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SOCIAL ENRICHMENT REVERSES THE ISOLATION-INDUCED DEFICITS OF NEURONAL PLASTICITY IN THE HIPPOCAMPUS OF MALE RATS

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Abstract

Environmental enrichment is known to improve brain plasticity and protect synaptic function from negative insults. In the present study we used the exposure to social enrichment in order to ameliorate the negative effect observed in post weaning isolated rats in which neurotrophic factors, neurogenesis, neuronal dendritic trees and spines were markedly altered in the hippocampus. In male rats exposed to 4 weeks of post-weaned social isolation followed by 4 weeks of reunion, different neuronal growth markers as well as neuronal morphology have been evaluated using different experimental approaches. Social enrichment was able to restore the reduction of BDNF, NGF and Arc gene expression in the whole hippocampus of social isolated rats. This effect was paralleled by an increase in density and morphology of dendritic spines, as well as in neuronal tree arborisation in granule cells of the dentate gyrus. All these changes were associated with a marked increase in neuronal proliferation and neurogenesis in the dentate gyrus of hippocampal formation that were reduced by social isolation stress. These data further suggest that exposure to social enrichment, by abolishing the negative effect of social isolation stress on hippocampal plasticity, may improve neuronal resilience with a beneficial effect on cognitive function.

Keywords: Social enrichment, Social isolation, Adolescence, Stress, Neuronal plasticity, Hippocampus

1. Introduction

Adolescence is one of the most critical periods during the lifespan in which brain functions exhibit remarkable changes making individuals more vulnerable or resilient to perturbations caused by great stress insults (Jankord et al., 2011).

In rodents, the exposure to prolonged environmental stress results in molecular, structural and functional alterations in different brain areas, including hippocampal formation (Hip). In fact, decreases of Nerve Growth Factor (NGF) and Brain-derived neurotrophic factor (BDNF) and parallel reduction in dendritic arborisation, spines density and neurogenesis, have been largely reported after these stressful conditions (see Cameron and Schoenfeld, 2018 for review). All these effects are accompanied by changes in emotional state, where the anxiety-like profile is the most common (Cameron and Schoenfeld, 2018). In contrast, it has been widely demonstrated that environmental and specific social enrichment (SE) may exert beneficial effects antagonizing most of the impairments induced by stress (Fares et al., 2013; Laviola et al., 2004; Lu et al., 2003). Thus, living in an enriched environment may improve the correct brain development both in animals and humans exposed to chronic insults during adolescence.

Several authors have clearly described the crucial role of negative environmental conditions and life style during pregnancy, post-birth, childhood, and adolescence for brain physiology (Sale, 2018; McLaughlin et al., 2017). All these factors may interact with brain function, leading to a continuous remodelling of its complex and dynamic structure (see Sale, 2018; Kondo, 2017 for review). Medial prefrontal cortex (mPFC), amygdala and the Hip, are three main brain areas involved in the modulation of emotions, that may undergo to profound changes in both neuronal structure and function in response to stress (McEwen, 2005).

Due to their gregarious nature, the post-weaning isolation rearing model in rodents represents a useful condition of post-natal early stress capable to induce long-lasting changes in brain function, HPA axis activity and emotional behaviour (Murinova et al., 2017; Burke et al., 2017). These functional alterations have a pathophysiological relevance similar to that seen in schizophrenia, such as altered dopamine transmission in the mPFC and

nucleus accumbens (Hall et al., 1998) and deficits in neuronal plasticity (Pisu et al., 2011; Ibi et al., 2008, Serra et al. 2008). However, the exact mechanisms related to these changes appear to be still unclear.

Environmental enrichment is considered one of the best models to improve brain function by physical and social surroundings. Accordingly, the periodic use of toys, colours and different activities (e.g., running wheels inside of the animal cages), as well as SE, may stimulate curiosity and facilitate exploration, a behaviour associated with Hip neurogenesis (Tanti et al., 2013; Xu et al., 2015; Kempermann et al., 1997), and the integration of newborn cells into functional circuits (see Kempermann et al., 2010 for review), which have been modified in their expression by severe environmentally stressful stimuli.

One of the environmental enrichment protocols used widely to study the effect of this experience on rodent brain plasticity is the SE that consists of the possibility of interaction with 5-6 other con-specifics in the same cage. SE appears to be a good model to study the beneficial effects on the brain after a prolonged chronic stress such as social isolation (SI). Accordingly, SE elicits behavioural benefits paralleled by an improvement of neuronal plasticity in brain areas such as the Hip (Faherty et al., 2003; Foster and Dumas, 2001; Foster et al., 1996; Green and Greenough, 1986; Hullinger et al., 2015; Kumar et al., 2012; Leggio et al., 2005; Malik and Chattarji, 2012).

BDNF and NGF, two of the most important neurotrophins for brain development involving neuron survival, specialization, migration, synaptogenesis, spine density and dendritic arborisation (Park and Poo, 2013), also play a crucial role for Hip function and synaptic plasticity related to learning and memory processes (Kuipers and Bramham, 2006). Accordingly, long-term early-life adverse event exposure and environmental enrichment can effect, in opposite manners, BDNF, NGF, and Arc gene expression in the Hip (Fone and

Porkess, 2008; Pisu et al., 2011, Cirulli et al., 2000; Branchi et al., 2006), an effect that we have previously shown to be present in SI rats (Biggio et al., 2014; Pisu et al., 2011).

Here, we studied the putative effect of SE in post-weaned SI rats by evaluating BDNF and NGF content, neurogenesis, spine density and dendritic arborisation in the rat dentate gyrus that, together with the other Hip subregions, play a crucial role in controlling learning and memory formation.

We show that the decrease in the amount of BDNF, NGF and activity-regulated cytoskeletal associated protein (Arc) expression elicited by SI is associated with a significant reduction in dentate Hip neurogenesis, spine density and dendritic arborisation, an effect completely abolished by 4 weeks of SE rearing.

Our data further support the idea that SE is an effective environmental factor able to protect from stressful adverse effects during brain development.

2. Material and methods

2.1. Animals

The study was performed using male Sprague-Dawley rats (Charles River, Calco, Italy). All animals were housed under an artificial 12h light-dark cycle (8:00 a.m-8:00 p.m.) at a controlled temperature (23 ± 2 °C) and humidity (65%). Food and water were available *ad libitum*. Mating occurred in individual cages, using one adult male and one female, and verified by the presence of a sperm cap (plug). This day was considered as gestational day 0 (GD 0).

