Title page

Central relaxin-3 receptor (RXFP3) activation increases ERK phosphorylation in septal cholinergic neurons and impairs spatial working memory.

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ABSTRACT

The medial septum/diagonal band (MS/DB) is a relay region connecting the hypothalamus and brainstem with the hippocampus, and both the MS/DB and dorsal/ventral hippocampus receive strong topographic GABA/peptidergic projections from the *nucleus incertus* of the pontine tegmentum. The neuropeptide relaxin-3, released by these neurons, is the cognate ligand for a $G_{i/o}$-protein-coupled receptor, RXFP3, which is highly expressed within the MS/DB, and both cholinergic and GABAergic neurons in this region of rat brain receive relaxin-3 positive terminals/boutons. Comprehensive *in vitro* studies have demonstrated that a range of cell signaling pathways can be altered by RXFP3 stimulation, including inhibition of forskolin-activated cAMP levels and activation of ERK phosphorylation. In this study we investigated whether intracerebroventricular (icv) injection of RXFP3-A2, a selective relaxin-3 receptor agonist, altered ERK phosphorylation levels in the MS/DB of adult male rats. In addition, we assessed the neurochemical phenotype of phosphorylated (p) ERK-positive neurons in MS/DB after RXFP3-A2 administration by dual-label immunostaining for pERK and key neuronal markers. RXFP3-A2 injection significantly increased pERK levels in MS/DB, compared to vehicle at 20 and 90 min post-injection. In addition, icv injection of RXFP3-A2 increased the number of cells expressing pERK in the MS/DB after 90 min, with increases detected in cholinergic, but not GABAergic neurons. Moreover, we found that septal cholinergic neurons express RXFP3 and that icv infusions of RXFP3-A2 impaired alternation in a spatial working memory behavioral paradigm. The presence of the receptor and the specific RXFP3-related activation of the MAPK/ERK pathway in MS/DB cholinergic neurons identifies them as a key target of ascending relaxin-3 projections with implications for the acute and chronic inhibition of cholinergic neuron activity/function by relaxin-3/RXFP3 signaling.

Key words: Calcium-binding proteins, Choline acetyltransferase, GABA neurons, MAPK/ERK pathway, Nucleus incertus, Septum, working spatial memory, RXFP3 labeling

INTRODUCTION

The septal area is involved in the regulation of behavioural processes of arousal attention and spatial navigation/exploration particularly via connections from the medial septum/diagonal band (MS/DB) to the hippocampus (Vertes and Kocsis 1997). Functionally, attention, arousal, and locomotion related to navigation and exploration and mnemonic processes in humans (Morales et al. 1971; Bannerman et al. 2004) are associated with hippocampal theta rhythm, a synchronous 4-12 Hz oscillation of primarily principal neurons and with place cell configuration (Kemp and Kaada 1975; O'Keefe 1976; Leung and Yim 1986; Raghavachari et al 2001; Hasselmo 2005; Buzsáki and Moser 2013; Fuhrmann et al 2015). Notably, hippocampal theta rhythm can be generated and modulated from the septum (Morales et al 1971; Bannerman et al 2004), and different types of neurons within the MS/DB participate in this process (Sotty et al 2003).

Septal *cholinergic* neurons are slow-firing neurons that promote hippocampal theta rhythm (Sotty et al 2003; Yoder and Pang 2005; Vandecasteele et al 2014) and are responsible for transient arousal states and hippocampal activation (Zhang et al 2011). Septal *GABAergic* parvalbumin (PV)-positive neurons are fast-firing (Sotty et al 2003; Yoder and Pang 2005; Vandecasteele et al 2014) and inhibit inhibitory hippocampal interneurons (Freund and Antal 1988; Toth et al 1997; Freund and Gulyas 1997; Hangya et al 2009). Resultant disinhibition of hippocampal granular and pyramidal cells promotes and facilitates synchronicity in theta frequency (Freund and Antal 1988; Tóth et al. 1997; Freund and Gulyás 1997; Hangya et al. 2009b). In addition, the majority of septal *glutamatergic* neurons are also fast firing and have been reported to drive hippocampal pyramidal cells (Huh et al 2010). Furthermore, septal *glutamatergic* neurons were recently reported to excite interneurons at the CA1 stratum oriens/ alveus border in hippocampus, which regulate feedforward inhibition of Schaffer collateral and perforant path input to CA1 pyramidal neurons in a locomotion-dependent manner (Fuhrmann et al 2015).

Various strategies have been used to study the role of the MS/DB and its effect in memory processes. Most of studies are centered on activation or deactivation of specific cell types within this region. For instance, time dependent increases in acetylcholine levels have been observed in the hippocampus after acquisition of spatial memory tasks such as the T-maze or spontaneous

alternation task (Hepler et al 1985; Ragozzino et al 1996; Fadda et al 1996). In contrast, lesion studies of the MS/DB have reported to disrupt spatial working memory but not nonspatial working memory (Kelsey and Vargas 1993). On no case did the lesions impair working memory on reference memory visual discrimination tasks or simultaneous conditional discrimination task (Thomas and Gash 1986). In addition, when lesions are not electrolytic but with specific excitotoxins which target cholinergic neurons, an impairment in a variety of spatial working memory tasks is produced (Johnson et al 2002; Gibbs and Johnson 2007; Fitz et al 2008).

