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ORIGINAL ARTICLE

Brain STAT5 signaling modulates learning and memory formation

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Abstract

The signal transducer and activator of transcription 5 (STAT5) is a transcription factor recruited by numerous cytokines. STAT5 is important for several physiological functions, including body and tissue growth, mammary gland development, immune system and lipid metabolism. However, the role of STAT5 signaling for brain functions is still poorly investigated, especially regarding cognitive aspects. Therefore, the objective of the present study was to investigate whether brain STAT5 signaling modulates learning and memory formation. For this purpose, brain-specific STAT5 knockout (STAT5 KO) mice were studied in well-established memory tests. Initially, we confirmed a robust reduction in STAT5a and STAT5b mRNA levels in different brain structures of STAT5 KO mice. STAT5 KO mice showed no significant alterations in metabolism, growth, somatotropic axis and spontaneous locomotor activity. In contrast, brain-specific STAT5 ablation impaired learning and memory formation in the novel object recognition, Barnes maze and contextual fear conditioning tests. To unravel possible mechanisms that might underlie the memory deficits of STAT5 KO mice, we assessed neurogenesis in the hippocampus, but no significant differences were observed between groups. On the other hand, reduced insulin-like growth factor-1 (IGF-1) mRNA expression was found in the hippocampus and hypothalamus of STAT5 KO mice. These findings collectively indicate that brain STAT5 signaling is required to attain normal learning and memory. Therefore, STAT5 is an important downstream cellular mechanism shared by several cytokines to regulate cognitive functions.

Keywords Hippocampus · Hypothalamus · Signaling pathways · Cytokines · Growth factors

Introduction

The signal transducer and activator of transcription 5 (STAT5) is an intracellular signaling pathway recruited by several cytokines, including interleukin-2, interleukin-3, thrombopoietin, ciliary neurotrophic factor (CNTF), erythropoietin, growth hormone (GH), leptin, granulocyte macrophage colony-stimulating factor (GM-CSF) and prolactin (Grimley et al. 1999). Following phosphorylation and consequent activation, STAT5 is transported to the nucleus to regulate the expression of target genes involved in numerous cell functions, such as proliferation, differentiation and apoptosis (Grimley et al. 1999).

The physiological importance of STAT5 pathway has been studied in different tissues. Global knockout (KO) mice for *Stat5a* and *Stat5b* genes exhibit several abnormalities, including high lethality rate, growth failure, infertility and metabolic problems (Udy et al. 1997; Teglund et al. 1998). Additionally, STAT5 signaling is required

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for mammary gland development and lactogenesis (Liu et al. 1997; Cui et al. 2004). STAT5 in the liver regulates circulating insulin-like growth factor-1 (IGF-1) levels, body growth and the sexually dimorphic liver gene expression (Holloway et al. 2007; Udy et al. 1997). In addition, hepatic STAT5 controls lipid metabolism and contributes to liver regeneration (Cui et al. 2007; Hosui et al. 2017). STAT5 is also essential for normal lymphoid development and differentiation, as well as early B cell development (Yao et al. 2006; Dai et al. 2007). Furthermore, muscle-specific STAT5 deletion leads to lipid accumulation in the liver and skeletal muscle of mice exposed to high-fat diet (Baik et al. 2017).

Previous studies have assessed specific functional roles of STAT5 in the brain as well (Furigo et al. 2016). STAT5b-deficient mice exhibit increased prolactin levels due to defects in the hypothalamic negative feedback (Grattan et al. 2001; Ma et al. 2005). Although brain-specific STAT5 ablation does not affect fertility or the expression of postpartum maternal behaviors, lactation and offspring growth were impaired in mutant mice (Buonfiglio et al. 2015). A recent study found that STAT5 signaling in kisspeptin neurons regulates the timing of puberty (Silveira et al. 2017). Loss of STAT5 signaling in the brain also alters the energy balance and leads to late-onset obesity, primarily in female mice (Lee et al. 2008; Patterson et al. 2012). However, the role of brain STAT5 signaling may embrace a broader set of functions than previously assumed. Several STAT5-recruiting cytokines are involved in memory formation, neuroplasticity, behavior and other cognitive aspects (Oomura et al. 2006; Li et al. 2002; Paz-Filho et al. 2008; Wang et al. 2013; Larsen and Grattan 2012, 2010; Waters and Blackmore 2011; Nyberg and Hallberg 2013; Gisabella et al. 2016). For example, the deficiency of leptin or GH causes cognitive deficits, and hormone replacement has beneficial effects (Waters and Blackmore 2011; Nyberg and Hallberg 2013; Gisabella et al. 2016; Paz-Filho et al. 2008; Oomura et al. 2006). Importantly, prolactin (Furigo et al. 2014; Brown et al. 2010), leptin (Singireddy et al. 2013), GH (Furigo et al. 2017), GM-CSF (Lee et al. 2008), erythropoietin (Cheng et al. 2015) and possibly other cytokines are able to induce STAT5 phosphorylation in neurons, indicating that STAT5 potentially mediates at least part of the effects of these cytokines in the brain. However, the importance of STAT5 signaling in the regulation of cognitive functions has hitherto not been studied in detail. Thus, the objective of the present study was to investigate whether brain STAT5 signaling modulates learning and memory formation. We also investigated possible mechanisms involved in the effects of STAT5 in cognitive functions. Our results collectively suggest that brain STAT5 signaling is required to attain normal learning and memory.