On GD 21 each pregnant dam was individually housed in a single cage waiting for delivery. The experimental procedure was in accordance with the Department of Health (685/2015-PR) and approved by the Italian ethics committee.

2.2. Social isolation and enrichment protocol

At the weaning day (PND 21), male rats used for our investigations were randomly assigned into three experimental groups: group housed (GH) animals (5 per cage) that were housed in the same cage (60 cm x 38 cm x 18 cm) for 8 weeks, socially isolated (SI) reared (one per cage) in a smaller cage (42 cm x 26 cm x 15 cm) for 8 weeks, and socially isolated rats for 4 weeks and then re-united (5 per cage) for the further 4 weeks (SI-J) until PND 77, when the experiments were started.

2.3. Immunoblot analysis

The brain was rapidly removed, and the Hip was dissected on ice, weighed, and stored at – 80°C until analysis. Total Hip proteins were extracted with Biobasic Kit protein extraction (Membrane and Cytoplasmic Protein Extraction kit, Bio Basic Inc, Markham Ontario, Canada), to obtain both cytoplasmic and membrane fraction proteins. Membrane fraction protein concentration was determined with DC Protein Assay kit (Bio-Rad, Milan, Italy). The extract (40 µg of protein) was incubated for 10 min at 70°C and then fractionated by SDS-polyacrylamide gel electrophoresis (NuPAGE Novex 4–12% Bis-Tris Midi Gel, Life Technologies, Monza, Italy). The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Milan, Italy) with the use of a Criterion Blotter (Bio-Rad) and subjected to immunoblot analysis with rabbit polyclonal antibodies specific to detect BDNF, NGF and Arc protein (Santa Cruz, Heidelberg, Germany), in the cytoplasmic fraction. The cytoplasmic fraction was exposed to 5% nonfat dried milk in Tris-buffered saline containing 1% Tween 20 (TBS-T) before an overnight incubation at 4°C with the specific antibody of interest. All antibodies were diluted in TBS-T containing 5% nonfat dried milk.

The membrane was then washed with Tris-buffered saline containing 0.01% Tween-20 before incubation for 60 min at 25°C with horseradish peroxidase-linked secondary antibodies to rabbit (anti-rabbit, Sigma, Milan, Italy; anti-mouse, Millipore, Milan, Italy) or mouse (anti-mouse, Millipore, Milan, Italy) IgG in 5% non-fat dried milk. An ECL kit (Millipore, Little Chalfont, UK) was used for detection of immune complex. The relative changes in the amount of BDNF, NGF, and Arc proteins were evaluated by measurement of the intensity of the immunoreactive bands with a Molecular Analysis program (PerkinElmer Geliance Imaging) and normalization of the data relative to the amount of mouse monoclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, Milan, Italy). Immune complexes were detected with enhanced chemioluminescence reagents (Millipore). The intensity of immunoreactive bands was quantitated by Geliance 600 Chemi Imaging System (Perkin Elmer, Monza, Italy) and analyzed by the GeneTools software (Perkin Elmer). Values of optical density (OD) for BDNF, NGF factors and Arc protein were normalized by the corresponding GAPDH values.

2.4. Immunohistochemistry

Golgi impregnation: a modified Golgi-Del Rio Hortega protocol was used to process the tissue. Rats (n = 5 per group) were killed by an overdose of chloral hydrate and the brains were removed and sectioned by a brain slicer at 1 mm in sagittal plane. The slices were immersed in Del Rio Hortega's chromating original mixture (freshly prepared), consisting of 10% potassium dichromate and 6% chloral hydrate, dissolved in 4% of paraformaldehyde for 4 days at room temperature in the dark. Chromating solution was changed daily with freshly prepared solution. After 4 days of chromation, the slices were washed several times with a 1,5% silver nitrate solution in distillate water before incubation on silver nitrate for another three days at room temperature in the dark, for heavy metal

precipitation to occur. Tissue slices were blocked, super-glued to a metal chuck, and were cut at 50 µm by a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany) into a water bath. Slices were placed on slides and allowed to dry. They were next run through a dehydrating series of alcohols and xylenes and coverslipped with Eukitt (Sigma, Milan, Italy).

Image analysis and quantification: the serial sections were chosen and acquired by a microscope motorized x, y and z focus (AxioObserver Z.1, Zeiss, Germany) fitted with a high resolution cooled CCD camera and Axiovision Rec 4.8 software, for high-resolution image acquisition and digital quantitation. In combination with a 100X objective, using a sophisticated and well-established method, this should represent a 3D quantitative profile of the neurons sampled and prevent a failure to detect less prominent spines. Neurons in the dentate gyrus region which fulfit the following criteria were selected for the analysis: (i) the cells type must be identifiable; (ii) image resolution should be sufficient to visually distinguish dendritic spine formation from variably contrasting background, and (iii) completeness of Golgi impregnation of all dendrites. Neurons with incomplete impregnation or neurons with truncations due to the plane of sectioning were not analyzed. We also made sure there was a minimal level of truncation at the most distal part of the dendrites; this often happens in most of the Golgi studies, likely due to the plane of sectioning at top and bottom parts of the section. With consideration of the shrinkage factor after processing (generally 10-25% shrinkage), the visualization of the spine subclass is no issue as we used a 100X Zeiss objective lens with immersion oil, which is sufficient to resolve the details or subtype of the spines for laborious counting.

To ensure standardization of dendritic regions, only branch segments from neurons entering into the molecular layer of the hippocampal dentate gyrus region were used in the present analysis. A software package (Axiovision, Zeiss) was used to measure the length of each dendritic segment and to record the number of distinct spines in the z-stacks (step size

0,5 μm 100X objectives). A spine was defined in the present analysis as any clearly radiating segment running at a tangent to the branch, with or without a ‘‘head,’’ from the dendritic branch of a length of 1 A, and no longer than 10 A. Those spines directly protruding toward the observer from the dendritic spine segment, or those coursing away underneath the segment in view, and hence obscured by the dark fill of the Golgi stained dendritic branch, could not be counted in our preparation. Dendritic spine density was calculated as number of spines per 10 μm unit length of dendrite measured.