The MS/DB receives strong connections from mesencephalic and brainstem areas, including the posterior hypothalamus and supramammillary nucleus and brainstem, particularly from the *nucleus incertus* (NI) Goto et al. 2001; Olucha-Bordonau et al. 2003; Ryan et al. 2011; Olucha-Bordonau et al. 2012). Previous studies have shown that activation of these areas alter hippocampal theta rhythm and locomotion. For example, in urethane-anesthetized rats, electrical stimulation of the NI induces an increase in hippocampal theta rhythm and electrolytic lesion of the NI abolishes the hippocampal theta induced by stimulation of the *reticularis pontis oralis* (RPO) region (Nunez et al 2006). Moreover, infusion of R3/I5, a specific RLN3 agonist, into the medial septum promotes theta rhythm (Ma et al 2009). In addition, it has been proposed that the nucleus incertus may relay a general stress response over the telencephalon centers involved in memory processes (Rajkumar et al 2016) and feeding behavior (Calvez et al 2016).

The majority of NI neurons in the rat synthesize and release GABA (Ford et al 1995; Olucha-Bordonau et al 2003; Ma et al 2007) and a significant population of these neurons express the neuropeptide, relaxin-3, a member of the insulin/relaxin superfamily (Bathgate et al 2002; Burazin et al 2002; Ma et al 2007). The largest number of relaxin-3 neurons is located in the NI, but they are also present in the ventral periaqueductal grey, the pontine raphe nucleus and an area dorsal to the lateral substantia nigra (Tanaka et al 2005; Ma et al 2007). Relaxin-3 is the single cognate of the $G_{i/o}$-protein-coupled receptor, RXFP3 (Liu et al 2003), and RXFP3 mRNA and binding sites are strongly expressed in the brain in a topographical distribution that aligns with that of relaxin-3 containing axons and nerve terminals in rat (Sutton et al 2004; Ma et al 2007) and mouse (Smith et al 2010) brain.

In vitro studies in various cell lines (Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) 293 cells) stably expressing human RXFP3 and SN56 cholinergic neuroblastoma cells, which display endogenous RXFP3 expression, demonstrate that RXFP3 stimulation can consistently decrease forskolin-stimulated cAMP levels and activate the MAPK/ERK pathway, as reflected by changes in ERK1/2 phosphorylation and activation of immediate early gene/transcription factor expression (e.g. activator protein 1, AP-1, nuclear factor-κB, NF-κB and serum response element, SRE). However, while central relaxin-3 injections have been associated with changes in immediate early gene expression and there are preliminary reports of RXFP3 activation producing hyperphosphorylation of putative RXFP3-positive neurons in rat brain slices *in vitro* (Kania et al 2014) consistent with inhibition of cellular cAMP levels by Gi/o coupled receptors, no reports exist on the effect of central RXFP3 activation *in vivo* on relevant cell signaling pathways.

Therefore, in this study, we investigated the effect of RXFP3 stimulation in rat brain by central administration of the selective relaxin-3 receptor agonist, RXFP3-A2 (Shabanpoor et al 2012) on MAPK/ERK pathway-related signaling in the MS/DB. Cerebellum, which lacks RXFP3 expression (Sutton et al 2004), was used as a negative control tissue. Using immunoblotting, we quantified phosphorylated ERK (pERK) and total ERK levels, as described in earlier *in vitro* studies (van der Westhuizen et al 2007), to assess the impact of RXFP3 activation on overall septal MAPK/ERK activation. Subsequently, we used immunofluorescence staining to characterize the neurochemical phenotype of pERK-positive neurons by co-localizing pERK immunoreactivity with choline acetyltransferase as a marker for cholinergic neurons and the calcium-binding proteins (CaBP), parvalbumin, calretinin and calbindin for different populations of septal GABAergic neurons (Olucha-Bordonau et al 2012). In addition, we have assessed the neuronal target of RLN3 by double immunofluorescence of RXFP3 and medial septal markers. Finally, we have studied the behavioral effect of A2 icv administration using a working spatial memory task.

Our data demonstrate that cholinergic neurons in the MS/DB express RLN3 receptor, RXFP3. Activation of RXFP3 by agonist infusion increased ERK phosphorylation in cholinergic neurons

of the MS/DB. In agreement with a putative effect of cholinergic neurons silencing (Fitz et al 2008).

RXFP3 central activation impairs the spontaneous alternation task. These results provide novel neurochemical and anatomical evidence for the importance of the NI and relaxin-3 related regulation of MS/DB and hippocampal function, with implications for better understanding the ascending control by these neural networks of attention/arousal, navigation/exploration, and associated cognitive processes.

