Accepted Manuscript

Title: Adult social isolation leads to anxiety and spatial memory impairment: brain activity pattern of COx and c-Fos

Authors: Candela Zorzo, Magdalena Méndez-López, Marta Méndez, Jorge L. Arias

PII: S0166-4328(18)31767-4
DOI: https://doi.org/10.1016/j.bbr.2019.03.011
Reference: BBR 11842

To appear in: *Behavioural Brain Research*

Received date: 14 January 2019
Revised date: 19 February 2019
Accepted date: 5 March 2019

Please cite this article as: Zorzo C, Méndez-López M, Méndez M, Arias JL, Adult social isolation leads to anxiety and spatial memory impairment: brain activity pattern of COx and c-Fos, *Behavioural Brain Research* (2019), https://doi.org/10.1016/j.bbr.2019.03.011

TITLE: Adult social isolation leads to anxiety and spatial memory impairment: brain activity pattern of COx and c-Fos.

AUTHORS: Candela Zorzo, Magdalena Méndez-López, Marta Méndez, Jorge L. Arias.

Candela Zorzo, Fellowship (BS). Departamento de Psicología. Instituto de Neurociencias del Principado de Asturias (INEUROPA). Universidad de Oviedo. Plaza Feijoo s/n. 33003 Oviedo, Spain. UO223002@uniovi.es

Magdalena Méndez-López, PhD. IIS Aragón, Departamento de Psicología y Sociología, Universidad de Zaragoza, Zaragoza, Spain. mmendez@unizar.es

Marta Méndez, PhD. * Departamento de Psicología. Instituto de Neurociencias del Principado de Asturias (INEUROPA). Universidad de Oviedo. Plaza Feijoo s/n. 33003 Oviedo, Spain. mendezlmarta@uniovi.es

Jorge L. Arias, PhD. Departamento de Psicología. Instituto de Neurociencias del Principado de Asturias (INEUROPA). Universidad de Oviedo. Plaza Feijoo s/n. 33003 Oviedo, Spain. jarias@uniovi.es

*** Correspondence**

Marta Méndez, PhD.
Departamento de Psicología
Universidad de Oviedo
Plaza Feijoo s/n. 33003 Oviedo, Spain
Telephone: (+34) 985104183; Fax (+34) 985104144
Email: mendezlmarta@uniovi.es

Declarations of interest: none

Highlights:

- Adult rat social isolation leads to anxiety-like behaviour
- Adult rat social isolation impairs Working Memory processes.
- Less c-Fos learning-related activity is observed in adult socially isolated rats.
- Metabolic brain activity is disrupted in adult socially isolated rats

ABSTRACT

Social isolation during adulthood is a frequent problem that leads to a large variety of adverse emotional and cognitive effects. However, most of the social isolation rodent procedures begin the separation early post-weaning. This work explores locomotor activity, anxiety-like behaviour, and spatial working memory after twelve weeks of adult social isolation. In order to study the functional contribution of selected brain areas following a working memory task, we assessed neuronal metabolic activity through quantitative cytochrome oxidase histochemistry and c-Fos immunohistochemistry. Behaviourally, we found that isolated animals (IS) showed anxiety-like behaviour and worse working memory than controls, whereas motor functions were preserved. Moreover, IS rats showed lower levels of learning-related c-Fos immunoreactivity, compared to controls, in the medial prefrontal cortex (mPFC), ventral tegmental area (VTA), and nucleus accumbens shell. In addition, the IS group showed lower neuronal metabolic activity in the mPFC, VTA, and CA1 subfield of the hippocampus. These results indicate that twelve weeks of social isolation in adult rats leads to different behavioural and brain alterations, and they highlight the importance of social support, not only in development, but also in adulthood.

Key words: social isolation; adulthood; anxiety; spatial working memory; c-Fos; cytochrome c oxidase.

1. INTRODUCTION

Social psychology and behavioural neuroscience have extensively demonstrated the relevance of social interactions in guaranteeing the well-being of most species [1]. People are inherently social animals, and so a lack of social experiences constitutes severe deprivation and causes a variety of negative effects [2].

Social isolation and loneliness are common problems in today's society. Social isolation refers to the absence or weakness of the social network, and loneliness is defined as an unpleasant lack or low quality of certain relationships, considered to be a subjective experience of isolation [3]. Unfortunately, both are highly prevalent. Data report that only 22% of the population declare that they never feel lonely, 11% recognize feeling lonely often, and 42% have felt depressed because of feeling alone [3]. More specifically, 25% of adults aged 52 and over report being lonely sometimes, and 9% most or all of the time [4].

Social disconnection is considered a risk factor for some health problems, such as adopting sedentary lifestyles [5] or an increase in psychological disorders [6]. In social mammals, isolation is a potent stressor that can provoke negative effects on maintaining homeostasis, leading to disruptions in neuro-immuno-endocrine communication [7]. The hypothalamic–pituitary–adrenocortical (HPA) axis is sensitive to social alterations, and periods of social defeat can produce increased sympathetic tone and activation of the HPA axis [8]. Moreover, decreased inflammatory control and immunity have been related to social isolation

[9], as well as reduced levels of protective hormones, causing downregulations of the immune system [10].

Furthermore, social disconnection may be associated with poor cognitive functioning in later life [11]. Reduced frequency/quality of social contacts or closed interactions can lead to impairments in global cognitive function [12,13], executive function [14], episodic memory [15,16], and working memory (WM) [17]. Although a good social lifestyle has been found to protect against decline in some cognitive domains, its function is not totally clear [18].

Post-weaning isolation rearing is the common animal procedure used to explore the effects of social isolation in rodents. It involves isolation of the animals with no handling, starting at a very early stage (postnatal day 20–28) for an extended period of several months [19]. This isolation procedure has been shown to cause a wide range of behavioural, physiological, and molecular abnormalities [20,21]. However, it is unlikely to properly emulate the effects of reduced social interactions experienced by adult people. Some studies have shown that acute social isolation of adult rodents impairs specific types of behaviours, such as anxiety [22,23] and depressive-like behaviour [23] or social recognition memory [24].

Although social isolation in experimental animals has led to emotional and cognitive impairments [9,20,23–25], little research has focused on the cerebral correlates underlying these deficits. Moreover, relatively few studies have investigated the effect of social isolation in adult rats.

