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Hammels, Caroline; Prickaerts, Jos; Kenis, Gunter; VANMIERLO, Tim; Fischer, Maximilian; STEINBUSCH, Harry; van Os, Jim; van den Hove, Daniel L. A. & Rutten, Bart P. F. (2015) Differential susceptibility to chronic social defeat stress relates to the number of Dnmt3a-immunoreactive neurons in the hippocampal dentate gyrus. In: *PSYCHONEUROENDOCRINOLOGY*, 51, p. 547-556.

DOI: 10.1016/j.psyneuen.2014.09.021

Handle: <http://hdl.handle.net/1942/18666>

Differential susceptibility to chronic social defeat stress relates to the number of Dnmt3a-immunoreactive neurons in the hippocampal dentate gyrus

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running title: Dnmt3a after social defeat

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Abstract

The enzyme DNA methyltransferase 3a (Dnmt3a) is crucially involved in DNA methylation and recent studies have demonstrated that Dnmt3a is functionally involved in mediating and moderating the impact of environmental exposures on gene expression and behavior. Findings in rodents have suggested that DNA methylation is involved in regulating neuronal proliferation and differentiation. So far, it has been shown that chronic social defeat **might influence** neurogenesis, while susceptibility to social defeat stress is dependent on gene expression changes in the nucleus accumbens and the mesolimbic dopaminergic system. However, the role of Dnmt3a herein has not been fully characterized. Our earlier immunohistochemical work has revealed the existence of two types of Dnmt3a-immunoreactive cells in the mouse hippocampus, of which one represents a distinct type with intense Dnmt3a-immunoreactivity (Dnmt3a type II cells) co-localizing with a marker of recent proliferation. Based on this, we hypothesize that behavioral susceptibility to chronic social defeat stress is linked to i) Dnmt3a protein levels in the nucleus accumbens and hippocampus, and ii) to the density of Dnmt3a type II cells in the hippocampal dentate gyrus. While no differences were found in global levels of Dnmt3a protein expression in the nucleus accumbens and hippocampus, our stereological quantifications indicated a significantly increased density of Dnmt3a type II cells in the dentate gyrus of animals resilient to social defeat stress compared to susceptible and control animals. Further characterization of the Dnmt3a type II cells revealed that these cells were mostly doublecortin (25%) or NeuN (60%) immunopositive, thus defining them as immature and mature neurons. Moreover, negative associations between the density of Dnmt3a type II cells and indices of depressive-like behavior in the sucrose intake and forced swim test were found. **These correlational data suggest that DNA methylation via Dnmt3a in the**

hippocampus co-regulates adaptivity of the behavioral response to chronic social defeat stress, and set the stage for further experimental studies testing a mediating role of Dnmt3a in experience-dependent plasticity, neurogenesis and (mal)adaptation to severe stressors.

Key words

Social defeat

Neurogenesis

Resilience

DNA methylation

Epigenetics

Dnmt3a

Introduction

Epigenetic mechanisms are crucial for mediating environmental influences on gene expression and behavioral traits, impacting differential susceptibility to severe stressors in mental ill-health. A role for epigenetic modifications in stress-related mental disorders including major depression or posttraumatic stress disorder has been suggested based on factors such as the absence of complete concordance in monozygotic twins, the onset of disease later in life rather than in childhood or adolescence and the link to environmental stressors (Mill and Petronis, 2007). Fraga *et al.* demonstrated that DNA methylation profiles in monozygotic twin pairs diverge as twins grow older (Fraga et al., 2005). These changes in genetically identical individuals could be explained by the influence of external factors such as smoking, physical activity, diet and psychological stress. Similarly, genetically identical inbred animals show considerable epigenetic differences resulting in phenotypic variation (Uher, 2011). The social defeat stress model has been proposed as a model to study the mechanisms mediating differential susceptibility to chronic and severe stress (Golden et al., 2011). Krishnan *et al.* showed that this experimental paradigm elicits two distinct responses in the domain of social behaviour: a first group of mice displays social avoidance after the social defeat experience. In contrast to this, the second group of mice still show social interaction rates that are comparable to the control group, although all mice are of identical genetic background, and all had been exposed to similar conditions of social defeat stress (Krishnan et al., 2007). This type of stress resilience has been shown to be mediated by changes in chromatin modification, resulting in altered gene expression (Covington et al., 2009; Covington et al., 2011). Moreover, antidepressants can induce histone modifications that are normally observed in resilient individuals (Wilkinson et al., 2009). However, little is known about DNA

methylation, mediated by DNA methyltransferase enzymes Dnmt3a and Dnmt3b, in relation to the process of resilience. We recently found that common genetic variations in the gene for DNA methyltransferase 3a (Dnmt3a) moderate the impact of environmental stressors (daily life stressors and perinatal adversities) on paranoid ideation. Moreover, Dnmt3a is required for neurogenesis and neuronal differentiation as it is crucially involved in the transcription of neurogenic genes (Wu et al., 2010). In rodents, adult neurogenesis appears to be restricted to the subependymal cells of the ventricular system and the subgranular zone of the dentate gyrus (DG) in the hippocampus (Ma et al., 2010). Numerous studies have demonstrated that psychological stress impacts cellular proliferation and differentiation in these neurogenic zones (Lagace et al.; Mirescu and Gould, 2006). Previous studies have shown that cell proliferation is decreased by 50 to 75% after acute social defeat stress (Czeh et al., 2002). Additionally, it has been demonstrated that chronic antidepressant treatment increases neurogenesis (Czeh et al., 2001) and that ablation of neurogenesis by X-irradiation prevents the effectiveness of antidepressants to counteract stress-induced behavioral alterations (Santarelli et al., 2003). These findings suggest that resilience to social defeat stress may be moderated by altered neurogenesis, which in turn is mediated by altered DNA methylation in the brain. Our previous work has identified two distinct Dnmt3a immunoreactive cell types in the brain. Type I cells show moderate immunoreactivity visible in nearly all cells in the brain, and type II display intense immunoreactivity visible in neurogenic niches of the brain and co-label with markers of recent proliferation in the hippocampal DG (Chouliaras et al., 2011).

Consequently, we hypothesized that Dnmt3a is involved in differential susceptibility to chronic social defeat stress, and that animals resilient to chronic social defeat stress may show signs of adaptive epigenetic regulation of neurogenesis, while susceptible

animals would not. To test this hypothesis, C57Bl/6 mice were subjected to the chronic social defeat stress paradigm (Berton et al., 2006). Levels of Dnmt3a expression in two brain regions involved in the stress response, i.e. the hippocampus and nucleus accumbens, were determined both qualitatively and quantitatively. The nucleus accumbens was included as it has been shown that susceptibility to social defeat stress depends on the mesolimbic dopaminergic system (Berton et al., 2006). Fate characterization, determinations of immunoreactivity levels and detailed stereological quantifications of the Dnmt3a type II cells was performed.

Methods

Animals

Seven-week-old male C57Bl/6 and 30-week-old male CD1 retired breeder mice were purchased from Charles River (L'Arbresle, France) and used in the experiment. The animals were single housed in individually ventilated cages in a humidity (60% RH) and temperature controlled (21°C) environment, with an inversed 12h light/dark cycle (lights on at 1900h). Standard rodent food and water was available at libitum. **Body weight of the animals was measured every day at the same time.** All experimental procedures were approved by the local ethical committee for animal experiments of Maastricht University, according to Dutch governmental guidelines.

Social defeat procedure

The social defeat procedure was performed as described by Berton *et al.* (Berton et al., 2006; Golden et al., 2011). In short, 9-week-old mice (n=20) were submitted to social defeat stress for 10 consecutive days. Every day, each experimental mouse was introduced into the home cage of an unfamiliar CD1 resident for 10 min and was

physically defeated. After 10 min of physical interaction, residents and intruders were maintained in sensory contact for 24h using a perforated Plexiglas partition dividing the resident home cage in two halves. Control animals (n=10) were housed by pair, one on each side of a perforated Plexiglas partition, and were handled daily.