To analyze the dendritic spine morphology, the images were deconvolved with software package (Axiovision, Zeiss) and spine analyses were performed using a semi-automated software NeuronStudio (http://research.mssm.edu/cnic/tools-ns.html), which is able to perform a 3D analysis of dendritic length, spine density, and morphometric features (i.e., head/neck diameter, length, subtype) for each dendritic spine. Spines were classified as thin or mushroom if the ratio of the head diameter/neck diameter was >1/1. If their ratio exceeded this value, spines with a maximum head diameter >0.4 μm were classified as mushroom or else were classified as thin. Spines with head-to-neck diameter ratios <1.1 were also classified as thin if the ratio of spine length-to-neck diameter was >2.5; otherwise, they were classified as stubby. A fourth type, called filopodial spines, was considered to have a long and thin shape with no enlargement at the distal tip, was very seldom observed, and was classified as thin. Finally, data readouts from the spine analysis algorithm were visually compared by the experimenter for each optical stack to verify accurate subtype classifications for dendritic spines. We associated different dot colors (mushroom = ciano; thin = yellow; stubby = red) from those proposed by Neuron studio (mushroom = orange; thin = yellow; stubby = red) in order to make more evident the differences between spine morphology.

All dendritic segments selected for spine density estimations had similar diameter (~ 1 μm) to minimize the effect of hidden spines above or below the dendrite. Five dendritic branches, at least 15 μm in length, were analyzed per neuron, and from 5 to 7 were analyzed per experimental brain. Spine counts were averaged and spine density was expressed as the number of total spines or spine subtypes per 10 μm of dendrite length.

To analyze the dendritic tree morphology the images of Golgi-labeled cells were obtained using a Z-stack (step size 1 μm, 20X objective) from five to seven cells and reconstructed in ImageJ (ImageJ 1.48) for each animal.

Neurogenesis and BrdU injection: the study of proliferation and survival of newborn adult hippocampal neurons were performed using the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) at a dose equimolecular of 100 mg/kg. The length of the S phase was estimated to be approximately 8h (Nowakowski et al., 1989). Thus, three injections (i.p.) of BrdU were given at 8-h intervals over 24h. The rate of cell proliferation was determined by killing the animals 24h after the first injection of BrdU. To track the fate of BrdU-labelled cells, animals were killed 14 days after the last injection of BrdU.

For tissue preparation, rats were deeply anaesthetized with equithesin (1 g sodium pentobarbital, 4.251 g choral hydrate, 2.125 g MgSO4, 12 ml EtOH, 43.6 ml propylene glycol, adjusted to a total volume of 100 ml with distilled water) and then perfused intracardially at 0.3 ml/g with 0.1 M sodium phosphate buffer, pH 7.3 (PBS), followed by 4% paraformaldehyde in PBS. Brains were extracted and then post-fixed in the same fixative for 24h at 4°C before being transferred to 10% sucrose diluted in PBS. Brains were sliced at 50 μm of sagittal sections using a vibratome (VT1200S Leica Microsystems, Wetzlar, Germany). Sections were collected throughout the entire dentate gyrus of the left and right hemisphere and stored in antifreeze solution (0.1M PBS, 30% ethylene glycol and 20% glycerol) at -20°C until processing for immunohistochemistry.

Immunohistochemistry: experiments were carried out in free-floating sections under moderate shaking. To examine the effect of social isolation and social enrichment on hippocampal neurogenesis, a series of every 10 sections through the entire dentate gyrus was selected for each animal and immunostained for BrdU to label the proliferating population that incorporated BrdU, Ki-67 as a marker for proliferating cells and doublecortin (microtubule associated-protein DCX) for newborn immature neurons. BrdU was used to label proliferating cells and track the fate of newly proliferating cells combined with DCX; the nuclear Ki-67 is expressed by cells throughout the cell cycle with the exception of a short period at the beginning of G1; DCX is required for the initial steps of neuronal dispersion and is expressed exclusively in immature neurons within 2 weeks after cell birth, therefore, DCX is often used as a marker to measure the level of neurogenesis.

For immunohistochemistry with Ki-67, slices were removed from antifreeze solution, rinsed three times with 0.1 M PBS, incubated for 1 hr at room temperature in PBS-T immunoblocking buffer (3% donkey serum and 0.3% triton X-100 in PBS) and incubated overnight at 4°C with an antirabbit polyclonal antibody against Ki-67 (1:100, Merck, Darmstadt, Germany). For immunohistochemistry with BrdU, tissue was removed from antifreeze solution, rinsed three times with 0.1 M PBS, then incubated in 0.1 M citric acid for 5 min at 90 °C, rinsed with PBS and DNA was denatured in 2N HCl for 30 min at 37°C. After this step, sections were rinsed twice in PBS and then incubated for 1 h at room temperature in PBS-T. Sections were then incubated overnight, at 4°C, with an antirat monoclonal antibody against BrdU (1:200, Ab Serotec, Kidlington, UK) or, for double immunofluorescence staining, sections were incubated with antibody against BrdU and an antigoat polyclonal antibody against DCX (1:250, Santa Cruz Biotechnology, Dallas, TX). After rinsing in PBS-T, sections were incubated for 2 h at room temperature in goat anti-rabbit IgG AlexaFluor 594 (1:1000, Jackson ImmunoResearch, Cambridge House, UK) to

reveal immunoreactivity of Ki-67; chicken anti-rat IgG CY3-conjugated secondary antibody (1:1000 GeneTex, Alton Pkwy Irvine, CA, USA) to reveal immunoreactivity of BrdU, or, for double immunofluorescence staining, were incubated with CY3 chicken anti-rat and donkey anti-goat IgG Alexa594-conjugated secondary antibody (1:1000 Jackson ImmunoResearch, Cambridge House, UK). After rinsing several times with PBS-T, all the sections were incubated with 1 μl/ml DAPI (Enzo Life, Farmingdale, NY, USA) for 10 min at room temperature, for nuclear staining. Finally, the sections were washed with PBS, mounted on glass slides, dried and then coverslipped under gelatin-glycerol. Negative controls were the slides incubated without primary antibody.

For cell counting, every ten sections throughout the entire extent of the hippocampus were used to determine the number of, Ki-67, BrdU and BrdU+DCX positive cells within the dentate gyrus. The same area and number of sections were studied for all the animals and all the experimental groups. The number of cells were verified using a confocal microscope (SP5, Leica, Heildeberg, Germany) and viewed using a 63X objective. Images were captured with the Leica application suite advanced fluorescence (Leica, Heildeberg, Germany). The area of dentate gyrus was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). To calculate the volume of each region, Cavalieri's principal (Gundersen and Jensen, 1987) was applied, in which the cells were counted bilaterally and the sum of the measured areas was multiplied by the distance between sections (500 μm). Colocalization with BrdU+DCX was confirmed by examining multiple optical planes for each cell on the z axis. The number of BrdU-positive cells (double for DCX) was determined.

2.5. Statistical analysis

Quantitative data are presented as means ± SEM and were compared by analysis of variance (ANOVA) followed by the Newman-Keuls post-hoc test (Statistica 6.0, StatSoft Inc.)

or a t-Test. A $p<0.05$ value was considered statistically significant.

