Systemic administration of a fibroblast growth factor receptor 1 agonist rescues the cognitive deficit in aged socially isolated rats

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ABSTRACT

Social isolation predominantly occurs in elderly people and it is strongly associated with cognitive decline. However, the mechanisms that produce isolation-related cognitive dysfunction during aging remain unclear. Here we evaluated the cognitive, electropgysiologial and morphological effects of short- (4 weeks) and long-term (12 weeks) social isolation in aged male Wistar rats. Long-term but not short-term social isolation increased the plasma corticosterone levels and impaired spatial memory in the Morris water maze. Moreover, isolated animals displayed dampened hippocampal long-term potentiation (LTP) *in vivo*, both in the dentate gyrus (DG) and CA1, as well as a specific reduction in the volume of the *stratum oriens* and spine density in CA1. Interestingly, social isolation induced a transient increase in hippocampal basic fibroblast growth factor (FGF2), while fibroblast growth factor receptor 1 (FGFR1) levels only increased after long-term isolation. Importantly, sub-chronic systemic administration of FGL, a synthetic peptide that activates FGFR1, rescued spatial memory in long-term isolated rats. These findings provide new insights into the neurobiological mechanisms underlying the detrimental effects on memory of chronic social isolation in the aged.

1. INTRODUCTION

In humans, social relationships are not only critical for physical well-being, but also for mental health (Cacioppo et al., 2010; Nicholson, 2012). In fact, social isolation is considered a stressful situation that can cause deterioration in an individual's psychological and physical health (House et al., 1988; Cacioppo et al., 2015). Indeed, social isolation is associated with an increased risk of morbidity and mortality (Berkman and Syme, 1979; Eng et al., 2002; Steptoe et al., 2013; Holwerda et al., 2016). Although it can occur in any period of life, social isolation is particularly prevalent in old age, reaching 40% in community-dwelling elderly people (Smith and Hirdes, 2009; Nicholson, 2012). Several epidemiological studies have indicated that social relationships and social support are protective factors for cognitive decline (Seeman et al., 2001; Barnes et al., 2004; Fratiglioni et al., 2004), whereas social isolation and/or a feeling of loneliness are related to age associated cognitive decline and the onset of dementia (Bassuk et al., 1999; Fratiglioni et al., 2000; Zunzunegui et al., 2003; Holwerda et al., 2014).

Social interactions in non-human primates and other social mammals are known to critically affect behavior (Welch and Welch, 1965; Valzelli, 1973). However, preclinical studies investigating the effects of social isolation on behavior have mainly focused on isolation during rearing, which leads to profound neurobiological and behavioral alterations if prolonged, these resembling some of the core symptoms of schizophrenia and that are reproduced in "isolation syndrome" (Hall, 1998; Martin and Brown, 2010). However, when social isolation takes place in adulthood, distinct behavioral, physiological and neuroendocrine changes have been described, depending on the length of isolation, the species and gender (Cruces et al., 2014; Arakawa, 2017). Elevated blood pressure and increased hypothalamic-pituitary-axis (HPA) activity are common effects of the social isolation of adult animals (Hall, 1998; Hawkley et al., 2012; Martin and Brown, 2010; Cacioppo et al., 2015), along with aggressiveness, enhanced anxiety and

depressive-like behavior (Martin and Brown 2010; Cruces et al., 2014). Together, these behavioral and neurobiological alterations support the view that social isolation can be considered a psychosocial stress.

Our current understanding of the effects of social isolation in hippocampal dependent tasks is largely based on studies of weaned or juvenile animals deprived of social contact, although the results obtained have been quite consistent relative to the gender, species and protocols used (Wongwitdecha and Marsden, 1996; Schrijver et al., 2002; Hellemans et al., 2004; Chida et al., 2006; Ibi et al., 2008; Pisu et al., 2011; Cruces et al., 2014; Oliveras et al., 2016; Arakawa, 2017). Strikingly, only three studies into long-term social isolation have focused on aged laboratory animals, studying how ageing influences spatial learning and memory. Accordingly, it appears that spatial learning and memory in the Morris water maze (MWM) deteriorates in isolated rodents (Arranz et al., 2009; Kumar et al., 2011; Huang et al., 2015). However, the neurobiological events that underlie the deleterious effects on cognitive function provoked by social isolation in aged adults remain largely unknown.

To investigate the effects of social isolation on spatial learning and memory, we evaluated how short (STI) and long-term social isolation (LTI) affects the performance of aged male Wistar rats in the MWM. Accordingly, we found that LTI, but not STI impaired spatial memory. To better understand the temporal dynamics of the changes induced by chronic social isolation in hippocampal structure and function, we assessed different parameters in these animals: 1) volume of the DG, CA3 and CA1 hippocampal sub-regions; 2) spine density on DG granule cells and CA1 pyramidal neurons; 3) hippocampal LTP *in vivo*, a mode of synaptic plasticity thought to be crucial for learning and memory (Bliss and Collingridge, 1993); and 4) the hippocampal changes in neural cell adhesion molecule (NCAM), PSA-NCAM, FGF2 and FGR1. In addition, we evaluated whether systemic

administration of FGL, a synthetic NCAM mimetic peptide that activates FGFR1 (Neiiendam et al., 2004; Chen et al., 2010), could efficiently reverse the memory impairment induced by social isolation.

