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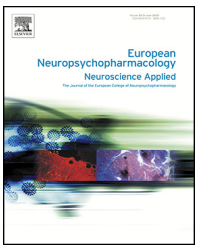

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RESEARCH PAPER

Sex differences in the antidepressant-like potential of repeated electroconvulsive seizures in adolescent and adult rats: Regulation of the early stages of hippocampal neurogenesis

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Abstract
Age and sex are critical factors for the diagnosis and treatment of major depression, since there is a well-known age-by-sex difference in the prevalence of major depression (being females the most vulnerable ones) and in antidepressant efficacy (being adolescence a less responsive period than adulthood). Although the induction of electroconvulsive seizures (ECS) is a very old technique in humans, there is not much evidence reporting sex- and age-specific aspects of this treatment. The present study evaluated the antidepressant- and neurogenic-like potential of repeated ECS across time in adolescent and adult rats (naïve or in a model of early life stress capable of mimicking a pro-depressive phenotype), while including a sex perspective. The main results demonstrated age- and sex-specific differences in the antidepressant-like potential of repeated ECS, since it worked when administered during adolescence or adulthood in male rats (although with a shorter length in adolescence), while in females rendered deleterious during adolescence and ineffective in adulthood. Yet, repeated ECS increased cell proliferation and

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vastly boosted young neuronal survival in a time-dependent manner for both sexes and independently of age. Moreover, pharmacological inhibition of basal cell proliferation prevented the antidepressant-like effect induced by repeated ECS in male rats, but only partially blocked the very robust increase in the initial cell markers of hippocampal neurogenesis. Overall, the present results suggest that the induction of the early phases of neurogenesis by ECS, besides having a role in mediating its antidepressant-like effect, might participate in some other neuroplastic actions, opening the path for future studies.

1. Introduction

Age and sex are critical factors to consider for the diagnosis and treatment of major depression, since there is a well-known age-by-sex difference in the prevalence of major depression (e.g., Klein et al., 1999; Cyranowski et al., 2000; Costello et al., 2002; Coryell et al., 2009; see Editorial by Hofmann (2020)). While major depression affects around 2–8% of children (6–12 years old) and 5–6% of adolescents (13–18 years old; Costello et al., 2006), there is a latent difference in the puberty-depression relationship for the sexes (Beltz, 2018; Beltz et al., 2019), with equally common rates among boys and girls during childhood and early adolescence (e.g., Costello et al., 2002; Hofmann, 2020), but higher rates in girls and women at later ages (e.g., Kessler, 2003; Marcus et al., 2005; LeGates et al., 2019). Moreover, males and females also seemed to exhibit some differences in response to certain antidepressants efficacy, although a consensus regarding these sex disparities has not been established (LeGates et al., 2019; Herzog et al., 2019). Additionally, antidepressants have been shown to differ in efficacy depending on the age of exposure, being adolescence a less responsive period than adulthood (e.g., Bylund et al., 2007; Bis-Humbert et al., 2020), during which time its use might even be harmful (Cipriani et al., 2016). In fact, all antidepressant prescriptions include a black box warning that its use in children or adolescents with depressive disorders might increase the risk of suicidal ideas and/or suicidal behaviors.

An alternative non-chemical antidepressant treatment used in psychiatry that offers a safe and really potent therapeutic response in major depressive disorders is the induction of electroconvulsive seizures (ECS) (UK ECT Review Group, 2003). Some studies have suggested that age and sex might influence the electrical charge needed to induce an effective convulsion (Sackeim et al., 1987): for the same age, women seem to require less charge than men to induce an optimal convulsion, and for both sexes the charge needs to be increased with age (Salvador Sánchez et al., 2017). Moreover, although ECS would not be a first-line approach to treat young people, some recent evidence supports its use in this age population (see revision in Weiner and Reti (2017) and Karayağmurlu et al. (2020)). Therefore, more research is needed to evaluate ECS efficacy and/or safety in adolescence (as compared to adulthood) and with a sex perspective.

In terms of how ECS exerts its antidepressant-like effects (see Commentary by Krishnan (2016)), a special attention has been given in the past 20 years to the role of neuroplasticity markers in the hippocampus, such as neurogenesis (e.g., Madsen et al., 2000; Malberg et al., 2000; Scott et al., 2000; see revisions in Segi-Nishida (2011), Jonckheere et al. (2018) and Ueno et al. (2019)), whose modulation has been considered as a possible cellular substrate for the treatment of depression (e.g., Nakamura et al., 2013). Adult hippocampal neurogenesis is a highly dynamic process that responds to neuronal activity and can be regulated at the level of neural stem cell recruitment and activation, progenitor proliferation, and newborn cell survival and differentiation. While the generation of new neurons in the human adult brain is a topic of recent debate, with some studies supporting it (Boldrini et al., 2018; Moreno-Jiménez et al., 2019; Tobin et al., 2019) and others in direct conflict with it (Cipriani et al., 2018; Sorrells et al., 2018), its existence in rodents is clear for at least two brain regions (the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus) (see recent revision in Petrik et al. (2019)). In fact, ECS robustly triggers adult hippocampal neurogenesis in rodents (Madsen et al., 2000; Malberg et al., 2000; Scott et al., 2000) and increases neural stem cells recruitment and activation (e.g., Segi-Nishida et al., 2008). Although hippocampal neurogenesis decreases with age (Kuhn et al., 1996) and is differently regulated by sex (e.g., Yagi and Galea, 2019), not much is known about how sex differentially affects ECS neurogenesis regulation in adolescence and adulthood or whether neurogenesis is necessary for ECS antidepressant-like effects (see Schloesser et al. (2015) and Olesen et al. (2017) for positive and negative results on this topic).

Against this background, the present study evaluated the antidepressant- and neurogenic-like potential exerted by repeated ECS across time in adolescent and adult rats (naïve or in a model of early life stress capable of mimicking a pro-depressive phenotype; Levine, 2005; Marco et al., 2015) and including sex as a biological variable. Moreover, this study evaluated how cell proliferation inhibition would impact the antidepressant- and neurogenic-like effects induced by ECS.

2. Experimental procedures

2.1. Animals

For the present study we used a total of 251 Sprague-Dawley rats (211 males and 40 females) bred in the animal facility at

the University of the Balearic Islands. Note that since the earlier results pointed out at sex-differences in ECS response (Figs. 1 and 2), in an attempt to reduce the overall number of rats used, considerably fewer female rats were utilized in this study (i.e., the last experiments were only performed in male rats; Figs. 3-5). Rats were housed in standard cages with *ad libitum* access to a standard diet and tap water in a temperature- (22 °C) and humidity- (70%) controlled vivarium (12:12 h light/dark cycle). Procedures followed the ARRIVE guidelines (McGrath et al., 2015), the EU Directive 2010/63/EU of the European Parliament and of the Council, and were approved by the Local Bioethical Committee (University of the Balearic Islands) and the regional Government (Conselleria Medi Ambient, Agricultura i Pesca, Direcció General Agricultura i Ramaderia, Govern de les Illes Balears).