Behavioral testing

Social approach-avoidance test

One day after the last social defeat session, the social approach-avoidance test was performed as described by Berton *et al.* (Berton et al., 2006). Each experimental mouse was introduced into the open field arena (42x42cm) and its trajectory was tracked for two consecutive sessions of 2.5 min. During the first session (“no target”) the open field contained an empty wire mesh cage (10 x 6.5 cm) located at one end of the field. During the second session (“target”), the conditions were identical except that an unfamiliar CD1 mouse had been introduced into the cage. Between the two sessions, the experimental mouse was removed from the arena, and was placed back into its home cage for approximately one minute. The videotracking data from both the “no target” and “target” conditions were used to determine the total distance moved, the time spent in the interaction zone and the corners of the open field opposite to the location of the target cage. For defeated animals, the interaction ratio was calculated as $100 \times (\text{time spent in interaction zone, target present}) / (\text{mean time all defeated mice spent in interaction zone, target absent})$. Mice with an interaction ratio > 100 were labeled as “unsusceptible or resilient”, mice with an interaction ratio < 100 were labeled “susceptible”. For control animals, the interaction ratio was calculated as $100 \times (\text{time spent in interaction zone, target present}) / (\text{mean time all control mice spent in interaction zone, target absent})$. Because of the rather small sample size (n=20 for

defeat, n=10 for control) in this study, we did not calculate the interaction ratio as described earlier (Krishnan et al., 2007). To avoid exclusion of animals by marginal times spent in interaction zones or corner zones, the group average of the time spent in the zone was used, rather than the individual measurement for each animal.

Sucrose intake test (SIT), elevated zero maze (EZM) and forced swim test (FST)

After social approach-avoidance behavior testing, mice were subjected to the SIT (day 3-5 after last defeat session), EZM (day 7 after defeat) and FST (day 9 after defeat). For the SIT, mice were first habituated to a 1% sucrose solution by replacing their drinking water with the sucrose solution for 24 h. On the day of the SIT, mice were deprived of food and water for 6 h (starting at 0700h), after which they received a bottle of 1% sucrose solution for 1 h. Sucrose intake and general water and sucrose consumption were recorded by weighing the bottles before and after the intake. Sucrose intake was corrected for body weight. The EZM consisted of a circular platform (50 cm in diameter), elevated 50 cm above floor level, with two opposite enclosed parts (50 cm high side walls) and two open parts equally divided along the circular runway (5 cm). Falls from the open parts were prevented by a 5 mm high edge. Both the side walls and the maze itself were made of black plastic, transparent for infrared light, and connected via an infrared video camera to a video tracking system (Ethovision Pro, Noldus Wageningen, The Netherlands). The experimental mouse was placed in the middle of one of the open parts, and allowed to explore the maze for a total time of 5 min. Total time spent in closed parts and total distance travelled was video tracked. For the FST, transparent Plexiglas cylinders (50 cm in height × 19 cm in diameter) were filled with warm water (32 ± 2 °C) up to 20 cm. Four mice were tested in parallel and videotaped from above. Mice were not able to see one another due to grey separation panels placed

between the four cylinders. A 10 min pre-test session preceded the 5 min test-session 24 h before. After the swim sessions, mice were dried with paper towels and returned to their home cages. A camera was placed above the cylinders and connected to a video tracking system (Ethovision Pro, Noldus, Wageningen, The Netherlands), which allowed automated recording of individual immobility times and total distance moved. Settings within Ethovision were adjusted based on manually recorded sessions of ten randomly chosen animals (immobility/mobility threshold 11%).

Plasma corticosterone measurements

In order to determine HPA axis responsiveness in the face of a stressor, blood samples were taken to measure plasma corticosterone concentrations in these mice. First, the mice were taken out of their home cage, immediately after which a blood sample was drawn from the vena saphena (basal corticosterone concentration) using heparinized blood collection tubes (Microvette CB300, Sarstedt, Germany). Subsequently, the mouse was exposed to five minutes of forced swim stress. After 20 minutes of swim stress, another blood sample was drawn from the vena saphena (to measure stress-induced corticosterone concentration). Animals were then placed back in their home cage and housing room and allowed to recover for 60 minutes after which a third sample was taken (to measure “recovery” corticosterone concentration). Blood samples were processed as described earlier (Sierksma et al., 2013) and plasma corticosterone was measured using the ImmuChem Double antibody corticosterone ¹²⁵I RIA Kit for rodents (MP Biomedicals, Orangeburg, NY, USA) according to the manufacturer’s instructions.

Tissue processing

After behavioral testing (i.e. 11 days after exposure to the social defeat paradigm),

animals were deeply anesthetized with an overdose of sodium pentobarbital (CEVA, Libourne, France) and intracardially perfused with tyrode solution and ice-cold Somogyi fixative (4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in 0.1M phosphate buffer, pH 7.6). Brains were dissected and post-fixed for 2h in the same fixative and overnight in Somogyi fixative without glutaraldehyde. Next, brains were stored at 4°C in sterile Tris buffered saline containing 0.1% sodium azide until further processing. Brains were serially cut in 30µm free floating coronal sections using a Leica VT1200S vibratome (Leica Microsystems, Wetzlar, Germany) and divided into 10 subseries of every 10th section, which were stored at 4°C in sterile Tris buffered saline containing 0.1% sodium azide.

Immunohistochemical detection

One series of free-floating sections was incubated in rabbit polyclonal anti-Dnmt3a primary antibody (dilution 1:200, Santa Cruz Biotechnology, Heidelberg, Germany) overnight, on a constant shaker at room temperature, after antigen unmasking with 10 mM sodium citrate buffer (pH 6.0) in a water bath for 20 min at 80°C, and incubation of 0.1% hydrogen peroxide for 1h, to quench endogenous peroxidase activity. The sections were then rinsed again with TBS and TBS-Tween, followed by incubation with the secondary antibody, i.e. donkey anti-rabbit biotine (dilution 1:100; Jackson, Westgrove, PA, USA) in TBS-T with 0.1% bovine serum albumin for 2h. After rinsing with TBS and TBS-T, the sections were incubated with avidin-biotin-peroxidase complex (diluted 1:400; Vector laboratories, Burlingame, CA, USA) for another 2h. To visualize the horseradish peroxidase reaction product, the sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma Aldrich, Uithoorn, The Netherlands). The reaction was stopped after 10 min by rinsing with TBS. The sections

were mounted on gelatin-coated glasses, dehydrated, and coverslipped using Pertex (HistolabProducts ab, Göteborg, Sweden). Immunofluorescent double labeling of Dnmt3a and Ki-67, GFAP, DCX and NeuN was qualitatively assessed. Ki-67 (proliferating cells), glial fibrillary acidic protein (GFAP, glial and neural progenitor cells), doublecortin (DCX, immature neurons) and the neuronal nuclei antigen (NeuN, mature neurons) were used as target for immunohistochemistry. Following antigen unmasking (see above), sections were incubated with anti-Dnmt3a primary antibody and mouse anti-Ki67 (BD Biosciences, Franklin Lakes, NJ, USA), biotinylated mouse anti-GFAP (Sigma Aldrich, Uithoorn, The Netherlands), goat anti-DCX (Santa Cruz Biotechnology, Heidelberg, Germany) or mouse anti-NeuN (Merck Millipore, Billerica, MA, USA). After 2h incubation with donkey anti-rabbit Alexa 488 (Invitrogen, Paisley, UK), sections were incubated with donkey-mouse Alexa 594, donkey anti-goat Alexa 594 or streptavidin Alexa 594 (all from Invitrogen, Paisley, UK). After selection of immunoreactive cells, image stacks of 16 μ m thick and consisting of 80 confocal images (with a distance of 0.2 μ m in between) were made with a 40x magnification (Olympus UPlanSApo) and the SISD system (MBF Bioscience, Magdeburg, Germany). The system consisted of a modified Olympus BX51 fluorescence microscope with customized spinning disk unit (DSU; Olympus, Zoeterwoude, The Netherlands), computer-controlled excitation and emission filterwheels (Olympus Zoeterwoude, The Netherlands), three-axis high-accuracy computer-controlled stepping motor specimen stage (4x4 Grid Encoded Stage, Ludl Electronic Products, Hawthorne, NY, USA), linear z-axis position encoder (Ludl Electronic Products, Hawthorne, NY, USA), ultra-high sensitivity monochrome electron multiplier CCD camera (1000x1000 pixels, C9100-02, Hamamatsu Photonics, Hamamatsu City, Japan) and controlling software (StereoInvestigator; MBF Bioscience, Magdeburg, Germany).