2. MATERIALS AND METHODS

2.1. Animals

Adult male Wistar rats (Harlan, France) were purchased at 3 months of age and kept pair-housed in transparent Plexiglas cages on a 12-hour light/dark cycle. When the animals reached 18 months of age, a group of rats was housed individually in shadow boxes for 12 weeks (to avoid visual contact with con-specifics: long-term isolation- LTI). Another group of rats was housed individually at 20 months of age for 4 weeks (short-term isolation-STI), while control animals remained in groups of three pair-housed in transparent Plexiglas cages. All animals were weighed weekly. Immediately after sacrifice adrenal glands of animals´ were weighed and corticosterone levels measured. All animal protocols were approved by the Committee of Ethics of the Universidad Nacional de Educación a Distancia (UNED) following the "Principles of laboratory animal care", and were carried out in accordance to the European Union Directive (2010/63/EU).

2.2. Experimental designs

2.2.1. Experiment 1. Effects of short- and long-term social isolation on spatial learning and memory in the Morris Water Maze

At 21 months of age, the spatial learning and memory abilities of STI (n= 10) and LTI (n=10) animals, as well as those of the control rats (n=10), were evaluated in the Morris water maze. The animals were coded with random numbers without following a specific

sequence for the experimental group to ensure that the observers were blind to the experimental conditions.

2.2.2. Morris Water Maze

The water maze was a black circular pool (2 m diameter, 45 cm high) filled with water (30 cm depth, at 24±1 °C) and divided into four virtual quadrants of equal size, with a hidden escape platform placed in the middle of the target quadrant. The testing room contained numerous spatial cues. The acquisition phase was divided into 3 training sessions (day 1–3), 90s trials per day, with a 60s inter-trial interval. Each training session consisted of four trials (90s), using four different starting positions, distributed equally around the perimeter of the maze (north, south, east and west). Each rat was placed in the water facing the tank's wall at one of the four designated starting points. A 60s probe memory session (day 4) was performed 24 h after the last trial of the learning period, during which the platform was not present. The trials and test were performed during the light period (09:00–15:00 h) to avoid the influence of circadian hormonal fluctuations. Since performance in the water maze may be influenced by other non-spatial learning factors, such as the sensory, motivational, emotional or motor behavior of the subjects, the rat's swimming speed was recorded. In addition, after the memory probe trial the animals were tested with a visible platform in the water maze, in which no spatial learning is involved. The behavioral data were acquired and analyzed using an automated tracking system (Ethovision, Noldus Wageningen, The Netherlands).

2.2.3. Blood Sampling: Plasma corticosterone measurement

Blood samples were obtained by the tail nick procedure, within 2 min of the animals being taken from the animal room. Plasma corticosterone levels were measured by ELISA with a sensitivity of 2.5 ng/mL (DRG Instruments GmbH, Germany).

2.2.4. Experiment 2. Effects of short- and long-term social isolation on hippocampal volume and on the spine density on hippocampal neurons

The effects of chronic social isolation on dorsal hippocampal volume were evaluated in animals trained in the MWM. Due to technical problems with the perfusion/fixation procedure in some cases, the final number of animals used in Figure 3 were Control n=7, STI n=10 and LTI n=6. Spine density in hippocampal granule cells and CA1 pyramidal neurons was assessed in a total of 7 animals per group. The animals were transcardially perfused with phosphate buffer followed by 4% paraformaldehyde (PFA: 0.1M, pH 7.4) and their brain were extracted and fixed again for 48 h in 4% PFA. Subsequently, the brains were cryoprotected in graded sucrose solutions (10%, 20%, 30%) for a total of 48 h. Stereological analysis of hippocampal volume and estimates of spine density were performed on serial vibratome brain sections.

For all morphological, electrophysiological and biochemical experiments, the experimenter was blind to the experimental condition.

2.2.5. Stereological analysis of hippocampal volume

Serial coronal vibratome sections (40 µm, every 10th section: Leica VT1000S) were stained with cresyl violet. The volume of the different dorsal hippocampal sub-regions (granular and molecular layer of Dentate Gyrus (DG), and *radiatum*, *pyramidal* and *oriens* layers of CA1, CA2 and CA3) was quantified stereologically, distinguishing each sub-region under a light microscope (Leica DRIV microscope equipped with a Optronics

MicroFire digital camera) according to the rat brain atlas (Paxinos and Watson, 2007). The volume was calculated with the assistance of StereoInvestigator software (MicroBrightField Inc., Williston, VT, USA), according to Cavalieri's estimator.