MATERIALS AND METHODS

Animals and surgical procedures

Procedures were in line with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes and approved by the Ethics Committee of the University Jaume I. Adult male Sprague-Dawley rats between 300-350 g were used. All rats were maintained on a 12 h light cycle and provided with food and water *ad libitum*.

For surgical procedures rats were first deeply anesthetized with ketamine (Imalgene 55 mg/kg i.p.; Merial Laboratories SA, Barcelona, Spain) and medetomidine (Xilagesic 20 mg/kg i.p.; Lab Calier, Barcelona, Spain). Cannula and holding-screw holes were made in the skull using a surgical drill. Stereotaxic coordinates used to insert a cannula into the right lateral cerebral ventricle were AP 0.48 mm, ML 0.1 mm, DV 4 mm from bregma (Paxinos and Watson, 2014).

Experimental groups

One week after surgery rats were injected with a total volume of 1 µL of RXFP3-A2 peptide (5 µg/µL) or vehicle (aCSF) was at a rate of 0.5 µL/min through the implanted cannula by means of a Harvard syringe injector (Harvard PHD 2000 Syringe Pump; Harvard Apparatus, Holliston, MA, USA). The 'RXFP3-A2' agonist ([R3A(11–24,C15→A)B]; kindly provided by Dr Mohammad Akhter Hossain, The Florey Institute of Neuroscience and Mental Health, Melbourne, Australia) has been shown to display high affinity for the cognate relaxin-3 receptor, RXFP3 with higher selectivity for RXFP3 *c.f.* the relaxin receptor, RXFP1; and has been shown to alter anxiety-like and other behaviours following central adminstration (Shabanpoor et al 2012; Ryan et al 2013). In the current studies, multiple experimental groups of rats were used with one cohort (n = 6-9/treatment group) used for measurement of pERK and ERK levels in different brain areas by Western blotting; and a second cohort (n = 6-7/group) used to assess cellular levels of pERK immunoreactivity in different types of neurons in the MS/DB – 'RXFP3-A2' rats were injected with 5ug/uL (~1nmol) of the peptide analogue; 'sham' rats were injected with artificial cerebral spinal fluid (aCSF) vehicle; and time-matched 'naïve' rats had not undergone any surgery (Table 1).

T-maze procedure

Rats from Sham and A2 groups were handled by researchers daily during a week prior to the behavioral test after the surgeries. On the day of behavioral test and sacrifice, both groups were habituated for 30 minutes to the behavioral room before the infusions (vehicle or RXFP3-A2). Five minutes after the infusion rats were left to explore the T-maze with one of the short arms closed (familiarization phase) for 5 min. Throughout the whole procedure the closed arm was balanced, alternating the closed arm every two cases (one vehicle, one RXFP3-A2) to avoid side bias. The closed arm is refereed as the *novel* arm and the open one which has been already explored during familiarization is the *familiar* arm. After familiarization, rats were left to rest in their home cages for 95 minutes before tested again (for 3min) on the T-maze with all entries open to evaluate the alternation between familiar and novel arms.

Immunoblotting

In Western blotting studies, pERK levels were assessed at 20 and 90 min after peptide injection. Rats were lightly anesthetized (Dolethal, 200 mg/Kg Vetoquinol S.A., Madrid, Spain) and then killed by decapitation. Brains were rapidly removed and frozen in cold isopentane (Sigma-Aldrich, St Louis, MO, USA). Brain areas of interest (septum and cerebellum) were dissected with 1 mm diameter disposable biopsy punches (Interna Miltex, Ratingen, Germany) from 20 µm brain slices cut using a cryostat at -15ºC to preserve protein phosphorylation. For the immunoblot assays, where necessary, we pooled tissues from 3 rats in each experimental group. Tissue was lysed in RIPA buffer containing protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor, Thermo Scientific, Waltham, MA). Mechanical cell lysis was achieved using a Sonicator (Hielsher Ultrasound Technology, Teltow, Germany).

Forty (40) µg of total protein was subjected to SDS-PAGE, transferred to Immobilon-P membranes (MERCK Millipore, Darmstadt Germany), which were blocked for non-specific binding and incubated with primary antibodies: anti-phospho MAPK/Erk1/2 (Cell Signaling, Danvers, MA, USA 1:2,000); and anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000) overnight at 4ºC. After several washes with washing buffer containing 0.1% Triton X-100, membranes were incubated for 1 h at RT with peroxidase-conjugated secondary antibodies

(anti-rabbit and anti-mouse, Jackson Immunoresearch, Suffolk, UK). Staining was developed using ECL (BioRad, Hercules CA, USA) and digital images were captured with charge-coupled device (CCD) imager (IMAGEQUANT LASc4000, GE Healthcare Little Chalfont, UK). Immunoreactive bands were quantified with ImageJ blots toolkit software (National Institutes of Health, Baltimore, MD, USA). Data was analysed by one way-ANOVA, followed by Bonferroni post-hoc test.