3. Results

3.1. Social isolation induces decrease of BDNF, NGF and Arc expression in the rat hippocampus: reversal by social enrichment

Hippocampal expression of BDNF, NGF and Arc protein was evaluated by immunoblot analysis (Fig 2). As expected, based on our previews reports (Biggio et al., 2014; Pisu et al., 2011), two months of SI induced a marked decrease in BDNF (Fig. 2A; $p < 0.005$), NGF (Fig. 2B; $p < 0.005$) and Arc expression (Fig. 2C; $p < 0.05$) in this brain area with respect to GH counterparts. SE completely restored the effect of SI on BDNF (Fig. 2A; $p < 0.005$), NGF (Fig. 2B; $p < 0.001$) and Arc expression (Fig. 2C; $p < 0.05$). One-way ANOVA revealed a significant effect of SI for BDNF [$F (2, 63) = 17.9040$, $p = 0.0001$]; NGF [$F (2, 71) = 8.4582$, $p = 0.0005$] and Arc [$F (2, 55) = 3.6346$, $p = 0.0329$].

3.2. Social enrichment enhances newly divided cell proliferation in the dentate gyrus of rats exposed to social isolation.

To examine the effect of 8 weeks of SI on newly divided cell proliferation in the Hip, BrdU was injected on the last day of isolation, and the number of BrdU labeled cells was counted in slices obtained 24 h after the BrdU injection. SI animals show a significant ($p < 0.0005$) decrease in BrdU-positive cells/mm^3 with respect to the GH counterpart group. Conversely, in SI-J group the number of BrdU-positive cells was significantly ($p < 0.0005$) increased not only when compared to SI animals but also to GH control group (Fig. 3C). One-way ANOVA revealed a significant effect of SI for BrdU-positive cells [$F (2, 12) = 105.676$, $p = 0.0001$]. (Fig. 3A-C). In order to strengthen the BrdU data, we also measured the number of Ki-67-positive cells in the three experimental groups. Ki-67-positive cells in SI animals show a significant ($p < 0.005$) decrease similar to BrdU-labeled cells (Fig. 3E), while

in SI-J the number of Ki-67-labeled cells was significantly ($p < 0.005$) different from the SI group and thus restored to values observed in GH animals (Fig. 3E). One-way ANOVA revealed a significant effect of SI for KI-67-positive cells [$F (2, 12) = 10.2753$, $p = 0.0025$] (Fig. 3D, E). In addition, DCX was measured in neurons after 14 days from BrdU injection (Fig. 3A) and calculated as DCX/BrdU-positive cells in order to evaluate cell maturation. The number of DCX/BrdU-positive cells was significantly ($p < 0.0005$) decreased in the Hip of SI animals compared to their GH counterparts. In contrast, animals exposed to SE showed a significantly higher DCX/BrdU ratio (Fig. 3G; $p < 0.001$) compared to the SI animals and the values were identical to the GH control group. One-way ANOVA revealed a significant effect of SI for DCX/BrdU-positive cells [$F (2, 12) = 18.1291$, $p = 0.0002$] (Fig. 3F, G).

3.3. Changes of dendrite and spines morphology in the dentate gyrus granule neurons of isolated rats: reversal by social enrichment

To evaluate the alterations in dendritic morphology and spine density elicited by SI protocol and the effects of SE, we used a modified protocol of Golgi-Del Rio Hortega staining.

In order to establish the changes in dendritic arborisation (Fig. 4A), we measured: dendritic length (Fig. 4B), total branch number (Fig. 4C), intermediate branch length (Fig. 4D), number of branch point/neurons (Fig. 4E), and relative number of tips/neuron (Fig. 4F). SI elicited a significant ($p < 0.001$) reduction in almost all measured parameters (Fig. 4B-E), except for intermediate branch length which resulted in an increase compared to GH. All these effects were completely restored by SE to values not different from those observed in GH ($p < 0.001$; Fig. 4B-E). One-way ANOVA revealed a significant effect of SI for dendrite branch length [$F (2, 26) = 10.748$, $p = 0.0004$], total branch number [$F (2, 26) = 19.9138$, $p = 0.0001$], intermediate branch length [$F (2, 26) = 9.288$, $p = 0.0009$], number of branch

point/neurons [F (2, 26) = 13.0465, p = 0.0001] and number of tips/neurons [F (2, 26) = 17.9790, p = 0.0001].

In addition, SI animals present a significant ($p < 0.0001$) reduction of dendrite spine density compared to GH counterpart animals (Fig. 5A), an effect completely restored to control values in SI-J animals (Fig. 5B). One-way ANOVA revealed a significant effect of SI on dendritic spine density [F (2, 278) = 21.817, p = 0.0001].

In a more detailed evaluation, we analysed spines morphology (Fig. 5C) and assigned them to the different typology: mushroom, thin and stubby, in GH, SI and SI-J animals. The graph of Fig. 5D shows that only mushroom spines were significantly ($p < 0.05$) decreased in SI compared to GH rats. In fact, the number of both stubby and thin spines was unaltered in all 3 experimental groups. It is worth noting that the amount of mushroom spines detected in SI-J rats, although significantly higher than that of SI animals, was still lower than those observed in GH. One-way ANOVA revealed a significant effect of SI for mushroom density spines [F (2, 12) = 17.776, p = 0.0001].

4. Discussion

Different environmental experience-dependent changes modify and shape the brain structure and its function with a consequent alteration of behaviour during development in both humans and rodents (Gao et al., 2018; Branchi et al., 2006; Ohline and Abraham, 2018). In this regard adolescents are markedly sensitive to stress events that may lead to long-lasting psychological disorders (McEwen, 2003; Conger and Petersen., 1984; Masten, 1987).

Environmental enrichment is considered one of the best translational models to investigate how positive life experiences and well-being can allow young and adolescent animals to recover certain molecular, functional and behavioural impairments induced by

environmentally stressful conditions (Nithianantharajah and Hannan, 2006). In fact, environmental enrichment may promote adult Hip neurogenesis (Kempermann et al., 1997), the integration of newborn cells into functional circuits (Tanti et al., 2013) and more in general neuronal plasticity. Moreover, several reports highlight the importance of such kinds of enrichment when given early in life, towards improving cognitive ability in a similar manner to what was observed when animals were environmentally enriched during the lifespan (Cortese et al., 2018; Fuchs et al., 2016; Harati et al., 2011).

