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ORIGINAL ARTICLE

Central relaxin-3 receptor (RXFP3) activation impairs social recognition and modulates ERK-phosphorylation in specific GABAergic amygdala neurons

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Abstract

In mammals, the extended amygdala is a neural hub for social and emotional information processing. In the rat, the extended amygdala receives inhibitory GABAergic projections from the *nucleus incertus* (NI) in the pontine tegmentum. NI neurons produce the neuropeptide relaxin-3, which acts via the $G_{i/o}$-protein-coupled receptor, RXFP3. A putative role for RXFP3 signalling in regulating social interaction was investigated by assessing the effect of intracerebroventricular infusion of the RXFP3 agonist, RXFP3-A2, on performance in the 3-chamber social interaction paradigm. Central RXFP3-A2, but not vehicle, infusion, disrupted the capacity to discriminate between a familiar and novel conspecific subject, but did not alter differentiation between a conspecific and an inanimate object. Subsequent studies revealed that agonist-infused rats displayed increased phosphoERK(pERK)-immunoreactivity in specific amygdaloid nuclei at 20 min post-infusion, with levels similar to control again after 90 min. In parallel, we used immunoblotting to profile ERK phosphorylation dynamics in whole amygdala after RXFP3-A2 treatment; and multiplex histochemical labelling techniques to reveal that after RXFP3-A2 infusion and social interaction, pERK-immunopositive neurons in amygdala expressed vesicular GABA-transporter mRNA and displayed differential profiles of RXFP3 and oxytocin receptor mRNA. Overall, these findings demonstrate that central relaxin-3/RXFP3 signalling can modulate social recognition in rats via effects within the amygdala and likely interactions with GABA and oxytocin signalling.

Keywords Arousal · Emotion · Nucleus incertus · Oxytocin receptor

Introduction

The capacity to identify and recall familiar conspecifics is crucial for appropriate social interaction and its absence is associated with impaired social abilities ranging from borderline personality disorder (Servan et al. 2017) to schizophrenia (Davis et al. 2014; Green et al. 2015) and autism

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spectrum disorders (Happé and Conway 2016). Social identification of conspecifics is intimately related to social recognition memory (SRM), a key process underlying long-term, socially-related behaviours, including pair bonding, aggression and mating (Dantzer et al. 1987; Gheusi et al. 1994; Williams et al. 2006; Maski et al. 2015). The amygdala is responsible for recognition of emotional facial expressions in humans (Baxter and Murray 2002; Seymour and Dolan 2008; Vuilleumier and Sander 2008; Gupta et al. 2011; Benarroch 2015; Bonnet et al. 2015) and social relationship pair-bonding in humans and rodents (Gobrogge et al. 2009; Hurlemann et al. 2010).

In rodents, conspecifics are recognized by olfactory information entering the amygdala from the main and accessory olfactory bulbs (Scalia and Winans 1975; Ferguson et al. 2001; Pro-Sistiaga et al. 2007; Trainor et al. 2010). Within the amygdala, the medial (MeA) nuclei and the bed nucleus of the stria terminalis (ST),[1] process social cues (Alheid et al. 1998; Rasia-Filho et al. 2000; Alheid 2006; Pereira et al. 2013; Fox et al. 2015) and, in turn, send projections to the hypothalamus (Hatalski et al. 1998; Smith and Vale 2006; Veenema 2008) and hippocampus (Hitti and Siegelbaum 2014; Okuyama et al. 2016). Ascending monoaminergic projections from neurons located in the brainstem also modulate aspects of social behaviour (Korzan et al. 2001; Arakawa 2017).

In a similar fashion, neurons in the *nucleus incertus* (NI), located in the pontine tegmentum, innervate the amygdala and other regions involved in social behaviour (Olucha-Bordonau et al. 2003; Ryan et al. 2011; Santos et al. 2016). The NI is characterized by GABA neurons that co-express relaxin-3 (RLN3), a member of the insulin/relaxin superfamily (Bathgate et al. 2002; Burazin et al. 2002; Ma et al. 2007, 2017). The impact of the NI GABA/RLN3 system on amygdala and extended amygdala-related behaviours has been described in various paradigms, including fear acquisition (Lee et al. 2014), extinction (Pereira et al. 2013), anxiety (Ryan et al. 2013a), feeding (Lenglos et al. 2014), and alcohol-seeking (Ryan et al. 2013b).

RLN3 is the cognate ligand of relaxin-family peptide receptor 3 (RXFP3), which is a $G_{i/o}$-protein-coupled receptor (Bathgate et al. 2002; Liu et al. 2003). Recent studies indicate that RXFP3 activation predominantly produces hyperpolarization of RXFP3 mRNA-positive neurons in rat brain slices, including magnocellular and parvocellular oxytocin neurons (Blasiak et al. 2013; Kania et al. 2017), consistent with inhibition of adenylate cyclase and reduced cellular cAMP levels associated with $G_{i/o}$-coupled receptor activation in cell-based systems (Liu et al. 2003; Halls et al. 2007). RXFP3 activation rapidly activates cellular externally-regulated kinases (ERKs) *in vitro* (Van der Westhuizen et al. 2007, 2010; Kocan et al. 2014); and *in vivo*, intracerebroventricular (icv) RXFP3 agonist injection produced increased levels of phosphorylated ERK (pERK) in rat medial septal cholinergic neurons (Albert-Gascó et al. 2017). However, this specific effect is thought to be indirect, as septal and diagonal band cholinergic neurons have been confirmed to be RXFP3 mRNA-negative (Albert-Gascó et al. 2018). Nonetheless, altered pERK levels in the amygdala have been associated with social interaction behaviour (Richter et al. 2005; Peng et al. 2010; Giese and Mizuno 2013) and changes in pERK levels have been used to assess brain activity changes underlying behavioural social deficits (Faridar et al. 2014).

We hypothesized that RXFP3 activation could modulate social recognition and/or recall of SRM. Thus, we examined the effects of icv infusion of the selective RXFP3 agonist, RXFP3-A2 (Shabanpoor et al. 2012; Zhang et al. 2015), on social discrimination performance in the 3-chamber paradigm; and levels of pERK in the extended amygdala. Subsequently, we studied the relationship between behavioural responses and the occurrence of pERK immunoreactivity in RXFP3 mRNA-expressing neurons. In addition, we investigated the anatomical association of RLN3-positive nerve fibres with activated neurons in the amygdala, reflected by neuronal pERK immunostaining. Finally, we assessed the neurochemical phenotype of RXFP3 mRNA-positive neurons in the extended amygdala by assessing the relative co-expression of *Rxfp3* mRNA with *vesicular GABA-transporter* (*SlC32a1*) and *oxytocin receptor* (*Oxtr*) mRNA. Overall, our findings suggest a role for RLN3/RXFP3 signalling in modulating social memory, which might occur via rapid alterations in ERK and related signalling in *GABAergic* and *Oxtr*-positive neurons within specific regions of the extended amygdala.

Materials and methods

Animals and surgical procedures

The Ethics Committee of the University Jaume I approved all procedures (#2015/VSC/PEA/00091). Male, Wistar rats (300–380 g) were maintained on a 12–12 h light–dark cycle with lights on at 0700 h GMT and behavioural procedures were conducted between 10.00 and 12.00 h GMT.

For surgical procedures, rats were anesthetised with ketamine (Imalgene 25 mg/kg i.p., Merial-Laboratories-SA, Barcelona, Spain) and xylazine (10 mg/kg, i.p., X1251

[1] The *bed nucleus of the stria terminalis* is abbreviated as BNST, BST or ST. However, when describing subdivisions of the area, the abbreviations become long and less useful. Thus, in the 7th Edition of The Rat Brain in Stereotaxic Coordinates, the whole *bed nucleus of the stria terminalis* was abbreviated as ST (Olucha-Bordonau et al. 2014; Paxinos and Watson 2014).