Materials and methods

Generation of brain-specific STAT5 knockout mice

To induce brain-specific deletion of the *Stat5a* and *Stat5b* genes, Nestin-Cre strain was bred with mice carrying loxP-flanked *Stat5a/b* alleles, as described earlier (Lee et al. 2008; Buonfiglio et al. 2015). Brain-specific STAT5 knockout (STAT5 KO) mice were homozygous for the loxP-flanked allele and hemizygous for the Nestin-Cre transgene, whereas control group was composed of littermate animals carrying only the loxP-flanked allele. Mice were weaned at 3–4 weeks of age and genotyped through PCR using DNA extracted from the tail tip (REDExtract-N-Amp™ Tissue PCR Kit, Sigma-Aldrich, St. Louis, MO, USA). Mice were bred and maintained in standard conditions of light (12-h light/dark cycle) and temperature (23 ± 2 °C). In all experiments, animals received a regular rodent chow diet (2.99 kcal/g; 9.4% calories from fat; Quimtia, Brazil). The animal procedures were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo.

Characterization of brain-specific STAT5 knockout mice

The hippocampus, cerebral cortex and hypothalamus of control and STAT5 KO mice were dissected and collected to determine the mRNA levels of STAT5a and STAT5b. Approximately 4-month-old male mice were studied to determine possible changes in body weight, food intake, nose–anus length, femur length, and the mass of perigonadal (periepididymal), subcutaneous and retroperitoneal adipose tissue. The masses of the brain, heart, liver, spleen and kidneys were also measured. The trunk blood was collected to assess serum GH and IGF-1 concentrations by the Rat/Mouse Growth Hormone ELISA kit (Millipore, St. Charles, MO, USA) and the Quantikine ELISA Mouse/Rat IGF-I kit (R&D Systems, MN, USA), respectively. Hypothalamic somatostatin (SST), growth hormone-releasing hormone (GHRH) and tyrosine hydroxylase (TH) mRNA expression was determined by relative gene expression analysis to evaluate possible changes in somatotropic and prolactin axes. Further relative gene analyses were performed in the hippocampus and hypothalamus to determine possible changes in the expression of transcripts related to memory formation and learning. The tissues used in these analyses were collected from mice that underwent the novel object recognition task, followed by the Open Field. Mice were allowed to recover for several weeks before the euthanasia.

Relative gene expression

The tissues were quickly dissected and frozen (− 80 °C) for relative gene expression analysis. Total RNA was extracted with TRIzol reagent (Invitrogen). Assessment of RNA quantity and quality was performed with an Epoch Microplate Spectrophotometer (Biotek). Total RNA was incubated in DNase I RNase-free (Roche Applied Science). Reverse transcription was performed with 2 µg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen) and random primers p(dN)6 (Roche Applied Science). Real-time polymerase chain reaction was performed using the 7500TM Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems). Specific primers were designed for each target gene according to sequences taken from GenBank (Table 1). Relative quantification of mRNA was calculated by $2^{-\Delta\Delta Ct}$. Data were normalized to the geometric average of β-actin, GAPDH and cyclophilin A and reported as fold changes compared to values obtained from the control group (set at 1.0).

Behavioral testing

Novel object recognition paradigm

For the novel object recognition task, 5-month-old control and STAT5 KO male mice were placed in an open field arena [0.3 m (w) × 0.3 m (d) × 0.45 m (h)], similar to previous studies (Kincheski et al. 2017; Lourenco et al. 2013; Figueiredo et al. 2013; Brito-Moreira et al. 2017), without habituation phase. Training session consisted of a 5-minute-long session during which animals were placed at the center of the arena in the presence of two identical objects [3 cm (w) × 3 cm (d) × 4 cm (h)] and the time spent by mice exploring each object was recorded by the experimenter. Approximately 24 h after training session, animals were reinserted into the arena for the test session, in which one of the two objects used in the training session was replaced by a new one with the similar size. Then, the time exploring familiar and novel objects was measured. Sniffing and touching the object were considered to be exploratory behavior. During behavioral sessions, objects were fixed with tape to the floor so that the animals could not move them and none of the objects used evoked innate preference. The arena and stimulus objects were cleaned thoroughly between trials with 40% alcohol (vol/vol) to ensure minimal olfactory cues. The results are expressed as the percentage of time

Table 1 Primer sequences

Target gene	Reference sequence	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	NM_031144.3	cctctgaaccctaaggccaa	agcctggatggctacgtaca
Cyclophilin A	NM_008907.1	tatctgcactgccaagactgagt	cttcttgctggtcttgccattcc
GAPDH	NM_017008.4	gggcagcccagaacatcat	ccgttcagctctgggatgac
STAT5a	NM_011488.3	gacgtgggctcctcacactga	cgctggactccatgcttctc
STAT5b	NM_011489.3	tccatcgtgtcttccagatcg	ggactccgtccttgataccg
SST	NM_009215.1	ctgcagaaactgacggagtct	ctgtcctgccgtctccagt
GHRH	NM_010285.3	atccttgggaatccctgcaaga	tatgcccggaaagtgatccag
TH*	Mm00447557_m1	–	–
Synaptophysin	NM_009305.2	actctccgtcttgttggcac	gcagtgggtctttgccatct
BDNF	NM_007540.4	gccttcatgcaaccgaagta	atgttccaccaggtgagaag
PSD-95	NM_007864.3	cccagacctgagttacccctt	agttgcaggtgaacggaaca
CREB	NM_009952.2	cctgaggcagcttgaacaac	actcagccgggtactaccat
IGF-1	NM_010512.5	gtacttcctttccttctcctttgc	ccacactgacatgcccaaga
VGAT	NM_009508.2	gcacgaacatgccctgaatg	gggtcacgacaaacccaaga
VGLUT-1	NM_182993.2	cccagcataggaaccgcaa	gtcacataccctgcttgcca
AMPAR GluA1	NM_001113325.2	ccaatcccagccctccaatc	cggaagtaaggacaagaccagt
NMDAR GluN1	NM_008169.3	cccggtgctcgtgtcttt	cgtgaacgtgtggaggaaga
NMDAR GluN2A	NM_008170.2	ccatctcaccgtcaccaacaa	aattgctctgcagaagggctc
NMDAR GluN2B	NM_008171.3	accaaatcgctttgccgatg	ttacaaccggtgcctagctg
$GABA_AR1$	NM_010250.5	gtccagcagtcggtccaaaa	agcacactgtcgggaagaag
$GABA_BR1$	NM_019439.3	atactgcacgccgttctgag	tcccggagcatctgtagtca
$GABA_BR2$	NM_001081141.1	gttgtactcgccgaccttca	agtcacgggtcaagttgtgtt

* TaqMan® Gene Expression Assay (Applied Biosystems)

exploring each object during the training or test session in relation to total exploration time in both objects and were analyzed using an unpaired Student's *t* test comparing the mean exploration time in novel object between control and STAT5 KO mice.

