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# Chronic Retinoic Acid Treatment Suppresses Adult Hippocampal Neurogenesis, in Close Correlation With Depressive-Like Behavior

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**ABSTRACT:** Clinical studies have highlighted an association between retinoid treatment and depressive symptoms. As we had shown before that chronic application of all-trans retinoic acid (RA) potently activated the hypothalamus-pituitary-adrenal (HPA) stress axis, we here questioned whether RA also induced changes in adult hippocampal neurogenesis, a form of structural plasticity sensitive to stress and implicated in aspects of depression and hippocampal function. RA was applied intracerebroventricularly (i.c.v.) to adult rats for 19 days after which animals were subjected to tests for depressive-like behavior (sucrose preference) and spatial learning and memory (water maze) performance. On day 27, adult hippocampal neurogenesis and astrogliosis was quantified using BrdU (newborn cell survival), PCNA (proliferation), doublecortin (DCX; neuronal differentiation), and GFAP (astrocytes) as markers. RA was found to increase retinoic acid receptor-α (RAR-α) protein expression in the hippocampus, suggesting an activation of RA-induced signaling mechanisms. RA further potently suppressed cell proliferation, newborn cell survival as well as neurogenesis, but not astrogliosis. These structural plasticity changes were significantly correlated with scores for anhedonia, a core symptom of depression, but not with water maze performance. Our results suggest that RA-induced impairments in hippocampal neurogenesis correlate with depression-like symptoms but not with spatial learning and memory in this design. Thus, manipulations aimed to enhance neurogenesis may help ameliorate emotional aspects of RA-associated mood disorders. © 2016 Wiley Periodicals, Inc.

**KEY WORDS:** all-trans retinoic acid; neurogenesis; depression; Morris water maze; learning and memory

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## INTRODUCTION

The retinoid family consists of vitamin A and its metabolites 13-cis-retinoic acid (13-cis-RA) and all-trans retinoic acid (ATRA, RA). They are crucial contributors to the developing (Durston et al., 1989; Janesick et al., 2015) and adult brain (Chiang et al., 1998; Misner et al., 2001; Lane and Bailey, 2005; Nomoto et al., 2012; Bonhomme et al., 2014). Retinoid levels in humans can be elevated through, e.g. dietary intake of vitamin A, or from, e.g. accutane treatment for acne. Elevated retinoid levels were recently shown to confer a risk factor for mood disorders (Bremner et al., 2012), a notion further strengthened by changes recently found in the endogenous retinoid signaling pathways in brain tissue of depressed patients (Chen et al., 2009; Qi et al., 2015).

Both RA and its ligand-activated nuclear retinoic acid receptors (RARs) are particularly abundantly present in the hippocampal dentate gyrus (DG) of adult mice and humans (Misner et al., 2001; Sakai et al., 2004; Fragoso et al., 2012; Goodman et al., 2012). The adult DG is a unique brain region where stem cells reside that continue to undergo division and proliferation into adulthood. After their subsequent migration into the granular cell layer (GCL), these stem cells differentiate into fully functional new neurons, a process referred to as adult neurogenesis. This important form of structural plasticity occurs in the brains of adult mammals, including humans (Eriksson et al., 1998; Bergmann et al., 2015; Ernst and Frisen, 2015) and is well regulated by various (gene-)environmental (Koehl, 2015) and hormonal factors like stress or exercise, that inhibit and

stimulate neurogenesis, respectively (Lucassen et al., 2010; Lucassen et al., 2015). Functionally, the adult-born neurons have been implicated in spatial information processing and in aspects of emotion (Snyder et al., 2005; Dupret et al., 2007; Kee et al., 2007; Dupret et al., 2008; Kempermann, 2008; Jessberger et al., 2009; Revest et al., 2009; Sahay et al., 2011; Tronel et al., 2015). Neurogenesis has further been implicated in the etiology of major depression and stress-related disorders (Vogel, 2000; Kempermann and Kronenberg, 2003; Santarelli et al., 2003; Lucassen et al., 2010; Fitzsimons et al., 2013; Aimone et al., 2014). For recent reviews, see (Lucassen et al., 2015; McEwen et al., 2016) and references therein.

Given our previous findings that chronic RA application potently activated the main neuroendocrine stress axis, that is, the hypothalamus-pituitary-adrenal (HPA)-axis (Hu et al., 2013), we expected neurogenesis to be altered as well. Therefore, we here test the hypothesis that RA application may alter hippocampal neurogenesis. To this end, we studied neuronal and astrocytic changes, e.g. cell proliferation, newborn cell survival, and neurogenesis. We subsequently studied whether changes in neurogenesis correlated with alterations in hippocampal spatial learning and memory, as measured with the Morris water maze (MWM), or with depressive-like symptoms like (an)hedonia, as measured with the sucrose preference test.

# EXPERIMENTAL PROCEDURES

## Animals

All animal procedures presented were approved by the animal ethical committee of University of Science and Technology of China and in accordance with the guidelines for care and use of laboratory animals of the National Institutes of Health. A total of 24 male adult Sprague–Dawley rats were randomly assigned into a vehicle (VEH) or ATRA group (RA) ($n = 12$ each). Half of them ($n = 6$ for each VEH and RA group) were used for behavior and immunohistochemistry. The other half of the animals were used for western blot analysis of their hippocampi.

Before the start of experiment, all animals (8–10 weeks of age, body weights of 300–350 g at the start of the experiment) were housed in pairs per cage under controlled conditions of a 12/12 h light/dark cycle with standard diet and water ad libitum. A 12 h light cycle was present from 07:00 am to 07:00 pm. The animals were allowed to adapt to the experimental setting for one week during which they were handled daily. Temperature and humidity were kept constantly at 20–22°C and 50–55%, respectively.