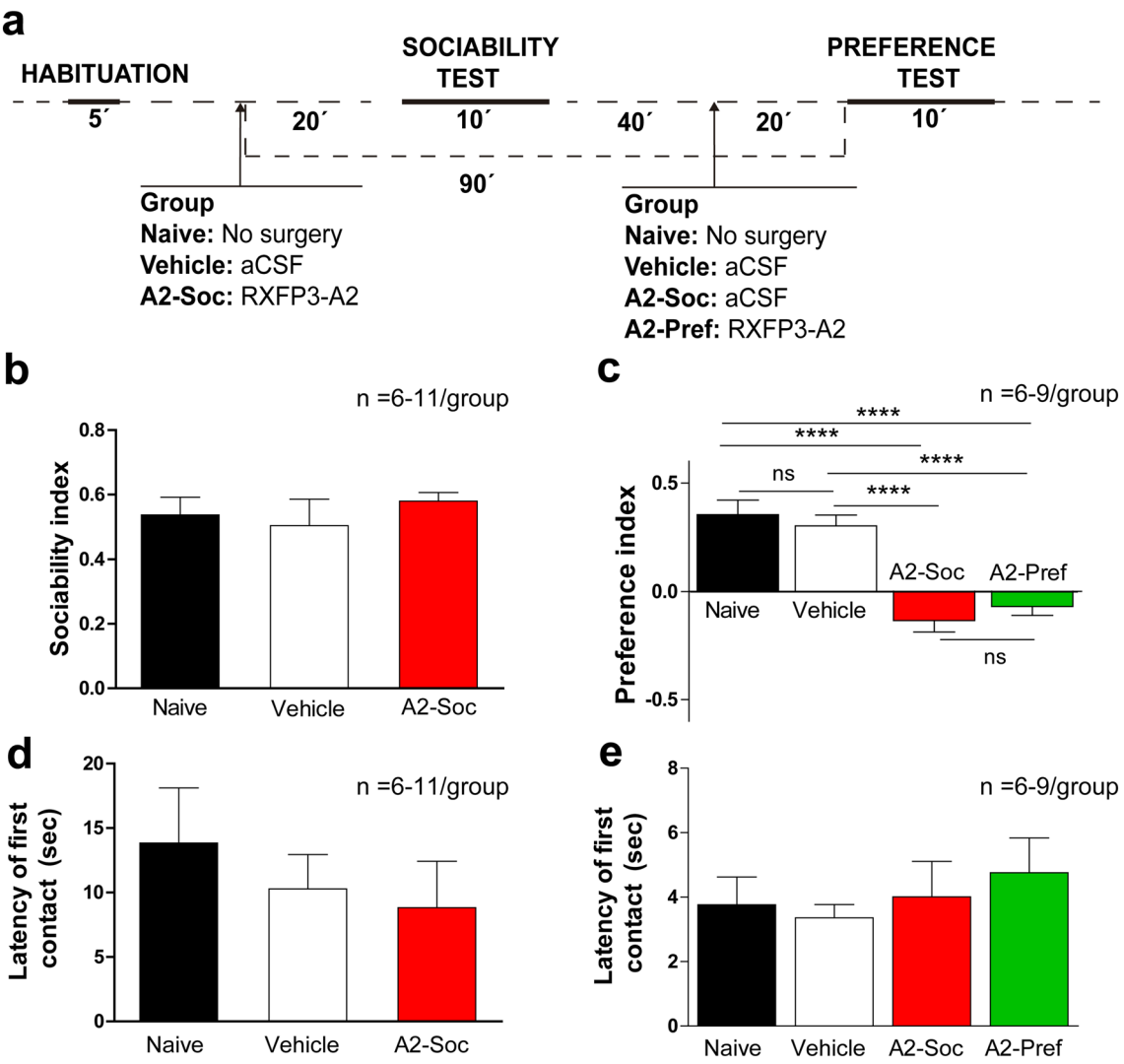


Fig. 1 Social recognition memory is impaired after RXFP3 agonist infusion. **a** Timeline of 3-chamber social interaction and memory and sociability tests, with infusion times indicated (black arrows). Preference index for the **b** sociability and **c** preference tests of experimental groups. First contact latency for the **d** sociability and **e** preference tests. *ns* not significant; *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$

Sigma-Aldrich, St Louis, MO, USA), and then a cannula was implanted at stereotaxic coordinates AP 0.48 mm, ML 0.1 mm, DV − 4 mm from bregma (Paxinos and Watson 2014) and secured with a screw attached to the skull.

Experimental groups

In this study, six experimental treatment groups were used. Apart from those in the *naïve* group, all rats had a cannula implanted targeting the right lateral cerebral ventricle.

Agonist-infused and vehicle groups used for immunoblotting studies, which did not undergo behavioural testing, were killed 20 and 90 min after peptide or vehicle infusions. Alternatively, infusions of agonist and vehicle rat groups which did undergo a 3-chamber social interaction behavioural test, were made either 20 min prior the sociability test, agonist infused rats were named RXFP3-A2-Soc (*A2-Soc*), or 20 min prior to the preference test, agonist infused rats were named RXFP3-A2-Pref (*A2-Pref*) (Fig. 1a).

On all cases, infusions were done 1 week after surgery, when the vehicle experimental group received 1 µl of artificial cerebrospinal fluid (aCSF) and agonist experimental groups received 1 µl of 5 µg/µl of RXFP3-A2 [R3A(11–24, C15 → A)B] specific RXFP3 agonist solution (Shabanpoor et al. 2012). Infusions were done at a rate of 0.5 µl/min over 2 min using a Harvard syringe injector (Harvard PHD2000 syringe pump; Harvard Apparatus, Holliston, MA, USA). After infusions, the injector needle was left in place for 5 min to avoid reflux of agonist or vehicle.

RXFP3-A2 agonist was kindly supplied by A/Prof M Akhter Hossain (The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia).

Three-chamber social interaction and memory test

All rats were handled daily during the week after surgery. On the day of the behavioural test, rats were habituated to the behavioural room for 30 min before the vehicle or peptide infusions 20 min prior to each trial. The behavioural test was conducted in a three-chamber apparatus. Each chamber measured 22 × 39 × 40 cm. On the first trial, following a 5-min habituation, each rat was allowed to explore either a conspecific or an inanimate object for 10 min ('Sociability Test'). After a 1 h inter-trial period, in the 'Preference Test' each test rat was allowed to explore a familiar conspecific (from the sociability test) or a novel one for 10 min. The chamber in which the novel (stranger) rat or object placed were balanced every two cases (agonist infused and control subject) in all experiments. In addition, the rat acting as stranger was balanced every two cases. Finally, rats acting as subject on the sociability test were then swapped to the opposite position on the preference test. Behaviour was analysed using video-tracking (Supplementary Fig. 1a, b). Rats were killed immediately after the preference test. Data were expressed as either a "sociability index", which was calculated as the time spent sniffing the subject minus the time sniffing the object (*sociability test*), or the "preference index", calculated as the time sniffing the novel conspecific minus the time sniffing the familiar conspecific (*preference test*) (Fig. 1a). Data were analyzed by one way-ANOVA followed by a Bonferroni *post-hoc* test. First contact latencies, as a measure of social anxiety, was used for both for the social interaction test and the preference test. Contact was considered when rats being tested sniffed a conspecific, was expressed in seconds and compared between the different behavioural groups.

Immunoblotting

In western blot studies, pERK levels were assessed in the amygdaloid complex, as described (Albert-Gascó et al. 2017). In brief, rats were lightly anesthetized (Dolethal, 200 mg/Kg Vetoquinol S.A., Madrid, Spain) and then killed by decapitation. Brains were removed and frozen. The amygdala was dissected using 1 mm diameter disposable biopsy punches (Interna Miltex, Ratingen, Germany) from 20 µm brain slices cut using a cryostat at − 15 °C to preserve protein phosphorylation. Brain tissue samples were lyzed in RIPA buffer containing protease and phosphatase inhibitors (Halt #78440 protease and phosphatase inhibitor, Thermo Scientific, Waltham, MA, USA).

Equal amounts of total protein were loaded onto a polyacrylamide gel, resolved and transferred to Immobilon-P membranes (MERCK Millipore, Darmstadt Germany), followed by blocking with bovine serum albumin. Membranes and primary antibodies (anti-pERK antibody (E-4) Santa Cruz Biotechnology sc-7383, Santa Cruz, CA, USA; 1:500) and anti-ERK (Santa Cruz Biotechnology SC-7383; 1:1000), were incubated overnight at 4 °C and for 1 h at room temperature with peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse, Jackson Immunoresearch, West Grove, PA, USA). Bands were developed using enhanced chemiluminescence (BioRad, Hercules CA, USA) and digital images were captured with a charge-coupled device imager (IMAGEQUANT LASc 4000, GE Healthcare Little Chalfont, UK). Immunoreactive bands were quantified with ImageJ blots toolkit software (National Institutes of Health, Baltimore, MD, USA). The intensity of the pERK bands was measured and normalized to the ERK band intensity, and data were expressed as the pERK/ERK ratio for each treatment, relative to that of the naïve rat group. Data were analysed by one way-ANOVA followed by Bonferroni *post-hoc* test.