Open field

Open field task was performed 3 days after the novel object recognition paradigm (in the same experimental group). Mice were placed in an open field arena [0.3 m (w) × 0.3 m (d) × 0.45 m (h)] for 5 min and monitored by the ANY-maze software, which tracks animal's position and recorded the distance traveled.

Barnes maze test

The Barnes maze protocol was adapted from Sunyer et al. (2007) and the maze consists of a white circular platform (92 cm of diameter) elevated 105 cm above the floor with 20 equally spaced holes (5 cm diameter and 7.5 cm between roles) along the perimeter with a darkened escape box in a constant position under one of the holes at the edge of the platform. Mice are presumed to learn the location of an escape hole using extra-maze visual cues placed on the walls of the room. Another group of 3.5-month-old control and STAT5 KO male mice were initially subjected to one habituation phase in the first day prior to the initial trial. This phase was performed by placing the animal in a cylindrical chamber in the middle of the maze for 10 s. Afterwards, the chamber was removed, and mice were gently guided to the escape box. Animals were allowed to remain in the escape box for 2 min and then they were returned to their original cage. The acquisition phase consisted of four trials per day for 4 days. For each trial, mice were placed in the cylindrical chamber in the middle of the maze for 10 s and then they could explore the maze for 3 min. After trial's completion (i.e., mice either entered the escape box or remained exploring the maze for 3 min), animals were gently guided to and remained inside the escape box for 1 min. Subsequently, mice were returned to their home cages until the next trial, with a 15-minute interval between trials. Time (primary latency) and number of errors (primary errors) to reach the escape hole for the first time were measured. Errors were considered as the number of head deflections into incorrect holes of the maze and were counted by the experimenter. Test sessions were performed 24 h and 1 week after the last trial (days 5 and 13, respectively) and consisted of 90 s exploring the maze with the escape box closed. In all experimental steps, the maze was cleaned with 40% ethanol and air-dried before each trial and between mice. The platform was monitored by the ANY-maze software, which recorded the animal's position and the distance the animal moved (total distance traveled).

Contextual fear conditioning paradigm

The contextual fear conditioning apparatus consisted of a chamber (0.25 × 0.25 × 0.25 m; Panlab Apparatus) with a grid floor connected to a precision-regulated shocker that delivered the electric footshock stimuli. The contextual fear conditioning paradigm was conducted in the same experimental group from the Barnes maze test within a 4 day-interval. Four days after the Barnes maze test, 4-month-old control and STAT5 KO male mice were allowed to freely explore the training chamber for 3 min, after which they received two 2-s long 0.25 mA footshocks with a 30-s interval. Animals were removed from the apparatus after 30 s. Twenty-four hours thereafter, they were reinserted into the box for 5 min and contextual fear memory was assessed with percentage freezing time during test session determined by "Freezing v1.3.04 Ink" software (PanLab). The chamber was cleaned with 40% ethanol between trials.

Evaluation of neurogenesis

Proliferating cells in the subgranular zone (SGZ) of the dentate gyrus (DG) were evaluated in a new group of naïve 5-month-old male mice. These animals received six intraperitoneal injections of bromodeoxyuridine (BrDU; 50 µg/g of body weight in 0.9% sterile saline; 10 µg/ml; Sigma), with a 12-h difference of each. Twenty-four hours after the last BrDU injection, mice were deeply anesthetized and perfused transcardially with saline, followed by a 10% buffered formalin solution (150–200 mL per mouse). Brains were collected and post-fixed in the same fixative for 1 h and cryoprotected overnight at 4 °C in 0.1 M PBS containing 20% sucrose, pH 7.4. Brains were cut (30-µm thick sections) in the frontal plane using a freezing microtome. To assess neurogenesis in the SGZ of the DG, one-in-four series of each brain were rinsed in 0.1 M PBS, pH 7.4, followed by pretreatment in an acid solution containing 16.4% hydrochloric acid (2N) for 1 h to produce DNA denaturation. After that, the neutralization of the acid solution was performed with 0.1M borate buffer (0.31% of boric acid and 0.475% of sodium tetraborate), pH 8.5, and then the sections were rinsed in PBS. Next, sections were blocked in 2% albumin and 2% milk for 1 h, followed by incubation in anti-BrDU primary antibody (1:100; Abd Serotec) overnight. Subsequently, sections were rinsed in PBS and 0.5% Tween 20, and then incubated for 2 h in AlexaFluor^{488}-conjugated anti-rat IgG antibody (1:250, Molecular Probes). The slides were successively washed in PBS containing 0.5% Tween 20, and then coverslipped with Fluoromount mounting medium (Electron Microscopic Sciences, Hatfield, PA). Photomicrographs

were acquired with a Zeiss Axiocam HRc camera coupled to a Zeiss Axioimager A1 microscope (Zeiss, Munich, Germany) and images were digitized using Axiovision software (Zeiss). BrDU positive cells were counted by a researcher unaware of the experimental groups, throughout the rostrocaudal extent of the DG (10 to 12 sections). Results were multiplied by four to obtain the total number of the DG. Photoshop image-editing software was used to combine photomicrographs into plates.

Statistical analysis

The results were expressed as mean ± SEM. Differences between groups were compared using an unpaired two-tailed Student's *t* test. Degrees of freedom (DF) for *t* statistics were marked as $t_{(DF)}$. Statistical analyses were performed using GraphPad Prism software.