Tissue immunofluorescence

Treated and naïve rats used for immunofluorescence (IF) assays were euthanized with an intraperitoneal (i.p.) injection of sodium pentobarbital (120 mg/Kg Eutanax, Fatro, Barcelona, Spain) 90 min after either RXFP3-A2 or vehicle icv infusions. Rats were transcardially-perfused 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). After perfusion, brains were removed and immersed in the same fixative for 4 h at 4°C. After fixation, brains were cryoprotected in 30% sucrose in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 3 days. Coronal sections (40 µm) were obtained using a freezing slide microtome (Leica SM2010R, Heidelberg, Germany). For each brain (n = 10 per group), 6 series of sections between Bregma +0.8 to 0.4 mm (Paxinos and Watson, 2014) were collected in 30% sucrose in 0.01 M PBS.

The neurochemical phenotype of pERK-positive neurons was assessed by dual-labeling with a pERK1/2 antiserum and antisera against neuronal markers in MS/DB sections from rats sacrificed 90 min after agonist or vehicle infusion. Coronal brain sections were incubated in blocking solution (4% NDS, 2% BSA in 0.05 M TBS, 0.3% Triton X-100, pH 8) followed by primary antibody incubation; rabbit anti-phospho MAPK/ERK (Cell Signaling), 1:200; goat anti-ChAT (MERCK Millipore, Darmstadt Germany) 1:700; mouse anti-calbindin (CB) (Swant, Marly, Switzerland) 1:2,000; mouse anti-calretinin (CR) (Swant) 1:2,000 and mouse anti-parvalbumin (PV) (Swant) 1:2,000 in 0.05M TBS, 0.3% Triton X-100 (2% NDS, 2% BSA, pH 8) overnight at RT. After several rinses, sections were incubated for 2 h at RT with either goat anti-rabbit Cy™3 and goat anti-mouse Alexa Fluor 488, or donkey anti-rabbit Cy™3 and donkey anti-goat Alexa

Fluor 488, secondary antibodies (Jackson Immunoresearch). Following further rinsing sections were mounted on slides and coverslipped using glycerol.

The neurochemical phenotype of RXFP3-positive neurons was measured by dual-labeling with a rabbit anti-RXFP3 (Atlas Antibodies, Stokholm Sweeden) 1:400 antiserum and antisera goat anti-ChAT (MERCK Millipore, Darmstadt Germany) 1:700 and mouse anti- PV (Swant) 1:2,000. After several rinses, sections were incubated for 2 h at RT with either goat anti-rabbit Cy™3 and goat anti-mouse Alexa Fluor 488 or donkey anti-rabbit Cy™3 and donkey anti-goat Alexa Fluor 488, secondary antibodies (Jackson Immunoresearch). Following further rinsing sections were mounted on slides and coverslipped using glycerol.

Image analysis and statistics

Fluorescence images were taken with a confocal scan unit with a module TCS SP8 equipped with argon and helio-neon laser beams attached to a Leica DMi8 inverted microscope (Leica Microsystems). Excitation and emission wavelengths for Cy3 were 433 nm and 560-618 nm respectively; Alexa488-labeled excitation wavelength was 488 nm and its emission at 510-570 nm. Serial 0.2 μm scans were obtained in the Z-plane and a maximal projection of 20 μm was generated with Image J.

For quantification of dual-labeling we used a 20× lens. With Image J software combined with *loci.tools* plugin (BIO-FORMATS, University of Wisconsin-Madison), we counted the number of positively-labeled cells in 15-18 sections from 4 brains from the different experimental groups. Data was expressed as the percentage of double-labeled neurons (neuronal marker + pERK), divided by the total number of marker-positive (ChAT, PV, CR or CB) neurons. All analyses were conducted by an observer blinded to the experimental conditions of the samples. Statistical analyses for significant differences in the pERK-positive neurons comparing sham and RXFP3-A2 groups, employed the Student's t-test with probability set at $\alpha < 0.05$ (GraphPad Prism V5 software, GraphPad, La Jolla, CA, USA).

RESULTS

RXFP3 activation by RXFP3-A2 increases ERK phosphorylation *in vivo*

ERK and pERK proteins were reliably detected by immunoblot in tissue extracts of septum and cerebellum from rats sacrified 20 and 90 min after infusion of RXFP3-A2 or aCSF vehicle (sham) and from naïve untreated rats (Fig. 1). ERK activity was quantified by measured the pERK and ERK band intensity and normalization to the ERK signal in each sample. In order to compare RXFP3-A2, sham and naïve samples within and between experiments, we expressed the data as a percentage of the naïve group pERK/ERK ratio and all samples followed a normal distribution according to the Shapiro-Wilk test. Data from septal samples collected 20 min after agonist or vehicle infusion were analyzed by one-way ANOVA followed by Bonferroni post-test ($F_{20,21}$ = 8.128, p = 0.0031; Fig. 1B**)** and pERK/ERK levels in the RXFP3-A2 group were significantly higher (1.5 ± 0.16, n = 7) than in the sham (1.0 ± 0.08, n = 7) and naïve (0.93 ± 0.07, n = 7) rat groups. In contrast, cerebellar levels of pERK/ERK from matched samples were not different from each other (one-way ANOVA followed by Bonferroni post-test ($F_{21,22}$ = 0.4348, p = 0.65; Fig. 1D)) - RXFP3-A2 (0.93 ± 0.14); sham (0.95 ± 0.05) and naïve (1.0 ± 0.06; n = 7/group).