Immunohistochemistry and immunofluorescence staining

Immunohistochemistry and immunofluorescence analysis were performed as described (Albert-Gascó et al. 2017). Briefly, the RXFP3-A2 and naïve groups ($n = 6$–8/group) were euthanized with sodium pentobarbital (120 mg/kg, Eutanax, Fatro, Barcelona, Spain) and transcardially with saline (0.9% NaCl) followed by fixative (4% paraformaldehyde in 0.1 M PB, pH 7.4) for 30 min (*600 mL per rat). Brains were then removed and immersed in fixative for 4 h at 4 °C. Subsequently, brains were cryoprotected in 30% sucrose in 0.01 M phosphate-buffered saline p H7.4 for 3 days.

Coronal sections (40 µm) from bregma 0.36 mm to − 3.6 mm (Paxinos and Watson 2014) were obtained using a freezing slide microtome (Leica SM2010R, Heidelberg, Germany) and stored in sucrose-PBS. After incubation in blocking solution (10% NGS, in 0.1 M PBS, 0.3% Triton X-100, pH 7.6) sections were incubated overnight at room temperature with rabbit anti-phospho-MAPK/ERK (#9101 Cell Signalling, Danvers, MA, USA; 1:200) and mouse anti-RLN3 (Tanaka et al. 2005; 1:5) in blocking solution.

For immunochemistry of pERK, after several rinses in PBS, sections were incubated for 1 h in 1:200 biotinylated goat anti-rabbit secondary antibody (Jackson Immunoresearch, 111-065-003), rinsed and transferred to avidin–biotin complex (Vectastain-Elite, Cat No. PK-6100; Vector Laboratories, Burlingame, CA, USA). Labelling was visualized with 0.025% DAB, 0.0024% H_2O_2 in Tris–HCl buffer, pH

7.6. After final washes, sections were mounted, air-dried, dehydrated and coverslipped with DPX (Sigma-Aldrich). Quantification of pERK-positive neurons was conducted using ImageJ (National Institutes of Health). Data were expressed as the number of pERK-positive neurons/area and normalized to values observed in vehicle rats.

Double-labeling immunofluorescence, was completed as described (Albert-Gascó et al. 2017) Briefly, sections were incubated with primary antisera and then with goat anti-rabbit Cy3 and goat anti-mouse Alexa Fluor 488 (Jackson Immunoresearch; #111-165-003, #115-545-003). Following further rinsing, sections were mounted on slides and coverslipped using Fluoromount-G (#0100-01, Southern Biotech, Birmingham, AL, USA).

Multiplex in situ hybridization

In studies to evaluate ERK activation in RXFP3 mRNA-positive neurons, we combined detection of pERK immunofluorescence with in situ hybridization (ISH) detection of *Rxfp3* mRNA-positive neurons, using fluorescence ISH [RNAscope™; Advanced Cell Diagnostics (ACD); Newark, CA, USA]. After behavioural testing, rats were transcardially perfused, as described (Albert-Gascó et al. 2017). After 18 h post-fixation at 4 °C, brain sections (30 µm) were collected using a vibratome (Leica VT 1200S, Wetzlar Germany) and transferred to a cryprotectant medium (30% ethylene glycol, 30% glycerol in phosphate buffer, pH 7.4) and stored at − 20 °C.

For the detection of *Rxfp3* mRNA, probes covered ~ 1000 bp of the target mRNA. Sections were mounted onto Superfrost Plus Slides (Fisher Thermo Scientific, Hampton, NH, USA, Cat#12-550-15) and air dried. The next day, sections were fixed in 4% formaldehyde for 10 min at 4 °C, and rinsed in PBS. Once dry, a hydrophobic barrier was drawn around the sections (ImmEdge hydrophobic PAP pen, Vector Laboratories, #310018). Sections were incubated with protease pretreatment-4 (ACD, Cat #322340) for 30 min at 40 °C. After a distilled water rinse, sections were incubated for 2 h at 40 °C with *Rxfp3* mRNA probe (ACD, #316181). Following incubation, sections were rinsed with wash buffer (#310,091) and the signal was amplified with ACD amplifier reagents. After several rinses in wash buffer and PBS, sections were incubated for immunofluorescence against pERK, with anti-phospho-MAPK/ERK (Cell Signaling 1:50) for 90 min at room temperature and Cy3 goat anti-rabbit IgG for 30 min.

The neurochemical phenotype of *Rxfp3* mRNA-expressing neurons was assessed using multiplex fluorescence ISH (RNAscope, ACD) using probes targeting *Rxfp3* (#316181), *Oxtr* (#483671) and *Slc32a1* (#415681) mRNA, as described (Albert-Gascó et al. 2018).

Confocal analysis

Confocal analysis was performed as described (Albert-Gascó et al. 2017). Briefly, immunofluorescence images were captured with a Leica DMi8 inverted microscope (Leica Microsystems) at 0.3 µm interval stacks. Immunofluorescent neurons were counted using ImageJ software (National Institutes of Health). Percentages of co-expression between pERK and Rxfp3 mRNA positive neurons in brains from the A2-pref group were compared to the vehicle-treatment group, using a Mann–Whitney test ($n = 4$).

Similarly, ISH images were captured using an LSM 780 Zeiss Axio Imager 2 confocal laser-scanning microscope (Carl Zeiss AG, Jena, Germany). This system is equipped with a stitching stage, and Zen software (Carl Zeiss AG) was used to stitch tiled images taken with a 20× objective or 40× objective. Quantification of co-expression (1 section per level and per rat) was conducted manually using Fiji (Schindelin et al. 2012), with labeling with each probe counted separately, relative to DAPI-stained nuclei, to avoid bias.

Results

Icv RXFP3-A2 infusion impaired social recognition memory

Previous studies have described robust effects of icv infusion of RXFP3 agonists within 20 min post–infusion (Ryan et al. 2013a; Albert-Gascó et al. 2017; de Ávila et al. 2018). Thus, we designed a schedule in which icv infusion of the agonist, RXFP3-A2 or vehicle, was administered 20 min prior to the social or preference tests.

RXFP3 agonist treatment did not alter responses in the *sociability test*, as all groups of rats spent an average 78.4 ± 2.6% of the 10 min duration of the test sniffing the conspecific rat and the sociability index was not significantly different across all groups ($F_{2,23} = 0.30$; $p = 0.74$, Fig. 1b; Supplementary Fig. 1c). However, RXFP3 agonist infusion before the sociability test (A2-Soc) or the preference test (A2-Pref) significantly altered the preference index, which was positive for control groups (naïve: 0.35 ± 0.06, $n = 6$; vehicle: 0.33 ± 0.07, $n = 5$), but negative for the A2-Pref (− 0.14 ± 0.05, $n = 5$) and A2-Soc (− 0.13 ± 0.06, $n = 6$) groups, ($F_{3,21} = 19.14$, $p < 0.0001$; Fig. 1c); indicating that agonist-treated rats had no preference for novelty. This response can be interpreted as a deficit in recalling the familiar rat. Control groups spent > 60% of the test duration exploring the novel conspecific, which is indicative of social memory of the familiar rat and preference to explore the novel rat. In contrast, agonist-treated rats spent a significantly longer time exploring the familiar rat (Supplementary Fig. 1d). Furthermore, the time exploring the

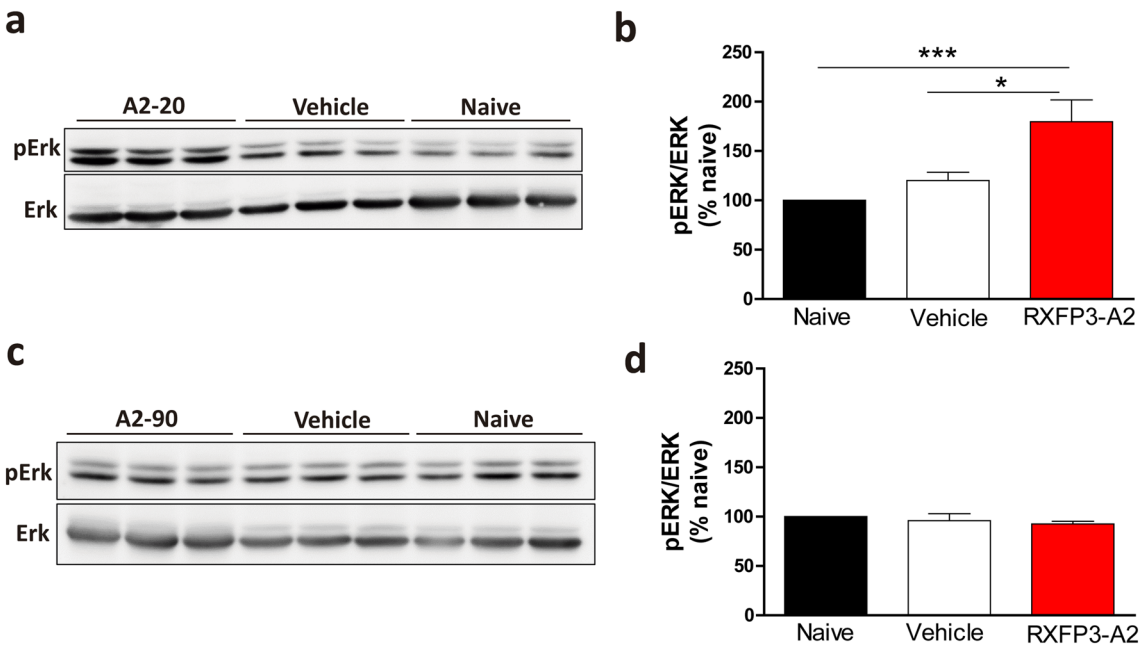


Fig. 2 pERK immunoreactivity is increased in amygdala after RXFP3 agonist infusion. **a, b** Rats killed 20 min after icv infusion of RXFP3-A2 (~1 nmol; red bar) displayed significantly increased pERK in the amygdala area, compared to vehicle-treated (aCSF, white bar) and naïve rats (black bar). **c, d** Rats killed 90 min after infusion exhibited similar pERK levels to those in the control groups. $^{*}p<0.05$; $^{***}p<0.001$