Results

Confirmation of brain-specific STAT5 deletion

To confirm the tissue-specific ablation, the mRNA levels of STAT5a and STAT5b were evaluated in the hippocampus, prefrontal cortex and hypothalamus of control and brain-specific STAT5 KO mice (Fig. 1). Brain-specific STAT5 KO mice showed a significant reduction in STAT5a and STAT5b mRNA levels in the hippocampus (STAT5A, $t_{(15)} = 6.456$, $P < 0.0001$; STAT5B, $t_{(15)} = 16.24$, $P < 0.0001$), prefrontal cortex (STAT5A, $t_{(16)} = 4.862$, $P = 0.0002$; STAT5B, $t_{(16)} = 7.939$, $P < 0.0001$) and hypothalamus (STAT5A, $t_{(15)} = 2.807$, $P = 0.0133$; STAT5B, $t_{(15)} = 6.066$, $P < 0.0001$), compared to control animals. A residual transcription of these mRNAs was expected in our analysis since several non-neuronal cell types express these genes (Liva et al. 1999; Natarajan et al. 2004; Mutze et al. 2007) and our deletion targeted predominantly neurons (nestin-derived cells).

Brain-specific STAT5 KO mice have no changes in growth or somatotropic axis

Previous studies have shown that whole-body *Stat5a/b* KO mice have a suppressed somatotropic axis and growth failure (Udy et al. 1997; Teglund et al. 1998). Therefore, we determined the effects of central deletion of STAT5 in growth and somatotropic axis of 4-month-old male mice. Brain-specific STAT5 KO male mice exhibited no differences in body mass (Fig. 2a, $t_{(11)} = 0.7756$, $P = 0.4544$), food intake (Fig. 2b, $t_{(38)} = 0.7787$, $P = 0.4410$), body length (Fig. 2c, $t_{(10)} = 0.1071$, $P = 0.9169$) and femur length (Fig. 2d, $t_{(9)} = 1.621$, $P = 0.1395$), compared to littermate controls. Additionally, control and STAT5 KO mice showed no difference in the masses of the brain, liver, spleen, heart and kidneys (data not shown). In accordance with previous studies indicating a phenotype of late-onset obesity (Lee et al. 2008), we observed a non-significant tendency towards a higher adiposity in STAT5 KO male mice (Fig. 2e–g). Serum concentrations of GH (Fig. 2h) and IGF-1 (Fig. 2i) were assessed to evaluate the somatotropic axis. Control and STAT5 KO mice showed similar GH ($t_{(13)} = 0.0778$, $P = 0.9391$) and IGF-1 ($t_{(16)} = 0.4011$, $P = 0.6937$) circulating levels, in comparison to control animals. Hypothalamic SST ($t_{(14)} = 0.6455$, $P = 0.5290$) and GHRH ($t_{(14)} = 0.6693$, $P = 0.5142$) mRNA expression was also similar between the groups (Fig. 2j), indicating that somatotropic axis was not affected by brain-specific STAT5 deletion. We also analyzed TH mRNA expression in the hypothalamus and no changes between groups were observed (Fig. 2j, $t_{(12)} = 0.3129$, $P = 0.7597$).

Impaired learning and memory in brain-specific STAT5 KO mice

To investigate the possible involvement of STAT5 signaling pathway in the regulation of memory and learning, first, we evaluated 5-month-old control and STAT5 KO male mice in novel object recognition memory test, and the groups showed no difference in the total time exploring the objects

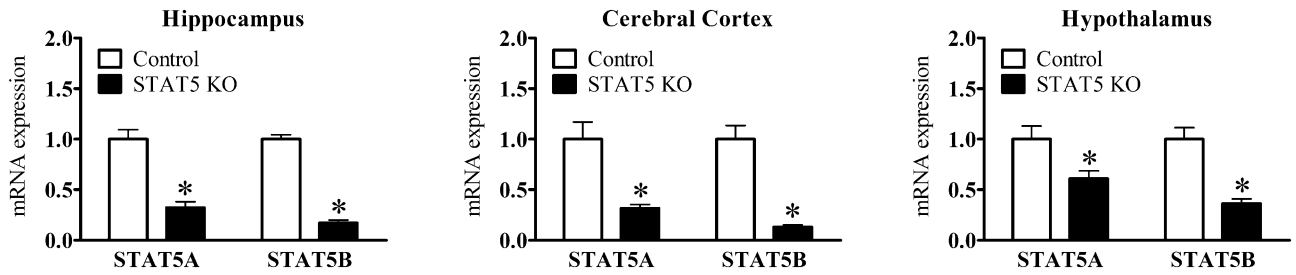

Fig. 1 Confirmation of brain-specific STAT5 deletion. Quantitative PCR analyses to determine the mRNA levels of STAT5a and STAT5b in the hippocampus, prefrontal cortex and hypothalamus of brain-specific STAT5 KO mice and their respective control groups ($n = 6$–11/group). *Significantly different ($P < 0.05$) versus control group