## ICV Surgery

Intracerebroventricular (i.c.v.) surgery was performed at least 7 days before drug administration started, to allow for postoperative recovery (Liu et al., 2012). All surgery was performed

under 30 mg/kg sodium pentobarbital (Sigma-Aldrich, St. Louis, MO) anesthesia (i.p.). After surgery, animals were housed singly to allow complete freedom of movement in each cage. With a standard Kopf stereotaxic apparatus, a unilateral i.c.v. guide cannula was placed into the lateral cerebral ventricle. The coordinates were: anterior posterior (AP), 1.0 mm; lateral, 1.5 mm; and 3.5 mm ventral from dura. Cranioplastic cement was used to secure the i.c.v. cannula and jugular and carotid outlets to four stainless-steel screws inserted into the skull. Location of the probe was checked afterwards in thionin-stained 40-μm cryostat sections. Only animals with a correct probe placement were used for further data analysis.

## Bromodeoxyuridine Labeling

To study survival of newborn cells, half of the animals ($n = 6$ for each VEH and RA group) received 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) intraperitoneally (200 mg/kg body weight, dissolved in 0.9% saline) (Hu et al., 2012) at 12:00 pm on the first day of chronic drug administration. This single injection paradigm aimed to ensure the delay between the first drug injection (of RA or VEH) and BrdU incorporation was comparable for all animals.

## Drug Administration

Drugs were given daily between 09:00 and 09:30 am. Drug administration was performed manually by using the guide cannula to insert the infusion probe cannula connected to a Hamilton syringe. During injections, rats were loosely held and disturbance of the animal was minimal. Injections were performed manually within 30 s. Thereafter, the infusion needle was kept in place for 1 min.

The RA group received 20 μg ATRA (Sigma-Aldrich, St. Louis, MO) dissolved in 2 μl 1:1 v/v DMSO: saline avoiding light (Hu et al., 2013) once daily for 19 consecutive days. The VEH group received a daily 2 μl 1:1 v/v DMSO: saline accordingly for 19 consecutive days. The scheme of the whole experimental design is shown in Fig. 1.

## Behavioral Tests

### Sucrose preference test

As indicated in Fig. 1, sucrose preference and MWM performance were tested in the same 12 rats ($n = 6$ per group) that had received BrdU injection. At the end of drug treatment on day 19, between 9:30 am and 12:00 pm the animals were trained to drink 1% (w/v) sucrose solution for 24 h adaptation. The next day (at day 20), they were given free access to two bottles containing normal water or sucrose solution. To avoid left/right preference, bottle order placement was alternated for each rat during both the adaptation and testing periods. Bottles were weighed at the beginning and end of the 24 h testing period. The percentage of sucrose solution from the total liquid consumed during the 24 h test phase was taken as a measure for anhedonia, a core symptom of depressive-like behavior (Loas, 1996).
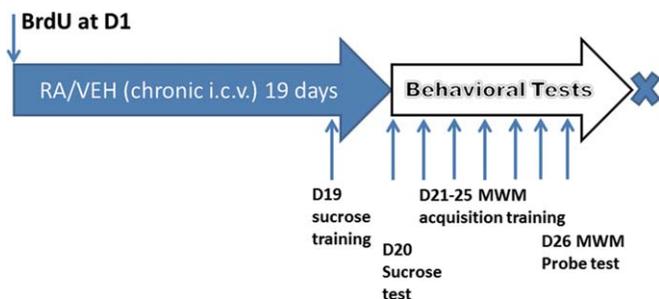
FIGURE 1. Schematic representation of the experimental design. 5-bromo-2-deoxyuridine (BrdU) was intraperitoneally injected on the first day of chronic drug administration. Animals received 2 µl all-trans retinoid acid (RA, dissolved in 1:1 v/v DMSO: saline) avoiding light or 2 µl vehicle (VEH, 1:1 v/v DMSO: saline) once daily for 19 consecutive days followed by a series of 7-day behavioral test. Sucrose training starts at the end of drug treatment on day 19 and lasts 24 h. On day 20, the percentage of sucrose consumption was measured during the testing 24 h. MWM test started at day 21. The acquisition training session lasted 5 days, while the probe test was done at day 26. Animals were sacrificed at day 27. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## Morris water maze test

The MWM test was used to test spatial learning and memory function as described before (Meng et al., 2011), starting on day 21 (shown in Fig. 1). Tests were performed between 9:30 am and 12:00 pm in a separate room. Before each test, animals were allowed 20 min adaptation in the test room.

The water maze (2.1 m in diameter) was filled with opaque water (22°C) with nontoxic paint added and surrounded by complex cues on the walls. The escape platform (12 cm in diameter) was placed in the center of the designated quadrant with its top located 1 cm below the water surface. During acquisition training, rats received 4 trials per day for 5 subsequent days. The starting point of each training trial was chosen at different locations according to literature (Vorhees and Williams, 2006). At the start of each trial, rats were placed in the water facing the wall of the pool. Rats were allowed 60 s to find the platform, and then to stay there for 30 s before they were taken off the platform, and returned to their home cages. If they failed to find the platform within 60 s, they were guided to the platform and allowed to stay there for 30 s. To assess reference memory at the end of the learning trials, a probe test was conducted on day 6 (at day 26), 24 h after the last acquisition training. During the probe test, the platform was removed from the pool and rats were allowed to swim for 90 s. Animals were placed in a novel start position in the maze, facing the wall of the pool to ensure its spatial preference is a reflection of the memory of the goal location rather than a specific swim path. All trials were recorded by a video camera coupled to a computer. The latency to find the platform in the acquisition trials and the time duration spent in the target quadrant during the probe test was analyzed by EthoVision software (Noldus, Wageningen, The Netherlands).