familiar rat was not > 60% of the test duration, ruling out any 'bonding' to the familiar conspecific. Lastly, RXFP3-A2-treated rats did not display increased locomotor activity compared to control groups (data not shown). When analyzing social interaction first contact latencies, A2-Soc groups (8.8 s ± 3.6 s, $n=5$) did not display a significative difference with either vehicle (10.27 s ± 2.7 s, $n=10$) nor naïve groups (13.84 s ± 4.3 s; $n=6$) ($F_{3,26}=0{,}41$, $p=0{,}67$; Fig. 1d). In a similar manner, in the preference test, A2-Soc (4.01 s ± 1.1 s, $n=5$) and A2-Pref (4.755 s ± 1.1 s, $n=8$) subjects did not show differential latencies with vehicle nor Naïve rats (3.8 s ± 0.9 s, $n=6$) ($F_{3,27}=0.59$, $p=0.63$; Fig. 1e). Rats did not display social anxiety, as no significative differences were observed when comparing the different experimental groups first contact latencies.

Icv RXFP3-A2 infusion rapidly increased ERK phosphorylation in amygdala

Using western immunoblotting, we quantified ERK and pERK levels in amygdala tissue extracts from rats killed 20 and 90 min after RXFP3-A2 or aCSF infusions, and from naïve, untreated rats (Fig. 2). All data followed a normal distribution according to a Shapiro–Wilk normality test. When compared to naïve rats (1.00 ± 0.07, $n=8$), rats receiving an aCSF infusion 20 min before perfusion did not display a significant increase in pERK levels (1.20 ± 0.08, $n=8$). In contrast, rats that received RXFP3 agonist infusion (A2–20) displayed a significant increase of pERK levels (1.79 ± 0.2, $n=7$) compared to naïve and aCSF-treated rats ($F_{2,22}=10.29$, $p=0.0008$; Fig. 2a, b). However, rats treated with the agonist 90 min prior to perfusion (A2–90) displayed a similar pERK/ERK ratio to naïve and aCSF-treated rats (0.9 ± 0.02, $n=5$; $F_{2,14}=1.00$, $p=0.39$; Fig. 2c, d).

Icv RXFP3-A2 infusion increased pERK immunoreactivity in discrete amygdaloid nuclei after the three-chamber social interaction and memory test

We assessed the effect of icv RXFP3-A2 infusion on cellular pERK levels in specific amygdaloid nuclei after the three-chamber social interaction test (Table 1). For analysis, the amygdala was considered in two parts: the rostral extension containing the bed nucleus of the stria terminalis (ST); and the temporal extension containing the medial amygdala (MeA) (Olucha-Bordonau et al. 2014). Within the MeA (Fig. 3a), significantly increased pERK (Fig. 3b) was observed in the anterior dorsal part (MeAD) in the A2-Pref group (190 ± 28.0%, $n=6$) compared to the vehicle-treated (100 ± 15.4%, $n=6$) and A2-Soc (101 ± 21.5%, $n=5$) groups ($F_{2,16}=5.49$, $p=0.017$; Fig. 3c–e). Similarly, significantly increased pERK (Fig. 3b) was observed in the posterior ventral part (MePV) of the A2-Pref group (177.3 ± 18.9%,

Table 1 Relative density of pERK-immunopositive neurons in different areas of amygdala

Brain area	Abbreviation	Bregma (mm)	Vehicle	RXFP3-A2-Pref	RXFP3-A2-Soc	*p* value
Anteromedial nucleus of the stria terminalis	STMA	0.84 to − 0.12	100 ± 18.5	136 ± 13.1	101 ± 7.9	0.078
Ventromedial nucleus of the stria terminalis	STMV	0.36 to − 0.36	100 ± 21.9	275 ± 27.2*	196 ± 49.0	0.004
Oval nucleus	OV	0.24 to − 0.24	100 ± 10.7	199 ± 29.0*	118 ± 8.5	0.021
Anteromedial medial amygdala	MeAV	− 1.8 to − 2.52	100 ± 23.6	85 ± 11.2	161 ± 44.6	0.207
Anterodorsal medial amygdala	MeAD	− 1.8 to − 2.4	100 ± 15.4	190 ± 28.0*	100.6 ± 21.5	0.017
Posteroventral medial amygdala	MePV	− 2.64 to− 3.6	100 ± 19.4	177 ± 18.9*	133.4 ± 22.3	0.037
Posteroventral medial amygdala	MePD	− 2.52 to − 3.48	100 ± 13.0	146 ± 20.7	122 ± 17.36	0.204
Central amygdala	CeA	− 1.56 to − 3.36	100 ± 13.3	197 ± 22.5*	110 ± 17.09	0.003
Basolateral amygdala	BLA	− 1.72 to − 3.12	100 ± 14.0	89 ± 12.1	75 ± 2.8	0.379

$n = 8$), compared to the vehicle-treated (100 ± 19.4%, $n = 6$) and A2-Soc (133.4 ± 22.3%, $n = 6$) groups ($F_{2,18} = 4.07$, $p = 0.037$; Fig. 3f–h). All other regions examined, did not exhibit significant differences in cellular pERK levels, across the groups (Table 1).

In the ST (Fig. 4a), rats in the A2-Pref group displayed a significantly higher density of pERK-positive neurons in the ventral part (STMV) (275 ± 27.2, $n = 8$; Fig. 4b) compared to the A2-Soc (196 ± 49.0, $n = 5$) and vehicle (100 ± 21.9, $n = 6$) groups ($F_{2,18} = 8.10$, $p = 0.04$; Fig. 4c–e). The A2-Pref group also displayed a significantly increased density of pERK-positive neurons in the oval nucleus (STOV) (198 ± 29.0, $n = 5$) compared to the vehicle (100 ± 10.7, $n = 5$) and A2-Soc (118.5 ± 8.50, $n = 5$) groups ($F_{2,11} = 6.10$, $p = 0.02$; Fig. 4f–h).

Using dual immunofluorescence detection of pERK and RLN3, we demonstrated putative close contacts between RLN3-immunoreactive fibres and pERK-positive neurons in the MeAD (Fig. 3i, i′), MePV (Fig. j, j′) and STMV (Fig. 4j, j′) in A2-Pref rats. (Densities of pERK-immunopositive neurons followed a normal distribution according to a S3hapiro–Wilk normality test).

Rxfp3 mRNA-positive neurons in amygdala display increased pERK immunoreactivity after three-chamber social interaction testing

We conducted the three-chamber social interaction test in a separate cohort of rats and assessed neuronal co-expression of *Rxfp3* mRNA and pERK immunofluorescence (Fig. 5a, b). In the MeA, the density of *Rxfp3* mRNA- and pERK immunopositive neurons, was significantly higher in the A2-pref group than the vehicle group, in both the MeAD (27.5 ± 2.9% A2-pref vs 7.25 ± 1.25% vehicle; $n = 4$; $p < 0.05$; Fig. 6a, b″), and the MePV (34.5 ± 5.7% A2-pref vs 6.75 ± 1.8% vehicle; $n = 4$; $p < 0.05$; Fig. 6c, d″).

Analysis of co-expression of *Rxfp3* mRNA and pERK immunolabeling in the OV detected a significantly higher level (Fig. 5a, b) in the A2-pref group than the vehicle group (29.5 ± 0.96% versus 1.5 ± 1.5%; $n = 4$; $p < 0.05$; Fig. 6e, f″). In contrast, although an increased density of pERK immunopositive neurons was observed in the STMV, co-expression of pERK and *Rxfp3* mRNA was not significantly different between the groups (A2-pref, 20.25 ± 5.41%; vehicle, 10 ± 1.8%; $n = 4$; $p = 0.25$; Fig. 5g, h″).