2.2. Maternal deprivation early in life

A single episode of maternal deprivation was carried out early in life for a set period of time (24 h from PND 9 to 10) (Ellenbroek et al., 1998; Marco et al., 2015). Pups from 3 litters ($n = 38$) were weighted right before (PND 9) and after (PND 10) maternal separation, during which each mother was placed in an adjacent independent cage while pups stayed in their home cage with no nutritional supplements. At weaning (PND 22), rats were separated in cages of 2-4 rats by sex (male: $n = 19$; female: $n = 19$) and were exposed to the experimental design described in Fig. 2a. Note that no control rats were run in parallel in this experiment since prior data from others (reviewed at Marco et al., 2015) and ours (Bis-Humbert et al., 2019) characterized the impact of early maternal deprivation on immobility in the forced-swim test. In particular, while maternal deprivation induced no effects in the forced-swim test during adolescence (as compared to control rats), it increased immobility during adulthood for both sexes (e.g., Bis-Humbert et al., 2019, and unpublished data). Thus, since we knew beforehand the impact of maternal deprivation on behavior (vs. control rats), in an attempt to reduce the number of animals tested at the same time (to improve behavioral logistics), we ascertained whether ECS would induce antidepressant- and neurogenic-like effects in rats previously exposed to early life stress (MD-SHAM vs. MD-ECS). Our aim was not to evaluate whether ECS would revert the effects of maternal deprivation (as compared to control rats; not exposed to maternal deprivation early in life) but to ascertain whether ECS would be beneficial in rats previously exposed to early life stress.

2.3. Electroconvulsive seizures

Randomly allocated rats from each age group (adolescence, Figs. 1a and 2a; adulthood, Figs. 2a, 3a, 4a and 5a) and sex (Figs. 1a and 2a) received daily ECS sessions via earclip electrodes or were connected to the electrodes with no electrical current (SHAM) during independent experimental studies (see particularities of each experimental design in Figs. 1-5). ECS animals received a total of 5 shocks using a pulse generator (ECT Unit 7801; Ugo Basile, Italy) (95 mA for 0.6 s at a frequency of 100 Hz square wave pulses, pulse width 0.6 ms) over a 5-day period (one shock per day) as described previously (García-Fuster and García-Sevilla, 2016), which daily produced the characteristic tonic and clonic convulsions. While some rats were exposed to behavioral testing and sacrificed 8 days post-treatment (as detailed in Figs. 1-5), other parallel experiments were performed in rats (with no behavioral testing) to collect brains at different time-points after treatment (1 and 3 days post-treatment or 15 and 30 days post-treatment, see Figs. 1, 3 and 5).

2.4. Temozolomide treatments

Temozolomide (TMZ) is an alkylating agent used for the treatment of multiform glioblastoma (Bahadur et al., 2019) that damages the DNA of dividing cells and stops the proliferation of tumors by causing cell death and thus also decreases hippocampal neurogenesis (e.g., Garthe et al., 2009; Niibori et al., 2012). To determine the optimal cyclic TMZ treatment conditions (e.g., Nokia et al., 2012) needed to reduce basal cell proliferation in the hippocampal dentate gyrus, a preliminary experiment was performed in male adult rats comparing the effect of 1 cycle (1x TMZ: 25 mg/kg, i.p., 5 days, 1 dose/day, $n = 7$) vs. 2 cycles (2x TMZ: 25 mg/kg, i.p., 5 days treatment per cycle, 1 dose/day, 10 days in total with 2 resting days in between cycles, $n = 7$) of TMZ. Control rats received 10 vehicle injections at the same times (1 ml/kg of DMSO in 2 cycles of 5 days, i.p., $n = 7$). Note that rats from the experimental group 1x TMZ received 5 injections of vehicle during the first cycle (similarly to control rats). All rats were killed by decapitation 1 day after the last TMZ dose (see Fig. 4a).

Then, in a separate experiment adult male Sprague-Dawley rats that were treated with 2x TMZ or vehicle (as described before), also received during the second cycle (3 h after each daily injections) repeated ECS (5 d) or SHAM treatment (as previously described, Fig. 5a). While these rats were exposed to behavioral testing and sacrificed 8 days post-treatment (see Fig. 5a), a parallel experiment was performed with no behavior, in which rats were sacrificed 1 day after treatment.

2.5. Forced-swim test

To evaluate the potential efficacy of ECS we relied on the forced-swim test, a behavioral tool useful in rodents to ascertain antidepressant-like responses under stress conditions. To do so, all rats were placed in individual tanks (41 cm high x 32 cm diameter, 25 cm depth) filled with water (25 ± 1 °C) during 15 min (pre-test session) followed, 24 h later, by a 5-min test session that was videotaped (for further details see García-Cabrerizo et al., 2015; García-Cabrerizo and García-Fuster, 2019a). Videos were blindly analyzed to determine individual basal levels of immobility (defined as the lack of movement except that which is necessary to keep the rat's nose above the water level) for each rat (Behavioral Tracker software, CA, USA) so later experimental groups were counterbalanced by immobility (see Figs. 1-3 and 5). Then, the antidepressant-like effect induced by repeated ECS was evaluated at different time-points after treatment (1, 3 and 7 days) by re-exposing rats to 5 min sessions in the forced-swim test (see Figs. 1-3 and 5). Similarly, prior studies from our group repeatedly exposed rats to the forced-swim test across time, demonstrating that the possible effects of learning due to repetition can be controlled if the same conditions are applied to all rats, and that this set-up allows for reliable measurements of the progression of this particular behavioral response (García-Cabrerizo and García-Fuster, 2019a, b; Jiménez-Romero et al., 2020; Bis-Humbert et al., 2020).

2.6. Tissue collection, immunohistochemical studies and cell counting

At the indicated times (see Figs. 1-5), rats were killed by rapid decapitation, the left half-brain was quickly isolated, frozen in −30 °C isopentane and stored at −80 °C until 30 μm serial sections were cryostat-cut throughout the whole extent of the hippocampus (−1.72 to −6.80 mm from Bregma) and slide-mounted (e.g., 8 tissue-sections per slide, 8 slides per series, 3 series per animal: series 1 being the most anterior part of the hippocampus, series 2

the middle part and series 3 the most posterior part of it) as previously performed (García-Fuster et al., 2010, 2011). The rate of cell genesis was evaluated for all experimental samples by immunohistochemical analysis with the following markers: Ki-67 for recent cell proliferation and NeuroD for early neuronal survival (García-Fuster et al., 2010, 2011) in the dentate gyrus. Briefly, as priory detailed (García-Fuster et al., 2010, 2011), 3 slides (1 from each series, 24 tissue-sections in total) per animal and marker were post-fixed (4% paraformaldehyde) and exposed to several steps (e.g., antigen retrieval, blocking in peroxidase solution and BSA) before overnight incubation with polyclonal rabbit anti-Ki-67 (1:40,000; kindly provided by Drs. Huda Akil and Stanley J. Watson, University of Michigan, MI, USA) or goat anti-NeuroD (1:25,000; Santa Cruz Biotechnology, CA, USA). The next day sections were incubated (1 h) with biotinylated anti-rabbit or anti-goat secondary antibody 1:1,000 (Vector Laboratories, CA, USA) and positive cells were visualized with an Avidin/Biotin complex (Vectastain Elite ABC kit; Vector Laboratories) and the chromogen 3,3′-diaminobenzidine (DAB) (with nickel chloride for NeuroD). Finally, tissue was counterstained with cresyl violet (only for Ki-67 detection), dehydrated in graded alcohols, immersed in xylene and cover-slipped with Permount®. All slides were coded so that the number of immunostained positive cells was blindly quantified (Leica DMR light microscope: 63x objective lens and 10x ocular lens = total magnification of 630x) by a modified unbiased procedure (Malberg et al., 2000; Malberg and Duman, 2003) that counts every 8th section throughout the entire extent of the hippocampal dentate gyrus (i.e., positive cells were located in the subgranular zone for Ki-67 and/or the granular cell layer of the dentate gyrus for NeuroD) to ensure an accurate count of individual cells. Two major considerations in this type of analysis are that no +labelled cell will be counted twice and that the area counted will be consistent in each section (see Malberg et al., 2000). Since every 8th section is quantified, the spacing ensures that the same positive cell will not be counted in two sections. Moreover, the quantification procedure allowed to distinguish cells within clusters and individual cells by focusing across the z-plane (thickness of the tissue: 30 μm). Cells were counted as being in the subgranular zone of the dentate gyrus if it was touching it or was in it. However, cells that were located more than two cells away from the subgranular zone were classified as hilar and were not included in the count. Then, the total number of +cells in each slice was multiplied by the sampling factor 8 to provide an estimate of the total number of +cells per marker and rat hippocampi (see García-Fuster et al., 2010, 2011).