## Brain Tissue Preparation

On the last day following the MWM tests (day 27), all animals were anesthetized in the morning (09:00–11:00 am) with 30 mg/kg pentobarbital sodium salt (Sigma-Aldrich, St. Louis, MO). All efforts were made to minimize their suffering. Twelve rats ($n = 6$ for each VEH and RA group) were perfused transcardially with saline followed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 M phosphate buffer (PB, pH 7.4). To prevent pressure artefacts, brains were additionally post-fixed overnight in the skull at 4°C. After washing, the brains were cryoprotected in 30% sucrose in PB. Frozen sections (40 µm thick) were cut and collected in PB with sodium azide (Hu et al., 2012).

The other 12 rats ($n = 6$ for each VEH and RA group) were quickly decapitated the next day after chronic drug application (at day 20). Hippocampi were dissected and quickly frozen in liquid nitrogen and stored at −80°C until use.

## Immunohistochemistry and Image Acquisition

Immunohistochemistry was performed with anti-BrdU (mouse, 1:100; sc-32323, Santa Cruz, CA), anti-PCNA (mouse, 1:100; sc-56, Santa Cruz, CA), anti-DCX (goat, 1:100; sc-8066, Santa Cruz, CA), anti-GFAP (rabbit, 1: 10,000; z0334, DAKO, Denmark), and anti-RAR-α (rabbit, 1:100; ab85058, Abcam, Cambridge, UK). Signal amplification was performed with biotinylated goat-anti-mouse (BA-9200), horse-anti-goat (BA-9500), and goat-anti-rabbit (BA-1000) IgG's (all 1:200; Vector Laboratories, Burlingame, CA), respectively, followed by incubation with avidin-biotin complex (1:800; Vector Laboratories, CA). Chromogen development was performed with diaminobenzidine (DAB, 20 mg/100 mL Tris buffer, pH 7.55, 0.01% $H_2O_2$). For BrdU/GFAP double-labeling, protocols were combined (Oomen et al., 2009). After first developing BrdU immunoreactive signal with nickel ammonium sulphate (0.02%) added to the DAB (20 mg/100 mL Tris buffer, pH 7.55, 0.01% $H_2O_2$), sections were subsequently incubated in anti-GFAP antibody (rabbit, 1: 10,000; z0334, DAKO, Denmark) overnight. The next day, the sections were incubated with biotinylated goat-anti-rabbit IgG's (BA-1000, Vector, CA) and avidin-biotin complex (1:800; Vector Laboratories, CA), followed by chromogen development performed with DAB (20 mg/100 mL Tris buffer, pH 7.55, 0.01% $H_2O_2$) alone.

Photographs were made with a Nikon E800u microscope (Nikon, Tokyo, Japan) equipped with a Canon Digital camera (PowerShotS 40, Tokyo, Japan).

## Quantification of BrdU, PCNA, DCX, and GFAP

Stereological quantification of the number of DCX⁺ and GFAP⁺ cells in the DG was performed unilaterally by systematic random sampling in every 8th hippocampal section using the StereoInvestigator system (Microbright field, Germany) according to stereological principles (Schmitz and Hof, 2005). Because of the relatively low numbers and the occurrence of

clusters, BrdU$^+$ and PCNA$^+$ cells were counted manually by means of a modified stereological procedure (Hu et al., 2012) in every 8th hippocampal section (Zeiss microscope, 200 × magnification) and multiplied by 8 to estimate the total cell number in the DG. Cell counting was performed by an investigator unaware of the experimental conditions.

To determine the amount of DAB-GFAP immunoreactive (ir) density, measurements were performed using ImageJ software (NIH, Bethesda, MD) system. Pictures were collected of the DG area taken at magnification 20 ×. Within the contour of the DG, a thresholding procedure is used to select only those pixels that contain DAB-GFAP-ir. The threshold is set such that it covers only the immunopositive cells within outlined area. Such thresholding procedure assures that background or surface areas not covered by GFAP signal are excluded from the quantification (Ishunina et al., 2007; Wang et al., 2013). Averaged optical density (OD) results of the RA group were expressed as percentage of the VEH group.

## Western Blot

Hippocampal protein samples were detected with anti-RAR-α (rabbit, 1:500; sc-551, Santa Cruz, CA) and HRP-conjugated goat-anti-rabbit IgG's (1:2,000; W4011, Promega, Madison, WI), using horseradish peroxidase-conjugated anti-β-actin (1:2,000; KC-5A08, Kangchen, Shanghai, China) as internal control standard (Chen et al., 2009). Signal was detected with an ECL chemiluminescence system (SuperSignal West Pico chemiluminescent Substrate, Pierce, IL). Immunoblots were quantified using a high-resolution scanner. Background subtraction was performed and density was calculated using Image-J software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD). Results were expressed as percentage of β-actin.

## Statistical Analysis

All statistics were performed with SPSS 17.0 software (SPSS Inc., NY). Data were expressed as mean ± S.E.M.

Immunohistochemical data, western blot data, sucrose preference percentage data and MWM probe test data were subjected to a one-way analysis of variance (ANOVA) for a comparison between two groups after tested for normal distribution. Latency data in the MWM acquisition trials were analyzed with a repeated-measure ANOVA, with treatment as between-subject factor and days as within-subject factor. Spearman's correlation was used to analyze: (1) correlations between sucrose preference and cell numbers of each neurogenesis marker; (2) correlations between the slope of the learning curve during MWM acquisition and cell numbers for each neurogenesis marker; and (3) correlations between the time spent in the target quadrant during the MWM probe test, and cell numbers for each neurogenesis marker. $P < 0.05$ was considered significant under all circumstances.