Here, we investigate temporary isolation in adulthood, assessing its behavioural consequences and brain function correlates. Behaviourally, we explore motor function, anxiety-like behaviour, and spatial WM. The latter, described as a system that provides temporary active maintenance of information [26], refers to the spatial information the animal needs to remember in order to perform successfully in a single trial and then actively forget it to avoid interference [27]. Regarding brain function, our aim is to assess neuronal metabolic activity through quantitative cytochrome c-oxidase (CO)

histochemistry, a reliable marker of neuronal energy demand [28] that can be used to map regional changes in brain activity related to spatial memory tasks [29]. Several brain regions support spatial WM. Some of them are the prefrontal cortex, the hippocampus, the striatum, and the ventral tegmental area, among others [30]. Traditionally, the prefrontal cortex, mainly the prelimbic (PL) and infralimbic (IL) subregions, is involved in WM [31] and connected to the hippocampus [32]. This is an essential brain region in spatial processing [33], and it is also involved in short-term and intermediate-term memory for space [34]. In addition to neuronal metabolic activity, we also aim to assess immediate early gene (IEG) expression in the key regions related to spatial WM. For this reason, we immunostained the c-Fos protein, the c-Fos-encoded protein, which is the product of the c-fos oncogene, an IEG whose expression is indicative of a change in neuronal activity and induced after some kinds of learning [35]. c-Fos can provide information about the neuronal plasticity required for spatial memory processes in discrete brain areas [36].

2. MATERIAL AND METHODS

2.1. Animals

A total of 16 young male (3 months old) Wistar rats (*Rattus norvegicus*) were used from the vivarium of the University of Oviedo. All the animals had ad libitum access to food and tap water, and they were maintained at constant room temperature (20-22ºC), with a relative humidity of 65-70% and a 12-hour artificial light-dark cycle (08:00-20:00/20:00-08:00 h). The procedures and manipulation of the animals used in this study were carried out according to the European Communities Council Directive 2010/63/UE and the Spanish legislation on the care and use of animals for experimentation (RD 53/2013), and the study was approved by the local committee for animal studies (University of Oviedo).

2.2. Experimental procedure

Initially, we randomly separated the animals into two groups. The experimental group, socially isolated (IS) (n=8), was composed of rats that remained grouped (4 per cage) (56x33x20 cm) until 3 months of age, when then they were then placed in individual cages (23x23x15 cm) for 12 weeks. After this period, we placed the animals in groups of 4 rats per cage (56x33x20 cm), and tests started two weeks later. The control group (C) (n=8) consisted of rats that remained grouped (4 per cage) in their home box (56x33x20 cm) and were housed in the same place for the same time period as the experimental group. Eight animals per group were used for the behavioural assessment and COx histochemistry, whereas 6 animals per group were randomly selected for c-Fos immunohistochemistry, based on previous literature [37]. Both experimental groups were handled weekly to perform the cleaning of the cages.

2.3. Determination of motor functions: Rotarod-accelerod test

We used the accelerating rotarod for rats 7750 by Ugo Basile (63x50x49 cm) (Ugo Basile Biological Research Apparatus, Italy). The rotarod had a 6 cm diameter, and the rod was elevated 40 cm above the floor. This motor performance test consists of a motor-driven rotating rod that enables us to assess motor coordination and resistance to fatigue [38]. The rat's body was placed perpendicular to the rotating axis, with its head against the direction of the rotation; therefore, the animal had to move forward in order to stay on the rod. The procedure followed has two parts. In the first part, we placed the animals in the apparatus, and we maintained the speed constant at 2 rpm for 60s. In the second part, we evaluated the rats for 5 minutes in the accelerod test session, where the rotation rate increases constantly until reaching 20 rpm. We recorded the latency to falling off the rod and the rotation speed. We cleaned the apparatus thoroughly between sessions.

2.4. Assessment of anxiety: Elevated plus maze

The elevated plus maze (EPM) is based on rodents' preference for small, dark, closed spaces and their tendency to avoid open well-lit spaces above the floor level of a room. Therefore, the open and closed arms of the apparatus produce an exploratory behaviour where

avoidance of the open arms is an index of the animals' anxiety [39]. Our maze (Panlab, Barcelona, Spain) consisted of four arms 45 cm long and 10 cm wide, two of which contained walls 50 cm high, situated in the centre of a well-lit room (two halogen lamps of 4000 lux) at 70 cm from the floor. A video camera (Sony V88E) and the EthoVision Pro programme (Noldus information Technologies, Wageningen, The Netherlands) recorded the animal's behaviour in the elevated plus maze. We always placed the animals at the end of one of the open arms, and we recorded the exploratory behaviour in a single 5-minute assay. We recorded the time of permanence and number of entries in the open and closed arms.

2.5. Evaluation of spatial working memory

2.5.1. Apparatus

Spatial WM was evaluated in the circular pool designed by Morris [40], also called the Morris Water Maze (MWM). The MWM was made of fiberglass and measured 150 cm in diameter with a wall 40 cm high. The water level was 30 cm, and its temperature was 22 ± 1 ºC. The platform used corresponded to a cylinder 10 cm in diameter and 28 cm high, of which 2 cm were below the surface of the water. The MWM was in the centre of a 16 m^2 lit room (two halogen lamps of 4000 lux) and surrounded by black panels on which the spatial clues were placed. These clues were yellow and consisted of a horizontal line measuring 22 x 76 cm, a 20 x 83 cm vertical line, and a 50 x 50 cm square rotated 45º. Four imaginary quadrants (quadrants A, B, C, and D) divided the pool. A video camera (Sony V88E) connected to a computer recorded the behaviour of the animal in the MWM. Each trial was analysed using a computerized video-tracking system (Ethovision Pro, Noldus information Technologies, Wageningen, The Netherlands).

2.5.2. Habituation

The same experimenter handled the animals daily for 3 days. In order to habituate animals to the testing contingencies of the MWM, they performed 3 trials in a small square water tank (47x75x38 cm). We used a platform located in different starting positions in these trials.

2.5.3. Spatial working memory task

The WM test consisted of a paired sample task. The task involved four sessions carried out on four consecutive days. Each session consisted of two trials (sample and retention). During both trials, we hid the platform 2 cm below the level of the water. The sample trial consisted of releasing the animal from one of the four starting points in the pool and letting it swim until it reached the hidden platform, or until 60 s had elapsed. If the animal had not reached the hidden platform in this time, it was placed on the platform and kept there for 15 s. Then, we removed the animal from the maze, and the retention trial started after 5 s. The task requires recalling the position occupied by the platform during the sample trial. The locations of the start positions and the platform were the same during one session, but varied on the different days (the location was pseudo-randomly assigned, so that the platform did not occupy the same place as on the previous three days). We recorded latency to reach the platform on each day, and we calculated the mean latency in the sample and retention trials for each animal, grouping the four sessions carried out.

2.6. Sacrifice and Brain Processing

Ninety minutes after the end of the WM task, the animals were decapitated, and their brains were removed intact, frozen rapidly in isopentane (Sigma-Aldrich, Germany), and stored at -40 ºC. We coronally (30 µm) sectioned the brain at -20 ºC in a cryostat (Leica CM1900, Germany). We mounted the sections on gelatinized slides for c-Fos immunohistochemical analysis and on non-gelatinized slides for cytochrome oxidase histochemistry. We anatomically defined the regions of interest according to Paxinos and Watson's atlas [41]. The regions of interest and their distances in mm counted from bregma were: +3.24 mm for the infralimbic cortex (IL) and prelimbic cortex (PL); +1.92 mm for the accumbens core (ACC) and accumbens shell (ACS); −3.24 mm for the CA1, CA3, and the dentate gyrus (DG) subfields of the dorsal hippocampus; and −4.92 mm for the ventral tegmental area (VTA).