2.7. Data analysis

Data was analyzed with GraphPad Prism, Version 8 (GraphPad Software, Inc., CA, USA). In line with the guidelines for displaying data and statistical methods in experimental pharmacology (e.g., Curtis et al., 2018; Michel et al., 2020), results are presented as mean values ± standard errors of the mean (SEM), and individual symbols are shown, when appropriate, for each rat within the bar graphs. Normal distribution for each measurement reported was evaluated with Shapiro-Wilk normality test. Student's *t*-tests were used to evaluate basal changes in the time spent immobile in the forced-swim test prior to treatment (Figs. 1a, 3a and 5a) and following adolescent treatment on PND 51 (Fig. 2a). The rest of changes in immobility were evaluated by two-way repeated measures ANOVAs followed by Sidak's multiple comparisons tests when appropriate, in which Treatment (SHAM vs. ECS) and Day (1, 3 or 7 days post-treatment) were used as independent variables. Given that some of the experiments in male and female rats were performed at different times, the differential response of ECS was evaluated for each sex separately. Since brains for each time-point (1–30 days post-treatment) were collected in separate independent experiments, changes in Ki-67+ and NeuroD+ cells, when comparing SHAM vs. ECS-treated rats at the indicated times, were evaluated by Student's *t*-tests. A one-way ANOVA test followed by Sidak's was used to evaluate the effects of TMZ (1x or 2x paradigm) over Ki-67+ or NeuroD+ cells. For the experimental design that includes the interaction of ECS and TMZ, a two-way ANOVA (Pre-treatment: vehicle vs. TMZ and Treatment: SHAM vs. ECS) and/or multiple *t*-tests (for pairwise comparisons) were used to ascertained neurogenic differences. The level of significance was fixed at $p \leq 0.05$.

3. Results

3.1. Antidepressant- and neurogenic-like effects induced by ECS during adolescence in male and female rats

Adolescent ECS exposure decreased the time male rats spent immobile in the forced-swim test (Treatment x Day interaction: $F_{2,36}$=8.03, p<0.01), suggesting an antidepressant-like effect observed 1 day post-treatment (−52±13 s, $^{**}p$<0.01 vs. SHAM; Fig. 1b). However, for female rats, although a significant Treatment x Day interaction was also observed ($F_{2,32}$=8.18, p<0.01), *post-hoc* analysis revealed that rats exposed to ECS showed increased immobility (prodepressant-like effect) 7 days post-treatment (+51±14 s, $^{**}p$<0.01 vs. SHAM; Fig. 1b).

Adolescent ECS exposure (vs. SHAM) increased recent cell proliferation 1 day post-treatment in male (+3317±826 Ki-67+ cells; t = 4.02, df=11, $^{**}p$<0.01) and female (+8152±776 Ki-67+ cells; t = 10.50, df=6, $^{***}p$<0.001) rats. However, a decrease in Ki-67+ cells was observed 8 days post-treatment in male (−1228±166 Ki-67+ cells; t = 5.31, df=18, $^{***}p$<0.001) and female (−453±151 Ki-67+ cells; t = 2.99, df=16, $^{**}p$<0.01) rats (Fig. 1c). When evaluating the effects of adolescent ECS exposure over early neuronal survival (NeuroD+ cells), the results showed increased rates 1 and 8 days post-treatment, both for male (1 d: +5449±1689 NeuroD+ cells; t = 3.28, df=11, $^{**}p$<0.01; 8 d: +24,355±2385 NeuroD+ cells; t = 10.21, df=18, $^{***}p$<0.001) and female (1 d: +9678±1690 NeuroD+ cells; t = 5.73, df=6, $^{**}p$<0.01; 8 d: +21,877±2058 NeuroD+ cells; t = 10.63, df=16, $^{***}p$<0.001) rats (Fig. 1d).

3.2. Antidepressant-and neurogenic-like effects induced by ECS across time following early life stress in male and female rats

As previously described for naïve rats (Fig. 1b), in rats exposed to maternal deprivation early in life (a model of prodepressive-like behavior; see Marco et al., 2015) adolescent ECS was capable of reducing immobility (i.e., antidepressant-like effect) in males (−38±193 s, t = 1.96, df=17, $^{*}p$<0.05) but not in females (−19±18 s, t = 1.04, df=17, p = 0.311), as measured 1 day post-treatment when comparing to MD exposed to SHAM (PND 51; Fig. 2b). Rats were left undisturbed for over 20 days and where then exposed to ECS or SHAM in adulthood (opposite treatment group than in adolescence) and the effects evaluated in the forced-swim test. ECS exposure in adulthood decreased the time male rats spent immobile in the forced-swim test (Treatment x Day interaction: $F_{2,34}$=3.91, p<0.05), suggest-