### Immunocytochemistry for BrdU, GFAP, PCNA, and DCX in the Adult Rat DG

BrdU$^+$, GFAP$^+$, PCNA$^+$, and DCX$^+$ cell numbers in the adult rat DG were quantified and compared between the VEH vs. RA-treatment groups. Similar to what has been described before (Hu et al., 2012), BrdU$^+$ cells prevailed in the subgranular zone (SGZ) bordering the hilus. Compared to the VEH (Fig. 2A) group, lower BrdU$^+$ cell numbers were found in the RA group (Fig. 2B). GFAP$^+$ cells were localized throughout the hippocampus with lower numbers present in the granule cell layer (GCL) (Figs. 2A,B). Seldom double-stained BrdU$^+$/ GFAP$^+$ cells were observed, indicating a general absence of astrogliogenesis. PCNA$^+$ cells also prevailed in the SGZ area and were clustered with less number in the hilus and GCL region. In the RA group, lower numbers of PCNA$^+$ cells were present (Fig. 2D) compared with the VEH group (Fig. 2C). For DCX, strong immunoreactivity was found in the SGZ and GCL of VEH controls (Fig. 2E). The somata of DCX$^+$ cells were located in the SGZ, with their dendrites and processes extending through the GCL into the molecular layer (ML) region. Compared to VEH-treated animals, generally lower numbers of DCX$^+$ cells were apparent with much shorter dendrites on individual DCX$^+$ cells in the DG of RA-treated animals, while more frequent gaps were seen along the SGZ as well (Fig. 2F).

### Chronic RA Exposure Suppressed Various Stages of Hippocampal Neurogenesis

Numbers of BrdU$^+$ cells in the DG were significantly decreased after chronic RA treatment (1,449 ± 209, $n = 6$) compared with VEH treatment (3,469 ± 255, $n = 6$) ($F(1,10) = 37.465$, $P < 0.001$) (Fig. 3A). Similarly, treatment with RA for 19 days yielded a significant reduction in the number of PCNA$^+$ cells (493 ± 27, $n = 6$) compared to treatment with VEH (1,403 ± 235, $n = 6$) ($F(1,10) = 14.868$, $P = 0.003$) (Fig. 3B). Similar results were found for DCX with lower numbers in the RA group (22,101 ± 2,350, $n = 6$) than in the VEH group (38,192 ± 2,314, $n = 6$) ($F(1, 10) = 23.803$, $P < 0.001$) (Fig. 3C). Regarding the number of GFAP$^+$ cells, no significant difference was found between the RA (105,953 ± 10,093, $n = 6$) and VEH group (81,760 ± 6,011, $n = 6$) ($F(1,10) = 4.241$, $P = 0.066$) (Fig. 3D). Further quantification of the average normalized optical density (OD) of GFAP-immunoreactivity in the DG has revealed no significant difference ($F(1,9) = 2.399$, $P = 0.160$) between the RA and VEH group (Fig. 3E) either.

Together, the above data demonstrate that chronic RA exposure robustly suppressed adult hippocampal neurogenesis, but did not significantly alter astrocytic morphology or numbers in the adult DG.
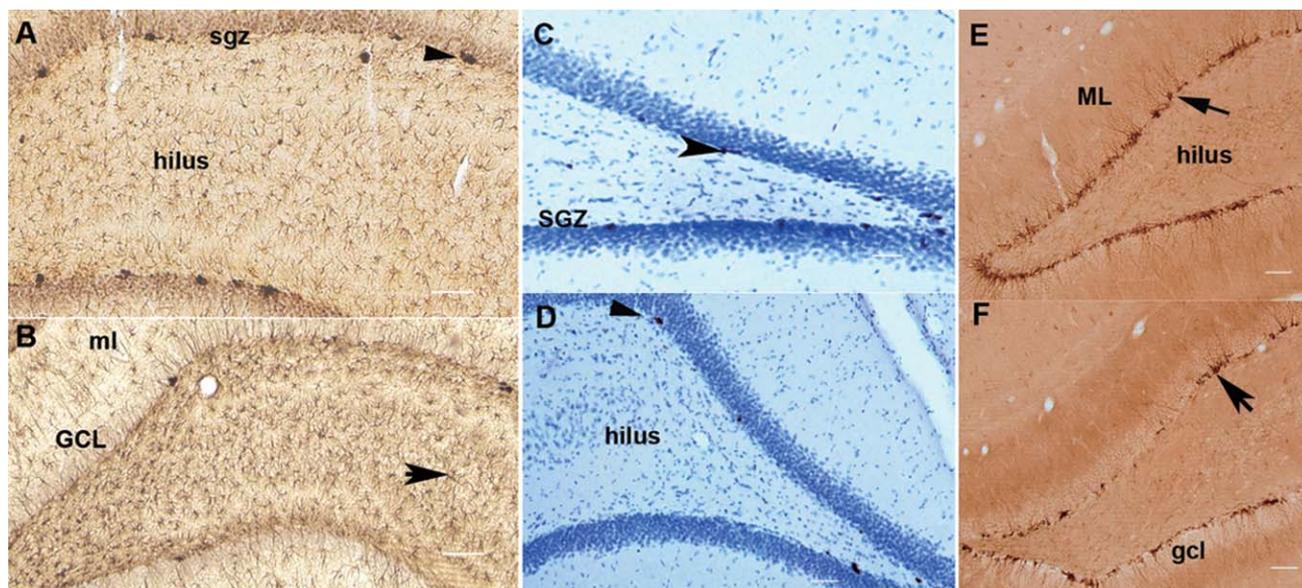
**FIGURE 2.** Immunohistochemical staining pattern of 5-bromo-2-deoxyuridine (BrdU), glial fibrillary acidic protein (GFAP), proliferating cell nuclear antigen (PCNA), and doublecortin (DCX) immunopositive cells expressed in the adult rat dentate gyrus (DG) compared between chronic vehicle (VEH) and all-trans retinoid acid (RA) group. (A): Distribution of double staining for BrdU$^+$/GFAP$^+$ cells in the DG of VEH and RA-treated (B) rats. BrdU$^+$ cells are mainly located in the subgranular zone (SGZ) (arrowhead). Some of them are found in the hilar region. Less BrdU$^+$ cells were found in the DG in the RA group. GFAP$^+$ cells (arrow) are mainly located in the hilus region and molecular layer (ML), except in the granular cell layer (GCL). They show stained processes and cytoplasm, while the cell nucleus is mostly without staining. No obvious difference was found in the number of GFAP$^+$ cells between the two groups. Calibration bars show 50 μm. (C): PCNA$^+$ cells in the DG of VEH and RA-treated (D) rats. They are clustered along the SGZ (arrowheads) and also in the hilus. RA treatment resulted in less PCNA$^+$ cells. Calibration bars show 50 μm. (E): Immunoreactivity of DCX shown in the DG of VEH and RA-treated (F) rats. DCX is mainly expressed in the SGZ and GCL, with cell body (arrows) located in the SGZ and dendrites extending through GCL into ML. DCX$^+$ staining after RA treatment shows shorter dendrites with more frequently found gaps along the SGZ-GCL border. Calibration bars show 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## Increased Hippocampal RAR-α Expression after Chronic RA Treatment