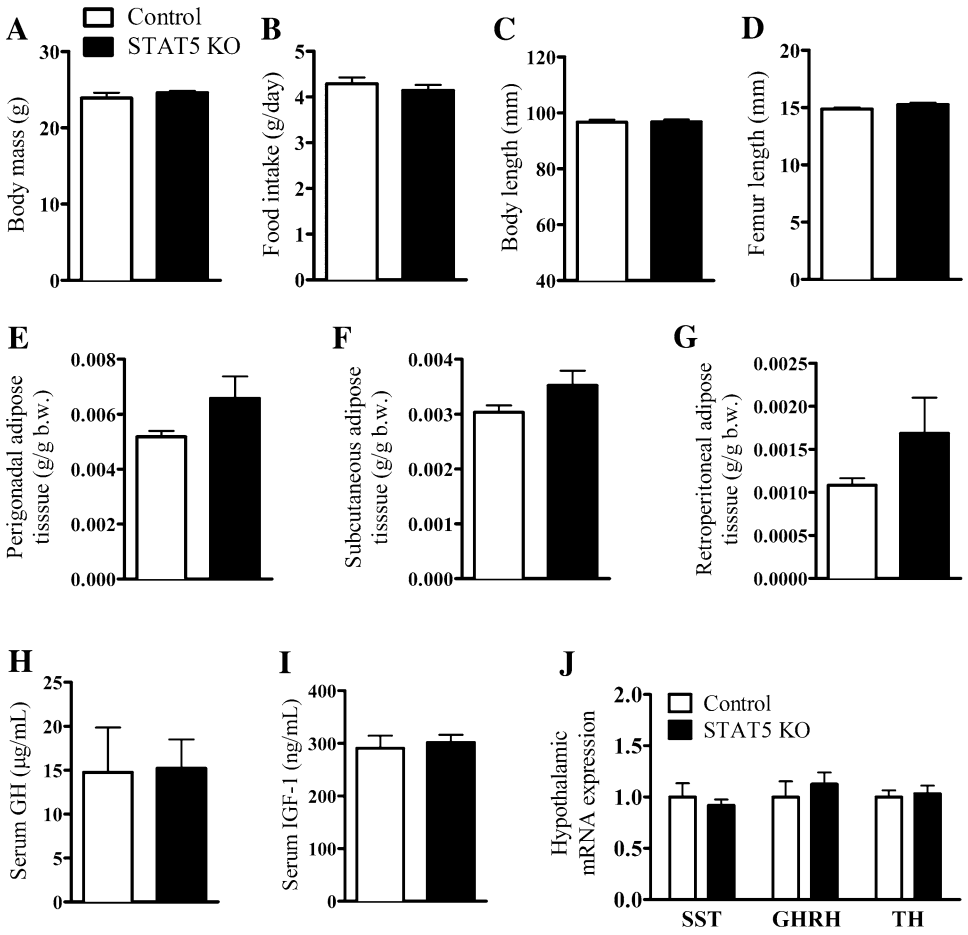

Fig. 2 Brain-specific STAT5 KO mice have no changes in growth or somatotropic axis. A-G Body mass (**a**), food intake (**b**), body length (**c**), femur length (**d**) and the masses of perigonadal (**e**), subcutaneous (**f**) and retroperitoneal (**g**) adipose tissue of 4-month-old male STAT5 KO ($n=5$) and control mice ($n=8$). Serum GH (**h**) and IGF-1 (**i**) concentration in adult male STAT5 KO and control mice ($n=8$–11/group). **j** Relative gene expression analysis in the hypothalamus of adult male mice ($n=6$–10/group)

during training or the test (Fig. 3a). During the training period, mice explored each object approximately 50% of the time, demonstrating no preference for any object presented to them (Fig. 3b). On the test day, STAT5 KO mice spent significantly less time exploring the new object when compared to control mice (Fig. 3c, $t_{(15)}=2.711$, $P=0.0161$). This result indicates that neuronal STAT5 ablation causes memory deficits in the novel object recognition test.

To evaluate potential changes in spontaneous locomotor activity, the same group of mice was subjected to an open field test and no significant difference in total distance traveled was observed between control and STAT5 KO mice (Fig. 3d, $t_{(15)}=1.278$, $P=0.2207$). These results suggest no deficits in spontaneous locomotor activity in STAT5 KO mice.

The Barnes maze was also used to evaluate spatial learning and memory in another group of 3.5-month-old control and STAT5 KO male mice. We observed that the animals decreased the primary latency (Fig. 4a) and the number of primary errors (Fig. 4b) to reach the escape box along the training period, indicating that both groups learned the task. The test sessions to access spatial memory retention were performed on the fifth and thirteenth days (Fig. 4c, d). No significant difference between the groups was observed on the fifth day ($t_{(19)}=0.3493$, $P=0.7307$; Fig. 4c). However, STAT5 KO mice spent less time in the target zone on the thirteenth day ($t_{(19)}=2.228$, $P=0.0382$), suggesting that brain-specific STAT5 deletion caused deficits in spatial memory retention (Fig. 4d). Next, the same animals were evaluated in the fear conditioning test (Fig. 4e). During the test, control animals that had been previously exposed to the electric footshock stimuli inside the chamber remained approximately 75% of the time in freezing (Fig. 4e). On the other hand, STAT5 KO mice spent significantly less time in freezing ($t_{(19)}=2.166$, $P=0.0432$), which indicates an impairment in contextual fear memory or aversive memory (Fig. 4e). Altogether, these results indicate that brain STAT5 signaling is important for learning and memory.

Brain-specific STAT5 deletion does not affect neurogenesis in the hippocampus of adult mice

STAT5 activation is particularly important to control cell proliferation in different tissues (Grimley et al. 1999). To unravel possible mechanisms that might underlie the memory deficits of STAT5 KO mice, we assessed neurogenesis