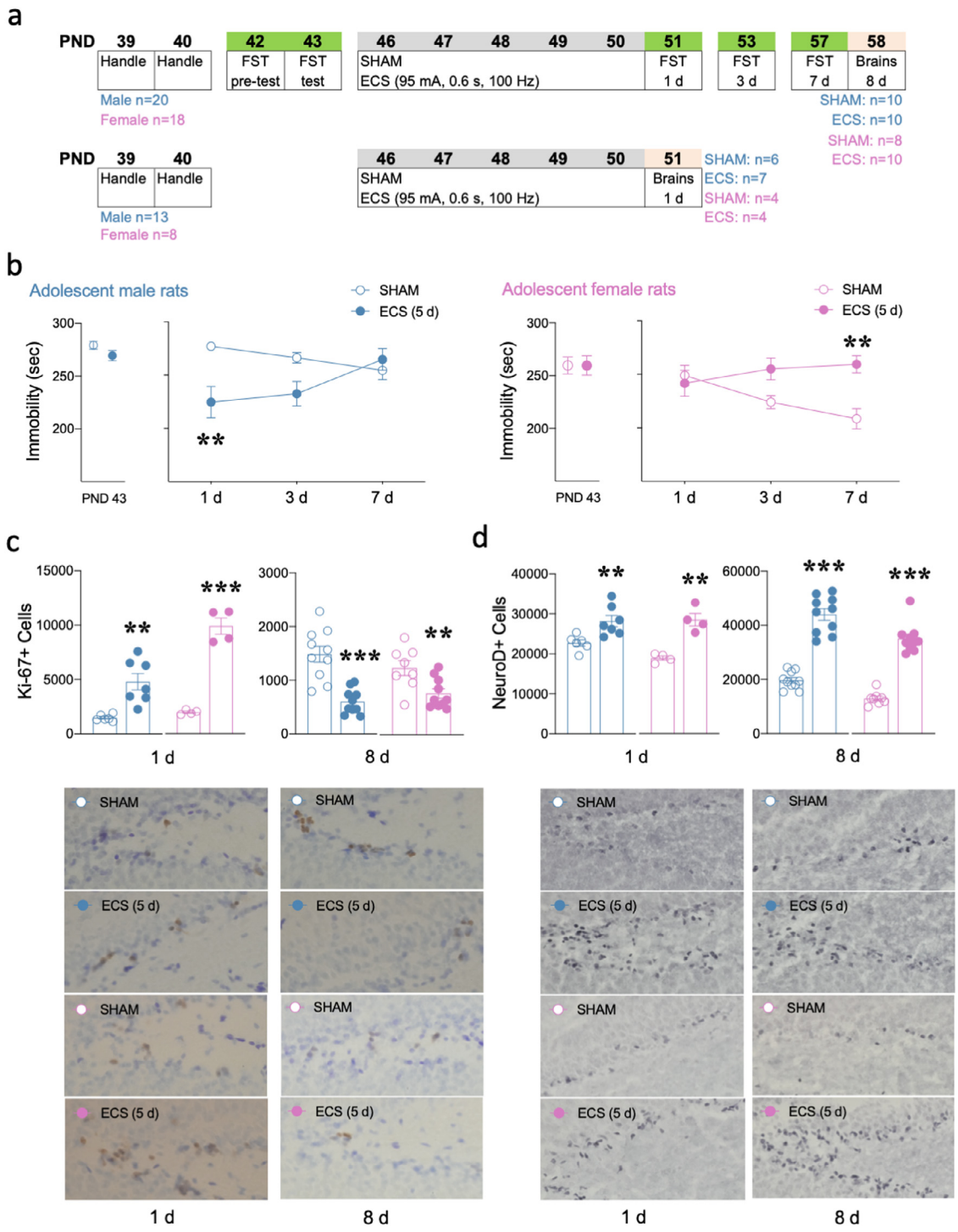

Fig. 1 Antidepressant- and neurogenic-like effects induced by ECS during adolescence in male and female rats. a Experimental design. ECS: electroconvulsive seizures; d: day; FST: forced-swim test; PND: postnatal day. **b** Immobility (s) in the FST in male and female rats: basal measurements during adolescence (PND 43) prior to treatment, and 1, 3 and 7 days post-treatment. Data represents mean ± SEM of the time (s) spent immobile. Two-way repeated measures ANOVAs followed by Sidak's multiple comparisons tests: $^{**}p<0.01$ vs. SHAM. **c** Quantitative analysis of Ki-67+ and **d** NeuroD+ cells in the left dentate gyrus. Data represents mean ± SEM of the number of + cells quantified in every 8th section throughout the entire extent of the hippocampal dentate gyrus and multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker (individual values are shown for each rat: symbols). Student's *t*-tests: $^{**}p<0.01$ and $^{***}p<0.001$ vs. SHAM. Representative images showing individual Ki-67+ (brown labeling in the blue granular layer) or NeuroD+ (dark blue labeling in the blue granular layer) cells taken with a light microscope (40x objective lens). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

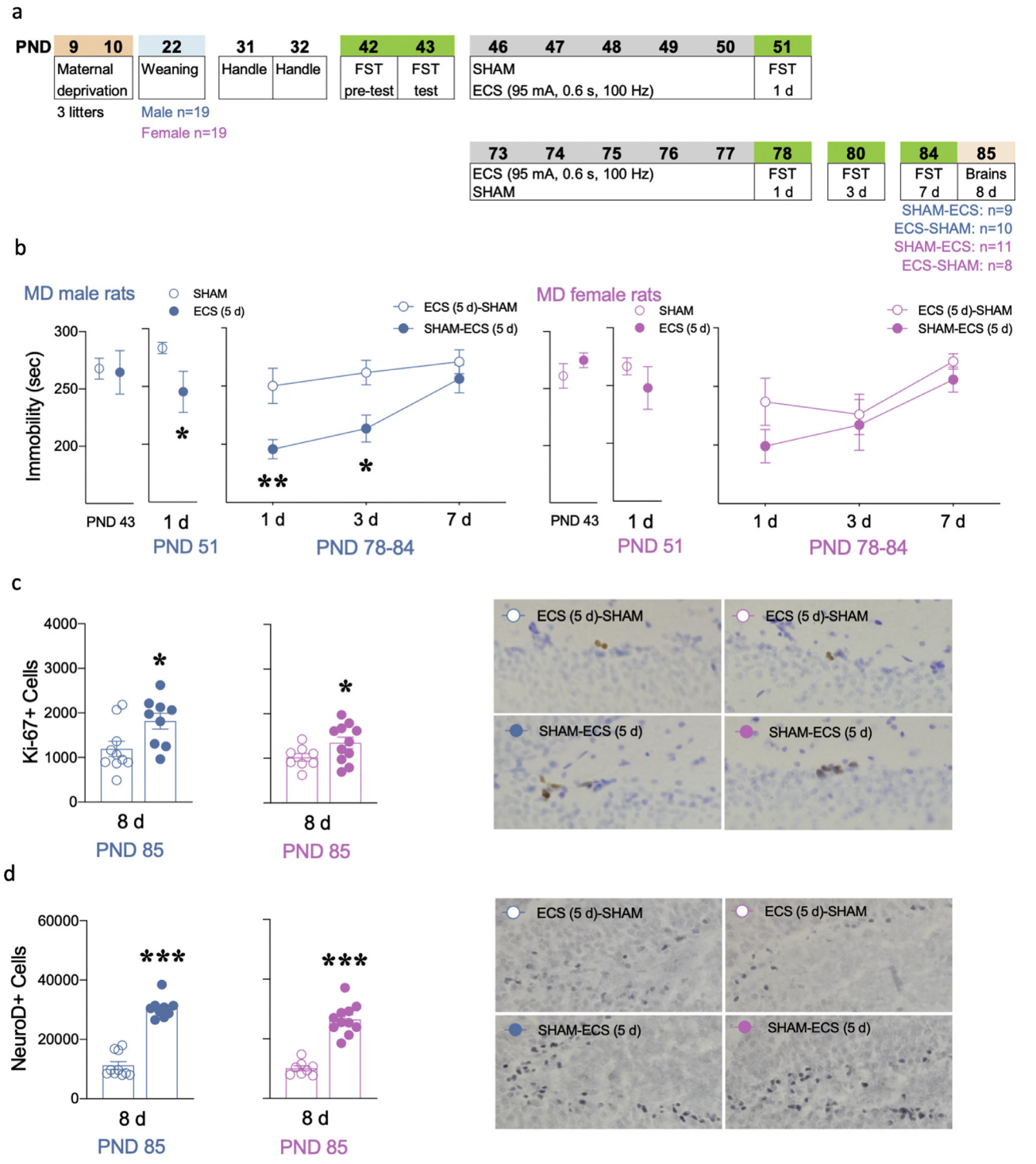

Fig. 2 Antidepressant- and neurogenic-like effects induced by ECS across time following early life stress in male and female rats. a Experimental design. ECS: electroconvulsive seizures; d: day; FST: forced-swim test; PND: postnatal day. **b** Immobility (s) in the FST in male and female rats following maternal deprivation early in life: basal measurements during adolescence (PND 43) prior to treatment, and 1, 3 and 7 days post-treatment. Data represents mean ± SEM of the time (s) spent immobile. Two-way repeated measures ANOVAs followed by Sidak's multiple comparisons tests: $^{*}p<0.05$ and $^{**}p<0.01$ vs. SHAM. **c** Quantitative analysis of Ki-67+ and **d** NeuroD+ cells in the left dentate gyrus. Data represents mean ± SEM of the number of + cells quantified in every 8th section throughout the entire extent of the hippocampal dentate gyrus and multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker (individual values are shown for each rat: symbols). Student's *t*-tests: $^{**}p<0.01$ and $^{***}p<0.001$ vs. SHAM. Representative images showing individual Ki-67+ (brown labeling in the blue granular layer) or NeuroD+ (dark blue labeling in the blue granular layer) cells taken with a light microscope (40x objective lens). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