Mean gray value and surface area of Dnmt3a-immunoreactivity

The levels of Dnmt3a-immunoreactivity in the hippocampus were assessed by measuring the mean gray value of Dnmt3a-immunoreactivity (reflecting the intensity) of delineated regions occupied by Dnmt3a staining as described earlier by Chouliaras *et al* (Chouliaras et al., 2011). In brief, the cell layers of the hippocampal areas DG, cornu ammonis 3 (CA3) and CA1-2 were identified and 8 images were taken (4 for the DG, 2 for the CA1-2 and 2 for the CA3) in 4 selected (bregma levels) yielding 32 images per hippocampal area per animal. For the nucleus accumbens core, 4 images were taken in two hemispheres in four selected sections per bregma (-1.94, -1.7, -1.42 and -1.1) according to Franklin and Paxinos (Paxinos, 1996). Thus, a total of 32 images in the nucleus accumbens per animal were analyzed. Please note that measurement of the absolute levels of Dnmt3a was not the intention of the present analysis, and that the present measurements reflect relative differences between immunoreactivity between the different groups.

Density of intense Dnmt3a immunoreactive cells

Quantification of the number of intense Dnmt3a immunoreactive cells, i.e. type II Dnmt3a immunoreactive cells, was performed with a computer-based stereology workstation as previously described by Chouliaras *et al*. (Chouliaras et al., 2011). In brief, the DG was delineated at a 10x magnification and estimates of the total cell number were carried out by means of the optical fractionator with a 40x lens. Cell densities were estimated for 5 sections per animal by calculating the ratio of the number of cells counted in each section, divided by the volume of the DG in the corresponding section.

Statistical analysis

All data are presented as mean and standard error of means. Comparisons between three groups (control, susceptible, resilient/unsusceptible) were performed with one-way analysis of variance (ANOVA). Statistical significance was established at $p < 0.05$. When significant differences were found, means were compared with a Bonferroni post hoc test for pair-wise comparisons. The Pearson correlation coefficient was used to correlate behavioral outcomes with Dnmt3a type II cell densities for all animals, and sensitivity analysis was performed by repeating the correlation analysis within the group of defeated animals. All statistical calculations were performed using the Statistical Package for the Social Sciences, (SPSS 16, SPSS Inc., Chicago, IL, USA). Graphs were built in GraphPad Prism (Version 4, GraphPad Software, San Diego, CA, USA).

Results

Social approach-avoidance and behavior testing

The mean interaction ratio of defeated animals was 118.7 ± 7.9 . Of the 20 mice subjected to social defeat stress, two animals were excluded because of severe biting wounds. Eight animals (44%) displayed the susceptible phenotype with a significant overall difference in time spent in the interaction zone ($F_{2,25} = 30.80$, $p < 0.0001$) and the corner zone ($F_{2,25} = 12.19$, $p = 0.002$) in the social interaction test. Post-hoc testing revealed that susceptible mice spent significantly less time in the interaction zone compared to control and resilient animals (figure 1A, $p < 0.05$ vs. control, $p < 0.01$ vs. unsusceptible), and more time in the corners zones (figure 1B, $p < 0.01$ vs. control and unsusceptible). No statistically significant differences were found in the sucrose intake

test (figure 1C, $F_{2,23} < 1$, $p = \text{ns}$), elevated zero maze test (figure 1D, $F_{2,25} < 1$, $p = \text{ns}$) and forced swim test (figure 1E, $F_{2,25} = 2.518$, $p = \text{ns}$) for the three groups of mice.

Body weight and plasma corticosterone

Body weight of the animals was recorded during the complete time lapse of the experiment. During the defeat (experimental day 1 till day 10), body weight of defeated animals was significantly higher compared to control animals (day 5 $t_{22} = 2.266$, $p = 0.032$; day 10 $t_{26} = 2.66$, $p = 0.0132$) After the defeat (experimental day 11 till day 20), the higher body weight of defeated animals returned back to normal levels, comparable with the control animals, see also Figure 2A. Alterations in HPA axis reactivity were explored by drawing blood samples during basal, stressful and recovery conditions and determining plasma corticosterone concentrations (figure 2B). Plasma corticosterone concentrations were significantly influenced by time point (i.e., basal, stress, recovery) ($F_{2,72} = 93.11$; $p < 0.0001$) and by group (i.e. defeat, control) ($F_{1,72} = 5.148$; $p = 0.0263$), but not by time point \times group. When analysing the effect of social defeat stress at each time point, baseline and stress plasma corticosterone levels did not differ between defeated and control mice, but recovery plasma corticosterone level was significantly higher in defeated animals compared to controls ($t_{22} = 2.179$, $p = 0.04$).

Qualitative analysis of Dnmt3a staining

Two qualitatively distinct types of Dnmt3a immunoreactive cells were found, in line with our previous findings (Chouliaras et al., 2011). The first type of Dnmt3a immunoreactive cells showed moderate levels of immunoreactivity (type I cells) and was observed in cell nuclei throughout the brain. The second type of Dnmt3a positive cells showed high levels of immunoreactivity (type II cells) and was detected in specific

regions in the brain: the granular zone of the DG, the subventricular zone, the olfactory bulb, and distinct white matter tracks such as the rostral migratory stream and corpus callosum, but not in the nucleus accumbens (see figure 3). In the immunofluorescent double labeling, no colocalization was detected for Dnmt3a and Ki-67 (figure 4A) or GFAP (figure 4B). Double labeling was observed for DCX (figure 4C) and NeuN (figure 4D). Semi-quantitative analysis of double labeling revealed that approximately 25% of Dnmt3a type II cells were doublecortin positive and 60% were NeuN positive.

Quantitative analysis of Dnmt3a immunoreactivity in type I cells

Gray scale measurements were performed for immunohistochemical staining in the nucleus accumbens and hippocampus. No significant differences in Dnmt3a staining intensity were found in the nucleus accumbens (figure 5B, $F_{2,23} < 1$, $p = ns$) or the DG, CA3 or CA1-2 areas of hippocampus (figure 5A, DG $F_{2,24} = 1.114$, $p = ns$; CA3 $F_{2,24} < 1$, $p = ns$; CA1-2 $F_{2,24} < 1$, $p = ns$)

Quantitative analysis of Dnmt3a type II cell density

The density of Dnmt3a type II cells showed significant differences ($F_{2,24} = 4.761$, $p = 0.0181$) between the groups. Bonferroni post-hoc testing showed that resilient animals face a higher density of Dnmt3a type II cells compared to susceptible ($p = 0.019$) and control animals ($p = 0.05$; figure 5C).