Our present findings highlight the impact of social enrichment to rescue molecular and structural impairments observed in the Hip of socially isolated rats. The reduction in the amount of BDNF, NGF and Arc elicited by SI which is associated with a significant reduction in dentate Hip neurogenesis, spine density and dendritic arborisation are completely abolished by a subsequent 4 weeks of social enrichment rearing.

The significant reduction of these neurochemical markers of neuronal plasticity, observed 8 weeks after weaning, suggests that this phenomenon lasts longer than previously published in rats (Murinova et al. 2017; Burke et al., 2017; Cacioppo et al., 2015; Biggio et al., 2014). Accordingly, previous reports from our and other research groups (Cho et al., 2017; Biggio et al., 2014; Pisu et al., 2011) have shown that the decrease of Hip, BDNF, NGF and Arc protein expression, elicited by SI reached the highest extent after 4 weeks of isolation.

Consistent with the evidence that BDNF and NGF are strongly involved not only in promotion of neuronal survival (Abbasi et al., 2018) but also in the control of neuronal plasticity in different brain areas, including the Hip (Peters et al., 2018; Bramham and Panja, 2014), the decrease in neurotrophic factors observed in the present study was accompanied by a reduction in dendritic arborisation, as induced by the fall of total dendritic branches of dentate gyrus granule neurons and by a parallel decrease in the density of mushroom, but

not thin or stubby spines. Interestingly, SE was capable of restoring these altered neurochemical and morphological measures to levels indistinguishable from those of GH animals.

Given that at Hip level mushroom spines are involved in the consolidation of long-term potentiation (LTP) (Bosh et al., 2014), and in agreement with other authors (Monteiro et al., 2014) our present results suggest that the opposite effect of SI and SE on the expression of neurotrophic factors and dendritic spine density may reflect parallel alteration in synaptic plasticity. In addition, our findings on changes in spine density are also in agreement with other studies performed in different brain areas, such as PFC and nucleus accumbens (NAcc) after Hip lesion (Flores, 2005). In particular, a neonatal ventral Hip (nVH) lesion induces similar effects on dendritic spine impoverishment on PFC pyramidal neuron and NAcc medium spiny neurons (Flores et al., 2005), suggesting the possibility that SI might promote a similar effect that probably involves losses of excitatory contacts which may participate in the remodelling processes on neuronal architecture and function. Although these mechanisms are not yet elucidated completely, the results suggest that the neuronal impoverishment in structure and function may represent a common mechanism involved in the negative effect induced by SI.

The decrease in dendritic spine density observed in isolated animals was paralleled by a reduction in dendritic arborisation in terms of dendritic branches and terminal tips suggesting a general fall in neuronal connections with surrounding cells. SE was able, as seen with the expression levels of neurotrophins, to almost totally reinstate the isolation-induced impairments in dendritic spine density, a condition necessary to obtain a recovery in cognitive processes. In line with our present findings, other authors have shown that while isolation rearing induces impairments in learning and memory formation, SE allows a complete recovery of cognitive performance (Lu et al., 2003).

In rodents, the dentate gyrus region is the principal area of the Hip formation deputized for neurogenesis processes both in young and adult animals (Duarte-Guterman et al., 2015). In fact, adult Hip neurogenesis plays a crucial role in both neuroendocrine and behavioural responses to stress, although it is unclear whether such phenomena, under certain cases, may influence the resilience or sensitivity to stress (Levone et al., 2014). In order to evaluate these aspects, we labelled the granule neurons of dentate gyrus with BrdU, KI-67 and DCX/BrDU markers and found that rearing animals in isolating conditions leads to a significant decrease in the number of newborn progenitor-derived cells, an effect that was promptly restored by 4 weeks of SE. These data are consistent with other findings from different research groups showing how environmental enrichment may increase the amount of neurogenesis in rodents (Monteiro et al., 2014; Speisman et al., 2013; Lu et al., 2003).

Overall, our results suggest that environmental enrichment may have a prominent role in reinstate the reduction of neuronal plasticity and some neurochemical deficits elicited by SI. Of great interest is the recovery in the morphology of dendrites and dendritic spines that are in the tight control of neuronal growth factors, such as BDNF, NFG and Arc. Finally, by measuring different parameters of neuronal plasticity, we can conclude how the increase in social interaction in such gregarious animals is able to completely reverse the profound brain impairments elicited by SI. Taken together, these results provide possible molecular and behavioral targets to limit stress and/or age-related cognitive decline.

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Figure legends

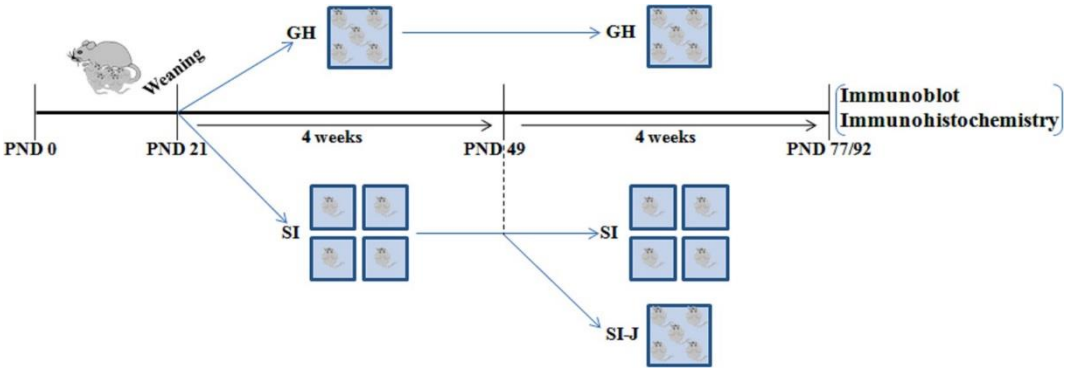

Figure 1. **Schematic overview showing the experimental protocol and the timing for biochemical and behavioral tests.** After weaning, on postnatal day 21 (PND 21), animals were reared in group (GH) or socially isolated (SI). At the end of 4 weeks (PND 49), a group of isolated animals were joined in group (SI-J) for a further period of 4 weeks. At PND 77/92 animals were sacrificed and immunoblot and immunochemistry analyses were assessed.