2.2.6. Neuronal injection and the measurement of dendritic spine density

Hippocampal neurons in the CA1 and DG area of freshly obtained coronal brain vibratome sections (150 µm: Leica VT1000S) were individually injected with Alexa 594 (Invitrogen, Eugene, OR) by passing a steady hyperpolarizing current through the electrode (-0.5 to -1.0 nA). The current was applied until the distal tips of each neuron fluoresced brightly, with images obtained on a confocal microscope (Zeiss LSM510 Meta). In each animal, 1 dendrite of 5 different *stratum oriens* pyramidal neurons was scanned from the CA1 (n=105 neurons) and from the somas of DG granular cells in the molecular layer (n=110 neurons). Dendritic spine density was determined by tracing the image of the dendrites acquired in three dimensions with Neurolucida, version 9 (MicroBrightField Inc., Williston, VT, USA) software. All protusions were considered to be spines, applying no correction factors to the spine counts. The reconstructed data were exported to Neurolucida Explorer, version 8 (MicroBrightField Inc., Williston, VT, USA) to analyze quantitatively and the spines were also analyzed in function of their distance from their origin (Sholl analysis).

2.2.7. Experiment 3. Effects of short- and long-term social isolation on hippocampal LTP induction *in vivo*

To investigate whether social isolation altered hippocampal synaptic plasticity, 21-month-old STI (n= 8) or LTI (n= 6) rats, and their controls (n=6), were anesthetized with urethane (1.6 g/kg i.p.), and LTP induction was studied in the DG and CA1 *in vivo*.

Animals were placed in a Kopf stereotaxic device in which the surgical procedures and recordings could be performed. The electrodes were introduced into the DG (−3.3 mm posterior, 2.0 mm lateral and 3.5 mm deep, relative to Bregma) and to the perforant pathway fibers (-3.3 mm posterior, 0.5 mm lateral and 4.0 mm deep) for the stimulating electrode), and into the CA1 (−3.3 mm posterior, 1.5 mm lateral and 3.0 mm deep) where the recording electrode activated the Schaffer collaterals pathway (-3.3 posterior, 3.0 mm lateral, 3.5 mm deep) for the stimulating electrode. Field potentials were obtained using nichrome macroelectrodes (<1 MΩ, 120 µm thick) and the perforant pathway was stimulated with a bipolar electrode (World Precision Instruments). The intensity was set to double the threshold intensity in order to elicit a response (10-50 µA).

The experimental protocol consisted of a control period of 10 min to ensure stable activity when the stimulation pathway was stimulated at 0.5 Hz. To induce LTP, a tetanic stimulation was delivered as three stimulation trains of 100 Hz during 500 ms, each separated by 2 seconds. Subsequently, the pathway was again stimulated at 0.5 Hz for 30 minutes and the average evoked field potential was calculated every minute (30 stimuli). The slope of the evoked field potential was measured and plotted considering 100% the mean slope during the control period.

2.2.8. Experiment 4. Effects of social isolation in aged rats on synaptic proteins in the hippocampus

The effects of social isolation on synaptic proteins were evaluated in 21-month-old control rats (n=15) or those previously submitted to STI (n=14) or LTI (n= 6). Animals were sacrificed by decapitation, and hippocampal brain tissues were collected to obtain homogenates and synaptosomes as described previously (Carlin et al. 1980). The amount

of protein was quantified by the Bradford method. The main NCAM isoforms were measured in Western blots, and PSA-NCAM, FGF2 and FGFR1 by ELISA.

2.2.9. Quantification of NCAM isoforms in Western blots

The three major NCAM isoforms were measured in Western Blots of crude synaptosomal preparations: NCAM120, NCAM140 and NCAM180. Hippocampal synaptosomal samples from each rat were incubated overnight at room temperature with Endo-N (AbCys) to selectively cleave the polysialic acid (PSA) moiety of NCAM and the resolved synaptosomes were probed in immunoblots with a polyclonal rabbit anti-rat NCAM antiserum (1:15,000) (a generous gift from Prof. Elisabeth Bock, University of Copenhagen, Denmark: Rasmussen et al., 1982) and an optimized protocol for western blotting published by Pereda-Pérez et al., (2013). The protein concentrations in each sample were estimated by the Bradford method. As loading controls, all blots were reprobed with a β-actin antibody and the signal for each band of interest was normalized to that of β-actin. The images were analyzed using NIH Image J software.

2.2.10. ELISA

Enzyme-linked immunoabsorbent assays (ELISA) were used to quantify the PSA-NCAM (AbCys Eurobio, France), FGF2 (NeoBiolab, Massachusetts, USA) and FGFR1 levels (Elabscience, Wuhan, P.R.C.) in the hippocampus.