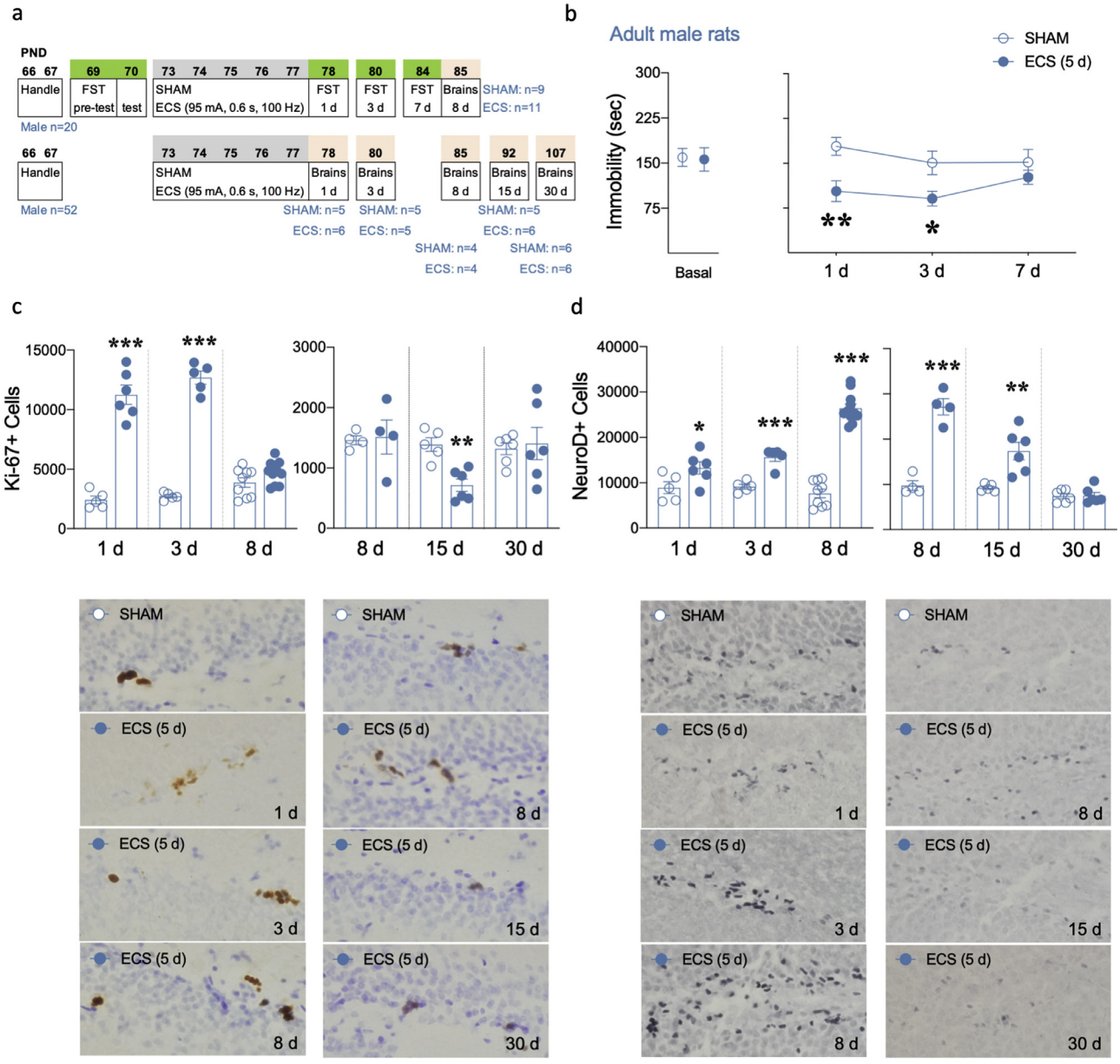

Fig. 3 Antidepressant- and neurogenic-like effects induced by ECS during adulthood in male rats. a Experimental design. ECS: electroconvulsive seizures; d: day; FST: forced-swim test. **b** Immobility (s) in the FST in male rats: basal measurements prior to treatment, and 1, 3 and 7 days post-treatment. Data represents mean ± SEM of the time (s) spent immobile. Two-way repeated measures ANOVAs followed by Sidak's multiple comparisons tests: $^{*}p<0.05$ and $^{**}p<0.01$ vs. SHAM. **c** Quantitative analysis of Ki-67+ and **d** NeuroD+ cells in the left dentate gyrus. Data represents mean ± SEM of the number of + cells quantified in every 8th section throughout the entire extent of the hippocampal dentate gyrus and multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker (individual values are shown for each rat: symbols). Student's *t*-tests: $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. SHAM. Representative images showing individual Ki-67+ (brown labeling in the blue granular layer) or NeuroD+ (dark blue labeling in the blue granular layer) cells taken with a light microscope (40x objective lens). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

ing an antidepressant-like effect observed 1 day (−54±16 s, $^{**}p<0.01$) and up to 3 days (−48±16 s, $^{*}p<0.05$) post-treatment (Fig. 2b). However, no significant Treatment x Day interaction was observed for female rats ($F_{2,34}=0.99$, $p = 0.382$; Fig. 2b).

In terms of the neurogenic-like effects induced by ECS, the results showed increased recent cell proliferation for male (+621±248 Ki-67+ cells; $t = 2.50$, $df=17$, $^{*}p<0.05$) and female (+326±167 Ki-67+ cells; $t = 1.95$, $df=17$, $^{*}p<0.05$) rats (Fig. 2c), as well as increased early neuronal survival for males (+19,290±1741 NeuroD+ cells; $t = 11.08$, $df=17$, $^{***}p<0.001$) and females (+16,400±1916 NeuroD+ cells; $t = 8.56$, $df=17$, $^{***}p<0.001$; Fig. 2d) 8 days post-treatment when compared to SHAM-treated rats.