Density of Dnmt3a type II cells correlates with behavior

The density of Dnmt3a type II cells in the DG showed statistically significant correlations with several behavioral phenotypes. It correlated with i) the time spent in the interaction zone (figure 6B, $R^2 = 0.2821$, $p = 0.023$) in the social approach-avoidance

test, ii) the sucrose intake (figure 6A, $R^2= 0.2209$, $p=0.012$), and iii) the distance moved in the elevated zero maze (figure 6D, $R^2= 0.3283$, $p=0.013$). No general locomotor differences were observed in the first trial of the social interaction test (data not shown). No significant correlation was found for the time spent in corner zones in the social interaction test, time spent in closed or open arms in the elevated zero maze and duration of immobility or latency to immobility in the forced swim test. When repeating the analysis within defeated animals instead of all animals, the correlation between time spent in the interaction zone (in the social approach-avoidance test) and the distance moved in the elevated zero maze with the Dnmt3a cell density remained statistically significantly.

Discussion

This is the first study linking Dnmt3a protein expression in the mouse hippocampus to social defeat stress. Our present study indicates that unsusceptible or resilient animals have a higher density of Dnmt3a type II cells, reflecting newly differentiated neurons, in the hippocampal DG than susceptible animals or control animals not exposed to social defeat. Our results furthermore show that the density of Dnmt3a type II cells in the hippocampal DG correlates with behavioral expression of resilience, i.e. increased social approach, a higher hedonic response in the sucrose intake test and more exploration in the elevated zero maze suggesting lower anxiety.

Dnmt3a is involved in neurogenesis

Our findings that significant proportions of the highly intense Dnmt3a immunoreactive cells in the brain are also immunoreactive for DCX and NeuN indicate that these cells are recently born neurons, which is in line with earlier work by Chouliaras et al.

showing colocalization of these cells with BrdU (Chouliaras et al., 2011). Feng et al. showed that Dnmt3a is present in both embryonic and postnatal central nervous system (CNS) tissues (Feng et al., 2005), with the highest levels of Dnmt3a expression were found in the embryonic mouse brain. In newborn animals, peak levels of Dnmt3a were found throughout the brain, which decreased to relatively low levels in 4-months old animals (Feng et al., 2005). Thus in the adult mouse brain, neurons express moderate levels of Dnmt3a. The authors therefore stated that Dnmt3a is particularly important in dynamic regulation of DNA methylation that is critical for prenatal neurogenesis and postnatal regulation of differentiation (Feng et al., 2005). In addition to these findings, Chouliaras et al. reported increased Dnmt3a immunoreactivity in the hippocampus of 24-months old mice compared to 12-months old mice (Chouliaras et al., 2011). However, in this study two different types of Dnmt3a positive cells are described. Type I cells show moderate Dnmt3a staining intensity and are widely distributed all over the brain. Type II cells display highly intense Dnmt3a staining and reflect a small subpopulation of hippocampal cells. It seems plausible that high levels of type II cells are present in the young brain, and that their number decreases as the animal grows older. As a result, it is possible that postnatal brain maturation involves the conversion of Dnmt3a type II cells to type I cells. This means that Dnmt3a expression in single neural cells decreases as the cell matures. We propose that during neuronal differentiation and maturation high levels of Dnmt3a are expressed (seen as intensely stained type II cells), which decrease to moderate levels in mature neurons. Along similar lines, Dnmt3a type II cells are only present in brain regions where adult neurogenesis takes place. Indeed, in the present study, we identified the highly intense stained type II cells in the subgranular zone of the hippocampal DG, the subventricular zone, olfactory bulb and rostral migratory stream, the main brain regions involved in

adult neurogenesis. To further characterize the Dnmt3a type II cells and to verify that they are in fact maturing neurons, we performed immunofluorescent double labeling of Dnmt3a with markers for different stages of neuronal development: GFAP (early neuronal progenitor cells), DCX (newly differentiated, immature neurons), NeuN (mature neurons) and Ki67 (marker for proliferating cells). While Chouliaras *et al.* found colocalization of Dnmt3a and BrdU immunoreactivity, suggesting that type II Dnmt3a cells had recently undergone proliferation (Chouliaras *et al.*, 2011), we did not find colocalization with either the Ki67 proliferation marker or with the early progenitor marker GFAP. Thus, although Dnmt3a type II cells are recently proliferated cells, they were not proliferating at the moment of analysis and had lost their progenitor status. However, high numbers of Dnmt3a type II cells showed double labeling with DCX or NeuN, indicating that they are maturing neurons. These data suggest that the Dnmt3a type II cells are newly born, maturing neurons and that they may serve as a marker for neurogenic processes. This also suggests that Dnmt3a plays a critical role in adult neurogenesis, extending previous studies showing that Dnmt3a is expressed in postnatal neural stem cells and is required for embryonic and adult neurogenesis (Wu *et al.*, 2010). The crucial role of Dnmt3a in neurogenesis is further highlighted by the fact that Dnmt3a knockout mice, while appearing grossly normal at birth, die prematurely because of severe abnormalities in postnatal brain development (Okano *et al.*, 1999), most likely due to impaired neuronal differentiation in neurogenic zones such as the subventricular and subgranular zone rather than by aberrant neural stem cell proliferation or survival (Wu *et al.*, 2010). This might confirm the regulative role of Dnmt3a in neural cell differentiation rather than cell proliferation and survival, as Wu *et al.* previously suggested (Wu *et al.*, 2010).

Stress susceptibility and adult hippocampal neurogenesis

The social defeat stress model elicits differential responses in social avoidance behavior which has previously been conceptualized in a dichotomous variable of susceptible versus unsusceptible animals, with approximately half of the animals displaying an unsusceptible or resilient phenotype (Krishnan et al., 2007). The present study is the first study to analyze the effects of chronic social defeat stress on neuronal proliferation and differentiation, while taking different expressions of social avoidance behavior into account. In this study, Dnmt3a type II cell density in the DG was used as a marker for neurogenic events, as we showed that these cells are recently differentiated, newborn neurons. We showed that social defeat stress does not influence Dnmt3a type II cell density in the hippocampal DG of susceptible animals. However, in resilient animals, type II cell density was increased compared to both control and susceptible animals, suggesting that hippocampal neurogenesis is enhanced in resilient mice. A considerable number of studies have demonstrated that psychological stress is associated with structural and functional changes in neurogenic regions of the adult brain. Previous studies have shown that hippocampal cell proliferation is transiently suppressed after exposure to social defeat stress (Lagace et al.). However, it seems that proliferating cells might habituate during chronic exposure to stress, as proliferation is only slightly decreased after chronic social defeat (Czeh et al., 2002). It has further been suggested that stress exposure induces neural progenitor cells to acquire a quiescent state (Fitzsimons et al., 2013). One limitation of these studies is that they did not take differential susceptibility to the effects of chronic stress and behavioral phenotypes into account.

Our study is the first to investigate the effects of differential susceptibility to social defeat stress on adult neurogenesis. We found increased numbers of Dnmt3a type II, newly born neurons in resilient animals compared to susceptible and control mice. Moreover, the density of the Dnmt3a type II cells in the hippocampal DG was positively correlated with the behavioral outcome measures of social interaction, hedonic responsiveness and negatively correlated with anxiety-like behavior, suggesting that an adaptive response to social defeat stress requires the ability to upregulate neurogenesis possibly through mediating effects of Dnmt3a. It should be noted here that we cannot rule out that baseline differences in the epigenotype and neurogenic potential, e.g. expressed as pre-defeat-existing variations in the generation of Dnmt3a type II cells, might partially determine the subsequent response to the chronic exposure to social defeat stress. However, more research is needed to determine whether changes in Dnmt3a type II cell density are acquired during or immediately after social defeat or that baseline Dnmt3a levels determine predisposition to stress susceptibility. It will furthermore be interesting to investigate DNA methylation profiles in Dnmt3a type II cells, for example using laser capture dissected or fluorescence-assisted cell sorting techniques.