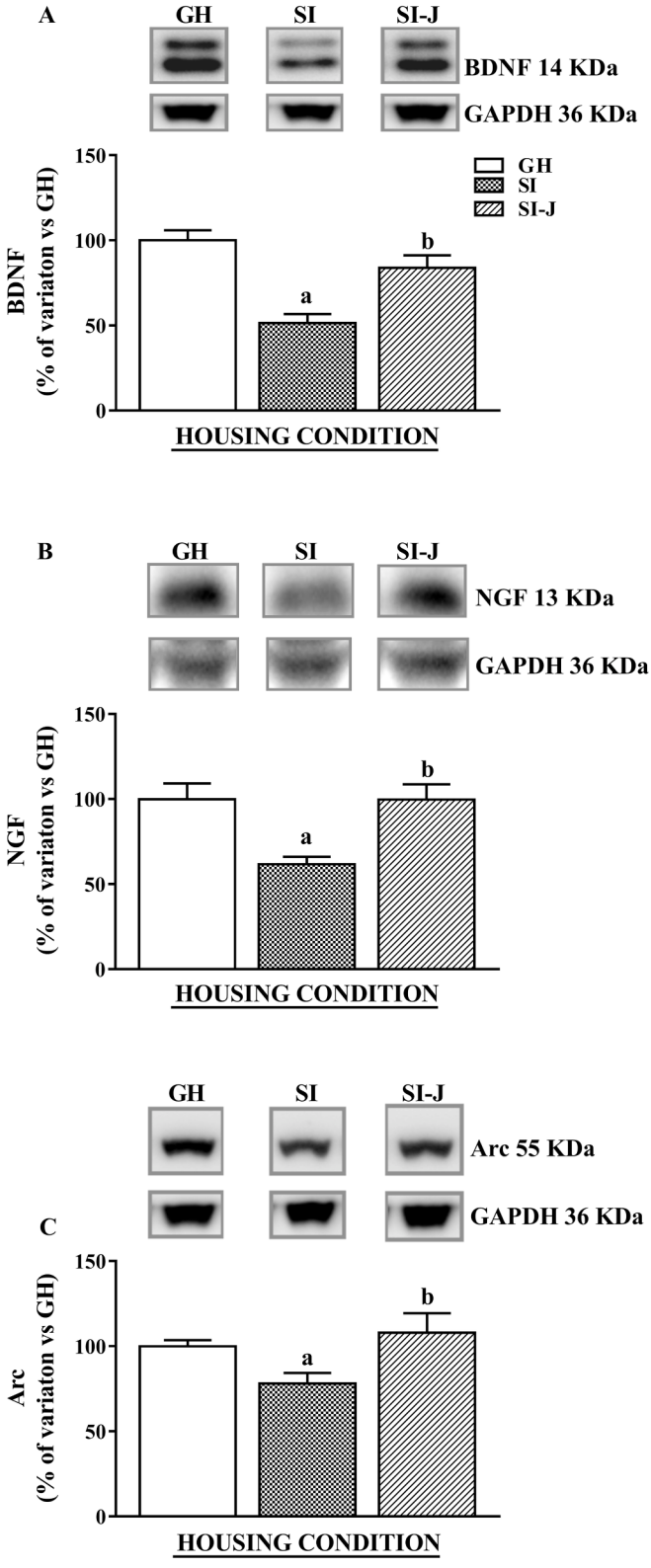

Figure 2. **Effect of SI and SE on BDNF, NGF and Arc expression in the rat hippocampus.** Protein extracts, prepared from the Hip of GH, SI and SI-J rats, were subjected to immunoblot analysis using GAPDH for normalization. Representative blots as

well as quantitation of the BDNF/ GAPDH, NGF/ GAPDH, Arc/ GAPDH ratio are shown as insets above bar graphs in Figs. A and C, respectively. Data are reported as means of OD ratio ± SEM and analyzed with one-way ANOVA followed by the Newman–Keuls post-hoc test. Statistical statement: (A) BDNF/GAPDH ratio from 22 to 25 rats: $^{a}p<0.005$ vs GH, $^{b}p<0.005$ vs SI. (B) NGF/ GAPDH ratio from 23 to 26 rats: $^{a}p<0.005$ vs GH, $^{b}p<0.001$ vs SI. (C) Arc/ GAPDH ratio from 19 to 23 rats: $^{a}p<0.05$ vs GH, $^{b}p<0.05$ vs SI.

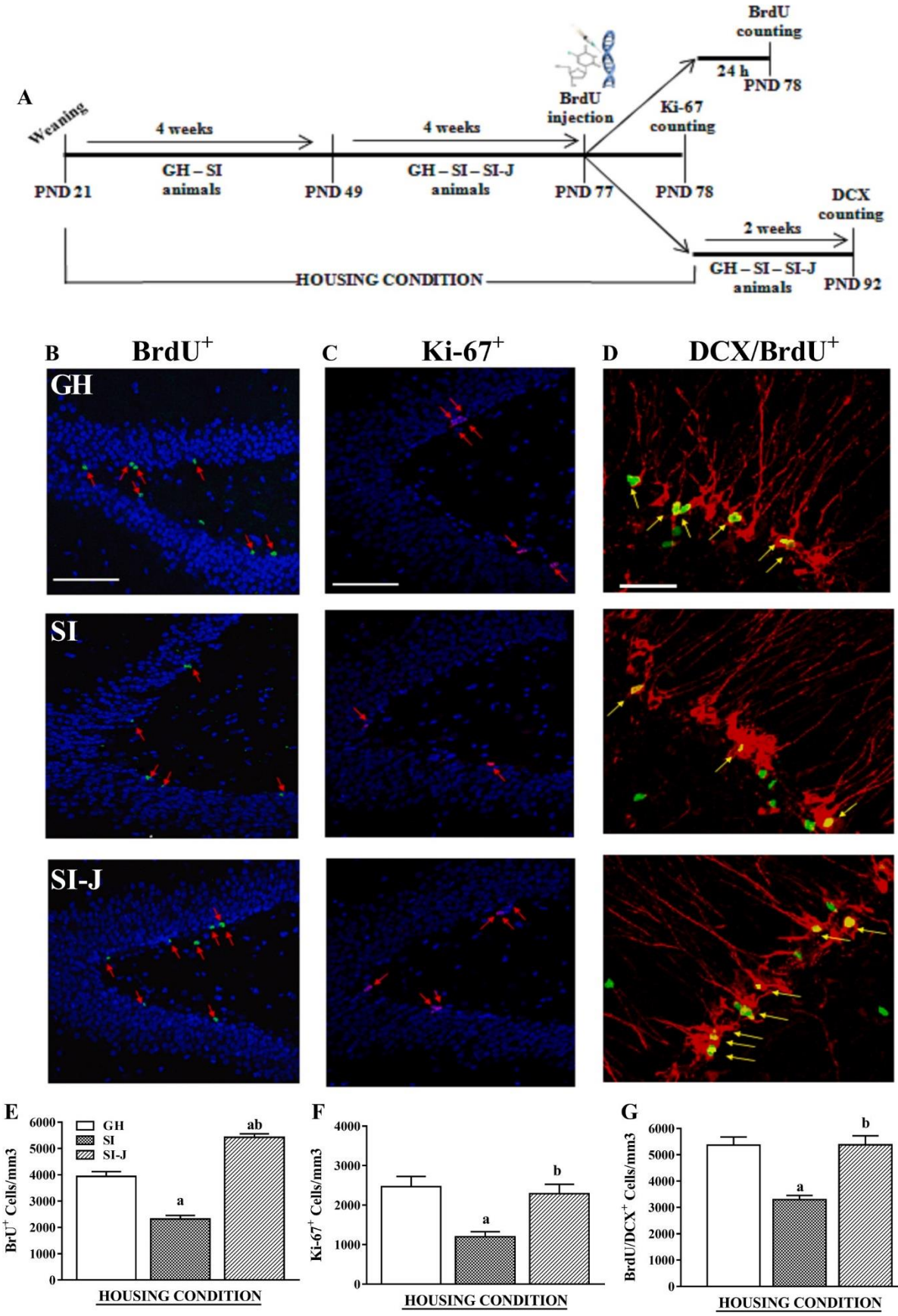
A
Weaning
4 weeks
4 weeks
BrdU injection
BrdU counting
24 h
PND 78
Ki-67 counting
PND 21
GH – SI animals
PND 49
GH – SI – SI-J animals
PND 77
PND 78
DCX counting
2 weeks
GH – SI – SI-J animals
PND 92
HOUSING CONDITION
B BrdU+
C Ki-67+
D DCX/BrdU+
GH
SI
SI-J
E
BrU+ Cells/mm3
GH
SI
SI-J
a
ab
HOUSING CONDITION
F
Ki-67+ Cells/mm3
a
b
HOUSING CONDITION
G
BrdU/DCX+ Cells/mm3
a
b
HOUSING CONDITION