As inconsistent results have been reported regarding to the presence of RAR-α expression in rat versus mice DG (Huang et al., 2008; Goodman et al., 2012), we further studied using immunohistochemistry whether the RAR-α cellular expression pattern in the DG was altered by RA treatment. Fig. 4A showed an example of explicit presence of RAR-α nuclear expression in the DG hilus and GCL of VEH-treated rat (example of DG full view was shown in Supporting Information Fig. S1). A much stronger immunoreactivity was observed after RA treatment (shown in Fig. 4C) compared with VEH. Examples of RAR-α cellular expression pattern in each highlighted hilar region are shown at a higher magnification in Fig. 4B (VEH group) versus 4D (RA group).

Western blots of RAR-α protein expression in the hippocampus confirmed the above finding. An example of hippocampal RAR-α protein bands from VEH vs. RA groups is shown in Fig. 4E. The average hippocampal RAR-α protein density in the RA group (78.20 ± 5.46% of β-actin) was significantly increased compared to that in the VEH group (44.62 ± 6.70% of β-actin; $P < 0.01$) (Fig. 4F), indicative of a possible activation of RA downstream signaling in the hippocampus after chronic RA application.

## Correlation between Adult Hippocampal Neurogenesis and Sucrose Preference

To examine depressive-like behavior, the sucrose preference test was performed to evaluate the degree of anhedonia induced by RA treatment. The percentage of sucrose preference in the RA group (23.78 ± 7.68%) was significantly decreased relative to the VEH group (84.74 ± 9.66%) ($F(1,9) = 25.087$, $P < 0.01$) (Fig. 5A), indicative of a typical depressive-like behavioral response.

To examine possible relationship between hippocampal neurogenesis and depressive-like behavior, we further analyzed the correlation between cell number of each neurogenesis measure and the individual corresponding sucrose preference score within both groups. Interestingly, highly significant positive correlations were present between the sucrose preference percentage and the number of: (1) BrdU$^+$ cells (Fig. 5B) (correlation coefficient $R = 0.964$, $P < 0.01$); (2) PCNA$^+$ cells (Fig. 5C; correlation coefficient $R = 0.908$, $P < 0.01$); and also (3) DCX$^+$ cells (Fig. 5D) (correlation coefficient $R = 0.983$,
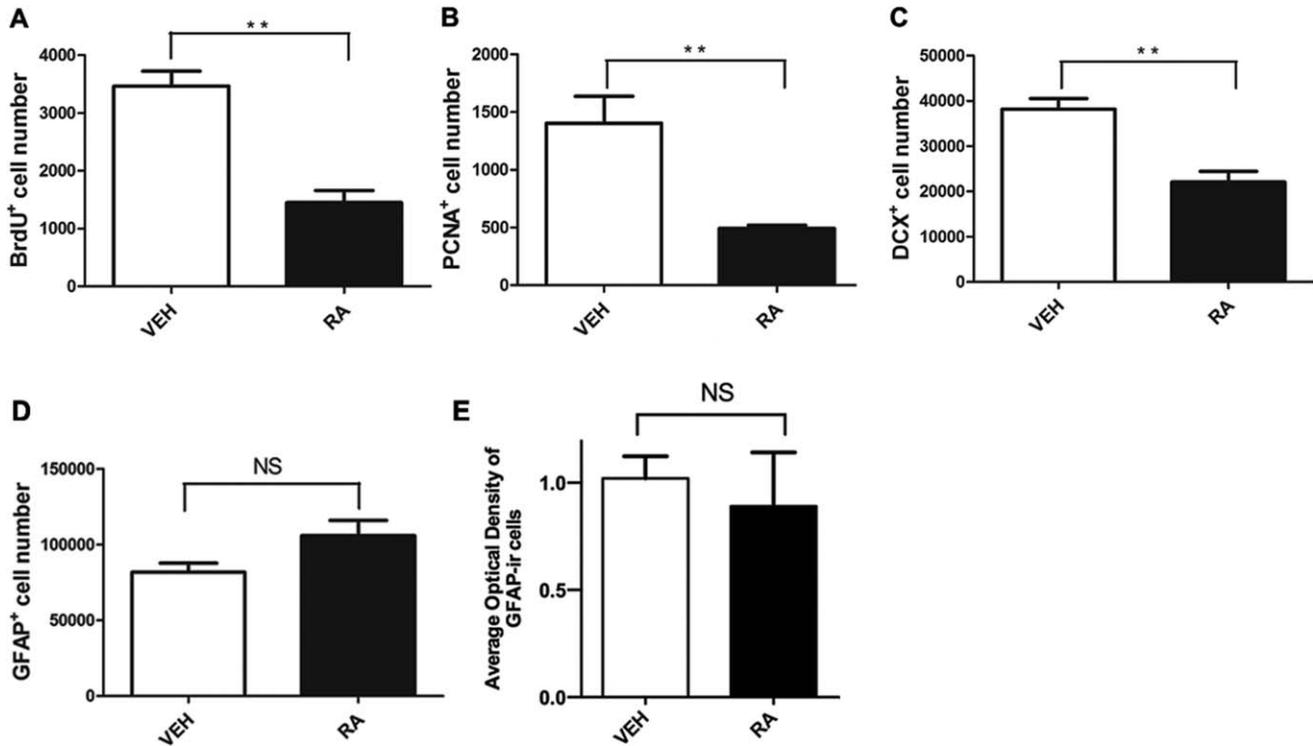
**FIGURE 3.** Chronic all-trans retinoic acid (RA) treatment robustly suppressed hippocampal neurogenesis, but did not affect the number and averaged optical density (OD) of GFAP$^+$ astrocytes in DG. (A): Significant reduction in the number of BrdU$^+$ cells was found in the dentate gyrus (DG) after 19-day RA treatment compared to vehicle (VEH; $P$ <0.01). (B): The number of PCNA$^+$ cells was significantly decreased ($P$ <0.01) after chronic RA treatment compared to VEH treatment. (C): Chronic RA treat- ment yielded a significant reduction ($P$ <0.01) in the number of DCX$^+$ cells compared to VEH. (D): Treatment with RA had no significant effect on the number of GFAP$^+$ astrocytes compared to VEH ($P$ >0.05). (E): Comparison of average normalized optical density (OD) of GFAP-immunoreactivity revealed no significant difference ($P$ >0.05) between RA and VEH group. Data are presented as mean ± SEM ($n=6$ animals per group). ** $P$ <0.01; NS: no significant difference.