Thus, our findings suggest that the altered number of Dnmt3a type II cells may also result in differential DNA methylation in the hippocampus, which by may modulate the response to severe adversities during life. Indeed, evidence accumulates that experience-driven alterations in DNA methylation during early life can regulate the HPA axis (McGowan et al., 2009) and hippocampal plasticity (Roth et al., 2011) and can have persistent influences on gene expression.

DNA methylation and traumatic stress

Although epigenetic studies on traumatic stress exposure in adult humans have been limited thus far, evidence suggests that exposure to severe stressors in adult life of humans is connected with altered DNA methylation profiles. For example, Ressler and coworkers observed PTSD-associated differential methylation in the gene ADCYAP1R1 in peripheral blood (Ressler et al., 2011), while other cross-sectional analyses have identified differential DNA methylation profiles using screening of methylation arrays. Uddin *et al.* and Smith et al. observed differential DNA methylation in PTSD, particularly in immune-related genes (Smith et al., 2011; Uddin et al., 2010). Together with our rodent findings showing a relation between Dnmt3a, i.e. one of the main enzymes responsible for DNA methylation, and differential susceptibility to chronic severe stress, it thus seems very attractive to further investigate the role of DNA methylation in susceptibility to severe stressors in humans. One could envision for example longitudinal studies on the impact of trauma exposure on DNA methylation in prospectively followed military cohorts such as the U.S. Marine Resiliency Study and the Dutch PRISMO study (ADD LATER refs to the manuscripts in the special issue on these cohorts).

In conclusion, we have demonstrated that Dnmt3a is expressed throughout the entire mouse brain. However, high intensity Dnmt3a cells (type II cells) are found only in neurogenic zones in the adult mouse brain and represent newly generated neurons. Animals unsusceptible to social defeat stress showed higher Dnmt3a type II cell densities in the hippocampal DG, which correlated with social approach, hedonic and anxiolytic behavior. Double labeling studies of these Dnmt3a type II cells revealed that they are newly generated, immature and mature neurons. Together with data in the

literature, these data suggest that susceptibility to the effects of chronic social defeat stress in mice is moderated or even mediated by Dnmt3a-related regulation of adult neurogenesis.

Acknowledgements

The authors would like to thank Dr. Herbert E. Covington III for his very helpful suggestions to set up the social defeat paradigm and M. Philippens for the technical assistance. B.P.F. Rutten has received funds via a VENI Award from the Netherlands Organisation for Scientific Research (NWO, grant number 916.11.086).

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Figure legends

Figure 1 Behavioral consequences of exposure to 10 days of chronic social defeat stress. **A** Time spent in the interaction zone when the CD1 target was present. **B** Time spent in the corner zone when the CD1 target was present. **C** Sucrose preference during 24h. Sucrose preference was calculated as the ratio of the amount of sucrose solution divided by the normal water intake in 24h. **D** Time spent in the closed zones of the EZM. **E** Duration of immobility in the FST. Bars represent mean and SEM. ** $p < 0.05$, *** $p < 0.01$. Control n=10, susceptible n=9, resilient n=9.

Figure 2 A Body weight of animals during the experiment. Social defeat stress was performed between experimental day 1 and 10. Behavioral testing was performed between experimental day 11 and 20. Control n=10, defeat n=18. **B** Plasma corticosterone levels at baseline ("baseline"), after 5 minutes of forced swim stress ("stress") and after 1h of rest ("recovery"). Control n=10, defeat n=18.

Figure 3 Dnmt3a immunoreactivity in brain of a control animal. **A** Rostral migratory stream **B** Olfactory bulb **C** Subventricular zone **D** Dentate gyrus. Scalebar represents 60 μm .

Figure 4 Representative merged images of the hippocampal dentate gyrus of a control animal showing fluorescent labeling of Dnmt3a (red) and cellular markers (green) **A** Ki-67 **B** GFAP **C** DCX **D** NeuN. Red arrows indicate Dnmt3a type II cells, green arrows indicate Ki-67, GFAP, DCX or NeuN positive cells respectively and yellow arrows indicate double positive cells. Scalebar represents 50 μm .

Figure 5 Quantitative analysis of Dnmt3a immunoreactivity in the hippocampus and nucleus accumbens (NAc). **A** Mean gray value measurements multiplied by surface area of Dnmt3a type I immunoreactivity in the CA1-2, CA3 and DG of the hippocampus. **B** Mean gray value measurements multiplied by surface area of Dnmt3a type I cell immunoreactivity in the core of the NAc. **C** Density of Dnmt3a type II cells in the dentate gyrus. Bars represent mean and SEM. (*) $p=0.05$, ** $p<0.05$. Control n=9, susceptible n=9, resilient n=9.

Figure 6 Pearson correlation of Dnmt3a type II cell density in the DG and behavioral outcome measures in all mice. **A** Time spent in interaction zone when the target is present. **B** Total sucrose intake in the SIT. **C** Distance moved in the EZM.

Reply to the Reviewers' comments:

Dear editor, please find below a point by point reply to the comments from the reviewers.

Reviewer #1: In this manuscript the authors study the effects of 10 days social defeat stress on behavior and Dnmt3a expression in relation to neurogenesis in the mouse hippocampus. The authors report that social defeat stress yields resilient animals and susceptible animals as tested in the social approach avoidance test but did not affect Dnmt3a expression. However, a correlation was found between Dnmt3a expression and behavior tested in the social avoidance task, sucrose intake and distance moved in the elevated zero maze. This is an interesting observation and interesting manuscript that tries to link Dnmt3a, neurogenesis and behavior after social stress.

1) The effects of chronic social stress are poorly described. Was body weight measured. Did the authors measure plasma corticosterone levels, adrenal weight or other parameters which are indicative of chronic stress?

Data on body weight and plasma corticosterone levels are added in the methods and results section. Figure 2 is added.

2) The authors want to link changes in Dnmt3a to neurogenesis and behaviour: Ki67 and DCX were used to examine the fate of cells. The quality of the discussion and paper would increase if effects on neurogenesis (Ki67/DCX) were included. Was social stress effective in reducing neurogenesis? Are there effects between susceptible/resilient animals?

Unfortunately, we have no more brain tissue available to quantify the expression of Ki67 and DCX. However, this will certainly be included in future studies.

3) Why were animals judged as resilient/susceptible as currently described? This should be explained and discussed?

We included this in the method section.

Why were no effects seen in sucrose preference test?

Earlier studies made use of the two-bottle choice test for sucrose preference. In this study, the sucrose intake test was used. This might explain why we were not able to find an effect in sucrose preference.

4) What do levels of Dnmt3a - as determined using immunocytochemistry- actually tell. This should be extended in the discussion.

This is included in the discussion.

5) I feel that the relationship between neurogenesis and depression should be introduced / discussed with more care.

We adapted this in the introduction and discussion.

Reviewer #4: This was a succinct study of the association between Dnmt3a immunoreactivity and susceptibility to social defeat stress. Mice that were resilient to defeat stress show higher Dnmt3 expression compared to controls (non defeat) and susceptible groups. There were some weak associations of dnmt3 immunoreactivity with social behavior, sucrose preference and locomotor activity.

Primary concern: The abstract concludes that "DNA methylation via Dnmt3 in the hippocampus coregulates adaptivity of the behavioral response to chronic social defeat stress". I think this is too much of a stretch from the actual data. It seems possible for example that the increase in Dnmt3 expression could simply be due to more neurogenesis in the resilient group, and the increase in Dnmt3 expression is just an epiphenomenon of a larger number of these cell types. These data are all correlational, thus actions of Dnmt3 on this phenotype cannot be inferred. This conclusion needs to be modified to fit better with the correlational not causal nature of these data.