Figure 3. **Effect of SI and SE on neuronal proliferation in the rat hippocampal dentate gyrus.** (A) Schematic representation indicating the timing for BrdU injection in order to evaluate neuronal proliferation in all experimental groups. (B-G) Proliferation and cells survival were measured in Hip dentate gyrus counting BrdU, Ki-67 and DCX labeled-cells in GH, SI and SI-J groups. Confocal representative images of BrdU positive cells (B; green) and DAPI staining (blue), Ki-67-labeled cells (C; purple) and DAPI staining (blue), and BrdU-positive cells (green) overlaid (yellow) with DCX (D; red). Arrows indicate positive cells; scale bar 100 μm. Data are reported as positive cells/mm^3 means ± SEM and analyzed with one-way ANOVA followed by Newman–Keuls post-hoc test. Positive cells were counted from 5 rats per group: cells/mm3 from 1440 to 3380 cells (GH), from 1920 to 2600 cells (SI) and from 1700 to 4460 cells (SI-J). (E) BrdU: $^{a}p<0.0005$ vs GH and SI-J, $^{b}p<0.0005$ vs SI. (F) KI-67: $^{a}p<0.05$ vs GH, $^{b}p<0.05$ vs SI. (G) DCX/BrdU: $^{a}p<0.0005$ vs GH and $^{b}p<0.001$ vs SI.

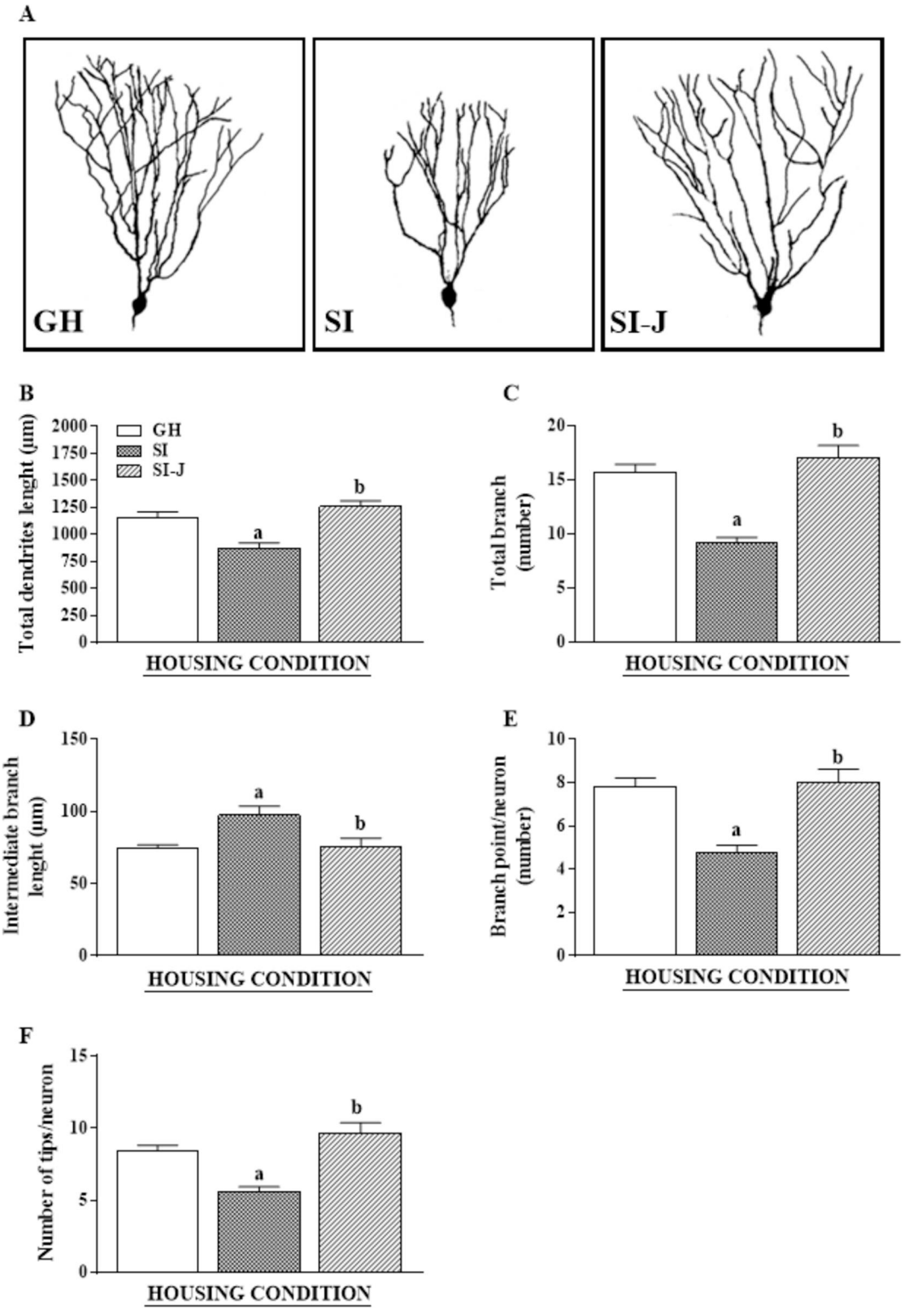
A
GH
SI
SI-J
B
Total dendrites lenght (µm)
2000
1750
1500
1250
1000
750
500
250
0
GH
SI
SI-J
a
b
HOUSING CONDITION
C
Total branch (number)
20
15
10
5
0
a
b
HOUSING CONDITION
D
Intermediate branch lenght (µm)
150
100
50
0
a
b
HOUSING CONDITION
E
Branch point/neuron (number)
10
8
6
4
2
0
a
b
HOUSING CONDITION
F
Number of tips/neuron
15
10
5
0
a
b
HOUSING CONDITION