Similarly, 90 min after agonist or vehicle infusion, normalized septal pERK levels in RXFP3-A2 treated rats (1.78 ± 0.16) were significantly higher than normalized pERK levels in sham (1.1 ± 0.15) and naïve (1.0 ± 0.06;) rats (n = 7-9/group; one-way ANOVA followed by Bonferroni post-test ($F_{26,27}$ = 11.66, p = 0.0003; Fig. 1F)). The pERK/ERK ratio in cerebellar samples from the same groups were similar (one-way ANOVA followed by Bonferroni post-test ($F_{26,27}$ = 0.6339, p = 0.5337; Fig. 1H)) - RXFP3-A2 (0.99 ± 0.13); sham (1.18 ± 0.20); naïve (0.92 ± 0.14) rats (n = 7/group).

RXFP3 activation by RXFP3-A2 increases pERK in cholinergic but not GABAergic neurons in MS/DB

pERK immunostaining was reliably detected within cells in different areas of the MS/DB. Relative levels of staining were assessed in coronal sections between 0.8 to 0.4 mm relative to Bregma (Paxinos and Watson 2014) in an area comprising MS and the vertical DB division (MS/vDB) and an adjacent area comprising the horizontal diagonal band (HDB) of the MS/DB

complex. pERK labeling in these areas followed a normal distribution (Shapiro-Wilk test) and the number of pERK-positive cells was increased in the HDB after RXFP3-A2 treatment (235.0 ± 28.82, n = 5) compared to sham vehicle treatment (111.9 ± 11.09, n = 5; Fig. 2; Student's t-test; p = 0.004). In the MS/vDB the density of pERK-positive neurons was also increased by RXFP3-A2 (137.6 ± 5.78) compared to sham (76.31 ± 5.083, n = 4/group; Student's t-test, p = 0.001).

In studies to characterize the neurochemical content of activated pERK-positive neurons in the MS/DB complex, we analysed dual immunofluorescence (IF) labeling in the MS/vDB (Fig. 3) and HBD (Fig. 4). The total numbers of pERK cells, marker-positive (ChAT, CR, PV and CB) cells and double-labelled cells were counted. The ratio of pERK + marker neurons divided by total number of marker-positive neurons was compared between RXFP3-A2 treated and sham rat groups. In the MS/vDB, normalized pERK/ChAT double-staining increased significantly in RXFP3-A2 treated compared to sham (Fig. 3C-H) rats (69.10 ± 5.587 and 20.93 ± 5.685, n = 6-7/group; Student´s t-test, p <0.0001; Fig. 3B). Under the same conditions very few pERK neurons were double-labeled with GABAergic cellular marker proteins (CB, PV and CR) in sham rats and no increase in double-staining occurred after RXFP3-A2 infusion (Fig. 3B).

Similarly in the HBD, pERK was present in ChAT-positive neurons in sham rats (25.16 ± 5.926, n = 6-7/group; Fig. 4C-E), and the level of double-staining was increased significantly after RXFP3-A2 infusion (59.65 ± 4.372, n = 6-7; Fig. 4F-H; Student's t-test, p = 0.0008; Fig. 4B). In contrast, little or no co-labeling of pERK and PV, CB or CR was observed, similar to observations in the MS/vBD (Table 2), as illustrated by images of labelling of pERK with CR (Fig. 5A-C), PV (Fig. 5D-F) and CB (Fig. 5 G-I) in MS/VDB; and pERK with PV in HBD (Fig. 5J-L).

The distribution of the different molecular markers within the MS/vDB was consistent with its onion-like structure (Wei et al 2012). CaBP positive neurons are arranged in three bands, from the midline outward – PV, CR and CB. Increased pERK staining induced by RXFP3-A2 was present lateral to PV neurons (Fig. 5A-C). Neurons containing increased pERK levels were found in the same regions as CB (Fig. 5D-E) and CR (Fig.5 G-H) neurons, but there was no co-localization of pERK with CB (Fig. 5C) or CR (Fig. 5I). Increased pERK labelling was found in

the superficial layers of the HBD (Fig. 5J), while PV labeled neurons were located deeper within the septum with respect to the brain ventral surface (Fig. 5K); and similarly no colocalization was observed (Fig. 5L). The number of CR- and CB-positive-neurons in this area was low and dispersed and no co-localization with pERK was observed (data not shown).

RXFP3 co-localizes with cholinergic neurons and not with parvalbumin GABAergic neurons

The distribution of RXFP3 expression co-localizes with cholinergic markers, suggesting that RXFP3 A2 infusion affected neurons expressing RLN3 receptor directly, and not through an indirect pathway.