We fully agree with the reviewer, and have adapted the conclusion in the abstract

Other comments:

Suggest removing all specific F values lower than the theoretical limit (1) be stated as $F < 1$, and no p value be given unless significant (i.e. p value > 0.05 be restated as ns). p values higher than the cut off are meaningless.

We changed this in the figure legends and in the results section.

Figure 5 correlations. It seems that the more critical question is WITHIN the social defeated animals, is dnmt3 expression associated with anxiety behaviors tested. If all mice are used in the correlation then it is basically just recapitulating the ANOVA as described in figure 4 across the 3 separate groups. By having all 3 groups in the correlation you can't distinguish between baseline differences in Dnmt3 expression (controls) and effects of social defeat vs. response to social defeat ACROSS the defeated animals.

This is further explained in the methods and results sections.

Is there an alternative interpretation that the "resilient" animals are actually cognitively impaired in some way? Why are they not avoiding the aggressor mouse after so many defeats? This could be interpreted as a memory problem just as easily as "resilience". Could neurogenesis be interfering with this memory? The way the

animals in the social defeat test have previously been validated as "susceptible" vs "resilient" is that they also differ in other behavioral protocols (forced swim, sucrose preference etc), but this was not the case in this cohort, so it is hard to say what categorizes these mice exactly if it is not anxiety/depression phenotype.

In this cohort, no cognitive testing was performed. However, earlier studies have shown that social defeat has a negative effect on cognitive performance (e.g. (Venzala et al., 2012; Wang et al., 2011; Yu et al., 2011)), but in these studies, no distinction between susceptible and resilient animals was made.

How many measures did not correlate with dnmt3 expression (i.e. time immobile, time spent in closed areas of the zero maze)? It would be better to show all the correlations that were done to get a better picture of what does and does not correlate with these expression levels in the defeated mice.

Other correlations are described in the results section.

Please state the N's in the figure legends

This has been added to the figure legends.

Suggest cutting the discussion down a bit, there is a lot of discussion that is gets pretty far from the specific data presented (e.g. first section of paragraph 2 of discussion is a lot of review of developmental effects of dnmt3).

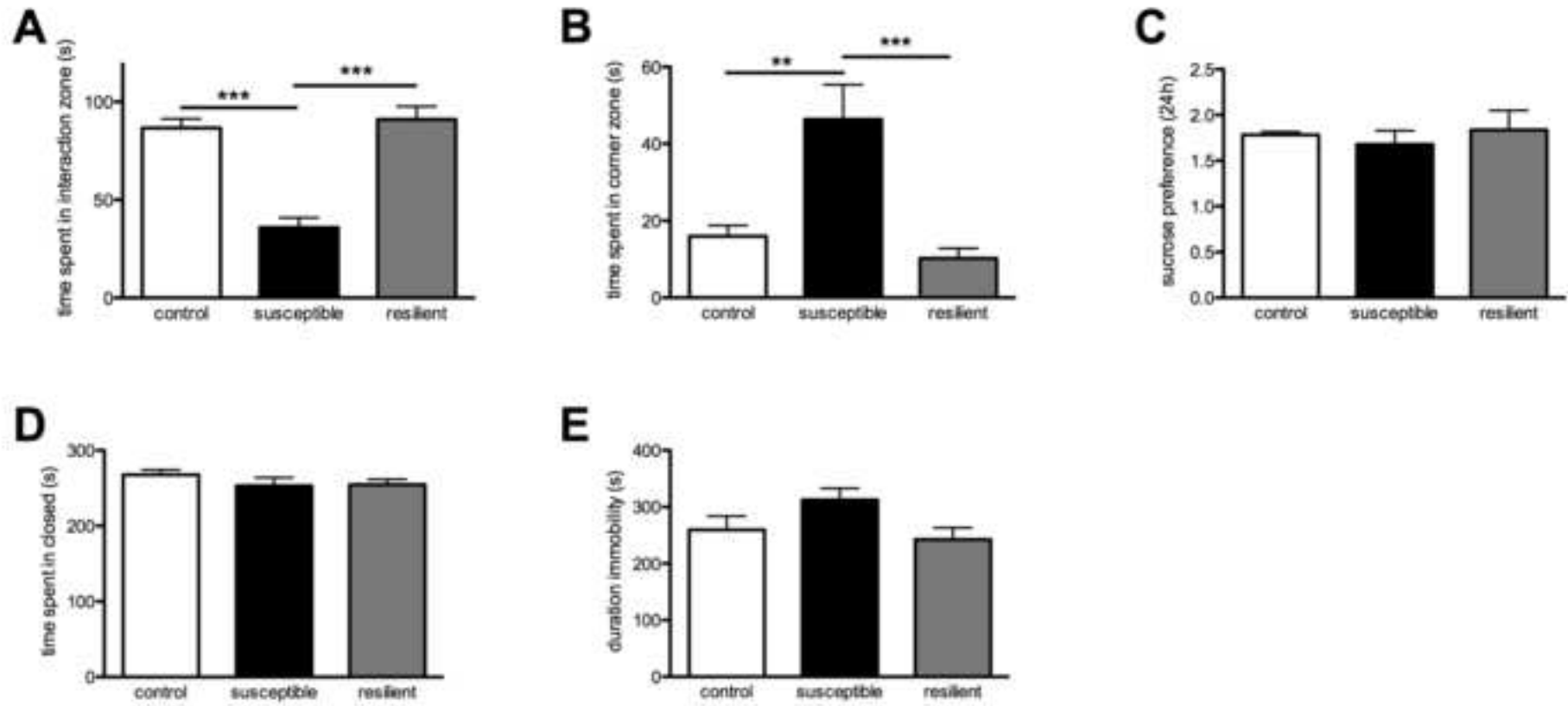
We adapted this in the discussion.

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Figure 1

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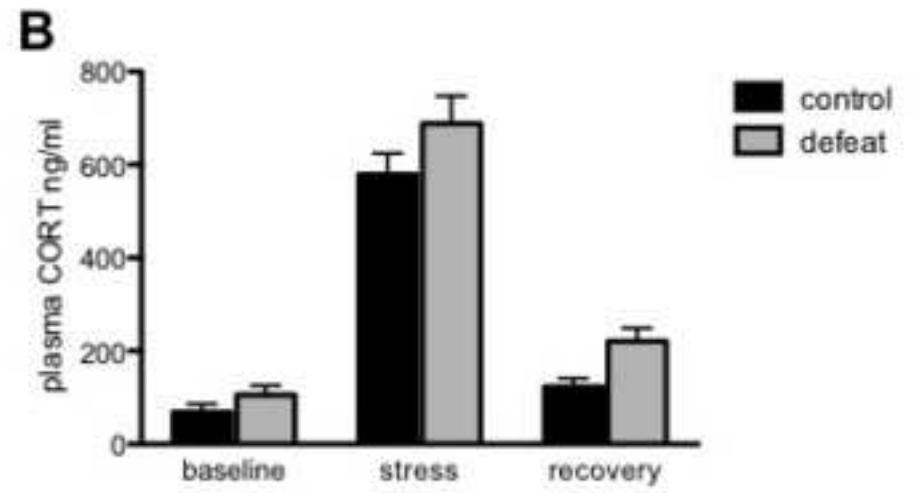
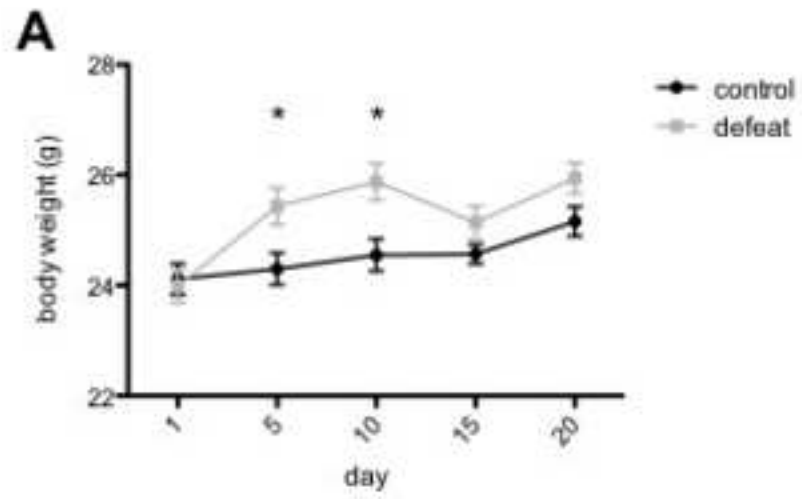


