (final volume: 50 uL). Parallel reactions containing no reverse transcriptase were used as negative controls to confirm the removal of all genomic DNA. The following murine-specific primers, previously reported in the literature (Gutkowska et al., 2009), were used: oxytocin forward primer 5'-CCT ACA GCG GAT CTC AGA CTG A-3', reverse primer 5'- TCA GAG CCA GTA AGC CAA GCA-3', oxytocin receptor forward primer 5'-CGA CTC AGG ACG AAG GTG GAG GA-3', and reverse primer 5'-AAG ATG ACC TTC ATC ATT GTT C-3'. Amplification and detection were performed with the ViiA7 Real Time PCR Detection System (Applied Biosystem, Monza, IT); the fluorescence signal was generated by SYBR Green I. Samples were run in triplicate in a 12 µL reaction mix containing 6 µL of 2×SYBR Green Master Mix (BIO-RAD Laboratories, Segrate, IT), 6 pmol of each forward and reverse primer, and 2 µL of diluted cDNA. The SYBR Green Master Mix includes ROX for passive reference. Each PCR experiment included serial dilutions of a positive control for the construction of the calibration curve, a positive and a negative DNA sample, and water blanks. The PCR program was initiated by 10 min at 95 °C before 40 cycles, each one of 1 s at 95 °C and 30 sec at 64 °C. A subsequent dissociation curve analysis verified the product's specificity. Gene expression levels were normalized to β Actin expression (forward primer: 5'-AGC CAT GTA CGT AGC CAT CC-3', reverse primer: 5'-CTC TCA GCT GTG GTG GTG AA-3') and data are presented as the fold change in target gene expression in p50 KO mice hypothalamic tissue normalized to the internal control gene and relative to WT mice hypothalamic tissue. The results were estimated as C_t values; the C_t was calculated as the mean of the C_t for the target gene minus the mean of the C_t for the internal control gene. The C_t represented the mean difference between the C_t of p50 KO minus the C_t of WT mice hypothalamic tissue. The *N*-fold differential expression in the target gene of p50 KO compared to WT mice hypothalamic tissue was expressed as $2^{-\Delta\Delta Ct}$. Data analysis and graphics were performed using GraphPad Prism 5 software and results were obtained employing 7-8 mice per group (WT and p50 KO hypothalamic tissue), and each sample was run in triplicate for each gene.

2.5. Reciprocal social interactions

Fine-grained measures of interactions between pairs of adult mice placed together in standard cages or arenas provide the most detailed insights into reciprocal social interactions (Silverman et al., 2010). For this behavioural analysis, 6-8 pairs of 4 to 6-month-old male age-matched mice for each genotype were used and a modified version of the Silverman protocol was applied. In particular, experimental mice were individually housed during 5 days preceding the behavioural test. Both mice in the pair were treated either with the same drug or with vehicle and used for the test. After a period of acclimation of 10 min into their cage in a quiet and dim experimental room, subjects were

exposed to an age, sex and strain matched unfamiliar subject (intruder subject) for 10 min. The 10 min social test was video recorded and later analysed by two independent observers on the video collected with the IC Capture software (Vision Link, Bremen, DE). Duration of social activities (that include first contact latency, anogenital sniffing, nose to nose sniffing, wrestling, following, mounting, and pushing past each other with physical contact) and non social activities (cage exploring, self-grooming, and climbing) were measured. During the test, the operator remained in an adjacent room, separated with a dark sliding door from the test room. Specific behaviours observed during the social test belong to well-defined ethograms used to analyse social interaction in laboratory mice (Grant and Mackintosh, 1962; Rodriguiz et al., 2011). Separate values were obtained for each individual in a pair, but since the two values cannot be considered statistically independent, pair means were used for statistical analysis.

2.6. Open Field

The open field test was performed on new batches of mice. After being acclimated to the procedure room for at least 10 min, each of WT and p50 KO mice (age-matched adult littermates, 4-6 month old, 6-8 mice/group) was individually videotaped during a 5-min exploration session in a 40 cm × 40 cm Plexiglas open field activity box. Mice were placed in the center of the arena at the beginning of the test period. Their movement around the arena was recorded by a portable video camera vertically mounted 1.5 meters above and remotely controlled by the experimenter. The Plexiglas box was cleaned after each individual test session to prevent subsequent mice from being influenced by odors deposited by previous mice. Testing was performed under normal room lights, during the light phase of the circadian cycle, between 09:30 and 17:30 h. Locomotor activity was recorded and total distance travelled, average speed and total time mobile were analysed and automatically scored with ANY-maze software.