Characterisation of *Rxfp3* mRNA-expressing neurons in amygdala

Multiplex fluorescence ISH was used to characterize key aspects of the neurochemical phenotype of *Rxfp3* mRNA-expressing neurons. In the temporal extended amygdala, expression of *Rxfp3, Oxtr* and *Slc32a1* mRNA was examined (Table 2). In the MeAD, ~ 78% of the *Rxfp3* mRNA-expressing neurons were *Slc32a1* mRNA-positive (GABAergic) (Fig. 7a, a′); 5% were *Oxtr* mRNA positive and ~ 12% were positive for *Oxtr* and *Slc32a1* mRNA. Only ~ 5% of *Rxfp3* mRNA-expressing neurons were negative for the other mRNA species (Fig. 7b).

In the MePV, 36% of *Rxfp3* mRNA-expressing neurons were *Slc32a1* mRNA-positive (GABAergic) (Fig. 7c, c′), ~ 20% were *Oxtr* mRNA-positive, and 11% expressed all three mRNA species. Furthermore, ~ 44% of *Rxfp3* mRNA-expressing neurons expressed *Oxtr* mRNA (Fig. 7d). These data indicate that although a small proportion of *Rxfp3* mRNA-expressing neurons in the MeAD express *Oxtr*, this population is larger in the MePV, where ~ 31–44% of the *Rxfp3* mRNA-expressing neurons express *Oxtr* mRNA. Conversely, almost half the *Oxtr* mRNA-expressing neurons in the MeAD expressed *Rxfp3* mRNA. Similarly, the percentage of *Oxtr* mRNA-expressing neurons that co-expressed *Rxfp3* mRNA in the MePV ranged from 42 to 69%, probably due to variation in the total number of *Rxfp3* mRNA-expressing neurons (Table 3).

In the STMV, ~ 43% of neurons co-expressed *Rxfp3* and *Oxtr* mRNA (Fig. 7g, h) and ~ 91% of these *Rxfp3* mRNA-positive neurons were *Slc32a1* mRNA-positive (Table 2; Supplementary Fig. 2). In the OV, ~ 38% of

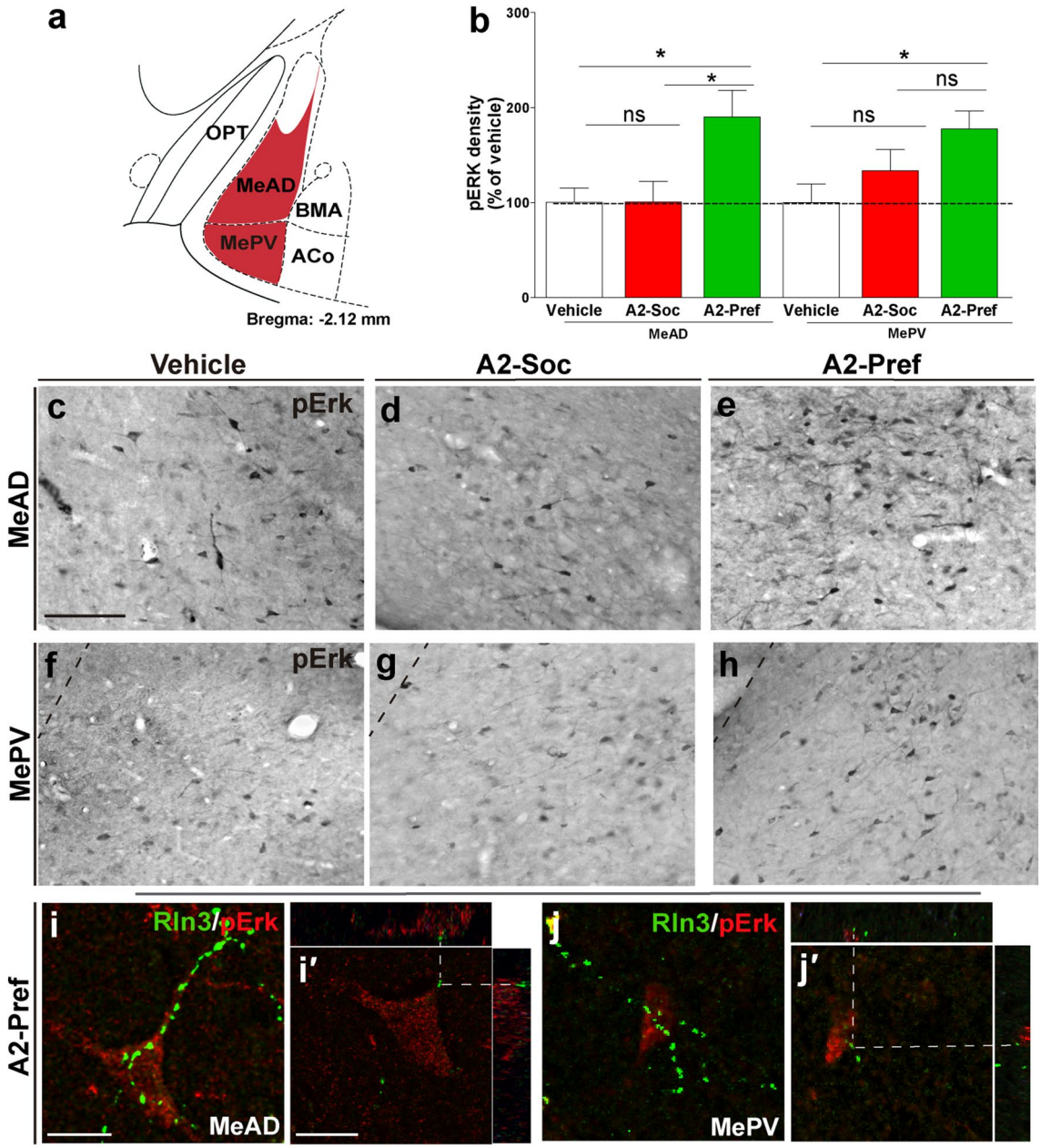


Fig. 3 pERK immunostaining in the MeA after social encounters. **a** Schematic illustrating the MeAD and MePV nuclei analysed. **b** Density of pERK-stained neurons was significantly increased in A2-Pref rats (green bar) compared to vehicle (dashed black line). Representative images of pERK immunostaining in the MeAD of **c** vehicle, **d** A2-Pref and **e** A2-Soc groups. Representative images of pERK immunostaining in the MePV of **f** vehicle, **g** A2-Soc and **h** A2-Pref groups. **i, j** RLN3-immunoreactive fibres (green) make close contact with pERK-positive neurons (red) in the **i** MeAD (**i′**, single plane orthogonal view) and **j** MePV (**j′**, single plane orthogonal view) of A2-Pref rats. *$p < 0.05$; **$p < 0.01$. Scale bars: 100 µm (**c**), 10 µm (**i, i′**)

Rxfp3 mRNA-expressing neurons expressed *Oxtr* mRNA. Furthermore, ~95% of *Rxfp3* mRNA-positive neurons expressed *Scl32a1* mRNA (i.e. GABAergic; Fig. 7e, f; Table 2; and Supplementary Fig. 2). These data indicate a significant proportion of *Rxfp3* mRNA-positive neurons express *Oxtr* mRNA (38–43%), and ~89% of *Oxtr* mRNA-positive neurons in the OV and ~39% in the STMV express *Rxfp3* mRNA (Table 3).