Most of RXFP3-positive neurons located in the lateral and central part of the MS/vDB area (Fig.6 A-C) and HDB (Fig.6 D-G) in coronal sections between 0.8 to 0.4 mm relative to Bregma (Paxinos and Watson 2014), similarly to ChAT positive neurons. Co-localization was observed by confocal labeling of both markers (insets Fig 6).

To confirm the specificity of the RXFP3 immunolabeling, we used PV-RXFP3 double immunofluorescence. PV-positive neurons at the MS/vDB where aligned throughout the midline while RXFP3-positive neurons where in the proximity but did not overlay them (Fig.6 H-I). At the HDB, PV-RXFP3 and PV neurons occupied the same space (Fig. 6 J-L) but similarly to the MS/vDB, co-localization with RXFP3 labeling was scarce.

RXFP3 activation by RXFP3-A2 impairs spatial working memory in a delayed spontaneous alternation T-maze test.

RXFP3-A2 infused subjects displayed memory impairment when compared to sham animals in the spontaneous alternation T-maze. We chose this paradigm since it has been shown a good method to evaluate spatial working memory (Fitz et al 2008).

Percentage of permanence time (%PT) in novel (52,17 ± 2,18) and familiar arm (47,83 ± 2,18) was similar in RXFP3-A2 subjects. In contrast, Sham subjects spent significantly more time in the novel arm (41,14 ± 3,28) than in the familiar one (58,86 ± 3,28) (n = 6-7/group; Kruskal-Wallis test, p= 0.007) (Fig.7A). Accordingly, when comparing the percentage of the number of entries to each arm, RXFP3-A2 animals did not show a significant difference between familiar

(48 ± 2,06) and novel arm (52 ± 2,06); whereas the sham subjects had significantly higher alternation, entering in the novel arm (58,86 ± 3,40) more often than in the familiar one (41,14 ± 3,4) (n = 6-7/group; Kruskal-Wallis test, p = 0,014) (Fig 7B). In addition, the analysis of the total number of entries to any of the 3 arms in the T-maze (Start + novel + familiar), suggested that the RXFP3-A2 rats maybe hyperactive (27,17 ± 1,96), compared to sham individuals (18,38 ± 1,46). Data analyzed by Student's t-test, p = 0,0031) (Fig 7C).

DISCUSSION

The current study has demonstrated that acute central activation of RXFP3 by icv infusion of the agonist peptide, RXFP3-A2 (Shabanpoor et al 2012) results in specific changes in the levels of phosphorylated ERK (pERK) in the septal area, assessed *in vivo* using Western blotting of pERK and ERK and immunohistochemical detection of pERK. RXFP3 is a metabotropic receptor and based on extensive characterization in *in vitro* cell-based systems (Liu et al 2003; Van der Westhuizen et al 2005; van der Westhuizen et al 2007) (, it is thought to routinely couple to $G_{i/o}$-proteins in mammalian cells, and to exert its actions on target cells via inhibition of adenylate cyclase and cellular cAMP levels and activation of phosphorylation of ERK (Liu et al 2003; Van der Westhuizen et al 2005; van der Westhuizen et al 2007). In line with these findings, in our *in vivo* studies in adult rats, we observed a rapid and sustained RXFP3-related increase in pERK levels in septal tissue, with no such effect in RXFP3-negative, cerebellar samples, consistent with the reported anatomical distribution of RXFP3 in rat brain (Sutton et al 2004; Ma et al 2007; Smith et al 2010).

Human (H3) relaxin-3 activation of human RXFP3, heterologously expressed in CHO cell lines (Van der Westhuizen et al 2005; van der Westhuizen et al 2007), induces ERK phosphorylation and a downstream up-regulation of AP1, NF-kB and SRE (van der Westhuizen et al 2007; van der Westhuizen et al 2010; Kocan et al 2014), which are involved in gene expression changes related to long-term synaptic plasticity (Impey et al 1999; Cammarota et al 2000; Davis et al 2000; Sweatt 2001; Mattson and Camandola 2001; Ramanan et al 2005; Ahn et al 2008). Thus, similar such changes if they occur *in vivo*, may contribute to some or all of the behavioral effects produced by acute central relaxin-3 or selective RXFP3 agonist administration in rats, such as altered anxiety-like behavior (Ryan et al 2013), increased feeding (McGowan et al 2005; Shabanpoor et al 2012) and altered retrieval of memory (Ma et al 2009). In the current study, we conducted studies in rats maintained in their home cage throughout the experiment. Further investigations of the nature of the septal activation following RXFP3-A2 are warranted, and whether the time-related increase in pERK levels is related to the duration of the peptide action and/or behavioral changes produced by the peptide.

The septal area plays a central role in controlling hippocampal function, and while the importance of the medial septum for 'pacemaking' of hippocampal theta rhythm was reported in very early studies (Stumpf et al 1962), there is still a great deal to learn about the role of different septal neuron populations and their interactions (Wu et al 2000; Sotty et al 2003) (see Introduction). Indeed, while there is both old and new evidence for the importance of septal glutamate, GABA and ACh neurons in regulating hippocampal activity and locomotion, navigation and exploration (Fuhrmann et al 2015), septal theta does not always precede hippocampal theta, suggesting that regulation of hippocampal theta activity is better viewed as occurring via a set of functionally interrelated components than via a single entity (Nerad and McNaughton 2006). A better understanding of how septal systems integrate the complex brainstem and telencephalic inputs/functions can be obtained by documenting the cytoarchitectonics and related circuits of the septum.