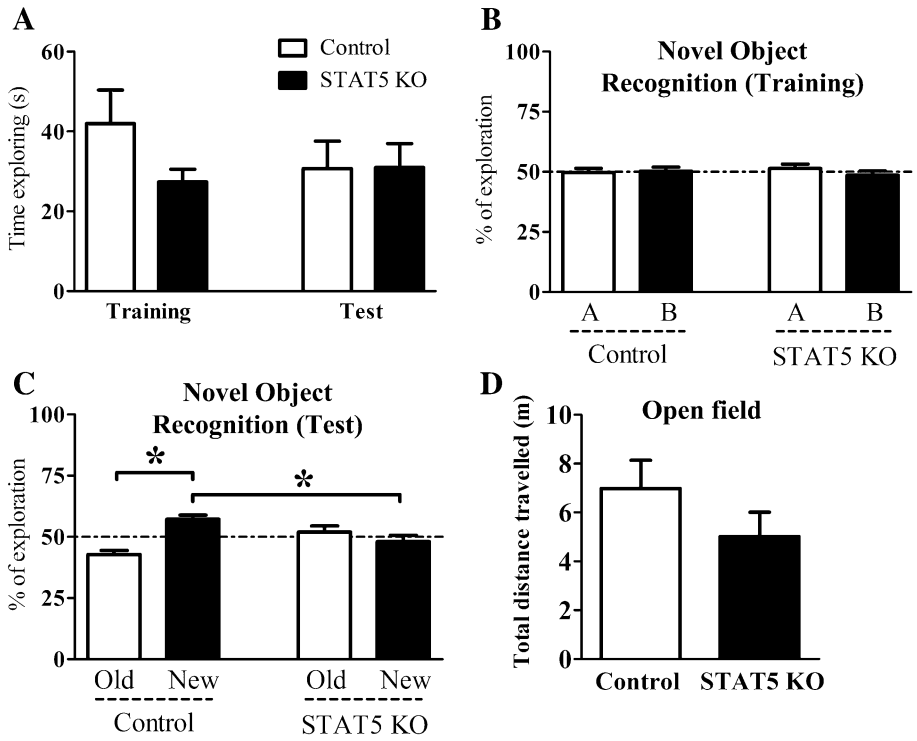

Fig. 3 Brain-specific STAT5 deficiency causes memory deficits in the novel object recognition test. Male 5-month-old STAT5 KO mice ($n=10$) spent a similar amount of time exploring the objects (**a**) compared to sex and age match controls ($n=7$) and showed no preference for the objects used during the training period (**b**). **c** Test session for novel object recognition paradigm demonstrates that STAT5 KO mice spent less time exploring the new object. *Significantly different ($P<0.05$). **d** STAT5 KO mice ($n=11$) showed no difference in the total distance traveled during the open field test compared to control animals ($n=8$)

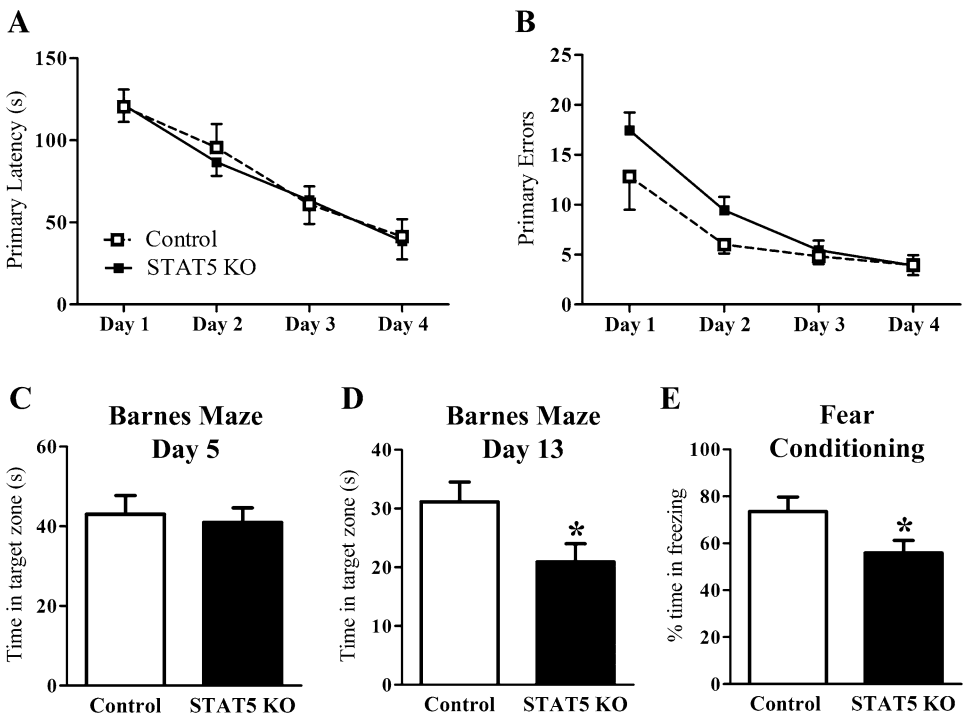

Fig. 4 Brain-specific STAT5 KO mice showed impaired memory in the Barnes maze and contextual fear conditioning tests. Primary latency (**a**) and primary errors (**b**) during the first 4 days of training in the Barnes maze. **c, d** During the Barnes maze test, male 3.5-month-old STAT5 KO mice ($n=12$) spent less time in the target zone at day 13 (**d**), but not at day 5 (**c**), compared to sex and age matched control animals ($n=9$). **e** Male 3.5-month-old STAT5 KO mice ($n=12$) spent a significantly lower percentage of the time in freezing during the fear conditioning test compared to sex and age matched control animals ($n=9$). *Significantly different ($P<0.05$) versus control group

in the SGZ of the DG of 5-month-old naïve male mice (Fig. 5a). Despite the importance of neurogenesis for learning and memory (Shors et al. 2001; Clelland et al. 2009; Deng et al. 2010), we found no significant difference in the number of BrDU positive cells in the DG of control (Fig. 5b) and STAT5 KO mice (Fig. 5c, $t_{(6)}=0.008$, $P=0.9939$).