Figure 4. **Effect of SI and SE on dendritic arborisation of granule neurons of the dentate gyrus.** (A) Representative images of Golgi impregnated pyramidal neurons of the Hip dentate gyrus from GH, SI and SI-J rats. (B-F) Bar graphs report the averaged total dendrite length (µm) (B), total branch number (C), intermediate branch length (µm) (D), branch/point neuron number (E) and number of tips/neuron of dendrites (F) in dentate gyrus granule neurons from GH, SI and SI-J rats. Data are reported as means ± SEM and obtained from 8 to 15 rats analyzed with one-way ANOVA followed by Newman–Keuls post-hoc test. (B) total dendrites length: $^{a}p<0.005$ vs GH and $^{b}p<0.0005$ vs SI; (C) total branch number, (D) branch/point neuron number and (E) number of tips/neuron dendrites: $^{a}p<0.0005$ vs GH and $^{b}p<0.0005$ vs SI; (F) intermediate branch length (µm): $^{a}p<0.005$ vs GH and $^{b}p<0.005$ vs SI.

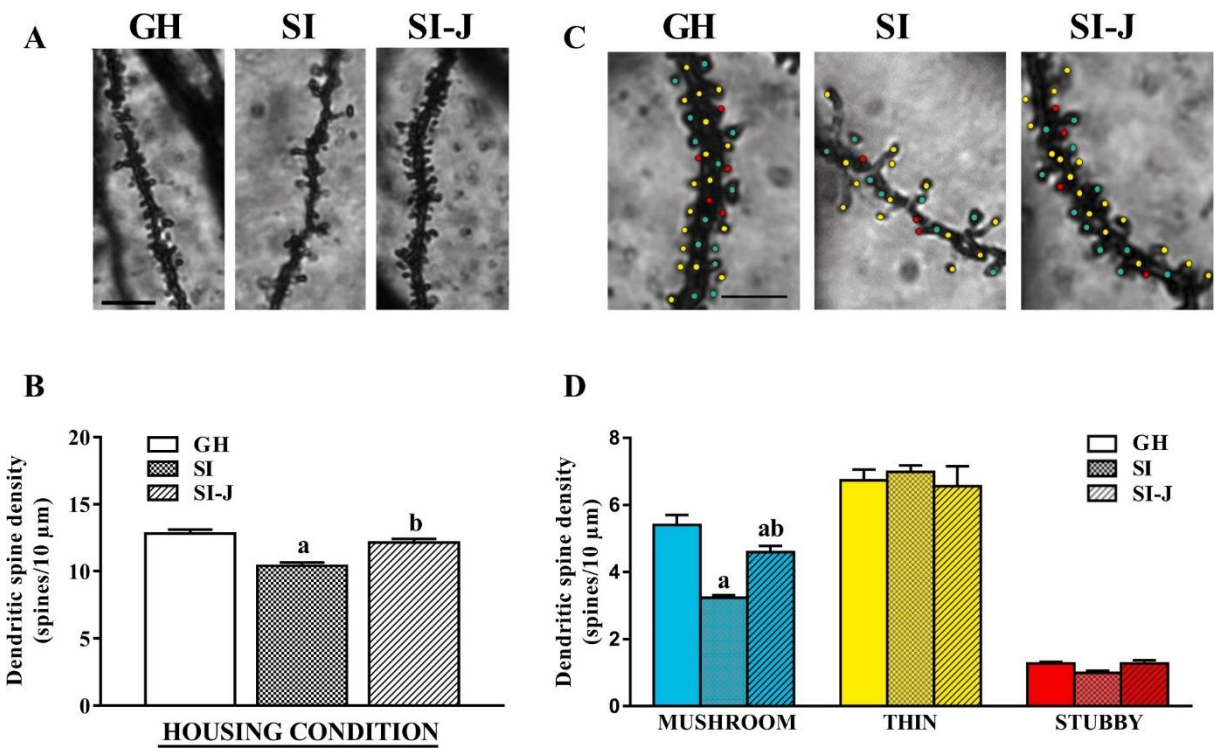

Figure 5. **Effects of SI and SI-J on dendritic spine density and morphology in the rat hippocampal dentate gyrus granule neurons.** (A) Representative images of Golgi impregnated dendrites of granule cells from dentate gyrus granule cells where dendritic spines are clearly evident (scale bar: 5 µm) from GH, SI and SI-J rats. Magnification 100x. (B) Bar graph summarizing the total dendritic spine density of Golgi-impregnated granule cells of the Hip dentate gyrus granule cells. Data are reported as means ± SEM of values from 5 rats/group (from 88 to 111 branches) analyzed with one-way ANOVA followed by Newman–Keuls post-hoc test. [a]$p<0.0001$ vs GH and [b]$p<0.0001$ vs SI. (C) Representative images of Golgi impregnated dendritic spines of different spines morphology divided in mushroom (cyan), thin (yellow) and stubby (red) (scale bar: 5 µm) from GH, SI and SI-J rats detected by Neuron Studios software. Magnification 100x + 10x optical zoom. (D) Bar graph summarizing the relative density of spines with different morphology as calculated in a 10µm dendritic section of the different experimental groups. Data are reported as means ± SEM

of values from 5 rats/group analyzed with one-way ANOVA followed by Newman–Keuls post-hoc test. [a]$p<0.05$ vs GH and [b]$p<0.001$ vs SI.