2.7. c-Fos immunohistochemical analysis

The slides containing the sections of the IS group (n=6) and the C group (n=6) were post-fixed in buffered 4% paraformaldehyde (0.1 M, pH 7.4) for 30 min and rinsed in phosphate-buffered saline (0.01M, pH 7.4). They were incubated for 15 min with 3% hydrogen peroxidase in PBS to remove endogenous peroxidase activity and washed twice in PBS. After blocking with PBS-T solution containing 10% Triton X-100 (Sigma, USA) and 3% bovine serum albumin for 30 min, the sections were incubated with a rabbit polyclonal anti-c-Fos antibody solution (1:10000) (Santa Cruz Biotech, USA) diluted in PBS-T for 24 h at 4ºC in a humid chamber. After that, slides were washed 3 times with PBS and incubated in a goat anti-rabbit biotinylated IgG secondary antibody (Pierce, USA; diluted 1:200 in incubating solution) for 2 h at room temperature. They were washed 3 times in PBS and reacted with avidin-biotin peroxidase complex (Vectastain ABC Ultrasensitive Elite Kit, Pierce) for 1 hour. After 2 washes in PBS, the reaction was visualized, treating the sections for about 3 min in a commercial nickel-cobalt-intensified diaminobenzidine kit (Pierce). The reaction was terminated by washing the sections twice in PBS. Finally, the slides were dehydrated through a series of graded alcohols, cleared with xylene, and cover-slipped with Entellan (Merck, USA) for microscopic observation. All immunohistochemistry procedures included sections that served as controls where we did not add the primary antibody. We coded the slides, so that the investigator who performed the entire analysis had no knowledge of the treatment of the individual subjects.

2.8. Quantification of c-Fos

We quantified the number of c-Fos positive nuclei in two alternate sections 30 μm apart. We performed the quantification by systematically sampling each of the regions selected using counting frames superimposed over the region. The sizes of the counting frames were 0.0576 mm^2 for PL and IL, 0.0648 mm^2 for ACC, 0.0576 mm^2 for ACS, 0.0864 mm^2 for CA1, 0.0648 mm^2 for CA3, 0.0324 mm^2 for DG, and 0.0144 mm^2 for VTA. The total area sampled by these frames per region and the percentage sampled with respect to the total mean area of the regions in the two sections selected were: 0.2304 mm^2 in PL (15%), 0.1728 mm^2 in IL (15%), 0.2592

mm^2 in ACC (24%), 0.1728 mm^2 in ACS (19%), 0.3456 mm^2 in CA1 (25%), 0.2592 mm^2 in CA3 (23%), 0.1296 mm^2 in DG (15%), and 0.0576 mm^2 in VTA (15%). We counted the c-Fos positive nuclei using a microscope (Leica DFC490, Germany) coupled to a computer with Leica's software installed (Leica application suite, Germany). We defined c-Fos positive nuclei based on homogenous grey-black stained elements with a well-defined border. Finally, we calculated the c-Fos positive nuclei per mm^2 for each brain area.

2.9. Cytochrome c-oxidase histochemistry

We processed section slides with quantitative COx histochemistry, as described by González-Lima and Cada (1994). To quantify enzymatic activity and control the staining variability across different baths, sets of tissue homogenate standards from the Wistar rat brains were cut at different thicknesses (10, 30, 40 and 60 μm) and included with each bath of slides. The sections and standards were incubated for 5 min in 0.1 phosphate buffer with 10% (w/v) sucrose and 0.5 (v/v) glutaraldehyde, pH 7.6. After that, baths of 0.1 M phosphate buffer with sucrose were given for 5 min each. Subsequently, 0.05 M Tris buffer, pH 7.6, with 275 mg/l cobalt chloride, sucrose, and 0.5 (v/v) dimethylsulfoxide was applied for 10 min. Then, sections and standards were incubated in a solution with 0.0075% cytochrome-c (w/v), 0.002% catalase (w/v), 5% sucrose (w/v), 0.25% dimethylsulfoxide (v/v), and 0.05% diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Madrid, Spain), in 0.1 M phosphate buffer, at 37°C for 1 h. The reaction was stopped by fixing the tissue in buffered 4% (v/v) formalin for 30 min at room temperature. Finally, the slides were dehydrated, cleared with xylene, and cover-slipped with Entellan (Merck, Germany).

2.10. COx optical density quantification

Quantification of COx histochemical staining intensity was done by densitometric analysis, using a computer-assisted image analysis workstation (MCID, Interfocus Imaging Ltd., Linton, England) made up of a high precision illuminator, a digital camera, and a computer with specific image analysis software. The mean optical density (OD) of each region was

measured on bilateral structures, using three consecutive sections in each subject (IS n=8; C n=8). In each section, four non-overlapping readings were taken, using a square-shaped sampling window that was adjusted for each region size. An investigator who was blind to the groups took a total of twelve measurements per region. These measurements were averaged to obtain one mean per region for each animal. Then, we converted OD values to COx activity units, determined by the enzymatic activity of the standards measured spectrophotometrically [28].

2.11. Data analysis

We analysed all the data with the Sigma-Stat 12.0 program (Systat, Richmond, USA). The results were considered statistically significant if $p<0.05$. We used a Shapiro-Wilk test to test the normality assumption ($p>0.05$). When the data fit a normal distribution, we used parametric tests. Otherwise, we used non parametric tests. We used the SigmaPlot 12.0 software program (SPSS Inc. and IBM Company, USA) for the graphic representation of the results. We presented data as mean+SEM.

2.11.1. Motor function

For the evaluation of the motor function on the rotarod-accelerod test, data (time on the roller and rotation speed) were analysed using T-tests for independent samples to examine differences between groups.

2.11.2. Anxiety

Anxiety level was explored in the elevated plus maze (time in open and closed arms, total entrances, and entrances in open and closed arms). Data were analysed using T-tests for independent samples to determine differences between groups for each variable.

2.11.3. Spatial working memory task

The learning criteria were determined by comparing the escape latencies during the sample trial with the retention latencies during the retention trial. Differences between escape

latencies in the sample and retention trials within the same group (C or IS) were compared with a paired T-test. Additionally, the mean latency to reach the platform was calculated for the sample and retention trials, grouping all sessions, and a T-test for independent samples was used to compare the trials within the same group. Finally, for between-group comparisons, a T-test for independent samples was used to examine if there were statistically significant differences between the sample and retention trials in each session of the task.

2.11.4. c-Fos data

The results obtained through c-Fos quantification were expressed as the number of c-Fos positive cells/mm^2 and analysed as follows: T-tests for independent samples were used to assess whether the number of c-Fos positive nuclei was different between the two behavioural groups in each brain region.

2.11.5. COx activity

T-tests for independent samples were used for statistical comparison of CO activity values between groups in each region of interest.