$P$ < 0.01) in the adult rat DG. This points to a higher degree of anhedonia and increased depressive-like behavior in those animals with lower levels of neurogenesis.

## Chronic RA Treatment Did Not Affect Performance in MWM Trials

Since newborn hippocampal neurons were suggested to be important for spatial learning (Kee et al., 2007) and contribute to hippocampal-dependent spatial memory function (Jessberger et al., 2009), we next questioned whether the impaired neurogenesis would relate to any changes in spatial learning and memory. First, in the MWM acquisition trials, latency data to reach the platform were analyzed. Both RA-treated rats and VEH controls showed improvements in spatial learning across the day-to-day acquisition trials (within-subject (day) $F(4,32)=13.421$, $P = 0.000$) (Fig. 6A). However, no significant difference was found between the two groups (between-subject (treatment) $F(1,8)=0.011$, $P = 0.917$). No interaction effect was found either (treatment × day $F(4,32)=0.550$, $P = 0.700$), suggesting that no change has occurred in spatial learning capacity after chronic RA application. Second, in the MWM probe test of spatial memory performance, we analyzed the time spent in the target quadrant in each group. No significant difference was found between the two groups either ($F(1,8)=0.047$, $P = 0.833$) (Fig. 6B), indicating unaltered spatial memory function after chronic RA application.