2.7. Statistical analysis

Unpaired t test with Welch's correction was used for RIA experiments in basal conditions (WT versus p50 KO mice). Multiple t test corrected for multiple comparison using Holm-Šidák method was used real time PCR experiments. After the treatments, analysis of variance (ANOVA) with two levels of genotype and four levels of treatment as between group factors have been performed. In particular, two-way ANOVA with Šidák multiple comparison tests was performed for RIA, open field and social interaction test. Statistical analyses were performed by GraphPad Prism 5 software. Data are presented as the means ± S.E.M, with the statistical significance level set at $p<0.05$.

3. Results

3.1. Oxytocin pathway in hypothalamus of p50 KO and WT mice

3.1.1. Oxytocin and oxytocin receptor

The *OXT* gene synthesizes an inactive precursor protein in the SON and PVN that includes the oxytocin carrier protein neurophysin I (Grinevich et al., 2015). Oxytocin, stored in vesicles, is released by axon terminals into the blood from the pituitary gland (Ludwig and Leng 2006). Moreover, the axon terminals show several connections with the mesolimbic area (including nucleus accumbens) that modulates the reward for social behaviour (Dumais and Veenema, 2016; Love, 2014). Here, first we analysed the levels of both oxytocin precursor and oxytocin receptor mRNA in WT and p50 KO mice. mRNA was obtained from hypothalamic tissue and retrotranscribed to cDNA; then, specific primers for oxytocin and oxytocin receptor were used for the qRT-PCR experiments. From this analysis emerged that both oxytocin and oxytocin receptor mRNA are significantly lower in p50 KO compared to WT mice (Fig. 1) (oxytocin, $t_{ratio} = 2.56$, df = 12.0, $p<0.05$; oxytocin receptor, $t_{ratio} = 2.84$, df = 13.0, $p<0.05$). To quantify the neuropeptide content we also measured oxytocin levels in hypothalamus of p50 KO and WT mice by RIA. We quantified oxytocin from hypothalamus-extracted samples. Sample extraction is a technique to remove the effect of potentially interfering molecules, useful to concentrate and enrich the analyte of interest (Szeto et al., 2011). It is still matter of debate the importance of sample extraction before oxytocin quantification. Several groups measured oxytocin levels from "non extracted samples" (Schneiderman et al., 2012, Feldman et al., 2012, Weisman et al., 2012) and this generated heterogeneous results compared to results obtained with different oxytocin quantification protocols (McCullough et al., 2013, Leng and Ludwig 2015, Leng and Sabatier 2016). According to Leng and Sabatier, that reported the importance of extraction to eliminate interfering factors, we decided to extract the samples. In line with qRT-PCR results, basal hypothalamic oxytocin levels were significantly lower in p50 KO compared to WT mice by RIA quantification ($t = 4.3$, df = 4.4, $p<0.05$, Figure 2A), suggesting a hypothalamic decrease in oxytocin activity based on reduced expression of neurohormone.

3.2. THIQ effect in WT and p50 KO mice

3.2.1. Oxytocin detection after THIQ treatment by RIA

Previous data showed that MC4R agonists improve the central oxytocinergic tone (Sabatier et al. 2003). In the present study, an acute treatment with THIQ, a MC4R agonist, was executed, and the neuropeptide content in the total hypothalamus was quantified by RIA 30 min after drug injection (Peñagarikano et al., 2015). THIQ treatment induced an increase in oxytocin level in WT and p50 KO mice ($F_{interaction}$ (3, 36) = 0.46, p=0.71; $F_{treatment}$ (3, 36) = 10.68, $p<0.0001$; $F_{genotype}$ (1, 36) = 3.48, p=0.07; Figure 2B). These data confirm an interaction between the oxytocin and melanocortin pathways and suggest a THIQ-dependent increase in oxytocin transit from the SON and PVN to the pituitary gland or other areas of the brain.