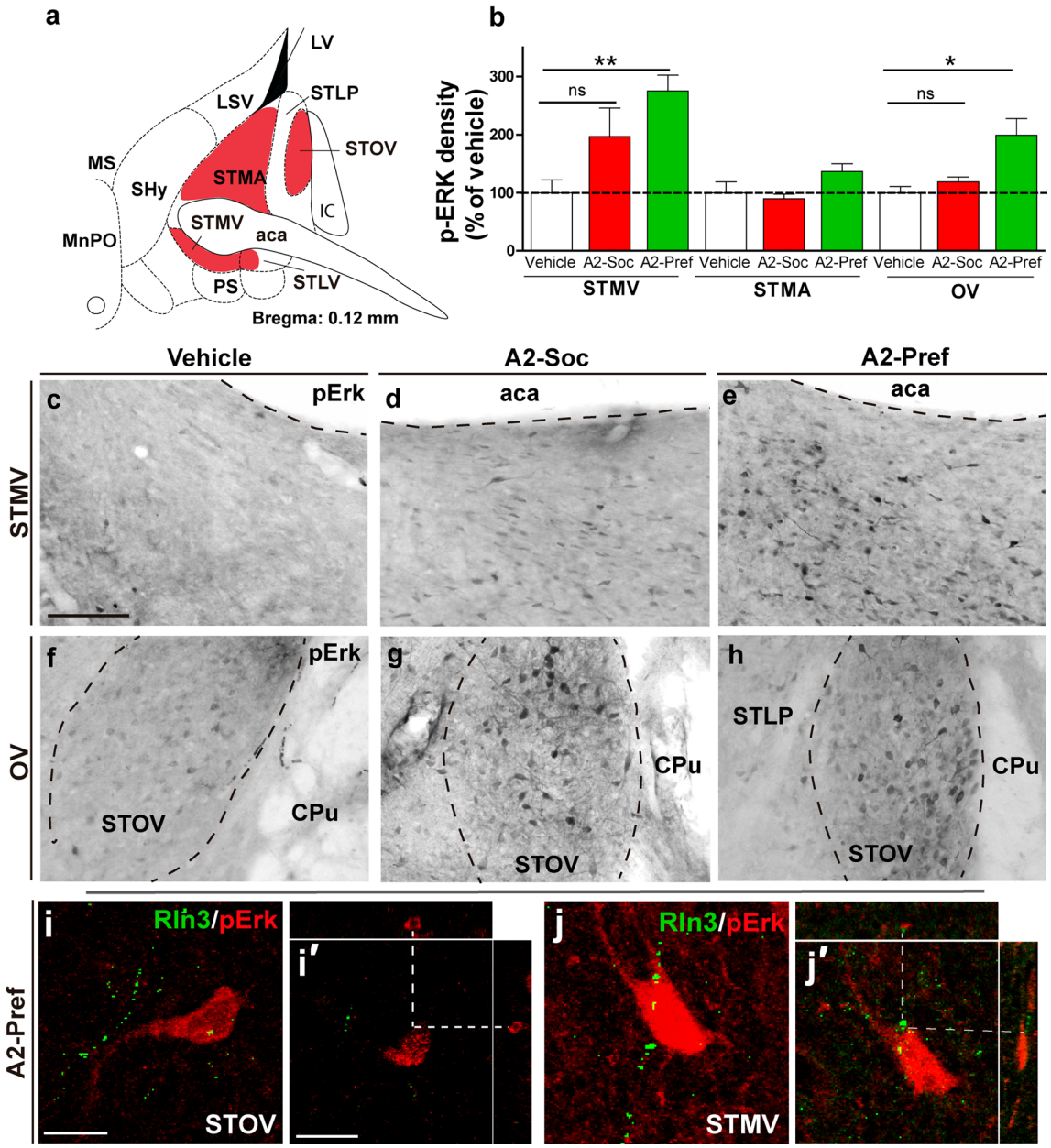


Fig. 4 pERK immunostaining in the extended amygdala after social encounters. **a** Schematic illustrating the STMV, STMA and OV nuclei in the extended amygdala analysed. **b** Density of pERK-stained cells was significantly increased in A2-Pref rats (green bars) compared to vehicle (dashed black line). Representative images of pERK immunostaining in the STMV of **c** vehicle, **d** A2-Pref and **e** A2-Soc rats. Representative images of pERK immunostaining in the OV of **f** vehicle, **g** A2-Soc and **h** A2-Pref rats. **i, j** RLN3-immunoreactive fibres (green) making close contact with pERK-positive neurons (red) in the **i** STMV (**i′**, single plane orthogonal view) and **j** OV (**j′**, single plane orthogonal view) of A2-Pref rats. *$p < 0.05$; **$p < 0.01$. Scale bars: 100 µm (**c**), 10 µm (**i, i′**)

Discussion

Social behaviour is characterized by a number of traits, including social recognition, motivation and reward, and is governed by complex brain processes (Dantzer et al. 1987; Gheusi et al. 1994; Hurlemann et al. 2010; Trezza et al. 2011; Pellissier et al. 2017). In this study, we demonstrate that activation of relaxin-3 receptor (RXFP3) signalling broadly throughout the forebrain impaired SRM of a familiar conspecific in adult male rats. We employed the truncated RXFP3 agonist, RXFP3-A2, which is selective for RXFP3 (Shabanpoor et al. 2012; Zhang et al. 2015) and has previously been administered icv to demonstrate RXFP3 influences on various modalities, including food intake (de Ávila

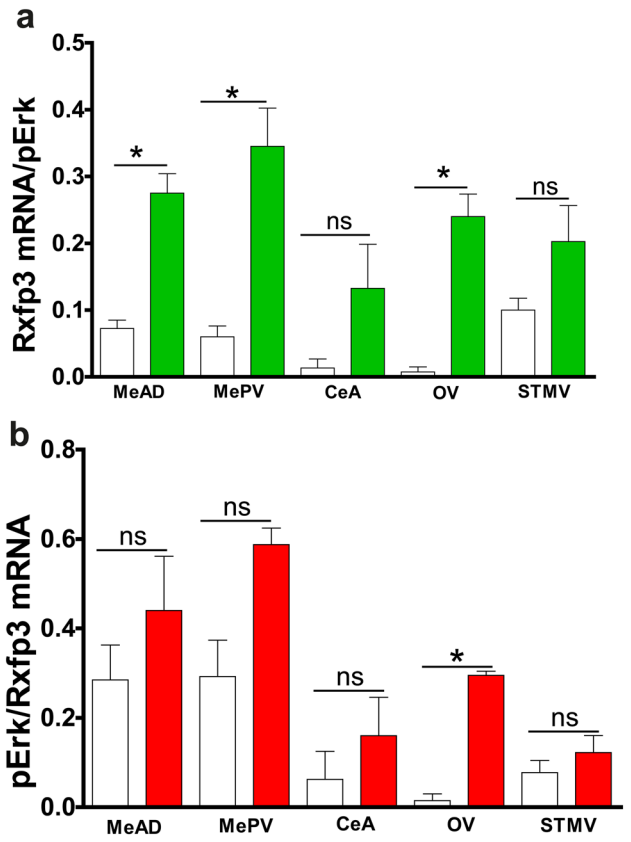


Fig. 5 pERK immunofluorescence and *Rxfp3* mRNA co-expression in neurons of the extended amygdala. **a** Ratio of Rxfp3 mRNA-positive neurons, which display pERK immunoreactivity, **b** ratio of pERK-immunopositive neurons that express Rxfp3 mRNA. *$p < 0.05$; ns: not significant

et al. 2018), anxiety-like behaviour (Ryan et al. 2013a; Ma et al. 2017) and hippocampal-dependent spatial memory (Albert-Gascó et al. 2017).

Global RXFP3 activation decreased discrimination between novel and familiar conspecific rats, but did not alter social discrimination between a rat and an object, as all experimental groups exhibited a similar preference to explore a conspecific rat rather than an inanimate object. Interestingly, RXFP3-A2 infusion prior to the sociability test also impaired SRM at both 20 and 90 min post-infusion. A likely explanation for these findings is that RXPF3 signalling can inhibit memory formation when the agonist is present during the sociability test, and it can interfere with memory recall when present prior to and during the preference test. In both cases, RXFP3 agonist-treated rats were impaired during memory retrieval of familiar conspecifics, due to impaired memory formation and/or recall.