To test the possible contribution of hippocampal neurogenesis to spatial learning and memory performance, we next analyzed the relationship between the slope of the latency curve during the MWM acquisition trials, and the cell numbers for each neurogenesis measure. We found no significant correlation between the slope of the acquisition latency curve and the number of: (1) either BrdU$^+$ cells (Fig. 6C; correlation coefficient $R = 0.287$, $P = 0.111$); or (2) PCNA$^+$ cells (Fig. 6D; correlation coefficient $R = 0.333$, $P = 0.081$); or (3) DCX$^+$ cells (Fig. 6E; correlation coefficient $R = 0.129$, $P = 0.308$) in the adult rat DG. Furthermore, we analyzed the relationship between each neurogenesis measure and the time spent in the target quadrant in the MWM probe test. No significant correlation was observed between the duration in the target quadrant during the probe trial and the number of: (1) BrdU$^+$ cells (Fig. 6F; correlation coefficient $R = 0.188$, $P = 0.603$); (2) PCNA$^+$ cells (Fig. 6G; correlation coefficient $R = 0.067$, $P = 0.855$); or (3) DCX$^+$ cells (Fig. 6H; correlation coefficient $R=-0.103$, $P = 0.777$) in the adult DG.
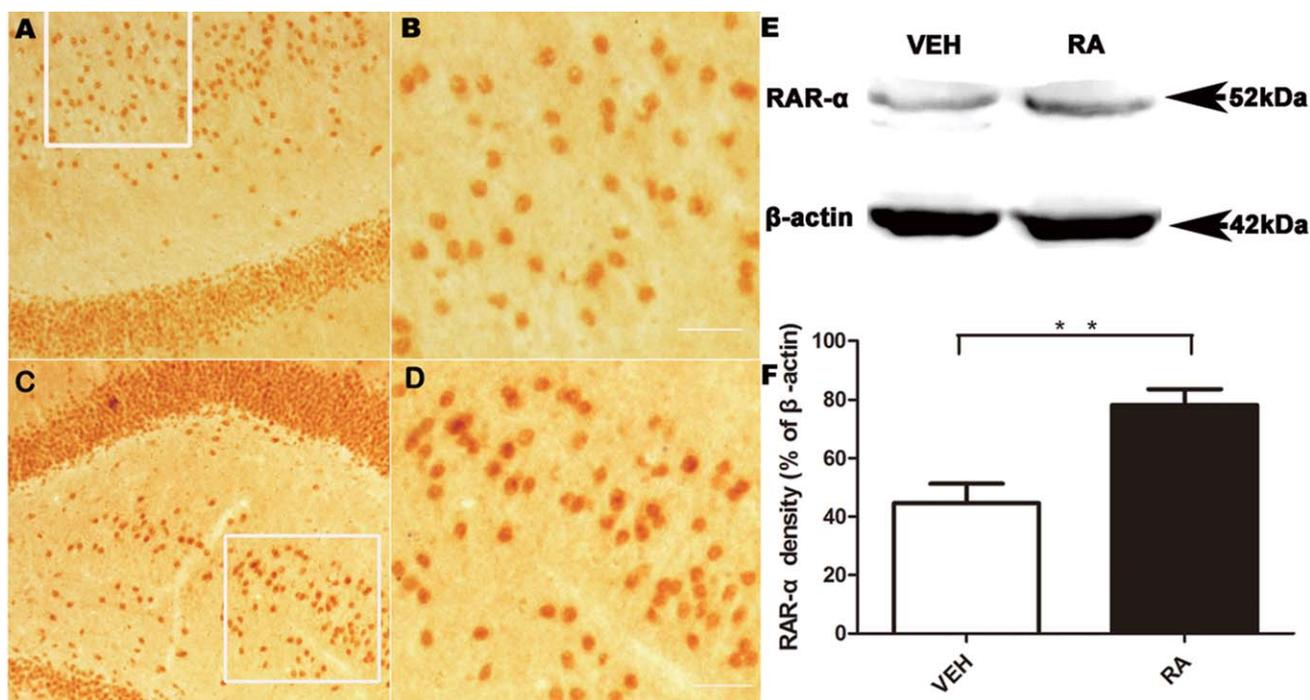
**FIGURE 4.** Immunostaining pattern of retinoic acid receptor-α (RAR-α) distribution in the adult rat dentate gyrus (DG) and effects of chronic all-trans retinoic acid (RA) treatment on hippocampal RAR-α protein expression. (A): Immunostaining pattern of RAR-α expression distributed in the DG of vehicle (VEH)-treated rats (10 × magnification). Selected square highlights typical RAR-α-immunopositive cells in the hilus area. (B): Amplified example of RAR-α-immunopositive cells in the selected square area of Figure 4A shown in a higher magnification (20 ×). Calibration bar: 50 μm. (C): Immunohistostaining pattern of RAR-α distribution in the DG of RA-treated rats (10 × magnification). Selected square highlights typical RAR-α-immunopositive cells in the hilus area. (D): Higher magnification (20 ×) of RAR-α-immunopositive cells shown in the selected square of Figure 4C. Calibration bar: 50 μm. (E): Western blot example of RAR-α protein expression in the hippocampus from chronic vehicle (VEH) vs. RA-treated animals. Arrows point to the molecular weight bands of RAR-α at 52 kDa and β-actin at 42 kDa respectively. (F): Average density of RAR-α protein expression (percentage of internal control β-actin) in the hippocampus was significantly higher in chronic RA group compared with VEH group. Data are presented as mean ± SEM ($n=6$ animals per group). ** $P <0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## DISCUSSION

In line with previous effects of RA on the HPA axis (Cai et al., 2010; Hu et al., 2013), we here show that chronic ICV treatment with RA reduces various measures of hippocampal neurogenesis, whereas no changes in astroglia or gliosis were found. Notably, neurogenesis changes correlated closely with depressive-like behavior, but not with hippocampal learning and memory in this experimental design.

Several clinical and preclinical studies have highlighted a role for RA in depression and depression-related models (O'Reilly et al., 2006; O'Reilly et al., 2009; Bremner et al., 2012), but how this is mediated mechanistically remains elusive. A prominent risk factor for depression is stress, and exposure to chronic stress or glucocorticoids suppresses various stages of hippocampal neurogenesis, an effect that has been implicated in the hippocampal changes seen in depression (Krugers et al., 2006; Mayer et al., 2006; Joels et al., 2007; Oomen et al., 2007; Lucassen et al., 2010; Hu et al., 2012; Loi et al., 2014; Lucas-

sen et al., 2014). Interestingly, RA is an endogenous regulator of hippocampal granule neuronal progenitors and proliferation (Jacobs et al., 2006; Schug et al., 2007; Bonnet et al., 2008; Goodman et al., 2012). This, together with our earlier results showing that RA potently activates the hypothalamus-pituitary-adrenal (HPA)-axis in a GR-dependent manner (Hu et al., 2013), prompted us to study whether RA also modifies neurogenesis and affects related cognitive and emotional measures. We selected the current RA dose and ICV application as we wanted to obtain a direct effect, avoid peripheral side-effects, and obtain clinically relevant concentrations (see below). The current dose further allowed for comparisons with earlier papers in which this dose range (Cai et al., 2010; Hu et al., 2013) induced depression/anxiety-related behavior. In humans, accutane is a comparable compound and its oral doses of 0.5–2.0 mg/kg per day were also linked to depressive symptoms before (Hazen et al., 1983; Wysowski et al., 2001; Bremner et al., 2012).