3.2.2. Behavioural effects after THIQ treatment

p50 KO mice are characterized by specific behavioural impairments, including hyperactivity and increased exploratory attitude (Bonini et al., 2016, Denis-Donini et al., 2008), reduced social interactions (Bonini et al., 2016) and decreased tendency to establish dominant subordinate

relationships among cage mates (Kassed et al., 2004). To modify or even revert some of these deficits and demonstrate the central role of MC4R agonism in increasing the oxytocinergic response, WT and p50 KO mice were acutely treated with 0.5, 1, or 2 mg/kg THIQ (i.p.) and tested in the reciprocal social interaction paradigm and in the open field test 30 minutes after drug administration. To better clarify the sort of social behaviour performed, a more detailed analysis of the different behaviours has been done. Social behaviours have been classified as follow: "social investigation", that include first contact latency, nose to nose sniffing, body sniffing, nose to anogenital sniffing, following, and "aggressive behaviours", that include wrestling, mounting and pushing past each other with physical contact. Since mice analysed spent minimal time in aggressive behaviours and no significant difference emerged between groups, only social investigation activities were reported in the graph. Regarding basal behaviours, as expected and shown in Figure 3A, p50 KO mice spent significantly less time (expressed in seconds) in social activities compared to WT mice. Conversely, p50 KO mice spent more time in non-social activities compared to WT mice (Figure 3B). Interestingly, treatment with THIQ significantly increased social attitude both in WT and in p50 KO mice at all the doses used [(Social investigation: $F_{interaction}$ (3, 56) = 1.37, p=0.26; $F_{treatment}$ (3, 56) = 16.87, p<0.0001; $F_{genotype}$ (1, 56) = 41.25, p<0.0001); (Aggressive behaviours: $F_{interaction}$ (3, 56) = 1.37, p=0.26; $F_{treatment}$ (3, 56) = 0.18, p=0.91; $F_{genotype}$ (1, 56) = 0.25, p=0.62); Figure 3A]. Concerning non social activity, the time spent in these actions was significantly decreased in THIQ-treated WT and p50 KO mice [(Non social behaviours: $F_{interaction}$ (3, 56) = 1.59, p=0.201; $F_{treatment}$ (3, 56) = 18.5, p<0.0001; $F_{genotype}$ (1, 56) = 43.06, p<0.0001); Figure 3B]. In both WT and p50 KO mice THIQ provoked a significant decrease in time spent doing exploring compared to vehicle treated mice. It is noteworthy that THIQ treatment did induce in both WT and KO mice an increase in time spent doing social interaction without affecting "aggressive behaviour".

Finally, we investigated the mice locomotor and exploratory activity by the open field test. We confirmed the increased motor activity of p50 KO mice compared to WT; indeed, p50 KO mice presented an increase in distance travelled and in walking speed compared to WT mice [(Distance travelled: $F_{interaction}$ (3, 45) = 1.23, p=0.31; $F_{treatment}$ (3, 45) = 0.93, p=0.43; $F_{genotype}$ (1, 45) = 13.53, p=0.0006); (Speed: $F_{interaction}$ (3, 45) = 1.37, p=0.26; $F_{treatment}$ (3, 45) = 1.01, p=0.4; $F_{genotype}$ (1, 45) = 12.0, p=0.001); (Total time mobile: $F_{interaction}$ (3, 45) = 2.86, p=0.05; $F_{treatment}$ (3, 45) = 1.98, p=0.13; $F_{genotype}$ (1, 45) = 3.01, p=0.09); Figure 4]. THIQ treatment did not manifest any significant effect on exploratory behaviour and motility in both WT and p50 KO mice. Finally, we performed a separate ANOVA in each dose with genotype as a between subject factor but it did not reveal any significant difference. These results suggest that THIQ treatment has a direct effect on social interaction in p50 KO mice without affecting "aggressive behaviours" or locomotor activity.