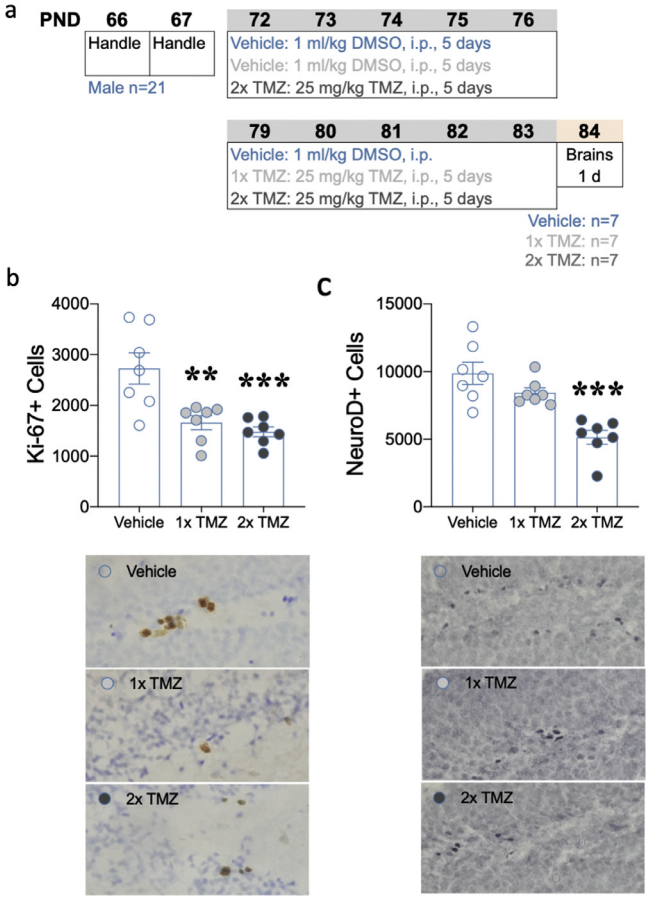

Fig. 4 Pharmacological inhibition of cell proliferation in male rats. a Experimental design. d: day; DMSO: dimethyl sulfoxide; TMZ: temozolomide. **b** Quantitative analysis of Ki-67+ and **c** NeuroD+ cells in the left dentate gyrus. Data represents mean ± SEM of the number of + cells quantified in every 8th section throughout the entire extent of the hippocampal dentate gyrus and multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker (individual values are shown for each rat: symbols). One-way ANOVA followed by Sidak's: $^{**}p<0.01$ and $^{***}p<0.001$ vs. Vehicle. Representative images showing individual Ki-67+ (brown labeling in the blue granular layer) or NeuroD+ (dark blue labeling in the blue granular layer) cells taken with a light microscope (40x objective lens). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3.3. Antidepressant-and neurogenic-like effects induced by ECS during adulthood in male rats

Since ECS exposure seemed to only induce beneficial behavioral effects in adolescent and adult male rats (naïve or exposed to maternal deprivation), the following experiments were solely centered in male rats. Adult ECS exposure decreased the time male rats spent immobile in the forced-swim test (Treatment x Day interaction: $F_{2,36}=4.38$, $p<0.05$), suggesting an antidepressant-like effect observed 1 day (−75±23 s, $^{**}p<0.01$) that lasted up to 3 days (−60±23 s, $^{*}p<0.05$ vs. SHAM) post-treatment (Fig. 3b), similarly to the effects observed in adult male rats exposed to maternal deprivation early in life (Fig. 2b).

Adult ECS exposure increased recent cell proliferation 1 day (+8821±945 Ki-67+ cells; $t = 9.34$, $df=9$, $^{***}p<0.001$) and 3 days (+9949±562 Ki-67+ cells; $t = 17.70$, $df=8$, $^{***}p<0.001$) post-treatment, and return to normal 8 days (+843±474 Ki-67+ cells; $t = 1.78$, $df=18$, $p = 0.092$) post-treatment vs. SHAM-treated rats (Fig. 3c). When repeating the experiment to incorporate longer time points of analysis, so we could evaluate the long-term impact of ECS on Ki-67+ cells, the results showed, as before, no changes 8 days post-treatment (+54±292 Ki-67+ cells; $t = 0.18$, $df=6$, $p = 0.860$), but a significant decrease observed at 15 days (−673±153 Ki-67+ cells; $t = 4.39$, $df=9$, $^{**}p<0.01$) that normalized 30 days post-treatment (+88±285 Ki-67+ cells; $t = 0.31$, $df=10$, $p = 0.764$) vs. SHAM-treated rats (Fig. 3c). In terms of the effects of adult ECS exposure over early neuronal survival (NeuroD+ cells), the results showed increased rates 1 day (+4343±1925 NeuroD+ cells; $t = 2.26$, $df=9$, $^{*}p = 0.05$), 3 days (+6488±1070 NeuroD+ cells; $t = 6.06$, $df=8$, $^{***}p<0.001$) and 8 days (+18,741±1371 NeuroD+ cells; $t = 13.67$, $df=18$, $^{***}p<0.001$) post-treatment (vs. SHAM; Fig. 3d). When replicating the experiment to evaluate the course of the observed NeuroD up-regulation, the results showed significant increases up to 15 days post-treatment (8 days: +17,396±2098 NeuroD+ cells; $t = 8.29$, $df=6$, $^{***}p<0.001$; 15 days: +8117±2161 NeuroD+ cells; $t = 3.76$, $df=9$, $^{**}p<0.01$) and an apparent normalization 30 days post-treatment (+57±932 NeuroD+ cells; $t = 10.06$, $df=10$, $p = 0.952$; Fig. 3d).

3.4. Pharmacological inhibition of cell proliferation

TMZ treatment decreased recent cell proliferation ($F_{2,18}=11.16$, $p<0.001$) and early neuronal survival ($F_{2,18}=16.47$, $p<0.001$). *Post-hoc* analysis revealed TMZ dose-dependently decreased Ki-67+ cells (1x TMZ: −1067±285, $^{**}p<0.01$; 2xTMZ: −1246±285, $^{***}p<0.001$; Fig. 4b) and NeuroD+ cells (1x TMZ: −1426±845, $p = 0.206$; 2xTMZ: −4729±845, $^{***}p<0.001$; Fig. 4c).

3.5. Pharmacological inhibition of cell proliferation prevents the antidepressant-like effect while only partially inhibits the neurogenic increase induced by ECS during adulthood

ECS exposure (in vehicle-treated rats) decreased the time male rats spent immobile in the forced-swim test (Treatment x Day interaction: $F_{2,32}=4.38$, $p<0.05$), suggesting, as previously reported in Figs. 2b and 3b, an antidepressant-like effect observed 1 day (−38±21 s, $^{**}p<0.01$) and up to 3 days (−80±21 s, $^{**}p<0.01$ vs. SHAM-vehicle) post-treatment (Fig. 5b). Interestingly, this antidepressant-like effect was non-existent in animals pre-treated with TMZ, since no significant Treatment x Day interaction was detected ($F_{2,38}=0.20$, $p = 0.819$).

In addition, when evaluating the effects on cell proliferation 1 day post-treatment a significant Pre-treatment x Treatment interaction was observed ($F_{1,17}=11.17$, $p<0.01$).