2.2.11. Experiment 5. Effects of systemic FGL treatment on spatial learning and memory after long-term social isolation at aging

To investigate whether the spatial memory impairment induced by long-term social isolation in aged animals can be reversed by central FGFR1 activation, 21-month-old

male Wistar rats previously submitted to long-term isolation (or pair-housed as controls) were systemically injected with FGL, an agonist of FGFR1 that can cross the blood brain barrier (Neiiendam et al., 2004; Secher et al., 2006). In the twelve days before and during the training in the Morris water maze, all the animals received sub-chronic treatment with subcutaneous injections of FGL (6.6 mg/kg b.w.) or the vehicle alone (10mM L-histidine and 30 mg/mL D mannitol adjusted to pH6) every two days. This peptide has been demonstrated to penetrate the brain after systemic administration and to remain detectable in cerebrospinal fluid (CSF) for up to 5 hours (Secher et al., 2006; Turner et al., 2018).

2.2.12. Statistical Analysis

The data were analyzed using SPSS version 22 Generalized linear models (McCulloch and Searle 2001). We performed the statistical analyses using a generalized linear model and generalized estimating equations due to the particular flexibility of those tests regarding the type of distribution and covariance structure (McCulloch and Searle, 2001; Hardin and Hilbe, 2003). When statistically significant interaction was found, additional pairwise comparisons (Bonferroni sequential adjustment) were made and the method of estimation used was the maximum likelihood (ML). Normality distribution and identity as a link function was always used. In all cases, the significance of the effects was determined with the Wald X^2 statistic. The data are presented as mean ± SEM and the statistical significance was set at $p<0.05$.

3. RESULTS

3.1. Effect of social isolation on body weight gain, morning plasma corticosterone levels and relative adrenal gland weight

There were significant differences in body weight gain between control and isolated animals, with group (Wald X^2 (2) = 10.28, p= 0.006), time (Wald X^2 (11) = 281.76, p<0.001) and interaction effects (Wald X^2 (22) = 238.36, p < 0.001: Figure 1A). Morning plasma corticosterone levels were significantly higher in the LTI animals compared to controls (group effect Wald X^2 (2) = 8.02, p =0.018: (Figure 1C), and the relative adrenal gland weight (adrenal glands to body weight ratio) was also significantly different between the two groups (Wald X^2 (2) = 112.46, p=0.002). Indeed, the adrenal glands were heavier in LTI animals than in the controls animals (p=0.001: Figure 1B).

3.2. Long–term social isolation impairs spatial memory in the Morris water maze

We examined the spatial learning abilities of the animals in the MWM. During the acquisition phase, the controls as well as STI and LTI rats progressively learnt the location of the platform as the training proceeded (trial and group x trial effect Wald X^2 (11) = 148.84, p < 0.001; Wald X^2 (22) = 55.52, p < 0.001, respectively: Figure 2A). Compared to controls, STI rats needed more time to reach the hidden platform in trials 3 (p=0.051) and 5 (p =0.047). We observed no significant differences in swimming speed between the control, STI and LTI rats (Wald X^2 (2) = 0.110, p = 0.951). In addition, we tested the animals with a visible platform in order to exclude any effects on sensory, motivational, emotional or motor functions. Again, we failed to detect any significant differences in escape latency of a visible platform among the control (23.2 ± 2.5s), STI (24.1 ±3.2s) and LTI rats (22.8 ± 3.8s) (Wald Wald X^2 (2) = 0.29, p = 0.834).

In the probe trial, compared to controls, the LTI, but not the STI rats, spent less time in the target quadrant where the platform had been located previously on the training days (Wald X^2 (2) = 12.11, p= 0.002: Figure 2B).

In addition, we tested the animals in a visible platform in order to exclude sensory, motivational, emotional, or motor functions. There were no significant differences in escape latency in a visible platform among controls (23.2 ± 2.5s), STI (24.1 ± 3.2s) and LTI rats (22.8 ± 3.8s) (Wald X^2 (2) = 0.29, p = 0.834).

3.3. Hippocampal volume changes after chronic social isolation

We investigated whether social isolation affected the volume of each sub-region in the dorsal hippocampus. A detailed analysis of the CA1 hippocampal volume showed a group effect in the *stratum oriens* of CA1 (Wald X^2 (2) = 12.01, p=0.002), with a significant decrease in volume STI (p=0.007) and LTI (p=0.001) rats relative to the controls (Figure 3A). A tendency towards a lower volume in the CA1 pyramidal layer relative to the controls was found in LTI rats (p=0.076). Conversely, there were no significant differences between the groups in the other hippocampal areas analyzed: CA2, CA3 and DG (p> 0.05: Figure 3 B, C and D).

3.4. Social isolation alters spine density in the DG and CA1

A Sholl analysis of the spine density on the DG granular cells showed significant effects for group, distance and group x distance interaction (Wald X^2 (2) = 9.32, p=0.009; Wald X^2 (14) = 2.032x10^8, p<0.001; Wald X^2 (14)= 309.74 p=0.001, respectively) but not for the group x distance interaction. Further analysis indicated a decrease in spine density in the STI and LTI rats compared to the controls (p=0.005: Figure 4A).