3.3. Prosocial effect is mediated by the oxytocin pathway

To establish if the THIQ prosocial effect was mediated by oxytocin, we took advantage of the availability of an oxytocin antagonist. The compound L-371,257 was administered at the dose of 300 µg/kg via intranasal administration 15 min before THIQ treatment. For THIQ we used the 0.5 mg/kg dose. As shown in Figure 5A, the antagonist alone did not generate a behavioural response

both in WT and p50 KO mice. On the contrary, when mice were cotreated with L-371,257 and THIQ, the prosocial effect of the MC4R agonist was blocked both in WT and p50 KO mice [(Social investigation: $F_{interaction}$ (3, 62) = 0.83, p=0.48; $F_{treatment}$ (3, 62) = 14.79, p<0.0001; $F_{genotype}$ (1, 62) = 33.44, p<0.0001); (Aggressive behaviours: $F_{interaction}$ (3, 62) = 1.97, p=0.13; $F_{treatment}$ (3, 62) = 0.74, p=0.53; $F_{genotype}$ (1, 62) = 0.502, p=0.48); Figure 5A]. In addition, WT and p50 KO mice spent more time in exploration and non social activities when treated with L-371,257-THIQ compared to THIQ only [Non social behaviours: $F_{interaction}$ (3, 62) = 0.96, p=0.42; $F_{treatment}$ (3, 62) = 14.15, p<0.0001; $F_{genotype}$ (1, 62) = 30.24, p<0.0001); Figure 5B]. Based on these results, acutely administered THIQ stimulates social interactions, but this behavioural effect is eliminated when the oxytocin receptor is blocked by L-371,257. Data obtained suggest that the THIQ prosocial effect is mediated by oxytocin pathway.

4. Discussion

In this work, we studied the effect of an acute treatment with THIQ, a selective melanocortin receptor agonist, on social behaviour and measured oxytocinergic tone in WT and p50 KO mice. In vertebrates, the oxytocin system innervates brain networks that modulate social behaviour and generates attitudinal trust (Kent et al., 2016, Nishina et al., 2016). Oxytocin has been proven to modulate the processing of social cues in a prosocial direction by increasing the reward value of positive social cues while buffering against experiences of negative emotionality in healthy subjects (Kanat et al., 2014). It emerged as a potent modulator of diverse aspects of interpersonal relationships (Patin et al., 2017). Interestingly, more than twenty years ago, a link between the oxytocin pathway and autism spectrum disorders (ASD) emerged. Indeed, several studies revealed a deficit of oxytocin/oxytocin receptor in ASD patients brain (Jacob et al., 2007; Gregory et al., 2009) and low plasma oxytocin levels (Modahl et al., 1998; Andari et al., 2010). Surprisingly, a single intranasal dose of oxytocin was able to improve social performance (Andari et al., 2010; Guastella et al., 2010).

We previously demonstrated that p50 KO mice exhibit behavioural impairments such as hyperactivity, increased locomotor and exploratory activities, and reduced social interactions (Bonini et al., 2016). Considering behavioural alterations found in p50 KO mice, we asked if hypothalamic oxytocin pathway could be involved in p50 KO mice behaviour impairments.

First, we found lower mRNA levels of both oxytocin precursor and oxytocin receptor in the hypothalamus of p50 KO when compared to WT mice. In this regard, it has been demonstrated that p65 NF-κB subunit interacts with the oxytocin pathway and modulates its expression (Soloff et al., 2006; Kim et al., 2015). Moreover, we previously evaluated p65 protein levels in p50 KO neuronal cortical cells and we found that p50 KO expressed significantly higher protein levels (+152%) compared with WT neurons (Bonini et al., 2011). Therefore, we hypothesize that the low expression of oxytocin pathway components could be associated to NF-κB pathway alteration.