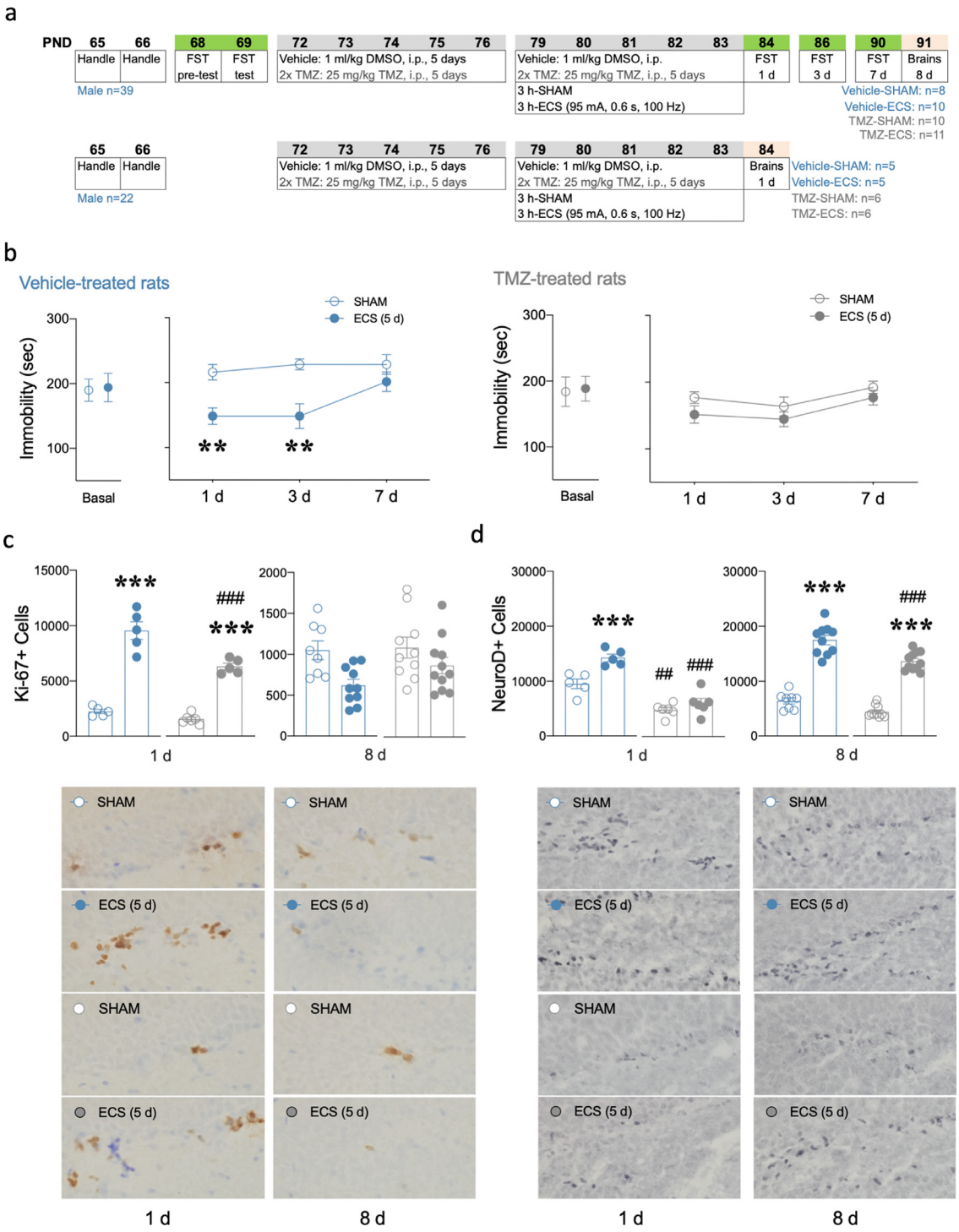

Fig. 5 Pharmacological inhibition of cell proliferation prevents the antidepressant- and partially the neurogenic-like effect induced by ECS in male adult rats. a Experimental design. ECS: electroconvulsive seizures; d: day; DMSO: dimethyl sulfoxide; FST: forced-swim test; TMZ: temozolomide. **b** Immobility (s) in the FST in male rats treated with vehicle or TMZ: basal measurements prior to treatment, and 1, 3 and 7 days post-treatment. Data represents mean ± SEM of the time (s) spent immobile. Two-way repeated measures ANOVAs followed by Sidak's multiple comparisons tests: $^{**}p<0.01$ vs. SHAM. **c** Quantitative analysis of Ki-67+ and **d** NeuroD+ cells in the left dentate gyrus. Data represents mean ± SEM of the number of + cells quantified in every 8th section throughout the entire extent of the hippocampal dentate gyrus and multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker (individual values are shown for each rat: symbols). Two-way ANOVAs followed by multiple *t*-tests: $^{***}p<0.001$ vs. corresponding SHAM; $\#\#p<0.01$ and $\#\#\#p<0.001$ vs. corresponding Vehicle-treated group. Representative images showing individual Ki-67+ (brown labeling in the blue granular layer) or NeuroD+ (dark blue labeling in the blue granular layer) cells taken with a light microscope (40x objective lens). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

In particular, although the expected increase in hippocampal cell proliferation induced by ECS 1 day post-treatment (+7307±615 Ki-67+ cells; $^{***}p<0.001$ vs. vehicle-SHAM) was still observed in rats pre-treated with TMZ (+4462±589 Ki-67+ cells, $^{***}p<0.001$ vs. TMZ-SHAM), its magnitude was significantly reduced (−3653±615 Ki-67+ cells, $\#\#\#p<0.001$ when comparing vehicle-ECS vs. TMZ-ECS; Fig. 5c). Moreover, no significant Pre-treatment x Treatment interaction was observed for cell proliferation 8 days post-treatment ($F_{1,35}=0.99$, $p = 0.327$). On the other hand, when measuring NeuroD+ cells, a significant Pre-treatment x Treatment interaction was observed 1 day post-treatment ($F_{1,18}=5.64$, $p<0.05$). The expected increase induced by ECS 1 day post-treatment (+4766±1092 NeuroD+ cells; $^{**}p<0.01$ vs. vehicle-SHAM) was blocked in rats pre-treated with TMZ (+1253±997 NeuroD+ cells, $p = 0.783$ vs. TMZ-SHAM). In fact, when directly comparing vehicle-ECS vs. TMZ-ECS a significant reduction in early neuronal survival was observed (−8175±1046 NeuroD+ cells, $\#\#\#p<0.001$; Fig. 5d). Moreover, although no significant Pre-treatment x Treatment interaction was detected for early neuronal survival 8 days post-treatment ($F_{1,35}=2.64$, $p = 0.113$), there was a significant effect of Pre-Treatment ($F_{1,35}=24.67$, $p<0.001$) and Treatment ($F_{1,35}=284.9$, $p<0.001$). In particular, ECS increased NeuroD+ cells (in vehicle and TMZ treated rats, $^{***}p<0.001$ for both comparisons vs. respective SHAM groups) and TMZ pre-treatment reduced the overall number of NeuroD+ cells ($\#\#\#p<0.001$) when comparing vehicle-ECS vs. TMZ-ECS (Fig. 5d).

4. Discussion

The present results demonstrated age- and sex-specific differences in the antidepressant-like potential of repeated ECS (in naïve and maternally deprived rats), since it worked when administered during adolescence or adulthood in male rats (although with different lengths of effective duration), while in female rats rendered deleterious in adolescence or ineffective in adulthood. Yet, repeated ECS increased cell proliferation and vastly boosted young neuronal survival in a time-dependent manner for both sexes and independently of age. Moreover, pharmacological inhibition of basal cell proliferation prevented the antidepressant-like effect induced by repeated ECS observed in male adult rats, but only partially blocked the very robust increase in recent cell proliferation and young neuronal survival. Overall, the present results suggest that the induction of the early stages of hippocampal neurogenesis by ECS, besides having a role in mediating its antidepressant-like effect, might participate in some other neuroplastic actions.

In terms of the antidepressant-like potential of ECS, prior results in male rodents showed that repeated ECS (14 sessions in Wistar rats) decreased immobility in the forced-swim test up to 3 days post-treatment (Li et al., 2007) and that 5 ECS-sessions were capable of inducing an effective antidepressant-like response in the Wistar-Kyoto rat model of depressive-like behavior up to 7 days post-treatment (Kyeremanteng et al., 2014). Bearing in mind that ECS effects vary among rat strains (Statler et al., 2008), and will likely be influenced by the settings used to induce the seizures, the current results go along the same lines for male rats while incorporates novel data during adolescence and its comparison with female rats. Particularly, male rats responded similarly to ECS when administered in adolescence or adulthood in terms of inducing an antidepressant-like effect (although it was shorter in length in adolescence), and its course was similar for naïve or maternally deprived rats. Furthermore, female rats were insensitive to the expected beneficial antidepressant-like effects of repeated ECS, and even showed opposite consequences when administered during adolescence. Therefore, repeated ECS administration during adolescence showed diminished behavioral effects for male rats, similarly to previous age-related studies from our group with other antidepressant-like drugs (Bis-Humbert et al., 2020), and even showed deleterious effects for female rats (i.e., induction of prodepressive-like effects), in line with the loss of efficacy and/or negative impact described for antidepressants in adolescence (e.g., Bylund et al., 2007; Cipriani et al., 2016). Interestingly, repeated ECS in adult male (but not female) rats, induced an antidepressant-like effect that lasted up to 3 days post-treatment. These results suggested that adult male rats responded better to ECS than adolescents, challenging the idea that higher electrical charges are needed to induce effective responses in Sprague-Dawley rats with age (Statler et al., 2008). Moreover, female rats seemed to be less affected in terms of the length of seizure and its recovery than their male counterparts throughout the experimental designs (personal observation), however, seizure duration does not appear to be associated with the antidepressant properties of treatment (Sackeim et al., 1991; Kales et al., 1997). In any case, future experiments will center in evaluating alternative dosing parameters for the electrical charge and seizure threshold that could potentially be more effective in female rats. Since these results demonstrated sex-specific differences in the antidepressant-like potential of repeated ECS in adolescent and adult rats, the next step evaluated the regulation of cell markers involved the early stages of hippocampal neurogenesis (i.e., recent cell proliferation and early neuronal survival) as a potential mechanism behind these sex-specific disparities.