3. RESULTS

3.1. Behavioural results

3.1.1. Motor functions

The isolated animals do not differ from the controls in the time spent on the roller at accelerated speed. There are no statistically significant differences between C and IS groups in time spent on the roll (t_{14}=-0.067, p=0.995) (see Fig. 1A). Moreover, control and isolated animals achieved the same revolutions per minute (t_{14}=-0.101, p=0.921) (see Fig. 1B).

3.1.2. Anxiety

A T-test for independent samples showed that there were significant differences between groups in the percentage of time the animals stayed in open and closed arms (t_{14}=-2.589, p=0.021; t_{14}=2.661, p=0.019, respectively) (see Fig 1C-D). Moreover, isolated animals

had a lower number of entrances in arms than controls (t_{14}=-2-255, p=0.041), showing a reduction in open arm entrances (t_{14}=-4.912, p<0.001), but not in closed arm entrances (t_{14}=0, p=1.000) (see Fig 1E).

3.1.3. Spatial working memory

The learning criteria were established on the second day in the C group. A statistically significant difference was found between the retention trial and the sample trial, (D1 (t_7=1.121, p=0.299), D2 (t_7=4.613, p=0.002), D3 (t_7=3.449, p=0.011), D4 (t_7=4.101, p=0.005) (see Fig. 2A). However, the IS group only showed a reduction between the sample and retention trials on days 2 and 4, and so it could not establish solid learning (D1 (t_7=1.993, p=0.086), D2 (t_7=2.64, p=0.033), D3 (t_7=1.278, p=0.242), D4 (t_7=3.458, p=0.011) (see Fig. 2B). Furthermore, there were no significant differences in the sample latencies between groups (D1 (t_7=0.829, p=0.421), D2 (t_7=0.451, p=0.659), D3 (t_7=-0.335, p=0.743), D4 (t_7=1.874, p=0.082), whereas the IS group showed a statistically significant higher retention latency than the control group on day 4 (t_7=4.093, p=0.001), but not on day 1 (t_7=-0.509, p=0.619), day 2 (t_7=0.484, p=0.636), or day 3 (t_7=1.135, p=0.275) (see Fig. 2).

3.2. C-Fos results

The number of c-Fos positive nuclei was lower in the IS group than in the C group in the inflalimbic and prelimbic regions of the prefrontal cortex (IL: t_{10}=3.08, p=0.012; PL: t_{10}=4.697, p≤0.01), the shell substructure of the nucleus accumbens (T=56, n_1=6, n_2=6, p=0.004), and the ventral tegmental area (t_{10}=2.919, p=0.015). These differences were not found in the dorsal hippocampus (CA1: t_{10}=0.767, p=0.461; CA3: T=35, n_1=6, n_2=6, p=0.589; DG: t_{10}=1.746, p=0.111) or the core region of the nucleus accumbens (t_{10}=0.143, p=0.889) (see Fig. 3).

3.2. CO activity

Densitometric analysis of COx activity reveals that the IS and C groups differ in cortical areas. The IS group showed lower activity in PL (t_{14}=-5.015; p≤0.001) and IL (t_{14}=-8.408;

$p \leq 0.001$). Furthermore, this group showed lower activity in CA1 (t_{14}=-3.688; p=0.002) and VTA (t_{14}=-5.574; $p \leq 0.001$). There were no differences between groups in the DG (t_{14}=-0.752, p=0.464), CA3 (t_{14}=-1.972; p=0.069), ACC (t_{14}=-1.574; p=0.138) or ACS (t_{14}=-1.963; p=0.07) (see Table 1).

4. DISCUSSION

The aim of the present study was to assess the effects of temporary adult social isolation on locomotion, anxiety, and cognition. Cognitive consequences of adult isolation were further examined by studying the cerebral function –through the exploration of c-Fos positive cell and COx activity- in specific brain limbic structures, after performing the spatial WM task. Most studies have focused on the effect of isolation in early developmental stages, whereas neurobehavioral implications in adult rodents have not been extensively examined.

Loneliness in adulthood has been found to be a risk factor for physical inactivity [5]. Consequently, several studies have focused on deciphering rodent locomotion and exploration after a period of social deprivation. Some of them showed that isolation can cause a reduction in spontaneous locomotor activity during night–time [42], but others did not find these alterations [43]. No consistent results have been found in locomotor exploration in the open field test (OFT) either: some authors found higher exploration rates [23,43], but others revealed no differences [44,45]. In order to evaluate the motor function, we used the rotarod-accelerod test. Our results indicate that the IS group did not show an impairment in motor coordination or resistance to fatigue. To our knowledge, only one previous study used the rorarod test in isolated animals to test motor skills [46]. Their isolation procedure took place post-weaning, and they found worse performance. However, this protocol and ours are not comparable because the isolation occurred at different ages.

Feeling lonely is a complex and unpleasant emotion that may result in several psychological alterations, such as anxiety and depression disorders or negative mood [10]. In animals, isolated housing can be associated with anxiogenic effects [47], which could promote

disruptions in the sympathetic nervous tone and the HPA axis [8]. Our results in the EPM indicate that the IS group shows a decrease in the time spent in open arms, thus showing anxiety-like behaviour by avoiding brightly lit open areas [39]. Our results agree with several social isolation studies that reported: anxiety-like behaviour after isolation in early stages of development [45,48–54], in adolescence [55–57], and also –but less described– in adulthood [22,47]. Moreover, chronic adult isolation reduced the duration of grooming and caused more urination and defecation [58]. Social deprivation is a potent emotional stressor, and it can alter the correct functioning of the immune and neuroendocrine systems [7]. Solitary housing in experimental studies can produce the "isolation syndrome", characterized by hyperadrenocorticism, reduced body weight, and enhanced pain responsiveness, among other alterations [59]. More recent studies have found additional physiological alterations after social isolation periods: social disconnection may activate stressor-specific pathways and disrupt redox homeostasis [9], which can result in the release of glucocorticoids (GCs) and, thus, increase the liberation of reactive oxygen species, causing mitochondrial dysfunction [60]. Moreover, the prefrontal cortex and hippocampus are considered the higher centres of HPA axis control; therefore, an HPA axis dysregulation provoked by social discomfort may result in negative brain feedback control [61]. Additionally, social stress can lead to overproduction of nitric oxide, and it is associated with some pathological inflammatory processes [62], as well as decreased expression of brain-derived neurotrophic factor in adulthood in both the hippocampus [23,25,63] and PFC [23].