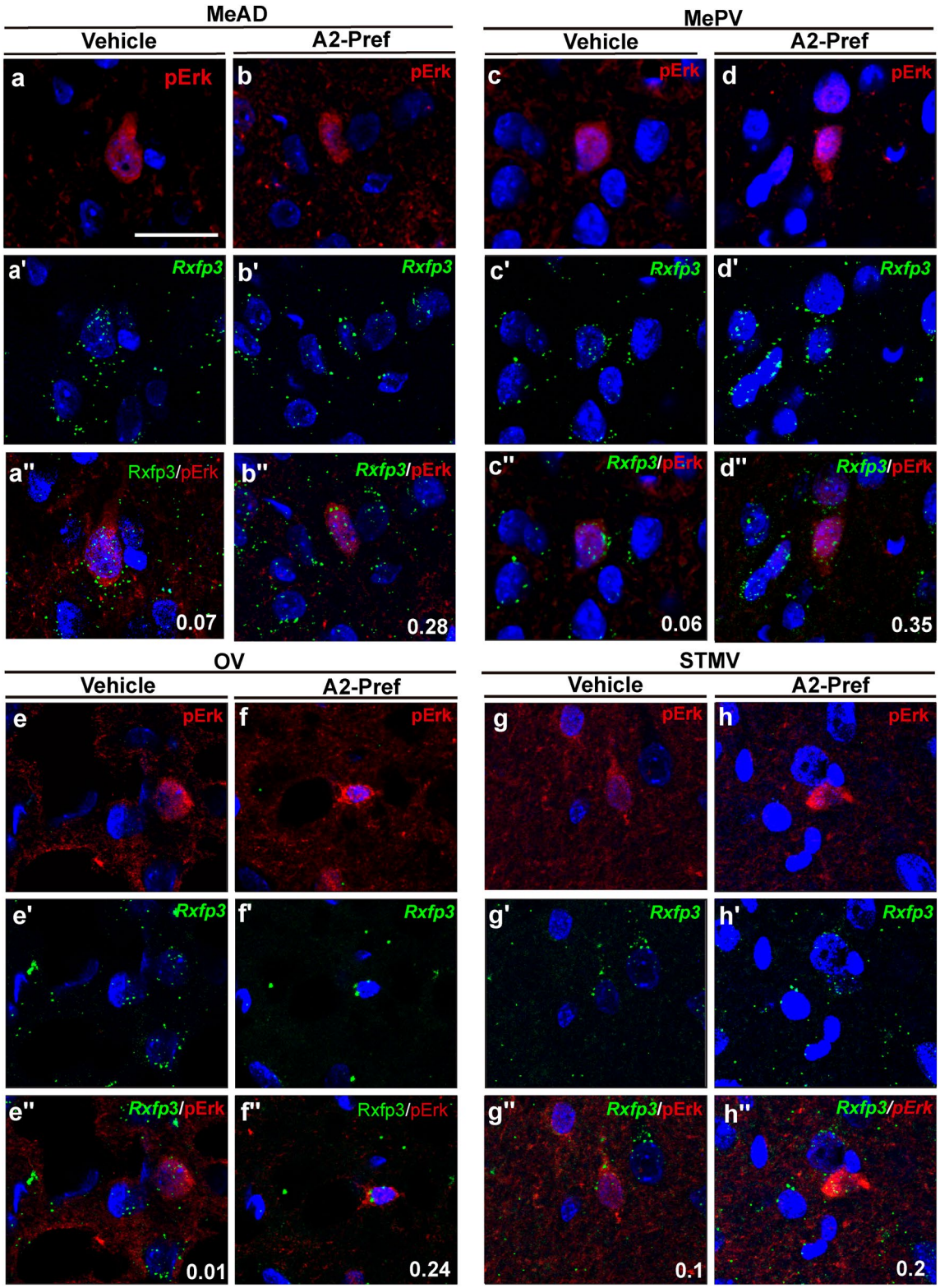
Complex brain circuits are involved in SRM processes (Scalia and Winans 1975; Landgraf et al. 1995; Alheid et al. 1998; Ferguson et al. 2001), and it is thought that the ventral CA1 is the primary repository or store for social-related memories (Okuyama et al. 2016). Amygdala nuclei

Fig. 6 pERK immunofluorescence and *Rxfp3* mRNA co-expression in neurons of the extended amygdala. **a, b** Representative images illustrating pERK-immunopositive neurons, **a′**, **b′** Rxfp3 mRNA and merged signal (**a″**, **b″**) from vehicle and A2-pref group rats in the MeAD. **c, d** Representative images illustrating pERK-immunopositive neurons, **c′**, **d′** Rxfp3 mRNA and merged signal (**c″**, **d′**) from vehicle and A2-pref group rats in the MePV. **e, f** Representative images illustrating pERK immunopositive neurons, **e′**, **f′** Rxfp3 mRNA and merged signal (**e″**, **f″**) from vehicle and A2-pref group rats in the OV. **g, h** Representative images illustrating pERK-immunopositive neurons, **g′**, **h′** Rxfp3 mRNA and merged signal (**g″**, **h″**) from vehicle and A2-pref group rats in the STMV. Scale bar: 25 µm (**a**)

and regions of ST are centrally involved in memory formation, and in particular, the MeA has been strongly associated with SRM regulation (Ferguson et al. 2001; Lukas et al. 2013; Gur et al. 2014), as it receives direct projections from centres involved in social processing (Scalia and Winans 1975). Notably, this social information is powerfully modulated by vasopressin (Everts and Koolhaas 1997; Veenema 2008; Gobrogge et al. 2009) and oxytocin (OT) systems. For example, OT gene knockout mice exhibit impaired SRM, and local infusions of OT into the MeA rescued this social behaviour (Everts and Koolhaas 1997; Winslow et al. 2000; Ferguson et al. 2001; Choleris et al. 2007; Veenema 2008). Furthermore, OT gene knockout mice exhibit decreased c-Fos expression in MeA, ST and medial preoptic area after a social interaction and memory test (Ferguson et al. 2001).

In an effort to determine whether RXFP3 signalling affected neural activity in the amygdala, we examined ERK activation patterns following icv RXFP3-A2 administration. Western blot analysis revealed increased pERK immunoreactivity in amygdala extracts 20 min post-agonist infusion, compared to levels in vehicle-treated and naïve, untreated rats. At 90 min post-agonist infusion, pERK immunoreactivity levels were equivalent to basal levels in these control rats. Thus, the peak in ERK phosphorylation in the amygdala was reached during social recognition and preference encounters, and highlights the relevance of ERK activation in social learning and memory (Peng et al. 2010; Giese and Mizuno 2013); as well as the consistent influence of the RLN3/RXFP3 system on the amygdala (Ma et al. 2007; Pereira et al. 2013; Santos et al. 2016).

Notably, in subsequent immunohistochemical studies, we detected an increase in pERK-positive neurons in the MeA. In line with this finding, pERK was increased in the MeA of mice with social deficits compared to controls, after a sociability and preference test (Faridar et al. 2014; Seese et al. 2014). Furthermore, in this model, oxytocin administration, improved social memory and reduced pERK levels in MeA, revealing a correlation between behavioural impairment and elevated pERK in MeA (Winslow et al. 2000; Winslow and Insel 2004). The observation that RXFP3 agonist treatment increased pERK levels in MeA and induced an impairment of SRM further



supports the involvement of MeA signalling in social behaviour regulation. In anatomical studies, we confirmed the nature of the RLN3 innervation pattern within the amygdala (Santos et al. 2016) and noted several RLN3-positive nerve fibres making close contacts with pERK-immunopositive neurons in MeAD and MePV after the SRM task.

In assessing the co-expression of RXFP3 mRNA and mRNA encoding other relevant neural markers, in the MeAD

Table 2 Proportion of *Rxfp3* mRNA-expressing neurons in different areas of amygdala that co-express other mRNA species

MeAD				
Rxfp3-alone	*Rxfp3-Oxtr*	*Rxfp3-Slc32a1*	Triple	Total *Rxfp3*
6	5	85	13	111
MePV				
Rxfp3-alone	*Rxfp3-Oxtr*	*Rxfp3-Slc32a1*	Triple	Total *Rxfp3*
29	18	32	10	89
Rxfp3-alone	*Rxfp3-Oxtr(1)*			Total *Rxfp3*
55	44			99
Rxfp3-alone	*Rxfp3-Oxtr(2)*			Total *Rxfp3*
27	37			64
OV				
Rxfp3-alone	*Rxfp3-Oxtr*			Total *Rxfp3*
47	29			76
Rxfp3-alone		*Rxfp3-Slc32a1*		Total *Rxfp3*
6		118		124
STMV				
Rxfp3-alone	*Rxfp3-Oxtr*			Total *Rxfp3*
13	10			23
Rxfp3-alone		*Rxfp3/Slc32a1*		Total *Rxfp3*
3		30		33

we observed that most *Rxfp3* mRNA-positive neurons were GABAergic (90% *Slc32a1* mRNA-positive), and 17% also expressed *Oxtr* mRNA. Furthermore, 46% of the *Oxtr* mRNA-expressing neurons in MeAD co-expressed *Rxfp3* mRNA. In contrast, in the MePV only 47% of *Rxfp3* mRNA-positive neurons expressed *Slc32a1* mRNA, while > 60% of *Oxtr* mRNA-positive neurons co-expressed *Rxfp3* mRNA. Activation of the oxytocin receptor (OTR), which is coupled to the excitatory $G_{q/11\alpha}$ class of G-proteins that together with Gβγ stimulate the activity of phospholipase C-β isoform proteins (Shojo and Kaneko 2000), has been shown to produce neuronal excitation in the MeA (Terenzi and Ingram 2005), and presynaptic OTR activation promoted glutamate release (Mairesse et al. 2015). In contrast, RXFP3 is coupled to inhibitory $G_{\alpha i/o}$-proteins (Liu et al. 2003; Van der Westhuizen et al. 2007, 2010) and has been reported to hyperpolarize OT (and vasopressin) neurons in vitro (Kania et al. 2017). Thus, OTR and RXFP3 likely have directly opposing effects on the activity of some populations of amygdala neurons, and bidirectional regulatory mechanisms may exist between OT and RLN3 neurons, to regulate peptide release (Mairesse et al. 2015) and postsynaptic actions (Tyzio et al. 2006, 2014).