Central oxytocin is released both axonally and somatodendritically from the magnocellular and parvocellular neurons in the PVN and in mesolimbic area such as ventral tegmental area and nucleus accumbens (Peris et al., 2017). In particular, the mesolimbic area acts as reinforcement for many behavioural responses, including social interactions (Salamone and Correa, 2012). The

reduced expression of hypothalamic oxytocin and oxytocin receptor could lead to a decreased mesolimbic response that could explain the poor social behaviour in the p50 KO compared to WT mice. Furthermore, we cannot rule out that the reduced sociality of p50 KO mice generates a down regulation of the oxytocin. Indeed, the same social interaction increases the release of oxytocin, therefore p50 KO mice might have a social deficit which could lead to oxytocin down-regulation. These findings are consistent with previous studies showing that the oxytocin pathway modulates prosocial behaviours and that deficits in the oxytocin system generate sociability impairments (Kirsch, 2015). Furthermore, several mouse models of neurodevelopmental disorders present an oxytocin and/or oxytocin receptor dysregulation (Rich, 2015). Finally, some drug treatments with oxytocin have been performed both in murine models and/or humans to ameliorate social impairments, but ambiguous results were obtained. Indeed, despite the central role of the oxytocin system in prosocial behaviour and promising results obtained with oxytocin by intranasal treatment, its clinical use presents some important limitations. In particular, only a minimal amount of the huge quantity of oxytocin applied intranasally reaches the cerebrospinal fluid; on the contrary, intranasal application produce large and prolonged increases in circulating oxytocin, that can trigger actions on reproductive organs, the heart, and gastrointestinal tract (Leng and Ludwig, 2016). Furthermore, intranasal oxytocin administration present wide variability of response between individuals, and this can be due to variations in anatomy and resultant airflow dynamic, vascularisation, status of blood vessels, mode of spray application, etc. (Guastella et al., 2012). For this reason we focused our attention on a small non-peptide molecule with high affinity and selectivity for the melanocortin 4 receptor (MC4R), THIQ (Wikberg and Mutulis, 2008). MC4R is known to play a central role in food intake and energy expenditure (Krashes et al., 2016). Moreover, several studies demonstrated the involvement of MC4R also in neuroinflammation and sexual activity (Muceniece et al., 2007; Lansdell et al., 2010). Recently, Modi and colleagues have found a link between MC4R activation and behaviours of partner preference associated with the oxytocin pathway in prairie voles (Modi et al., 2015). Indeed, MC4R activation interacts with several mesolimbic pathways such as oxytocin, dopamine and serotonin that modulate behaviours connected to reward, as mentioned above (Roseberry et al., 2015). Melanocortin agonism was reported to increase prosocial activity and to induce oxytocin release in the brain (Sabatier et al., 2003). More specifically, MC4R agonists were shown to induce substantial release of oxytocin from dendrites in isolated hypothalamic supraoptic nuclei (Sabatier et al., 2003). Our results demonstrated how, after THIQ treatment, oxytocin in both WT and p50 KO mice is significantly increased over basal values at all tested doses. Hence, we suggest a direct role for THIQ in increasing oxytocinergic tone by stimulating oxytocin release.

Our behavioural tests showed that 0.5 mg/kg THIQ acute treatment is able to improve social interactions between unfamiliar p50 KO mice. These findings are consistent with previous data showing that animals treated with melanocortin agonists exhibit more prosocial behaviours compared to control group (Peñagarikano et al., 2015; Modi et al., 2015). Indeed, we observed a strong prosocial effect also in WT mice treated with THIQ compared to basal WT mice.

Although the prosocial response of MC4R agonists could be explained by an oxytocinergic tone improvement, some mechanisms need to be better defined. In fact, some authors did not observe changes in the oxytocin content in microdialysated samples collected in the somatosensory hypothalamic area after treatment with αMSH analogues in rats (Paiva et al., 2016). Moreover,

although Modi and colleagues hypothesized that melanotan II (αMSH analogue) could increase hypothalamic concentration of oxytocin and its release in mesolimbic area, they failed to prove it experimentally in female prairie voles (Modi et al., 2015). On the other hand, Peñagarikano and colleagues (2015) reported a social improvement and an endogenous oxytocin release after MC4R agonist in an ASD mouse model. From our results on behaviour and RIA, we hypothesize that acute THIQ treatment could increase hypothalamic oxytocin level, affecting social behaviour in p50 KO mice that become more prone to socialize. The results obtained cotreating mice with the oxytocin receptor antagonist L-371,257 could validate this hypothesis. Indeed, L-371,257 blocked the prosocial action of 0.5 mg/kg THIQ both in WT and in p50 KO mice during behavioural testing. Moreover, mice treated with the oxytocin antagonist showed a social behaviour equal to that observed in basal groups. Overall, these results can contribute to better defining MC4R agonist's role in the regulation of social behaviour through oxytocin action.