Apart from emotional disturbances, social isolation can equally affect cognitive function [12,13,17,64]. Nevertheless, there are few studies investigating whether and how social isolation during adult periods affects cognition [25]. Our spatial WM task's results revealed that temporary adult isolation can impairs the ability to retain in short term memory the location of a submerged escape platform effectively. Controls achieved the learning criteria on day 2 meanwhile IS group where inconsistent in their execution, showing a weaker learning process. Moreover, IS animals needed more time than controls in the retention trial carried out the last

training day. The WM task has been defined as the capacity to temporarily holding and manipulate active information, as well as to use this information to guide decision-making. Thus, this cognitive process is required for successful execution of everyday tasks [26]. Our results are consistent with previous studies [53], although they used distinct isolating and working memory protocols. For this reason, this is the first study to demonstrate that temporary social deprivation during adulthood causes spatial WM impairment. In addition, this study demonstrates that the poor performance of the IS group was not due to a deterioration in their motor skills. To our knowledge, only one study found impairments in spatial WM following chronic isolation during adulthood. However, they used mice with a genetic predisposition to Alzheimer's disease, which makes it clear that environmental manipulations –such as isolation- enhance cognitive alterations in susceptible subjects [65].

It has been well established that the PFC is involved in WM processes [31]. This region is connected with the hippocampus [33], and together they are implicated in cognitive domains underlying this type of memory. c-Fos is commonly used as a brain activity marker [36]. We found that the isolated animals trained on the WM task presented a lower number of c-Fos labelled cells than the C group in the medial PFC (mPFC) areas (i.e., IL and PL). The reduction in c-Fos positive cells in the mPFC could be explained by inefficient acquisition of the WM task, previously described in other experimental models [66], showing that it is PFC-dependent [67]. It could be expected that, because as acute stress leads to activation of HPA axis [8], the subsequent release of GCs in PFC can affect WM processes [67,68]. Also, the corticotropin-releasing factor, which is a peptide hormone mainly secreted in response to stress, impaired goal-directed actions, such as those required in WM performance [69]. Similarly, a previous study have found a decrease of Fos activity in post-weaning isolated animals in several regions, including cortical regions, thalamus, amygdala, hippocampus and striatum [70]. We also found lower number of c-Fos positive cells in VTA and ACS limbic regions. It has been reported that stressful events perturb not only PFC but also nucleus accumbens function: isolation during adolescence enhances dopamine levels in the accumbens and increases the reinforced effects of

drugs (reviewed by [71]). Moreover, accumbens has a key role in the meso-cortico-limbic pathway, being essential for memory processes [72]. In this sense, an altered dopaminergic system in the VTA-ACS-PFC network can explain our behavioural results. As we know, dopamine signalling in the PFC, hippocampus and striatum modulates the WM function [73]. Therefore, a disrupted brain activity in these areas can alter this pathway. Altogether, prefrontal, accumbens and VTA lower c-Fos activity can explain the worse WM performance by adult social isolated rats compared to controls in the last day of the task.

With respect to COx analysis, we found similar results except for the ventral striatum, in which COx activity is similar between groups. This could be explained by the fact that COx histochemistry is a technique that shows accumulated neuronal response. Therefore, the inconsistent performance of the IS group in the WM task (sometimes well-performed while others not) is not reflecting an endurable metabolic dysfunction in a brain structure known to be involved in rewarding responses [74]. Both PFC and VTA were impaired not only in c-Fos expression, but also at metabolic level. Isolated animals showed a decreased metabolic activity in IL, PL, CA1 and VTA after the WM task. It has been reported that higher levels of GCs, that isolation may produce, cause mitochondrial dysfunction [9], that might lead to the altered neuronal metabolic activity found in these regions. The reduced metabolic activity in the areas mentioned above could be responsible for the poorest performance in the WM task, which is prefrontal-hippocampal dependent [75]. In addition, network oscillations between PFC-VTA-hippocampus can be essential during WM task [75,76]. Post-weaning isolation during an extended period of time (8 weeks) induces several effects in brain function: a decreased in antioxidant capacity, a higher production of reactive oxygen species in PFC and hippocampus and decreased levels of glutamate [53].

Our results are very similar to those of Shao et al., [53]. They also found anxiety-like behaviour and a poorer WM performance. However, in their study the animals were isolated in post-weaning meanwhile we separated them in the adulthood. This could reveal that social deprivation is such a potent psychological stressor that result in neurobehavioral changes in

several stages of life. Further studies need to clarify the molecular mechanisms altered after a period of social deprivation in adulthood.

5. CONCLUSIONS

Twelve weeks of social isolation in adult rats lead to different behavioural and brain alterations. Anxiety-like behaviour and working memory processes were impaired, motor functions were preserved. Regarding brain correlates, we found that isolated animals showed lower c-Fos activity in the PFC, ACS, and VTA, and less metabolic activity in the PFC, CA1 subfield of the dorsal hippocampus, and VTA, related to weak performance on the spatial WM task.

Acknowledgements

This study was supported by Project Grants of Gobierno de España PSI2017-83893-R, MINECO PSI2015-73111-EXP and PSI2017-90806-REDT, Programa "Severo Ochoa" de Ayudas Predoctorales de la CONSEJERÍA DE CULTURA Y DEPORTE del Principado de Asturias BP17-11 to C.Z. Other source of funding was the Gobierno de Aragón (Departamento de Innovación, Investigación y Universidad) and FEDER “Construyendo Europa desde Aragón”.

References

[1] Eisenberger NI. The pain of social disconnection: examining the shared neural underpinnings of physical and social pain. Nat Rev Neurosci 2012;13:421–34. doi:10.1038/nrn3231.

[2] Leigh-Hunt N, Bagguley D, Bash K, Turner V, Turnbull S, Valtorta N, et al. An overview of systematic reviews on the public health consequences of social isolation and loneliness. Public Health 2017;152:157–71. doi:10.1016/j.puhe.2017.07.035.

[3] Griff J. The Lonely Society? Mental Health Foundation. 2010.

[4] Gellis ZD, Kenaley BL, Have T Ten, Beaumont J. Measuring National Well-being - Older people and loneliness. J Am Geriatr Soc 2013:1–11. doi:10.1111/jgs.12776.

[5] Lindsay Smith G, Banting L, Eime R, O'Sullivan G, van Uffelen JGZ. The association between social support and physical activity in older adults: A systematic review. Int J Behav Nutr Phys Act 2017;14. doi:10.1186/s12966-017-0509-8.

[6] Cacioppo S, Grippo AJ, London S, Goossens L, Cacioppo JT. Loneliness: Clinical Import and Interventions. Perspect Psychol Sci 2015. doi:10.1177/1745691615570616.

[7] Cacioppo JT, Cacioppo S, Capitanio JP, Cole SW, Angeles L. The Neuroendocrinology of Social Isolation 2016:733–67. doi:10.1146/annurev-

psych-010814-015240.

[8] Steptoe A, Owen N, Kunz-Ebrecht SR, Brydon L. Loneliness and neuroendocrine, cardiovascular, and inflammatory stress responses in middle-aged men and women. Psychoneuroendocrinology 2004;29:593–611. doi:10.1016/S0306-4530(03)00086-6.

[9] Filipović D, Todorović N, Bernardi RE, Gass P. Oxidative and nitrosative stress pathways in the brain of socially isolated adult male rats demonstrating depressive- and anxiety-like symptoms. Brain Struct Funct 2017;222. doi:10.1007/s00429-016-1218-9.