In RXFP3-A2-treated rats, we also detected increased pERK levels in the STMV and OV nuclei, which are strongly interconnected with the cortical amygdala and the MeA (Gutiérrez-Castellanos et al. 2014; Cádiz-Moretti et al. 2016) and have been implicated in social interaction and social memory (Ferguson et al. 2001; Bannerman et al. 2004; Takahashi et al. 2004; Kuhlmann et al. 2005). In these regions, *Rxfp3* mRNA was predominantly expressed by GABAergic neurons (80% *Slc32a1* mRNA-positive), and ~ 30% were *Oxtr* mRNA-positive. A major (39%) and minor (16%) proportion were *Oxtr* mRNA-positive in the MePV and the MeAD, respectively (Table 2). These findings suggest the RLN3/RXFP3 and OT/OTR systems may interact in the ST. In this regard, OT gene knockout mice displayed a specific decrease in neuronal activation in ST after a social interaction and memory test (Ferguson et al. 2001). All areas studied in these experiments are also known to be in rodents important processing centres which precede elicitation of aggressive behaviours; these include MeA, ST, LS and hippocampus (Nelson and Trainor 2007). In addition, aggression is known to impair recognition of social cues and enhance impulsivity (Kruk 1991). Thus, there is a possibility that social recognition memory modulation by the RLN3/RXFP3 system could elicit aggressive cues. Specially interesting is if this effect is dimorphic given its impact over the ST (Everts et al. 1997; Calvez et al. 2016).

Taken together, the current findings have demonstrated that RXFP3 is expressed by GABAergic neurons in the extended amygdala, consistent with reports in other brain areas (Richter et al. 2005; Blasiak et al. 2013; Haidar et al. 2017; Albert-Gascó et al. 2017), which implies an overall disinhibitory effect of the RLN3/RXFP3 system within the amygdala. Furthermore, the presence and proportions of *Rxfp3* and *Oxtr* mRNA-expressing neurons was broadly consistent across the different amygdaloid nuclei, suggesting a functional interaction between RXFP3 and OTR signalling, which warrants further investigation. Notably, OT has been

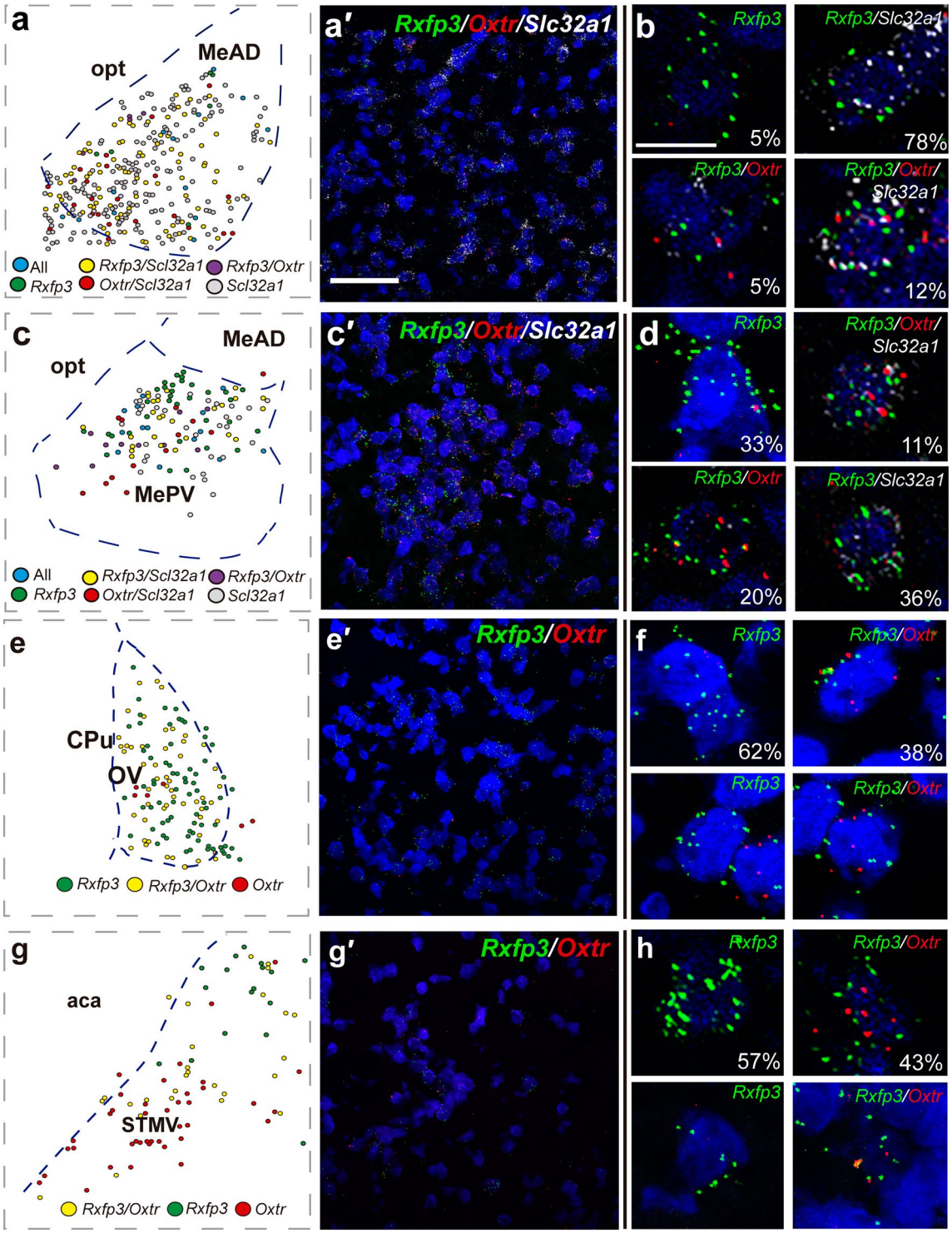


Fig. 7 Characterization of the neurochemical phenotype of *Rxfp3* mRNA-positive neurons in the extended amygdala. **a** Schematic illustrating *Rxfp3* mRNA distribution and co-expression with *Oxtr and Slc32a1* mRNA in the MeAD. **a′**, **b** Representative images of fluorescent ISH and quantification of co-expression percentages indicated (lower right corner). **c** Schematic illustrating *Rxfp3* mRNA distribution and co-expression with *Oxtr and Slc32a1* mRNA in the MePV (**c′**, **d**). **e** Schematic illustrating *Rxfp3* mRNA distribution and co-expression with O*xtr* mRNA in the STOV. **e′**, **f** Representative images of fluorescent ISH and co-expression percentages. **g** Schematic illustrating *Rxfp3* mRNA distribution and co-expression with O*xtr* mRNA in the STMV (**g′**, **h**). Scale bar: 100 µm (**a′**), 10 µm (**b**)

Table 3 Proportion of *Oxtr* mRNA-expressing neurons that co-express *Rxfp3* mRNA in different areas of amygdala

Probe combination	*Rxfp3*/*Oxtr*	Total *Oxtr*
MePV		
Rxfp3-Oxtr-Slc32a1	22	66
Rxfp3-Oxtr 1	52	77
Rxfp3-Oxtr 2	33	48
MeAD		
Rxfp3-Oxtr-Slc32a1	18	66
OV		
Rxfp3-Oxtr	52	58
STMV		
Rxfp3-Oxtr	27	68

implicated in the elicitation of social memory consolidation (Ferguson et al. 2001; Gur et al. 2014). As such, our results suggest a role for RLN3/RXFP3 signalling in modulating social memory, which might occur via rapid (direct and/or indirect) alterations in pERK and related signalling in GABAergic/peptidergic neurons within specific areas of the extended amygdala. Moreover, RXFP3 activation might be capable of modifying (antagonizing) the influence of OT/OTR signalling on MeA and ST neurons, in regulating (promoting) social behaviour, but further studies are now required to clarify the nature and complexity of this interaction.

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Author contributions HA-G, performed most experiments, wrote the first draft of the manuscript, compiled and edited the figures, and edited successive drafts of the manuscript. SS-S, helped perform the behavioural experiments, and conducted analysis of behavioural data. SM, helped design and perform the multiplex in situ hybridization experiments, and edited successive drafts of the manuscript. CG-D, helped perform the behavioural experiments and the combined multiplex in situ hybridization and immunofluorescence studies, and analysed these data. ALG, participated in the design of the experiments, and edited the figures and successive drafts of the manuscript. AMS-P, participated in the conception of the study and directed the research, and edited successive drafts of the manuscript. FEO-B, conceived the study and directed the research, designed the experiments, and edited successive drafts of the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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