There were significant differences across the groups in the total spine density in the proximal zone of the dendritic tree of granule cells (Wald X^2 (2) = 6.72, p =0.035). When compared to the controls, there was a decrease in total spine density in both STI (p=0.02) and LTI rats (p =0.034 (Figure 4B). Significant differences were also found between

groups in the total spine density in the distal zone (Wald X^2 (2) = 9.65, p < 0.005). Compared to the controls, there was a decrease in total spine density in the STI (p =0.026) and LTI rats (p =0.030: Figure 4C). In addition, there were significant differences in average total spine density across groups (Wald X^2 (2) = 12.08, p =0.002: Figure 4E). Further analysis indicated there was a decrease in the average total spine density in the STI (p=0.004) and LTI rats (p =0.002) relative to the controls.

A Sholl analysis of spine density in the *stratum oriens* of CA1 (Figure 4F) showed significant effects for group and distance qnd group x distance (Wald X^2 (12) = 1470.96, p< 0.001, Wald X^2 (17) = 40.46, p =0.001 respectively), but not for the group x distance interaction. Further analysis showed a decrease in spine density in the LTI rats compared to controls (p=0.039). No significant differences were found in STI rats compared with the controls. Moreover, there did no appear to be differences in the average total spine density in the *stratum oriens* of CA1 between the three experimental groups (Wald X^2 (2) = 4.04, p =0.133: Figure 4G).

3.5. Activity related plasticity changes induced by social isolation

To explore whether social isolation affected LTP induction in the DG *in vivo*, we compared the field excitatory postsynaptic potential (fEPSP) slope in STI and LTI rats with that in control animals. As a result, we found that each group of animals had similar basal synaptic transmission (control 1.8±0.61, STI: 2.4±0.3 and LTI: 2.1±0.3 mV/ms.; p =0.483 and p= 0.613 respectively *vs* control). However, a group effect was evident for (fEPSP) slope (Wald X^2 (2) = 15.50, p < 0.001; Figure 5A) and the LTI rats had a significantly lower LTP than the control animals (p < 0.01: Figure 5B). In the CA1, the analysis of fEPSPs after high frequency stimulation of Schaffer collaterals indicated

significant differences between the groups (Wald X^2 (2) = 21.38, p <0.001: Figure 5C). Again, LTI rats had a slower fEPSP than the control rats (p <0.001: Figure 5D).

3.6. Effects of social isolation on learning and memory related proteins in the hippocampus

In hippocampal crude synaptosomes there were no significant differences in the main NCAM isoforms between the groups (NCAM 180 kDa, Wald X^2 (2) = 0.24, p =0.888; NCAM 140 kDa, Wald X^2 (2) = 2.26, p =0.323; NCAM 120 kDa, Wald X^2 (2) = 0.46, p = 0.795: Figure 6A). However, there was a significant reduction in the synaptic PSA-NCAM content in the hippocampus in rats subjected to LTI (Wald X^2 (2) = 11.96, p=0.003: Figure 6B). Given that FGFR1 (Reuss and von Bohlen und Halbach 2003) is involved in hippocampal synaptic plasticity and memory consolidation (Zhao et al. 2007) and that FGF2 is abundantly expressed in the hippocampus, we decided to investigate their hippocampal levels. There were significant differences between the groups in terms of the hippocampal FGF2 levels (Wald X^2 (2) = 6.46, p =0.039), whereby STI rats had more FGF2 than the group-housed animals (p =0.037; Figure 6C). In addition, LTI rats had elevated levels of FGFR1 in the hippocampus (Wald X^2 (2) = 7.02, p =0.03: Figure 6D).

3.7. FGL treatment reverses the impairment in spatial memory induced by long-term social isolation

To investigate whether FGFR1 activation might be able to revert the impact of long-term isolation on spatial abilities, we administered FGL after 3 months of isolation. During systemic FGL treatment, significant differences in body weight gain were detected between control (4.0 ± 0.6 g) and isolated (0.4± 0.7 g) animals (Wald X^2 (1) = 13.51, p<

0.001), with no effect of FGL treatment (Wald X^2 (1) = 0.04, p = 0.839) or any interaction effect (Wald X^2 (1) = 0.68, p = 0.794). An analysis of the latency to find the platform during the acquisition phase in the MWM after long-term social isolation indicated the existence of significant differences in the trial and (trial x group) interaction (Wald X^2 (11) = 93.92, Wald X^2 (31) = 3750.86, respectively: p < 0.001). Further analysis revealed that LTI rats that received FGL needed more time to find the hidden platform in trial 5 than the control rats that received the vehicle alone (p=0.008). The LTI rats that received the vehicle alone dislayed a tendency to need more time to find the hidden platform than control rats that received FGL in trial 11 (p = 0.060: Figure 7A) and than LTI rats that received FGL in trial 12 (p=0.073).