IGF-1 mRNA levels are reduced in the hippocampus and in the hypothalamus of STAT5 KO mice

Several growth factors, synaptic proteins and receptors are considered key molecules involved in neuronal plasticity and long-term memory formation. Therefore, we performed a

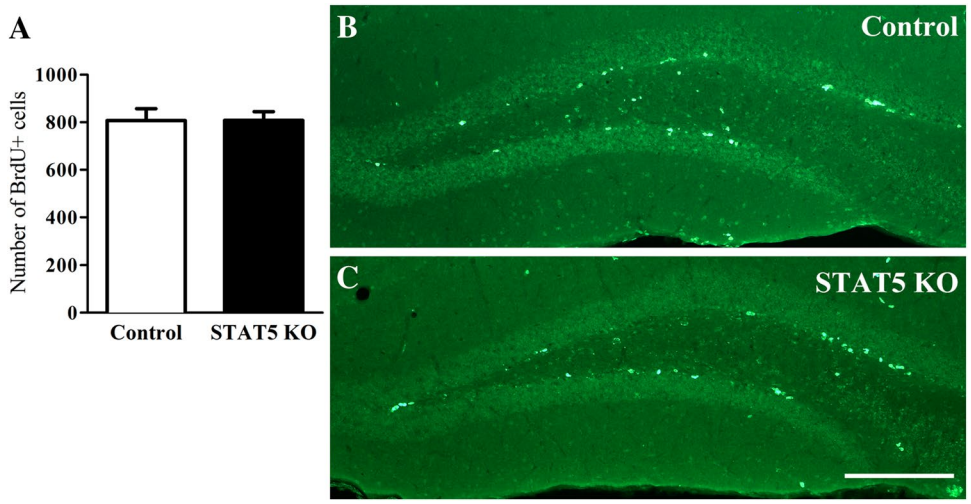

Fig. 5 Neurogenesis in the subgranular zone of the dentate gyrus. **a** Number of BrDU positive cells in the subgranular zone of 5-month-old control and STAT5 KO mice ($n=4$/group). **b, c** Epifluorescence photomicrographs illustrating the distribution of BrDU positive cells in the hippocampus of a control mouse (**b**) and a STAT5 KO mouse (**c**). Scale bar = 200 µm

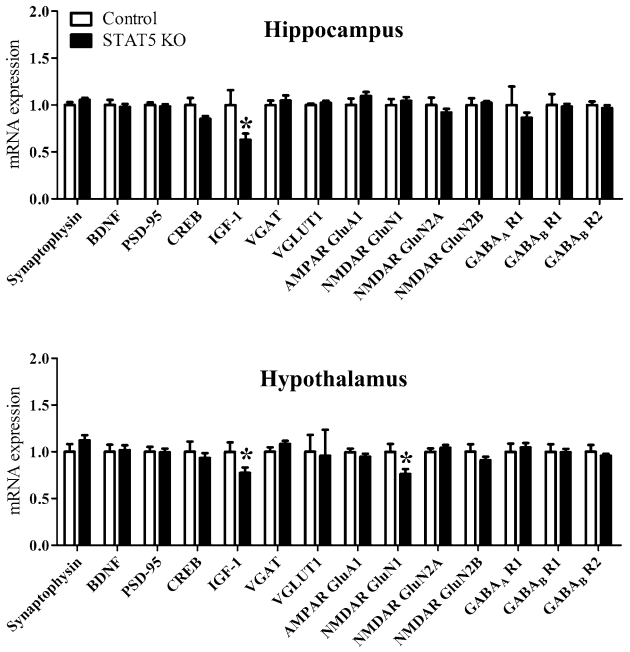

Fig. 6 Gene expression analysis in the hippocampus and hypothalamus of STAT5 KO mice. Quantitative PCR analyses to determine the hippocampal and hypothalamic mRNA levels of several factors involved in memory formation in 4-month-old control and STAT5 KO mice ($n=6$–11/group). *$P<0.05$ versus control group

gene expression analysis in the hippocampus of 4-month-old STAT5 KO and control mice (Fig. 6). No significant differences were observed in the expression of synaptophysin, brain-derived neurotrophic factor (BDNF), postsynaptic density protein-95 (PSD-95), cAMP response element-binding factor (CREB), VGAT and VGLUT1 (Fig. 6). The AMPA receptor (AMPAR) GluA1 subunit, the *N*-methyl-D-aspartate receptor (NMDAR) GluN1, GluN2A and GluN2B subunits, and the $GABA_A$ R1, $GABA_B$ R1 and $GABA_B$ R2 subunits also showed no difference between groups (Fig. 6). However, a lower IGF-1 mRNA expression ($t_{(15)}=2.395$, $P=0.0301$) was observed in the hippocampus of STAT5 KO mice, compared to control animals (Fig. 6). Gene expression analyses were also performed in the hypothalamus since STAT5 signaling is strongly recruited by several cytokines in this brain structure (Furigo et al. 2017, 2014; Nagaishi et al. 2014; Brown et al. 2010; Lee et al. 2008; Severi et al. 2015). STAT5 KO mice showed reduced IGF-1 ($t_{(15)}=2.134$, $P=0.0489$) and NMDAR GluN1 ($t_{(15)}=2.529$, $P=0.0231$) mRNA expression in the hypothalamus, compared to control animals (Fig. 6). No further differences were observed in the hypothalamus comparing control and STAT5 KO mice (Fig. 6).

Discussion

Although many studies have described the participation of several STAT5-recruiting cytokines in the regulation of neuroplasticity, cognitive functions, neuroprotection and behavioral expression (Oomura et al. 2006; Li et al. 2002; Paz-Filho et al. 2008; Wang et al. 2013; Larsen and Grattan 2010, 2012; Waters and Blackmore 2011; Nyberg and Hallberg 2013; Gisabella et al. 2016; Cheng et al. 2015), much less information is available about the molecular mechanisms and intracellular pathways recruited by them to affect cognitive functions. For example, STAT1 signaling in astrocytes is essential for an effective control of infection in the central nervous system (Hidano et al. 2016). Neural STAT3 expression is critical for energy and glucose homeostasis (Gao et al. 2004). Additionally, STAT3 is also involved in cognitive aspects. Dysfunction of microglial STAT3 alleviates depressive behavior via neuron–microglia interactions (Kwon et al. 2017). Previous studies also revealed that amyloid-beta causes memory impairment by disturbing the

JAK2/STAT3 axis in hippocampal neurons (Chiba et al. 2009), and STAT3 regulates maturation and terminal differentiation of mouse hippocampal neurons (Ma et al. 2017). The cAMP-dependent protein kinase, phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways are recruited by several factors, including IGF-1 and insulin, and also play a pivotal role controlling cellular processes in neurons and cognitive functions (Schafe et al. 2000; Sweatt 2001; Crowder and Freeman 1998; Sanna et al. 2002; Abel et al. 1997; Johannessen et al. 2004). Here, we described a previously unrecognized role of STAT5 signaling in regulating learning and memory.