Different types of neurons are distributed in an organized onion-like pattern within the septum from the medial to the most lateral regions – with neurotransmitters (acetylcholine, GABA, glutamate, nitric oxide) and a number of protein markers of different neuronal subpopulations (parvalbumin (PV), calretinin (CR) and calbindin (CB) expressed across the area (Gritti et al. 2003; Sotty et al. 2003; Ujfalussy and Kiss 2006). These various types of neurons are involved in an intricate pattern of connections with telencephalic (Freund and Antal 1988) and brainstem areas (Leranth and Kiss 1996; Borhegyi and Freund 1998; Olucha-Bordonau et al 2012; Sánchez-Pérez et al 2015) and also innervate each other and participate in the formation of the synchronous rhythmic output to the hippocampus (Mysin et al 2015).

In the current study, we observed that a population of cholinergic neurons within the MS/DB is targeted by central administration of an exogenous RXFP3 agonist. A comparison of pERK staining in the septum of RXFP3-A2 treated vs sham rats revealed a significantly higher number of pERK-positive neurons in the MS/vDB and HDB, where a topographic distribution of RXFP3 (GPCR135) mRNA (Sutton et al 2004); and RXFP3 agonist (R3/I5) binding sites are present, particularly in the lateral part of MS/VDB (Ma et al 2007) and where relaxin-3-positive fibers

make synapses with cholinergic, CB, CR and PV neurons (Ma et al 2009; Olucha-Bordonau et al 2012; Sánchez-Pérez et al 2015). These data suggest that relaxin-3/RXFP3 signaling targets septal cholinergic neurons and provide a putative mechanism by which NI relaxin-3 projections regulate the septo-hippocampal system (Olucha-Bordonau et al 2012).

In our samples, most (if not all) cholinergic septal neurons express RXFP3, in agreement with the observed increase of ERK phosphorylation in ChAT positive neurons. We also observed that parvalbumin GABAergic neurons scarcely express RXFP3. Moreover, RXFP3 inmunohistochemistry labeling shows similar pattern that mRNA RXFP3 observed by hybridization in situ (Ma et al 2007).

Very recent studies in transgenic mice, identified cholinergic neurons in medial septum as key elements in the septo-hippocampal connections modulating the shifting behavioral states in the brain (Lee and Dan 2012; Tsanov 2015). Cholinergic neuron activation can trigger the septo-hippocampal system during inactive behavioral states, whereas non-cholinergic septal cell activation regulates hippocampal function during explorative behavior (active states). Cholinergic neuron activity is, thus, necessary for the transition from inactive to active hippocampal-related behavior, but is inefficient in inducing significant change in the activity of the hippocampal neurons during arousal states (Mamad et al 2015). Moreover, optogenetic activation of septal cholinergic neurons increases hippocampal activity within the theta band (2-6 Hz) in anesthetized mice (Vandecasteele et al 2014), and exploratory tasks have been reported to increase AchC in hipoccampus (Roland et al 2014).

Supporting a role for relaxin-3/RXFP3 signaling in the modulation of septo-hippocampal activity, direct infusion of an RXFP3 agonist peptide (R3/I5, (Liu et al 2005) into the MS/DB enhanced hippocampal theta activity in urethane anesthetized rats and theta frequency in awake rats; while intra-septal RXFP3 antagonist injections impaired spatial working memory (Ma et al 2009). As mentioned, optogenetic activation of septal cholinergic neurons enhances hippocampal theta oscillations (Vandecasteele et al 2014), supporting the need for activation of medial septal cholinergic neurons to increase theta. However, RXFP3 is thought to couple to inhibitory $G_{i/o}$-proteins and inhibition of cAMP (Liu et al 2005; van der Westhuizen et al 2007), which would

imply a likely inhibition of septal cholinergic neurons. Indeed, there is independent evidence of the acute and reversible hyperpolarisation by RXFP3-A2 of putative, RXFP3-positive hypothalamic neurons *in vitro* (Kania et al 2014). In the present study, we show that RXFP3 is expressed in septal cholinergic neurons, therfore it is very likely that RLN3 exerts an inhibitory effect on cholinergic neruosn via activation RXFP3 metabotropic receptor, exrpressed in these cells.

Alternation in a T-maze have long been considered a paradigm for septohippocampal function (Brito and Thomas 1981). Moreover, septal cholinergic neurons have been reported to mediate working spatial memory (Kitabatake et al 2003; Fitz et al 2008). Therefore, we set out to determine if A2 icv infusions could exert an effect on the alterantion task in a T-maze. We observed that indeed A2 activation of RXFP3 induces an impairment of the spontaneous alternation task, which furher suggest that RXFP3 may play an inhibitory effect on the septal cholineric projections to hippocampus.