[10] Cacioppo JT, Hawkley LC. Social Isolation and Health, with an Emphasis on Underlying Mechanisms. Perspect Biol Med 2003;46:S39–52. doi:10.1353/pbm.2003.0063.

[11] Evans IEM, Martyr A, Collins R, Brayne C, Clare L. Social Isolation and Cognitive Function in Later Life: A Systematic Review and Meta-Analysis. J Alzheimer's Dis 2018:1–26. doi:10.3233/JAD-180501.

[12] Ellwardt L, Van Tilburg TG, Aartsen MJ. The mix matters: Complex personal networks relate to higher cognitive functioning in old age. Soc Sci Med 2015;125:107–15. doi:10.1016/j.socscimed.2014.05.007.

[13] Lee SH, Kim YB. Which type of social activities may reduce cognitive decline in the elderly?: a longitudinal population-based study. BMC Geriatr 2016;16:165. doi:10.1186/s12877-016-0343-x.

[14] Bourassa KJ, Memel M, Woolverton C, Sbarra DA. Social participation predicts cognitive functioning in aging adults over time: comparisons with physical

health, depression, and physical activity. Aging Ment Heal 2017;21:133–46. doi:10.1080/13607863.2015.1081152.

[15] Klaming R, Annese J, Veltman DJ, Comijs HC. Episodic memory function is affected by lifestyle factors: a 14-year follow-up study in an elderly population. Aging, Neuropsychol Cogn 2017;24:528–42. doi:10.1080/13825585.2016.1226746.

[16] Mousavi-Nasab SMH, Kormi-Nouri R, Nilsson LG. Examination of the bidirectional influences of leisure activity and memory in old people: A dissociative effect on episodic memory. Br J Psychol 2014;105:382–98. doi:10.1111/bjop.12044.

[17] McGue M, Christensen K. Social activity and healthy aging: A study of aging Danish twins. Twin Res Hum Genet 2007;10:255–65. doi:10.1375/twin.10.2.255.

[18] Kuiper JS, Zuidersma M, Oude Voshaar RC, Zuidema SU, van den Heuvel ER, Stolk RP, et al. Social relationships and risk of dementia: A systematic review and meta-analysis of longitudinal cohort studies. Ageing Res Rev 2015;22:39–57. doi:10.1016/j.arr.2015.04.006.

[19] Heidbreder CA, Weiss IC, Domeney AM, Pryce C, Homberg J, Hedou G, et al. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience 2000;100:749–68. doi:10.1016/S0306-4522(00)00336-5.

[20] Fone KCF, Porkess MV. Behavioural and neurochemical effects of post-weaning social isolation in rodents-Relevance to developmental neuropsychiatric disorders. Neurosci Biobehav Rev 2008;32:1087–102. doi:10.1016/j.neubiorev.2008.03.003.

[21] Hall FS. Social Deprivation of Neonatal, Adolescent, and Adult Rats Has Distinct Neurochemical and Behavioral Consequences. Crit Rev Neurobiol 1998;12:129–62. doi:10.1615/CritRevNeurobiol.v12.i1-2.50.

[22] Cuenya L, Fosacheca S, Mustaca A, Kamenetzky G. Effects of isolation in adulthood on frustration and anxiety. Behav Processes 2012;90:155–60. doi:10.1016/j.beproc.2012.01.003.

[23] Ieraci A, Mallei A, Popoli M. Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. Neural Plast 2016;2016. doi:10.1155/2016/6212983.

[24] Shahar-Gold H, Gur R, Wagner S. Rapid and Reversible Impairments of Short- and Long-Term Social Recognition Memory Are Caused by Acute Isolation of Adult Rats via Distinct Mechanisms. PLoS One 2013;8. doi:10.1371/journal.pone.0065085.

[25] Gong WG, Wang YJ, Zhou H, Li XL, Bai F, Ren QG, et al. Citalopram Ameliorates Synaptic Plasticity Deficits in Different Cognition-Associated Brain Regions Induced by Social Isolation in Middle-Aged Rats. Mol Neurobiol 2017;54:1927–38. doi:10.1007/s12035-016-9781-x.

[26] Baddeley A, Hitch G. Working Memory. The psychology of learning and motivation: advances in research and theory. New York Acad Press 1974;8:47–89. doi:10.1016/j.cub.2009.12.014.

[27] Futter JE, Aggleton JP. How rats perform spatial working memory tasks: Limitations in the use of egocentric and idiothetic working memory. Q J Exp Psychol 2006;59:77–99. doi:10.1080/02724990544000068.

[28] Gonzalez-Lima F, Cada A. Cytochrome oxidase activity in the auditory system of the mouse: A qualitative and quantitative histochemical study. Neuroscience 1994;63:559–78. doi:10.1016/0306-4522(94)90550-9.

[29] Méndez-López M, Méndez M, Sampedro-Piquero P, JL A. Spatial learning-related changes in metabolic activity of limbic structures at different posttask delays. J Neurosci Res 2013;91:151–9. doi:10.1002/jnr.23134.

[30] Aggleton JP, Brown MW. Episodic memory, amnesia, and the hippocampal-anterior thalamic axis. Behav Brain Sci 1999;22:425–44. doi:10.1017/S0140525X99002034.

[31] Miller EK. The prefontral cortex and cognitive control. Nat Rev Neurosci 2000;1:59–65. doi:10.1038/35036228.

[32] Jay TM, Witter MP. Distribution of hippocampal CA1 and subicular efferents in the prefrontal cortex of the rat studied by means of anterograde transport of Phaseolus vulgaris- leucoagglutinin. J Comp Neurol 1991;313:574–86. doi:10.1002/cne.903130404.

[33] Squire LR, Stark CEL, Clark RE. The medial temporal lobe. Annu Rev Neurosci 2004;27:279–306. doi:10.1146/annurev.neuro.27.070203.144130.

[34] Long JM, Kesner RP. The effects of dorsal versus ventral hippocampal, total hippocampal, and parietal cortex lesions on memory for allocentric distance in rats. Behav Neurosci 1996;110:922–32. doi:10.1037/0735-7044.110.5.922.

[35] Santín LJ, Aguirre JA, Rubio S, Begega A, Miranda R, Arias JL. c-Fos expression in supramammillary and medial mammillary nuclei following spatial reference and working memory tasks. Physiol Behav 2003;78:733–9.

doi:10.1016/S0031-9384(03)00060-X.

[36] Méndez-Couz M, Conejo NM, Vallejo G, Arias JL. Spatial memory extinction: A c-Fos protein mapping study. Behav Brain Res 2014;260:101–10. doi:10.1016/j.bbr.2013.11.032.

[37] Banqueri M, Méndez M, Arias JL. Why are maternally separated females inflexible? Brain activity pattern of COx and c-Fos. Neurobiol Learn Mem 2018;155:30–41. doi:10.1016/j.nlm.2018.06.007.

[38] Sampedro-Piquero P, Zancada-Menendez C, Begega A, Mendez M, Arias JL. Effects of forced exercise on spatial memory and cytochrome c oxidase activity in aged rats. Brain Res 2013;1502:20–9. doi:10.1016/j.brainres.2012.12.036.