Interestingly, the results showed that repeated ECS increased hippocampal cell proliferation and early neuronal survival (i.e., early stages of neurogenesis) for both sexes and it did so in a similar fashion in adolescence and adulthood, suggesting this mechanism does not seem to be responsible for the disparities observed in antidepressant-like efficacies. Prior studies have shown that maximal cell proliferation seemed to occur on days 3 and 5 post-ECS (Madsen et al., 2000) and that almost all proliferating cells that are increased during and after repeated ECS, survive for 1 month (Scott et al., 2000; Nakamura et al., 2013), 3 months (Madsen et al., 2000) or even up to 12 months (Olesen et al., 2017) post-ECS. Moreover, a prior study that examined the number of ECS sessions needed to yield a maximum stimulation of hippocampal cell proliferation showed that 10 days of a single ECS exposure per day had the greatest effects on increasing neurogenesis (Ito et al., 2010). In this context, our results showed that 5 sessions of ECS were capable of inducing robust increases in cell markers of the early stages of neurogenesis. In particular, repeated ECS induced an early increase in cell proliferation (observed 1

and up to 3 days post-treatment) that eventually led to a later decrease in the number of Ki-67+ cells, as observed 8 days post-treatment for adolescent rats and 15 days post-treatment for adult rats. Notably, since the 8 days data point was evaluated following a series of stressful forced-swim test, and given that stress is known to decrease hippocampal neurogenesis, one could not exclude that stress played a role in these effects. Moreover, our results evaluating early neuronal survival with NeuroD marker showed that repeated ECS induced an early and persistent increase in young neuronal survival (observed 1 and 8 days post-treatment) in rats during adolescence or adulthood and independently of sex. These results, which were extended for adult rats, showed a biphasic regulation of NeuroD+ cells content by ECS, with increases starting 1 and 3 post-treatment, a maximum response observed following 8 days (around 3-fold increase varying with age of exposure and sex), and then a drop in the magnitude of increase following 15 days, while returning to normal levels 30 days post-treatment.

So far, our data determined a temporal and parallel course regulation for the antidepressant-like effects and for the induction of recent cell proliferation by ECS (significant effects observed up to 3 days post-treatment). Since the involved mechanism of action should be affected for a time period that matches the average endurance of behavioral-like effects, and given that prior data suggested that ECS required hippocampal neurogenesis for its antidepressant-like efficacy in a neuroendocrine mouse model of depressive-like behavior (Schloesser et al., 2015), the present study evaluated whether the pharmacological inhibition of basal cell proliferation with TMZ would prevent the beneficial effects exerted by ECS on behavior and brain function. Although there are, more specific, alternative methods for inhibiting basal cell genesis selectively in the hippocampal dentate gyrus (i.e., image-guided cranial irradiation Rivera et al., 2019), TMZ was selected based on recent publications (i.e., Brozka et al., 2017; Cuartero et al., 2019; Luján et al., 2019) as an easier pharmacological approach to successfully inhibit cell proliferation and ascertain behavioral changes. The results showed that the expected antidepressant-like effect induced by ECS was abolished in rats pre-treated with TMZ. However, the effects on recent cell proliferation and early neuronal survival were only partly reduced. In particular, 1 day post-ECS treatment, while the expected increase in Ki-67+ cells was still observed (although its magnitude was significantly reduced), the increase in NeuroD+ cells was completely blocked in rats pre-treated with TMZ. Moreover, 8 days post-ECS treatment, the expected increase in NeuroD+ cells was still present in rats pre-treated with TMZ, though with a decreased magnitude. Similarly, prior data have suggested that while there is a requirement for adult neurogenesis in mediating some of the beneficial effects of antidepressants (Santarelli et al., 2003), decreasing neurogenesis alone is not sufficient to drive a depressive-like phenotype (Vollmayr et al., 2003; Samuels and Hen, 2011), similar to what we observed for TMZ-pretreated rats. In this line of results, a prior study concluded that neurogenesis was a crucial contributor to the beneficial effects of ECS, but the regulation of other neurotransmitter systems and/or effects might also be needed, since no mediation between ECS treatment and forced-swim test immobility rates was observed (Olesen et al., 2017). Prior literature suggested that ECS's great antidepressant-like efficacy was driven not only by increasing the proliferation of neural progenitor cells (like chemical antidepressants), but also by inducing the proliferation of neural stem cells (i.e., recruitment and activation) at early mitotic phases (Segi-Nishida et al., 2008). The present results showed that some, but not all of the newly generated proliferating and surviving cells appeared to be needed for ECS to exert its antidepressant-like response, suggesting that facilitating the early steps of neurogenesis by ECS may alter other functional roles in the hippocampal circuit (see Segi-Nishida et al., 2011). ECS also promotes structural hippocampal plasticity by enhancing other neuroplastic effects (reviewed pre-clinical and clinical data in Bouckaert et al., 2014), such as gliogenesis (Kaae et al., 2012), synaptogenesis (e.g., Chen et al., 2009) and angiogenesis (Hellsten et al., 2005) without apparently inducing cell death in the hippocampus (Vaidya et al., 1999). It is still unknown what the long-term consequences of this large induction of new young neurons might be at the behavioral level. In this regard, prior studies demonstrated that repeated ECS caused negative behavioral changes, such as impairment in short-term memory and increased activity in locomotion in the open field test (Hidaka et al., 2008). On the other hand, other studies suggested that repeated ECS increased newly generated neurons without negative behavioral change (Nakamura et al., 2013). This excitatory neuronal activity, since it is pro-neurogenic, has been generally considered beneficial, however it is worth mentioning that ECS is also used to model convulsive seizures in rodents as observed in epilepsy. While in animal models ECS markedly increases neurogenesis, in epileptic rodent models, seizures induce the generation of misplaced neurons with abnormal morphological and electrophysiological properties. In fact, there is a pile of literature explaining the contradictory effects of neuronal hyperexcitation on adult hippocampal neurogenesis and the possible responsible mechanisms (Pineda and Encinas, 2016; Bielefeld et al., 2019) for this abnormal process, which is referred to as aberrant neurogenesis. Thus, the exact role for the vast increase in early neuronal survival following repeated ECS deserves future investigations.

To sum up, these results add to the existing literature regarding the antidepressant-like potential of repeated ECS by providing evidence of a detailed time-course differential response in male and female rats during adolescence and adulthood, and by suggesting that the early neurogenic-like capabilities induced by ECS go beyond the regulation of its antidepressant-like effects and deserves broaden characterization.

Conflict of interest

The authors declare no conflict of interest.

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Contributors

Authors RG-C, SL-C and MJG-F were responsible for the study concept and design. Authors RG-C and SL-C conducted the experiments and analyzed the behavioral and molecular data, with the participation of CB-H. Author MJG-F wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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