A statistical analysis of spatial memory abilities of the four groups of animals revealed a significant group effect (Wald X^2 (3) = 18.47, p <0.001: Figure 7B). The LTI rats that received the vehicle alone spent less time in the target quadrant than their control counterparts (p = 0.001) or the control+FGL (p < 0.001) and LTI+FGL rats (p =0.003). Therefore, these results indicate that FGL treatment reverted the spatial memory deficits in LTI animals.

In order to study whether FGL affects the stress response of isolated animals, plasma corticosterone levels were measured two days after completion of the cognitive evaluation. Morning plasma corticosterone levels did not differ between LTI animals treated with FGL (74.9 ±10.1 ng/ml; p=0.810) or the vehicle alone (77.2 ±11.4 ng/ml). In addition, the relative adrenal gland weight was similar in LTI rats injected with FGL and in those that received the vehicle alone (0.101 ±0.070 mg/g b.w. and 0.106 ±0.061 mg/g b.w., respectively, p=0.748).

4. DISCUSSION

Our study reveals that aged animals submitted to LTI, but not STI, develop deficits in spatial memory. This cognitive dysfunction may be related to the impaired *in vivo* hippocampal LTP, and the loss of dendritic spines on DG granule cells and CA1 pyramidal neurons. Although chronic social isolation did not appear to modify the main NCAM isoforms in the hippocampus, it decreased the PSA-NCAM at synapses, and it transiently increased the FGF2 and FGFR1 content. Finally, systemic administration of FGL, a synthetic peptide that activates FGFR1 (Neiiendam et al., 2004), appeared to be an effective pharmacological treatment to recover spatial cognition in LTI animals.

While the body weight gain in aged rats submitted to either 4 or 12 weeks of social isolation was weaker than in control animals, the relative weight of the adrenal gland and the plasma corticosterone levels only increased in LTI animals. Loss of body weight, elevated blood pressure and enhanced HPA activity are frequently observed after separation of an animal from its conspecifics (Castro and Matt, 1997; Hall, 1998; Martin and Brown, 2010; Garrido et al., 2012; Hawkley et al., 2012; Zlatković and Filipović, 2012; Cacioppo et al., 2015). Indeed, these physiological and endocrine effects of social isolation are consistent with the effects produced by chronic stress (Daniels-Severs et al., 1973; Sapolsky et al., 1986) and in fact, social isolation is considered a stressful event in animals and humans (Grant et al., 2009; Cacioppo et al., 2015). Unlike other types of "active" stress (e.g., restraint stress, foot shock, learned helplessness or social defeat), social isolation can be considered a "passive" stress. Chronic exposure of adult male rats to "active" stress and social isolation frequently provokes anxiety- and depression-like behavior, and cognitive impairment (Cruces et al., 2014; Krishan et al., 2007). In addition, prolonged exposure to "active" stress and social isolation increases the number of cells that express c-Fos in many brain regions, including the hippocampus, medial prefrontal cortex, striatum and amygdala (Westenbroek et al., 2003; Stanisavljević et al., 2018).

However, some aspects of the neural response differ between these two types of stress. Thus, depression and anxiety-like symptoms induced by long term social isolation of adult mice and rats are mediated by a downregulation of CREB that dampens *nucleus accumbens* excitability, whereas active stressors upregulate CREB activity in this brain region (Carlezon et al., 2005; Wallace et al., 2006; Wilkinson et al., 2009). Here, we also observed slower acquisition of spatial learning in STI animals and impaired spatial memory after LTI. What underlies the effects of chronic social isolation on spatial abilities is not clear, although these effects do appear to be influenced by gender, species, length of isolation and whether rodents are isolated at weaning (Wongwitdecha and Marsden, 1996b; Hellemans et al., 2004; Chida et al., 2006; Ibi et al., 2008; Pisu et al., 2011; for review see Arakawa, 2017) or in adulthood (Coudereau et al., 1997; Moragrega et al. 2003; Chen et al., 2016). To our knowledge, only three studies have investigated the spatial learning of animals submitted to social isolation in old age. As such, isolated aged female mice acquired spatial learning more slowly (Arranz et al., 2009) and spatial memory was impaired in male aged APP/PS1 mice (Huang et al., 2015), a transgenic model of Alzheimer´s disease and long-term isolated male rats (Kumar et al., 2011). Interestingly, social isolation in elderly people has already been associated with poorer cognitive function (Shankar et al., 2013; Cacioppo and Cacioppo, 2014).