[39] Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987;92:180–5. doi:10.1007/BF00177912.

[40] Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60. doi:10.1016/0165-0270(84)90007-4.

[41] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. Acad Press San Diego 1997;3rd.

[42] Tsvirkun D, Bourreau J, Mieuset A, Garo F, Vinogradova O, Larina I, et al. Contribution of social isolation, restraint, and hindlimb unloading to changes in hemodynamic parameters and motion activity in rats. PLoS One 2012;7. doi:10.1371/journal.pone.0039923.

[43] Ishikawa J, Ogawa Y, Owada Y, Ishikawa A. Hyperlocomotor activity and stress vulnerability during adulthood induced by social isolation after early weaning are

prevented by voluntary running exercise before normal weaning period. Behav Brain Res 2014;264:197–206. doi:10.1016/j.bbr.2014.02.007.

[44] Pryce CR, Bettschen D, Bahr NI, Feldon J. Comparison of the effects of infant handling, isolation, and nonhandling on acoustic startle, prepulse inhibition, locomotion, and HPA activity in the adult rat. Behav Neurosci 2001;115:71–83. doi:10.1037/0735-7044.115.1.71.

[45] Weiss IC, Pryce CR, Jongen-Rêlo AL, Nanz-Bahr NI, Feldon J. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behav Brain Res 2004;152:279–95. doi:10.1016/j.bbr.2003.10.015.

[46] Ulupinar E, Erol K, Ay H, Yucel F. Rearing conditions differently affect the motor performance and cerebellar morphology of prenatally stressed juvenile rats. Behav Brain Res 2015;278:235–43. doi:10.1016/j.bbr.2014.10.003.

[47] Botelho S, Estanislau C, Morato S. Effects of under- and overcrowding on exploratory behavior in the elevated plus-maze. Behav Processes 2007;74:357–62. doi:10.1016/j.beproc.2006.12.006.

[48] Hellemans KGC, Benge LC, Olmstead MC. Adolescent enrichment partially reverses the social isolation syndrome. Dev Brain Res 2004;150:103–15. doi:10.1016/j.devbrainres.2004.03.003.

[49] Lukkes JL, Engelman GH, Zelin NS, Hale MW, Lowry CA. Post-weaning social isolation of female rats, anxiety-related behavior, and serotonergic systems. Brain Res 2012;1443:1–17. doi:10.1016/j.brainres.2012.01.005.

[50] Lukkes JL, Mokin M V., Scholl JL, Forster GL. Adult rats exposed to early-life social isolation exhibit increased anxiety and conditioned fear behavior, and

altered hormonal stress responses. Horm Behav 2009;55:248–56. doi:10.1016/j.yhbeh.2008.10.014.

[51] Bledsoe AC, Oliver KM, Scholl JL, Forster GL. Anxiety states induced by post-weaning social isolation are mediated by CRF receptors in the dorsal raphe nucleus. Brain Res Bull 2011;85:117–22. doi:10.1016/j.brainresbull.2011.03.003.

[52] Tanaś Ł, Ostaszewski P, Iwan A. Effects of post-weaning social isolation and environmental enrichment on exploratory behavior and anxiety in Wistar rats. Acta Neurobiol Exp (Wars) 2015;75:72–9.

[53] Shao Y, Yan G, Xuan Y, Peng H, Huang QJ, Wu R, et al. Chronic social isolation decreases glutamate and glutamine levels and induces oxidative stress in the rat hippocampus. Behav Brain Res 2015;282:201–8. doi:10.1016/j.bbr.2015.01.005.

[54] McCool BA, Chappell AM. Early social isolation in male long-evans rats alters both appetitive and consummatory behaviors expressed during operant ethanol self-administration. Alcohol Clin Exp Res 2009;33:273–82. doi:10.1111/j.1530-0277.2008.00830.x.

[55] Skelly MJ, Chappell AE, Carter E, Weiner JL. Adolescent social isolation increases anxiety-like behavior and ethanol intake and impairs fear extinction in adulthood: Possible role of disrupted noradrenergic signaling. Neuropharmacology 2015;97:149–59. doi:10.1016/j.neuropharm.2015.05.025.

[56] Yorgason JT, España RA, Konstantopoulos JK, Weiner JL, Jones SR. Enduring increases in anxiety-like behavior and rapid nucleus accumbens dopamine signaling in socially isolated rats. Eur J Neurosci 2013;37:1022–31. doi:10.1111/ejn.12113.

[57] Chappell AM, Carter E, McCool BA, Weiner JL. Adolescent rearing conditions influence the relationship between initial anxiety-like behavior and ethanol drinking in male long evans rats. Alcohol Clin Exp Res 2013;37. doi:10.1111/j.1530-0277.2012.01926.x.

[58] Spasojevic N, Gavrilovic L, Varagic VV, Dronjak S. Effects of chronic diazepam treatments on behavior on individually housed rats. Arch Biol Sci 2007;59:113–7. doi:10.2298/ABS0702113S.

[59] Valzelli L. The "isolation syndrome" in mice. Psychopharmacologia 1973;31:305–20. doi:10.1007/BF00421275.

[60] Papadopoulos MC, Koumenis IL, Dugan LL, Giffard RG. Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. Brain Res 1997;748:151–6. doi:10.1016/S0006-8993(96)01293-0.

[61] Mizoguchi K, Ishige A, Aburada M, Tabira T. Chronic stress attenuates glucocorticoid negative feedback: Involvement of the prefrontal cortex and hippocampus. Neuroscience 2003;119:887–97. doi:10.1016/S0306-4522(03)00105-2.

[62] Brown GC. Mechanisms of inflammatory neurodegeneration: iNOS and NADPH oxidase. Biochem Soc Trans 2007;35:1119–21. doi:10.1042/BST0351119.

[63] Ma J, Wu CF, Wang F, Yang JY, Dong YX, Su GY, et al. Neurological mechanism of Xiaochaihutang's antidepressant-like effects to socially isolated adult rats. J Pharm Pharmacol 2016:1340–9. doi:10.1111/jphp.12616.

[64] Santini ZI, Koyanagi A, Tyrovolas S, Haro JM, Donovan RJ, Nielsen L, et al. The protective properties of Act-Belong-Commit indicators against incident

depression, anxiety, and cognitive impairment among older Irish adults: Findings from a prospective community-based study. Exp Gerontol 2017;91:79–87. doi:10.1016/j.exger.2017.02.074.

[65] Huang HJ, Liang KC, Ke HC, Chang YY, Hsieh-Li HM. Long-term social isolation exacerbates the impairment of spatial working memory in APP/PS1 transgenic mice. Brain Res 2011;1371:150–60. doi:10.1016/j.brainres.2010.11.043.

[66] Méndez M, Méndez-López M, López L, Aller MA, Arias J, Arias JL. Working memory impairment and reduced hippocampal and prefrontal cortex c-Fos expression in a rat model of cirrhosis. Physiol Behav 2008;95:302–7. doi:10.1016/j.physbeh.2008.06.013.