Whole-body *Stat5a/b* KO mice exhibit robust metabolic and endocrine abnormalities (Udy et al. 1997; Teglund et al. 1998) that represent important confounders in studies about the specific role of STAT5 in the central nervous system. In this case, metabolic or endocrine defects per se could impair cognition, independently of brain STAT5 signaling (Etgen et al. 2010; Bomfim et al. 2012). Our brain-specific STAT5 KO mice showed no major growth or metabolic problems, as well as normal circulating IGF-1 concentrations. Consequently, this model allowed us to investigate the potential role of STAT5 in cognitive functions, without a possible interference of the complex problems of whole-body *Stat5a/b* KO mice.

STAT5 KO mice presented poor performance in the novel object recognition and contextual fear conditioning tests, both paradigms designed to access long-term memory, indicating that STAT5 is involved in long-term storage of declarative and associative memories. Nonetheless, learning throughout a spaced training session was not impaired in the STAT5 KO mice challenged to the Barnes Maze task. Animals still presented memory retention when tested 24 h after the last trial (day 5), but it was not sustained when mice were tested 1 week later (day 13). Collectively, behavioral data indicate that STAT5 is involved with long-term memory processes. All analyses in the present study were performed in male mice. Several reports implicate sex-related differences regarding memory performance under distinct conditions (Tronson and Collette 2017; Merz and Wolf 2017). Thus, further studies using females should be addressed to investigate if STAT5 is also implicated in long-term memory.

Earlier studies have investigated the role of several STAT5-inducing cytokines in learning and memory. For example, GH deficiency impairs cognitive functions and GH replacement improves these defects (Deijen et al. 1998; Maruff and Falleti 2005; van Dam 2005; Carroll et al. 1998). CNTF also regulates hippocampal neurogenesis, neuronal plasticity and spatial memory in mice (Blanchard et al. 2010; Garcia et al. 2010; Kazim et al. 2017; Chohan et al. 2011). Although several signaling pathways contribute to the regulation of cognitive functions, as previously mentioned, our findings indicate that STAT5 is an important downstream factor recruited by cytokines to modulate learning and memory. Whether the defects exhibited by brain-specific STAT5 KO mice can be explained by the lack of signaling of a specific cytokine or if it depends on the combined incapacity of several hormones to induce STAT5 activation is an issue that still needs to be clarified.

Although STAT5 is expressed in the hippocampus (Furigo et al. 2017; Sun et al. 2007), there is no information about the cell types and hippocampal regions which exhibit STAT5 signaling. However, some forms of cerebral stress, such as ischemia, can modulate STAT5 expression in the hippocampus. For example, cerebral ischemia induces an upregulation of STAT5 expression and downstream transcripts (Sun et al. 2007). Interestingly, STAT5 phosphorylation is increased in hippocampal CA1 at 1 and 3 h after cerebral ischemia and this activation has been suggested to have a neuroprotective effect by improving CA1 neuronal survival after cerebral ischemia (Zhang et al. 2007).

To identify possible mechanisms involved in the memory defects caused by brain-specific STAT5 deficiency, we performed several analyses in the hippocampus, since this brain region has been shown to be involved with the memory tests employed in the present study (Broadbent et al. 2010; Zheng et al. 2009; Sanders et al. 2003). Despite the important participation of neurogenesis, glutamate and GABA receptors, vesicles and other synaptic proteins in memory formation (Bliss and Collingridge 1993; Mantamadiotis et al. 2002; Kornau et al. 1995; Shors et al. 2001; Clelland et al. 2009; Deng et al. 2010), STAT5 KO mice showed no significant changes compared to control animals. In contrast, a reduced hippocampal IGF-1 expression was observed in STAT5 KO mice. Several studies have described that IGF-1 influences cognitive processes and has neuroprotective and neurodevelopmental effects (Miltiadous et al. 2011; O'Kusky and Ye 2012; Aberg et al. 2006; Witty et al. 2013; Molina et al. 2013). Since IGF-1 is a major STAT5 target gene (Lanning and Carter-Su 2006; Cui et al. 2007), a deficiency in STAT5-dependent IGF-1 transcription may have contributed to the memory impairment of STAT5 KO mice.

Although the importance of the hippocampus for memory is well-established, other brain areas may also contribute to the cognitive deficits observed in the STAT5 KO mice. Of note, several cytokines such as GH, CNTF and GM-CSF induce STAT5 phosphorylation in hypothalamic nuclei, affecting neuronal or glial cells (Severi et al. 2015; Lee et al. 2008; Furigo et al. 2017). The activation of STAT5 in hypothalamic cells may also modulate cognitive functions. Notably, we also found a reduced IGF-1 mRNA expression in the hypothalamus of STAT5 KO mice, indicating that STAT5 ablation induces similar molecular changes in different brain areas. A reduced expression of the NMDAR GluN1 subunit in the hypothalamus was also observed, but the importance of this finding for the cognitive deficits exhibited by

STAT5 KO mice is unknown. It is important to mention though that gene expression analyses were performed in the basal state, without any specific stimulus. Therefore, it remains unknown whether the transcripts analyzed could exhibit dynamic changes in STAT5 KO mice during cognitive challenges.

In summary, our findings identified STAT5 as an important transcription factor involved in learning and long-term memory formation. These findings unraveled the importance of a specific downstream cellular mechanism shared by several cytokines to regulate cognitive functions, and they contribute to the understanding of neurological problems caused by changes in the levels of different STAT5-recruiting hormones, cytokines and growth factors.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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