Since spatial navigation is highly dependent on correct hippocampal function, particularly the dorsal part (Wiener, et al., 1989; for rev. see Stranger et al., 2014), we investigated the structural and functional changes in the dorsal hippocampus of socially isolated aged animals. After 4 weeks of social isolation, spine density was reduced on granule cells of the DG, but not on CA1 pyramidal neurons, while LTP was not affected *in vivo*. When social isolation persisted for 12 weeks, spine loss was evident in both the DG and CA1, together with dampened LTP in these two hippocampal areas. Similarly, important

structural neuroplastic changes have been reported in the hippocampus of animals after chronic stress, including dendritic atrophy, reduced spine density and neurogenesis, and impaired LTP (Sousa et al., 2000; Pham et al., 2003; Joëls et al., 2004). While there is strong evidence of reduced spine density in hippocampal neurons of animals isolated post-weaning (Silva-Gómez et al., 2003), only two studies reported that chronic social isolation in adulthood reduces hippocampal volume in mice and degus (Pereda-Pérez et al., 2013; Huang et al., 2015). Chronic stress can affect structural plasticity differently in distinct brain structures, including the hippocampus, frontal cortex and amygdala (McEwen, 2000). The hippocampus is critical for declarative memory in humans (Squire and Zola-Morgan, 1991; Eichenbaum, 2000) and spatial memory in rodents (Morris et al., 1982), and it seems to be especially vulnerable to prolonged exposure to stress. Thus, a decrease in hippocampal volume was witnessed in patients suffering from stress-related psychiatric disorders, which was associated with memory deficits (Bremner et al., 1995; Shin et al., 2004). In addition, the expression of molecules involved in synaptic plasticity like NCAM and its polysialylated PSA-NCAM is also altered after chronic stress (Venero et al., 2002) or LTI (Pereda-Pérez et al., 2013).

It is well known that chronically stressed animals undergo dendritic atrophy and a loss of dendritic spines in the CA1 and CA3 areas (McEwen, 1999; Sousa et al., 2000), as well as impaired LTP in all hippocampal areas (Pavlides et al., 2002; Alfarez et al., 2003; for review see Fa et al., 2014). Interestingly, these morphological and electrophysiological changes in the hippocampus of chronically stressed animals seem to be associated with the deficits in spatial learning and memory tasks (Luine et al., 1994; Sandi et al., 2003; Rahman et al., 2016). Moreover, these stress-induced cognitive and plastic changes were reversed by treatment with an inhibitor of corticosterone synthesis or an antagonist of the glucocorticoid receptor (Krugers et al., 2006; Kvarta et al., 2015). Therefore, the decrease

that we observed in spine density in the DG and CA1, together with the impaired LTP in these hippocampal areas after LTI, might be related to the impaired spatial memory evident in LTI aged animals. It was previously shown that long-term potentiation (LTP) in the CA1 region of hippocampal slices was impaired in LTI aged male rats but not in isolated-aged animals that were maintained under an exercise regime, nor in environmentally-enriched paired-housed animals (Kumar et al., 2012). Moreover, spatial memory in the MWM was impaired in isolated animals.

Social isolation in aging induced a decrease in synaptic PSA-NCAM in the hippocampus of LTI but not STI animals. This reduction in hippocampal PSA-NCAM was not paralleled by a decrease in any of the main NCAM isoforms. We recently observed a similar drop in the hippocampal PSA-NCAM content in the female adult degus subjected to LTI (Pereda-Pérez et al., 2013). By contrast, an increase in hippocampal PSA-NCAM was also reported in young-adult male rats after STI (Djordjevic et al., 2012). These discrepancies in the polysialation state of NCAM in the hippocampus after social isolation may be related to the timing of social isolation and the age of the animals, particularly given that there is much less hippocampal PSA-NCAM in older rats than in young or adult animals (Regan and Fox, 1995; Seki, 2002).

While there was more hippocampal FGFR1 in LTI animals, the elevation of FGF2 in STI but not LTI animals may reflect a compensatory mechanism to dampen the detrimental effects of social isolation on spatial memory. FGF2 is expressed abundantly in the hippocampus and it has the highest binding affinity to FGFR1 (Reuss and von Bohlen und Halbach, 2003), a receptor involved in hippocampal synaptic plasticity and memory consolidation (Zhao et al., 2007). Here, we found that systemic administration of FGL, a synthetic NCAM mimetic peptide that activates FGFR1 (Neiiendam et al., 2004) and penetrates the brain after systemic administration (Secher et al., 2006; Turner et al., 2018),

rescues cognitive function in LTI animals. We previously demonstrated that FGL can improve the cognitive performance of adult and old rats through a mechanism that involves hippocampal FGFR1 phosphorylation, PKC activation and the facilitation of AMPA receptor delivery to synapses (Cambon et al., 2004; Borcel et al., 2008), provoking an increase in AMPAR-mediated synaptic transmission (Knafo et al., 2012). In addition, FGL administration facilitates the induction and maintenance of *in vivo* synaptic plasticity in the DG (Dallérac et al., 2011). Furthermore, in aged rats, FGL treatment attenuates the impaired hippocampal LTP (Downer et al., 2010), and increases the ratio mushroom to thin spines in the middle molecular layer of the dentate gyrus (Popov et al., 2008). Therefore, sub-chronic administration of FGL can initiate a cascade of biochemical, electrophysiological and morphological events within neurons, which ultimately rescue spatial memory in aged isolated animals.