On the other hand, we observed that RXFP3 activation induces a general increase in locomotor activity, these data are in in agreement with the hypoactivity effect observed following the knocking down of RLN3 gene expression (Smith et al 2012; Hosken et al 2015). This effect may or may not be associated directly with cholinergic septohippocmapal pathway. Therfore, futher studies addressing the effect of RLN3 on specific cell types may be necessary to elucidate the contribution of RLN3 in different brain circuitries.

In conclusion, this study provides biochemical, anatomical and behavioral evidence that relaxin-3/RXFP3 signaling can regulate MS/DB activity, likely via inhibitory effects on cholinergic neurons. The MAPK/ERK pathway in cholinergic neurons is activated for a sustained period *in vivo* by central RXFP3 agonist administration, and the assays used provide a paradigm to further assess RXFP3 signaling pathways underlying the outcomes of related physiological and behavioral studies. These findings have implications for the acute and chronic regulation of septal cholinergic neuron activity/function by relaxin-3/RXFP3 signaling.

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FIGURES LEGENDS

Fig.1 pERK/ERK detection by Western blot **(A-D)** 20 and **(E-H)** 90 min after icv infusion of RXFP3-A2 (~1 nmol) (black bars), aCSF vehicle (white bars) and in time-matched naïve rats (stripped bars) (n = 4-9 per group). Rats sacrificed 20 min after agonist infusion displayed an increased ERK activation in the septal area compared to sham and naïve groups (**A, B**), but did not display any differences in the cerebellum **(C, D)**. Likewise, rats sacrificed 90 min after RXFP3-A2 infusion also displayed higher pERK levels than control (sham and naïve) in the septum **(E, F)** but not in cerebellum **(G, H)**. * $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$.

Fig. 2 Density of pERK-positive neurons in septal MS/vDB and HDB regions of brains from rats killed 90 min after RXFP3-A2 (black bars) or aCSF vehicle (white bars) infusion. MS/vDB and HDB contain ≥ 2-fold higher pERK levels in RXFP3-A2 than sham rats. ** $p < 0.01$.

Fig. 3 pERK/ChAT immunofluorescence in the septal MS/vDB. (**A**) Schematic of MS/vDB region in which labeled cells were counted (adapted from Paxinos and Watson 2014)), **(B)** Levels of % dual-labeling in the MS/VDB of pERK and ChAT and different calcium-binding proteins, documenting a >3-fold increase in the % co-localization of pERK/ChAT in rats sacrificed 90 min after RXFP3-A2 (black bars) compared with aCSF (white bars) infusion. **(C-H)** Low- and high-power (insets) illustrations of ChAT and pERK staining and merged images from RXFP3-A2 treated (**C-E**) and aCSF treated **(F-H)** rats Scale bar, 100 µm, inset scale bar, 25 µm. **** $p < 0.0001$; *** $p< 0.001$.

Fig. 4 pERK/ChAT immunofluorescence in the septal HDB. (**A**) Schematic of HDB region in which labeled cells were counted (adapted from Paxinos and Watson 2014)), **(B)** Levels of % dual-labeling in the HDB of pERK and ChAT and different calcium-binding proteins, documenting a >3-fold increase in the % co-localization of pERK/ChAT in rats sacrificed 90 min after RXFP3-A2 (black bars) compared with aCSF (white bars) infusion. **(C-H)** Low- and high-

power (insets) illustrations of ChAT and pERK staining and merged images from RXFP3-A2 treated (**C-E**) and aCSF treated **(F-H)** rats Scale bar, 100 µm, inset scale bar, 25 µm. * $p < 0.05$.

Fig. 5 pERK and calcium-binding protein immunofluorescence in the septal MS/vDB and HDB. **(A-C)** pERK and parvalbumin, **(D-F)** pERK and calbindin and **(G-I)** pERK and calretinin staining in the MS/vDB in RXFP3-A2 treated rats processed after 90 min. **(J-L)** pERK and parvalbumin staining within the HDB in RXFP3-A2 90 min rats. Scale bar, 100 µm.

Fig. 6 RXFP3 inmmunostaining and colocalization with ChAT immunofluorescence in septal MS/vDB **(A-C),** and HDB **(D–F)** and with PV staining in MS/vDB (**G-I**) and HBD (**J-L**) areas from naïve rat brain slices. Scale bar, 100 µm; inset scale bar 25 µm.

Fig. 7 Spontaneous alternation in a T-maze paradigm. Sham group had a percentage permanence time significantly higher in the novel arm (white column) than in the familiar arm (black column), whereas RXFP3-A2 rats did not show different percentage of permanence time in familiar arm (horizontal stripes) versus novel arm (dotted columns) **(A)**. Sham rats showed an increased percentage number of entries in the novel arm compared to the familiar one, while RXFP3-A2 rats explored both arms equally **(B)**. Total number of entries in all T-maze arms was higher in the RXFP3-A2 compared to sham rats **(C)**. * $p < 0.05$;** $p < 0.01$