In conclusion, data obtained in this study demonstrated that p50 KO mice present an oxytocin pathway down regulation in the hypothalamus compared to WT mice. Moreover, THIQ treatment significantly increases social interactions both in WT and p50 KO mice, and this effect could be mediated by oxytocin since it is counteracted by an oxytocin receptor antagonist. At present, the mechanism by which THIQ affects oxytocin pathway requires further insights. Our results corroborate once again an important role for MC4R in social oxytocin-mediated behaviour. Moreover, future studies will be needed to better demonstrate the central role of MCR4 agonism to improve social deficits through the oxytocin pathway.

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Figure Captions

Fig. 1. mRNA expression of oxytocin and oxytocin receptor in the hypothalamus of WT and p50 KO mice. Both oxytocin (OXY) and oxytocin receptor (OXY-R) transcripts were significantly lower in p50 KO compared to WT mice (*$p<0.05$). Multiple t test analysis and statistical significance determined using the Holm-Sidak method. Data are expressed as the mean ± S.E.M. and relative quantification of target gene was expressed as $2^{-\Delta\Delta Ct}$. Target gene expression was normalized to β-actin expression.

Fig. 2. Oxytocin detection in WT and p50 KO hypothalamus by RIA. Under basal conditions, hypothalamic oxytocin levels were significantly lower in p50 KO compared to WT mice (A). $^{\S\S}p<0.05$ vs WT mice. Data are expressed as the mean ± S.E.M. Unpaired t test with Welch's correction was done. After THIQ administration, an oxytocin increase in both WT and KO mice was observed (B). **$p<0.01$ vs WT VH, ***$p<0.001$ vs WT VH, #$p<0.05$ vs KO VH. Data are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Sidak's multiple comparisons test was executed.

Fig. 3. Effect of THIQ treatment on social behaviour in WT and p50 KO mice by the reciprocal social interaction test. The experimental mice were individually housed during the 5 days preceding the behavioural

test. Both mice in the pair were treated either with the same drug or with vehicle and used for a 10-min social test. (A) Social investigation includes first contact latency, nose-nose sniffing, ano-genital sniffing, body sniffing and following. (B) The following non social activities were evaluated: exploring and self-grooming. *p<0.05 vs WT VH, **p<0.005 vs WT VH, ##p<0.005 vs KO VH, ###p<0.0005 vs KO VH, ####p<0.0001 vs KO VH, §§§p<0.0005 vs WT VH. Data are expressed as mean ± S.E.M. Two-way ANOVA followed by the Šidák's multiple comparisons test was executed.

Fig. 4. Effect of THIQ treatment on WT and p50 KO mice motility by Open Field Test. Graphic representation of data collected in the open field test by automatically measuring the total distance travelled (A), the speed (B) and the total time mobile (C). Data confirmed a higher exploratory behaviour and locomotor activity in p50 knock out (KO) compared to wild type (WT) mice treated with vehicle (VH). **p<0.01 by two-way ANOVA followed by the Šidák's multiple comparison test.

Fig. 5. Effects of L-371,257 treatment on social behaviour of WT and p50 KO mice. The compound L-371,257 was administered at a dose of 300 µg/kg via intranasal administration 15 min before THIQ treatment; subsequently, social interaction testing was executed. Duration of social activities (A) and non social activities (B) were measured. (A) Social investigation includes first contact latency, nose-nose sniffing, ano-genital sniffing, body sniffing and following. (B) The following non social activities were evaluated: exploring and self-grooming. *p<0.05 vs WT VH, **p<0.005 vs WT VH, °°°p<0.0005 vs THIQ WT, °°°°p<0.0001 vs THIQ WT, #p<0.05 vs KO VH, &p<0.05 vs THIQ KO, &&p<0.005 vs THIQ KO, £££p<0.001 vs WT VH. Data are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Šidák's multiple comparisons test was executed.