Overall, our data show that the cognitive function of aged animals is vulnerable to the deleterious effects of social isolation, and it defines some of the neurobiological mechanisms that produce this effect. In addition, we provide evidence that FGL may be a viable pharmacological agent to reverse the cognitive impairment induced by long-term social isolation.

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Figure Legends

Figure 1. Effects of social isolation on body weight gain, morning plasma corticosterone levels and relative adrenal gland weight. (A) Body weight gain (%). (**B)** Relative adrenal gland weight. **C**) Morning plasma corticosterone levels at sacrifice.

Mean and SEM are shown (10 animals per group). *p<0.05 STI *vs* control group; $^{@}$p<0.05, $^{@@}$p<0.01 LTI *vs* control group.

Figure 2. **Effects of short and long-term social isolation on spatial learning and memory abilities in the Morris water maze**. **(A)** Spatial training-escape latency. **(B)** Transfer memory test.

Means and SEM are shown (10 animals per group). *p<0.05 LTI *vs* control group; $^{@@}$ p<0.01 LTI *vs* control group.

Figure 3. Hippocampal volume changes after chronic social isolation. Dorsal hippocampal volume in CA1 **(A)**, CA2 **(B)**, CA3 **(C)** and DG **(D)**.

Mean and SEM are shown. Control n=7, STI n=10 and LTI n=6. *p<0.05 STI *vs* control group, $^{@@}$p<0.01 LTI *vs* control group.

Figure 4. Effects of short- and long-term social isolation on spine density in granule cells and in CA1 pyramidal neurons of the hippocampus. *Sholl* analysis showing spine density as a function of distance from the soma of granule cells DG **(A, B, C)** and CA1 pyramidal neurons **(F). (E, G)** Total spine density in DG and CA1 neurons, respectively. **(D)** Representative diagram of a granule cell of the hippocampus indicating the proximal and distal zones in the dendritic tree.

Means and SEM are shown. In DG, Control n = 7, STI n = 6, LTI n = 5 animals. In CA1, Control n = 7, STI n = 6 and LTI n = 7. *p<0.05 STI *vs* control group; **p<0.01 STI *vs* control group; ***p<0.001 STI *vs* control group; $^{@@}$ p<0.01 LTI *vs* control group; $^{@@@}$ p<0.001 LTI *vs* control group; $^{@}$ p<0.05 LTI *vs* control group.

Figure 5. **Effect of short and long-term social isolation on *in vivo* LTP induction in the hippocampus**. **(A)** LTP induction in DG region; Vertical arrow indicates the application of a stimulation train at the perforant pathway. Control n = 8, STI n= 5, LTI n =5 animals. **(B)** Representative EPSP slope average for CA1 region calculated during the 10 first minutes (basal activity; gray trace) and during the 10 last minutes of recording (black trace) Control n = 7, STI n= 6, LTI n =5 animals. **(C)** Mean % fEPSP, calculated during the 10 last minutes of recording, respect to basal activity in DG; black bar shows data from control animals, red bar from short-term isolation and blue bar from long-term isolation. (**D-F)** Same plots as in (A-C) by LTP induction in CA1 region.

Means and SEM are shown. @@p<0.01 and @@@ p<0.001 LTI *vs*. control group.

Figure 6. Effects of social isolation on learning and memory related proteins in the hippocampus. (A) Effects of chronic social isolation on protein levels of the three major NCAM isoforms (NCAM-120, -140, and -180) in hippocampal synaptosomes. Levels of PSA-NCAM **(B)**, FGF2 **(C)** and FGFR1 **(D)** in hippocampal homogenates.

Means and SEM are shown. Control n = 15, STI n = 14, LTI n = 6. *p<0.05 STI *vs* control group; $^{@}$ p<0.05; $^{@@}$ p<0.01 LTI *vs* control group.

Figure 7. FGL treatment reverses the impairment in spatial memory induced by long-term social isolation. MWM performance of vehicle injected undisturbed animals (Control-vehicle), FGL treated-undisturbed animals (Control-FGL), animals exposed to long-term social isolation plus FGL treatment (isolated-FGL) or vehicle injection (isolated-vehicle). **(A)** Training scape latency. **(B)** Transfer memory test.

Means and SEM are shown. Control n = 9, Control FGL n = 9, LTI Vehicle n = 9 and LTI FGL n = 8.

**p<0.001, $^{@@}$p<0.01 LTI-vehicle *vs* control-vehicle group; $^{\#\#}$ p<0.01 LTI+FGL *vs* control+vehicle group; ^{aaa}p<0.001 LTI+vehicle *vs* control+FGL; ^{bb}p<0.01 LTI vehicle *vs* LTI+FGL.