[67] Mika A, Mazur GJ, Hoffman AN, Talboom JS, Bimonte-Nelson HA, Sanabria F, et al. Chronic stress impairs prefrontal cortex-dependent response inhibition and spatial working memory. Behav Neurosci 2012. doi:10.1037/a0029642.

[68] Barsegyan A, Mackenzie SM, Kurose BD, McGaugh JL, Roozendaal B. Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. Proc Natl Acad Sci 2010;107:16655–60. doi:10.1073/pnas.1011975107.

[69] Hupalo S, Berridge CW. Working memory impairing actions of Corticotropin-Releasing Factor (CRF) neurotransmission in the prefrontal cortex. Neuropsychopharmacology 2016;41:2733–40. doi:10.1038/npp.2016.85.

[70] Ahern M, Goodell DJ, Adams J, Bland ST. Brain regional differences in social encounter-induced Fos expression in male and female rats after post-weaning social isolation. Brain Res 2016;1630:120–33.

doi:10.1016/j.brainres.2015.11.006.

[71] Watt MJ, Weber MA, Davies SR, Forster GL. Impact of juvenile chronic stress on adult cortico-accumbal function: Implications for cognition and addiction. Prog Neuro-Psychopharmacology Biol Psychiatry 2017;79:136–54. doi:10.1016/j.pnpbp.2017.06.015.

[72] Del Ferraro G, Moreno A, Min B, Morone F, Pérez-Ramírez Ú, Pérez-Cervera L, et al. Finding influential nodes for integration in brain networks using optimal percolation theory. Nat Commun 2018. doi:10.1038/s41467-018-04718-3.

[73] Miyoshi E, Wietzikoski S, Camplessei M, Silveira R, Takahashi RN, Da Cunha C. Impaired learning in a spatial working memory version and in a cued version of the water maze in rats with MPTP-induced mesencephalic dopaminergic lesions. Brain Res Bull 2002. doi:10.1016/S0361-9230(02)00754-2.

[74] Mayer D, Kahl E, Uzuneser TC, Fendt M. Role of the mesolimbic dopamine system in relief learning. Neuropsychopharmacology 2018. doi:10.1038/s41386-018-0020-1.

[75] Sigurdsson T, Duvarci S. Hippocampal-Prefrontal Interactions in Cognition, Behavior and Psychiatric Disease. Front Syst Neurosci 2015;9:190. doi:10.3389/fnsys.2015.00190.

[76] Fujisawa S, Buzsáki G. A 4 Hz Oscillation Adaptively Synchronizes Prefrontal, VTA, and Hippocampal Activities. Neuron 2011;72:153–65. doi:10.1002/9781119962083.ch1.

FIGURE LEGENDS

Figure 1. Rotarod- accelerod test (A-B) and Elevated plus maze (C-E) results. (A) Bar charts represent time spent on the rod. (B) Bar charts represent rotation speed on the rotarod-accelerod test. There were no statistically significant differences between the groups. (C) Bar charts represent percentage of time spent in the open arms. (D) Bar charts represent percentage of time spent in the and closed arms. There were significant differences between the groups (* $p<0.05$). (E) Bar charts represent the number of entries in the arms of the elevated plus maze. There were significant differences between the groups in the total number of entries and open arm entries (* $p<0.05$). Data are represented as mean ± SEM.

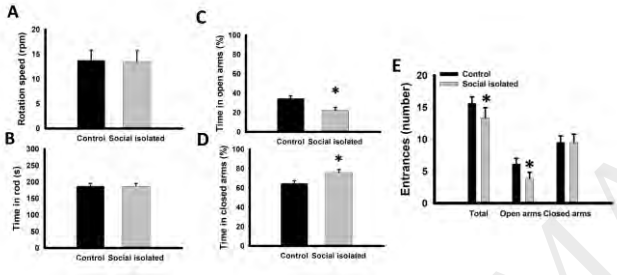

Figure 2. Mean escape latencies to platform on the spatial working memory test on days 1 to 4 (mean ± SEM of sample and retention trials) in the C group (A) and IS group (B). There were significant differences between the sample and retention trials (* $p<0.05$ sample vs. retention trial). There was a significant difference between the C and IS groups (+ $p<0.05$ C vs IS group).

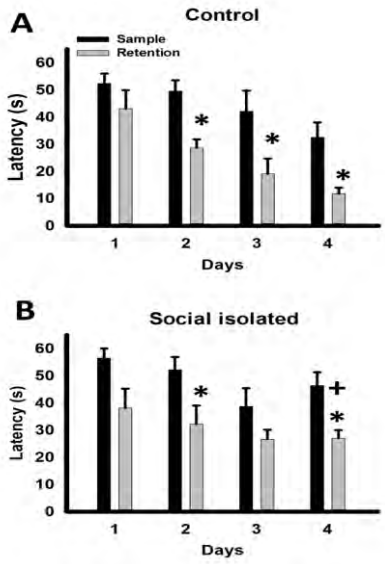

Figure 3. (A) Number of c-Fos-immunoreactive cells (c-Fos positive cells/mm2) present in prelimbic (PL) and infralimbic (IL) cortex, dorsal hippocampus (CA1, CA3, DG), nucleus accumbens core (ACC), and shell (ACS) and ventral tegmental area (VTA). There were significant differences between the groups in IL, PL, ACS and VTA (* $p<0.05$). Data are shown as mean ± SEM. (B-C) Representative photomicrographs showing sections of PL cortex that were immunostained for c-Fos protein from C (B) and IS (C) groups. Many immunopositive nuclei were observed in control rats, but less expression was noted in isolated rats. Scale bar is equal to 150 µm.

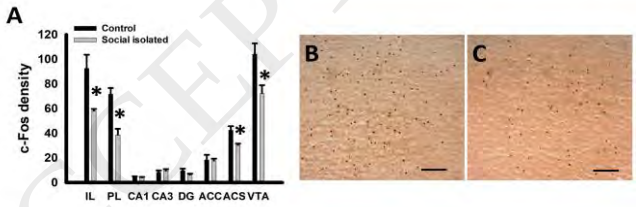

Table 1

COx values		
Structure	Control	Social isolated

IL	24.894±0.404	19.695±0.468*
PL	23.783±0.545	19.759±0.545*
CA1	21.461±0.632	16.381±1.224*
CA3	19,627±0.442	17.493±0.988
DG	24.488±0.974	23.329±1.195
ACC	23.367±0.492	21.838±0.837
ACS	28.631±0.827	26.246±0.89
VTA	14.387±0.403	10.322±0.608*

COx values (mean ± SEM) in C and IS groups. Infralimbic cortex = IL, Prelimbic cortex = PL, CA1, CA3, Dentate Gyrus = DG, Accumbens Core = ACC, Accumbens Shell = ACS, and Ventral Tegmental Area = VTA. There were significant differences between the groups in IL, PL, CA1 and VTA (* $p<0.05$).