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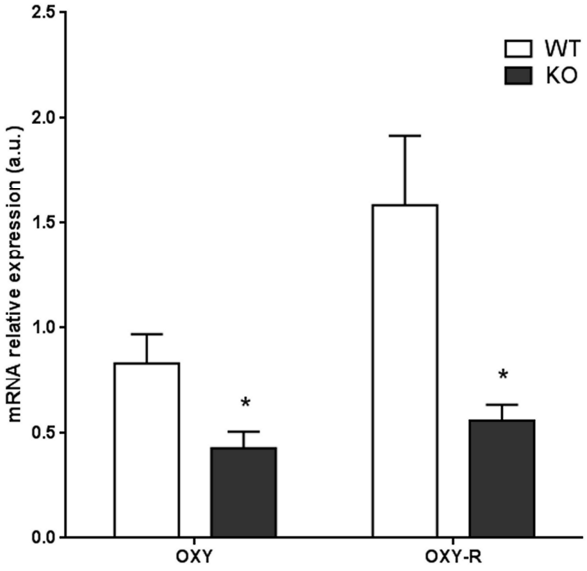

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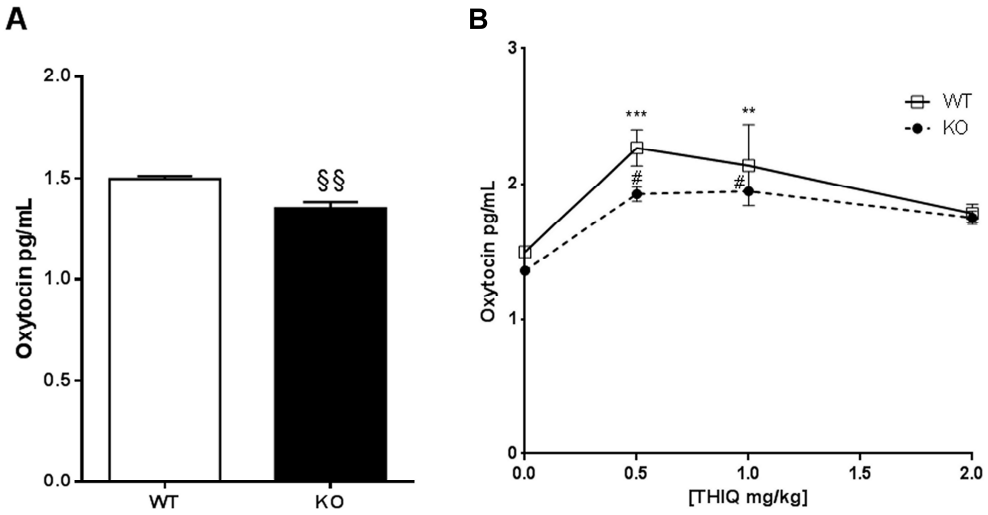

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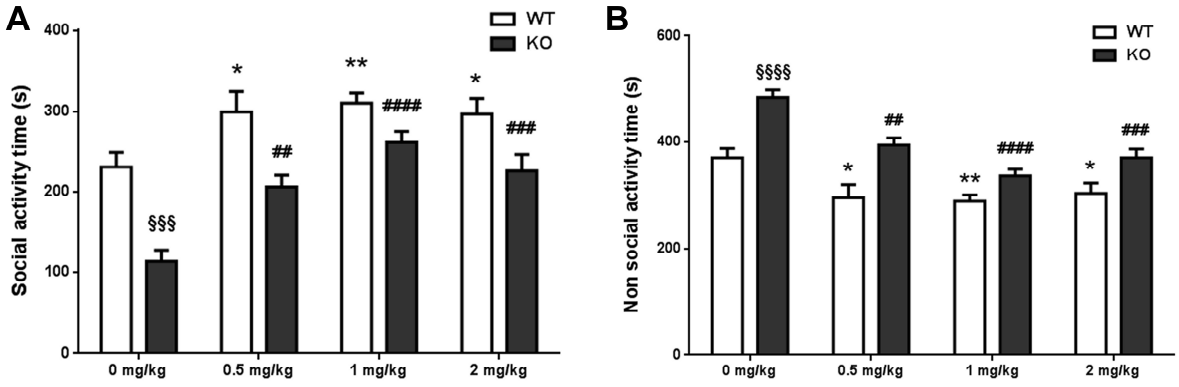