Figure3
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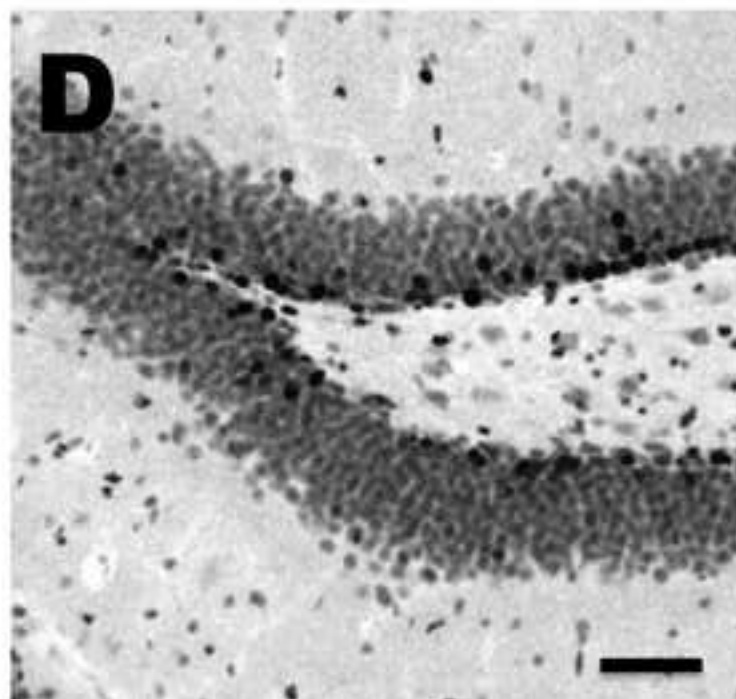
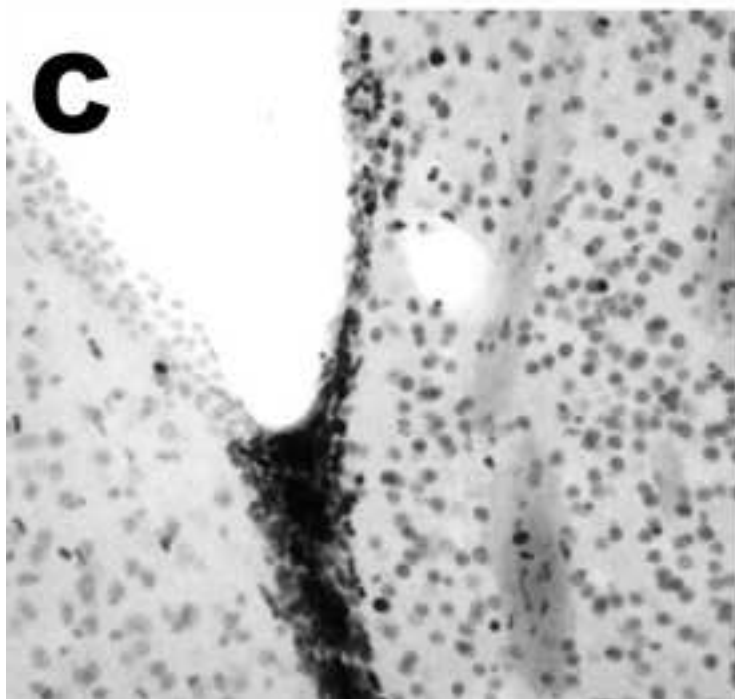
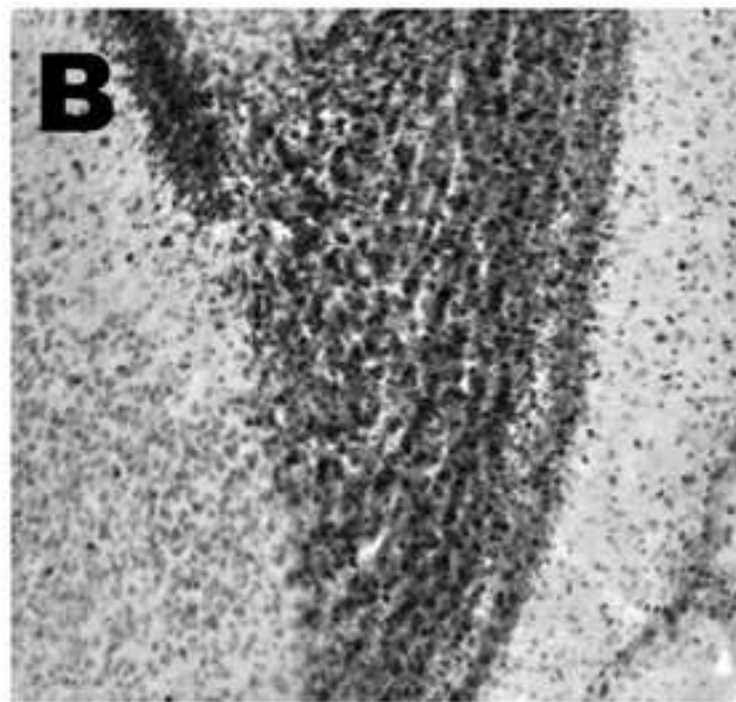
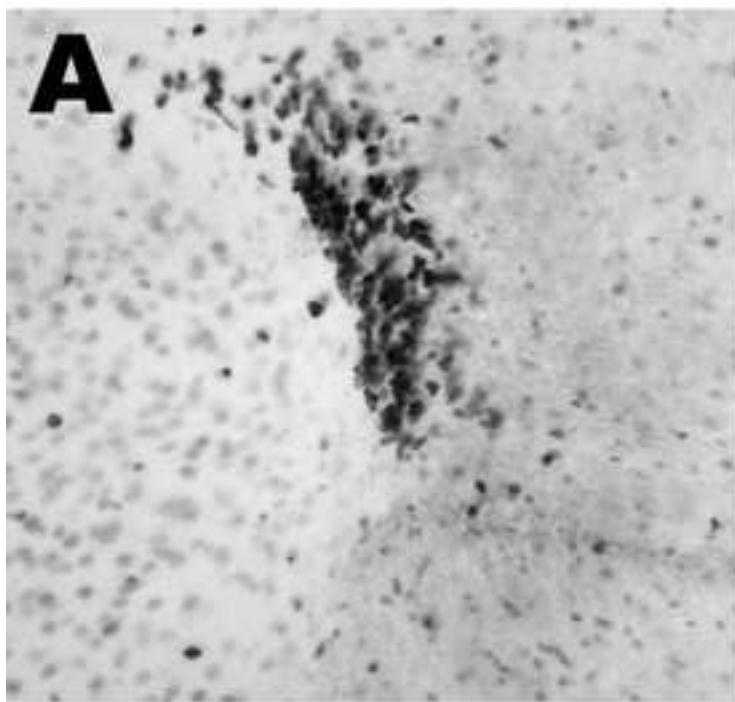