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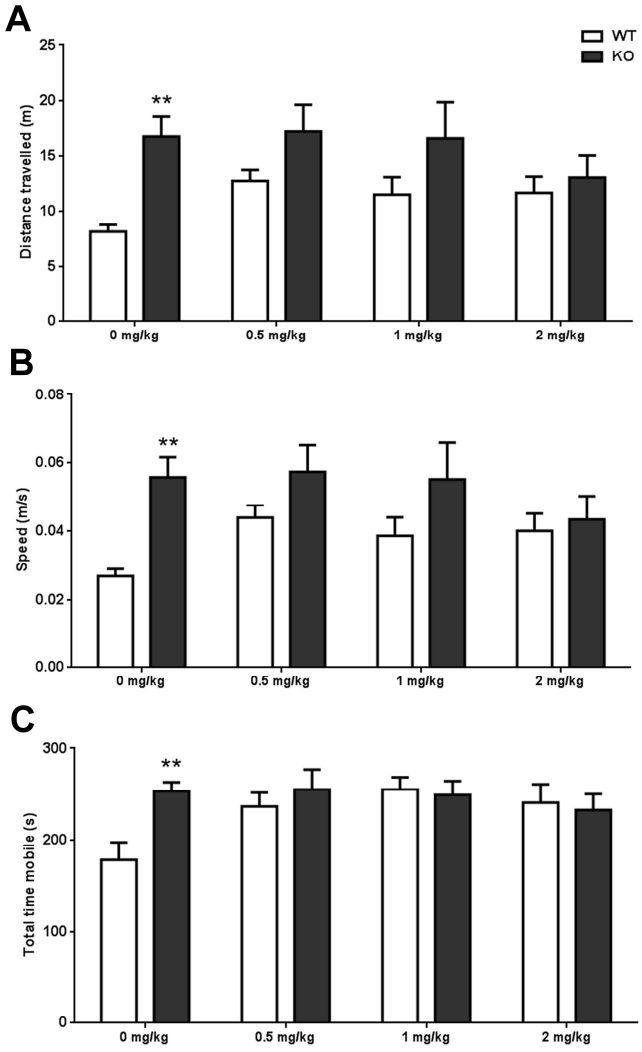

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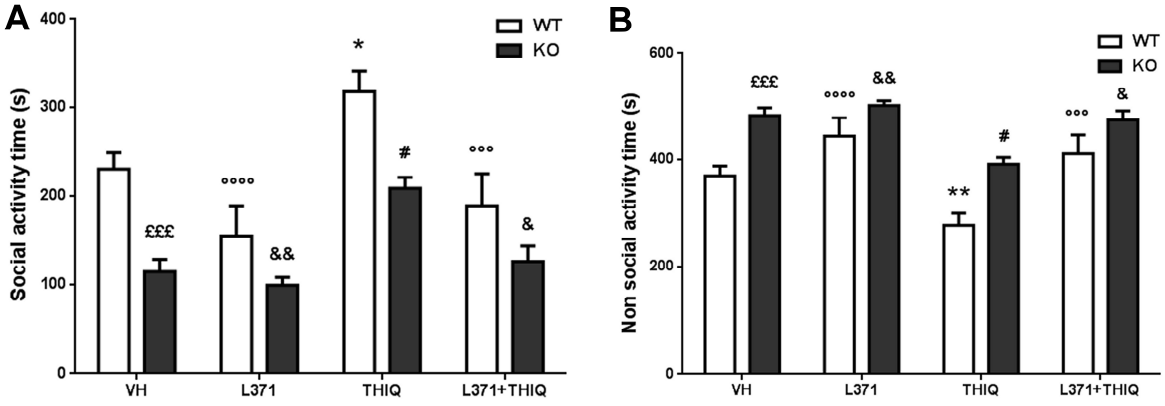

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Highlights:

- p50 KO mice display poor social behavior, with a reduction in social interaction
- p50 KO mice present both oxytocin and oxytocin receptor mRNA levels down-regulation
- MC4-R agonist THIQ is able to improve social interaction between unfamiliar p50 KO mice
- THIQ prosocial effects are mediated by oxytocin pathway

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