Figure4
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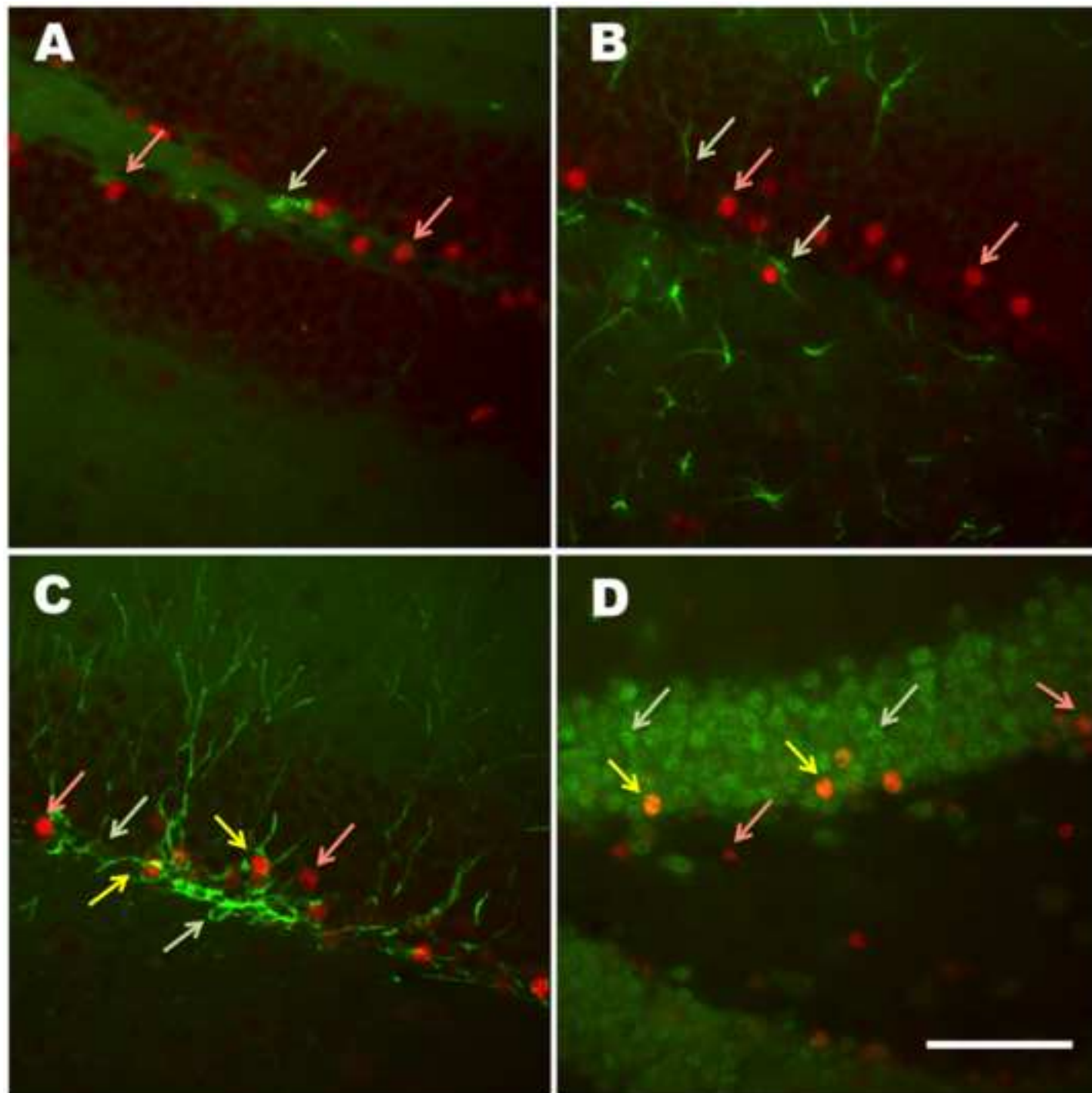


Figure5

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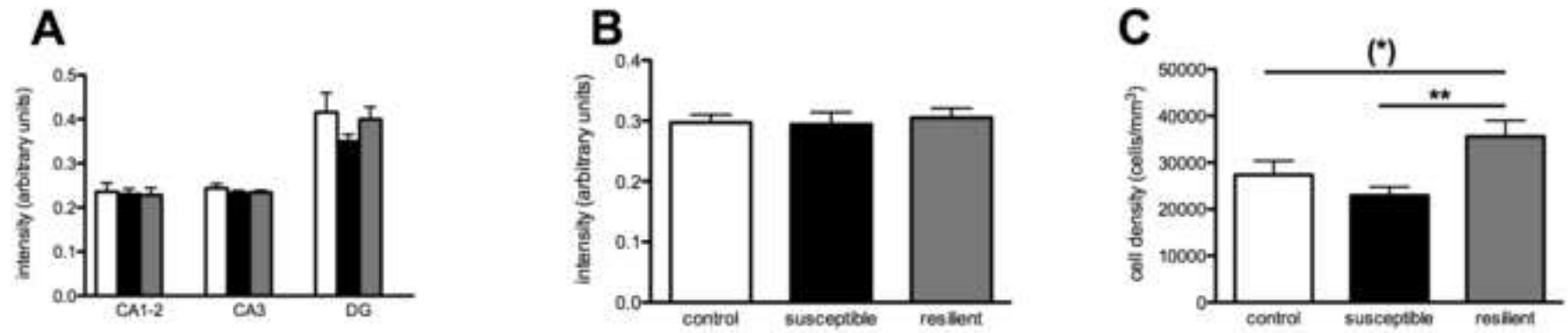
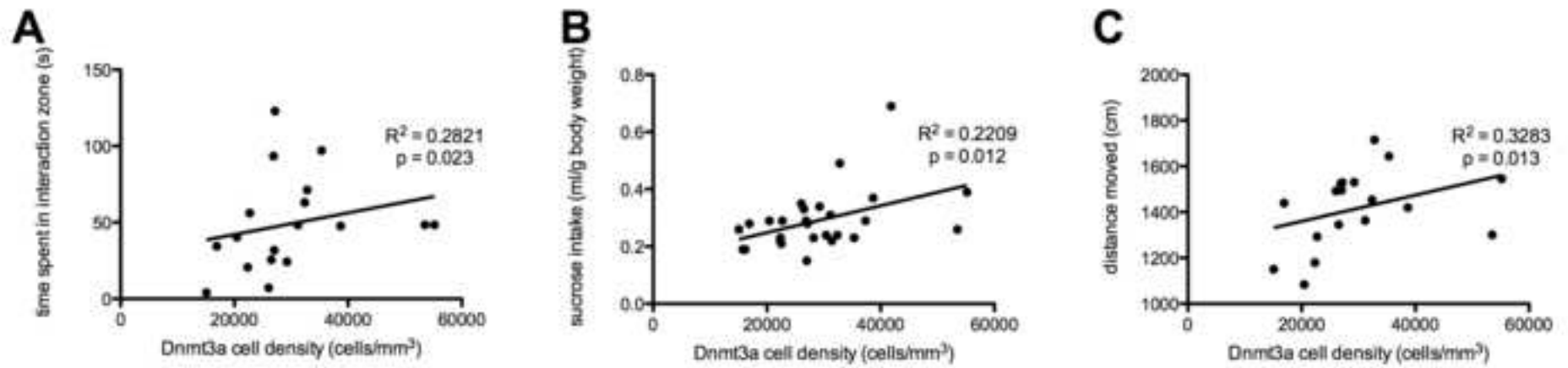


Figure6

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Dnmt3a type II cells are found in the brain regions involved in adult neurogenesis

High numbers of Dnmt3a type II cells showed double labeling with DCX or NeuN

Type II cell density was increased in resilient animals compared to control and susceptible animals

Type II cells density correlates with anti-depressive behavior