Although neurogenesis and structural plasticity was studied before in relation to RA, these studies used different
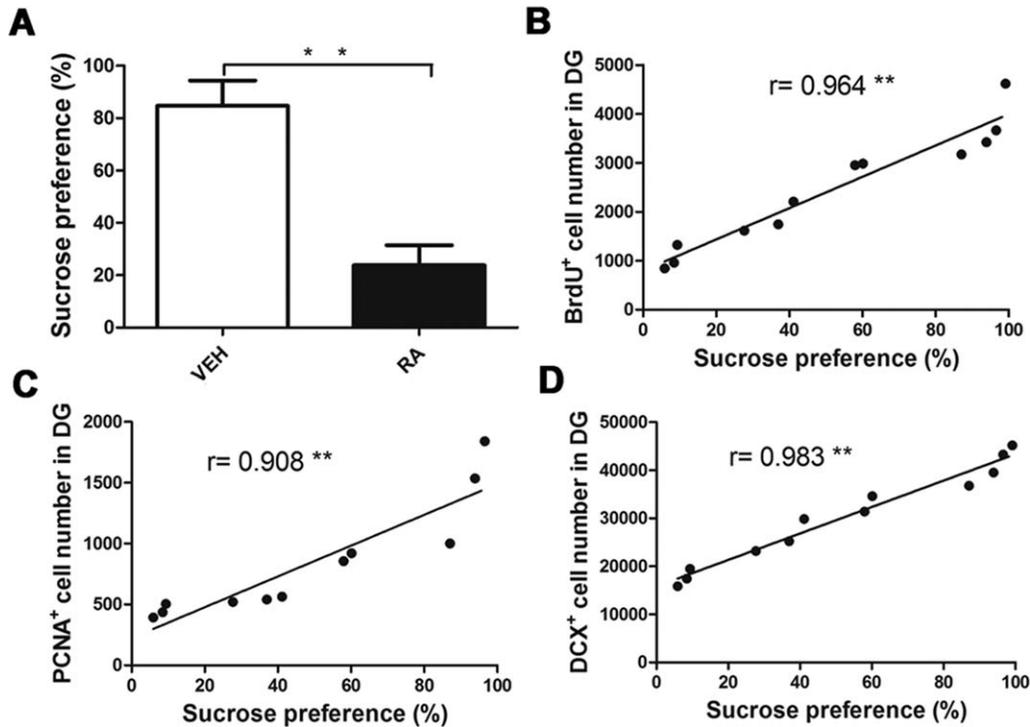
FIGURE 5.    Correlations between sucrose preference percentage and hippocampal neurogenesis measures in the adult rat dentate gyrus (DG). (A): Sucrose preference percentage was significantly decreased in chronic all-trans retinoic acid (RA)-treated animals compared to vehicle (VEH)-treated animals. Data are presented as mean ± SEM ($n=6$ animals per group). ** $P$ <0.01. (B): Significant positive correlation was present between BrdU$^+$ cell number in the DG and corresponding sucrose preference percentage ($n=12$; correlation coefficient $r=0.964$, $P$ <0.01). (C): Similarly, significant positive correlation was found between PCNA$^+$ cell number in the DG and corresponding sucrose prefer-ence percentage ($n=12$; correlation coefficient $r=0.908$, $P$ <0.01). (D): Again, significant positive correlation was found between DCX$^+$ cell number in the DG and corresponding sucrose preference percentage ($n=12$; correlation coefficient $r=0.983$, $P$ <0.01). ** $P$ <0.01. Note that all the number of BrdU$^+$, PCNA$^+$, and DCX$^+$ cells is based on pooled data collected from both VEH and RA groups analyzed throughout the entire DG as shown in Figures 3A–C. The value of individual corresponding sucrose preference percentage is also based on pooled data collected from both treatment groups as shown in Figure 5A.

compounds (i.e., 13-cis-RA) and dosing regimens and/or assessed only newborn cell division or survival (Crandall et al., 2004; Sakai et al., 2004). We here show that chronic ICV ATRA treatment robustly suppressed all main phases, that is, cell proliferation, newborn cell survival and neurogenesis, but not gliogenesis. The reduction we find with the proliferation marker PCNA (Moldovan et al., 2007) after RA treatment is consistent with earlier reports (Crandall et al., 2004; Goodman et al., 2012). The total number of surviving BrdU$^+$ cells at d27 is the net result of the balance between production and proliferation rate on d1, and their subsequent phenotypic specification and selection during subsequent rounds of divisions of daughter cells (Cameron and McKay, 2001; Dayer et al., 2003). In line with previous studies reporting the induction of cell loss by 13-cis-RA (Sakai et al., 2004; Griffin et al., 2010), also newborn cell survival was substantially reduced in our study. To identify neurogenesis, the microtubule-associated protein DCX was used, that is specific for immature, young neurons and expressed from 4 to 14 days after their birth (Brown et al., 2003). As possible explanation, the current decline in

DCX$^+$ cell numbers may result from: (a) a slowing down of the cell cycle, which would yield lower numbers of cells entering neuronal differentiation at any time-point, or (b) a putative decrease in cell survival, which is consistent with the decrease we found in newborn cell survival (Fig. 3A). A third option is (c) a shift in phenotype after RA treatment, which could suppress neuronal differentiation per se and more stem cells would form glia, which does not seem to be the case based on our GFAP data. Whereas astrocytes have been implicated in major depression (Banasr et al., 2010), overt gliosis was not apparent in postmortem hippocampal tissue of depressed cases (Muller et al., 2001). Also other contradictory findings exist as between rodent and human tissue; increased glial density was e.g. found in the DG of patients with major depressive disorder (Stock-meier et al., 2004), whereas glial atrophy was observed in the hippocampus and PFC of animal depression models (Liu et al., 2011; Czeh et al., 2013). In the current study, we found no cells double labeled for BrdU and GFAP, nor did we find significant differences in numbers and immunoreactivity of GFAP. This suggests that astrogliogenesis does not play a major
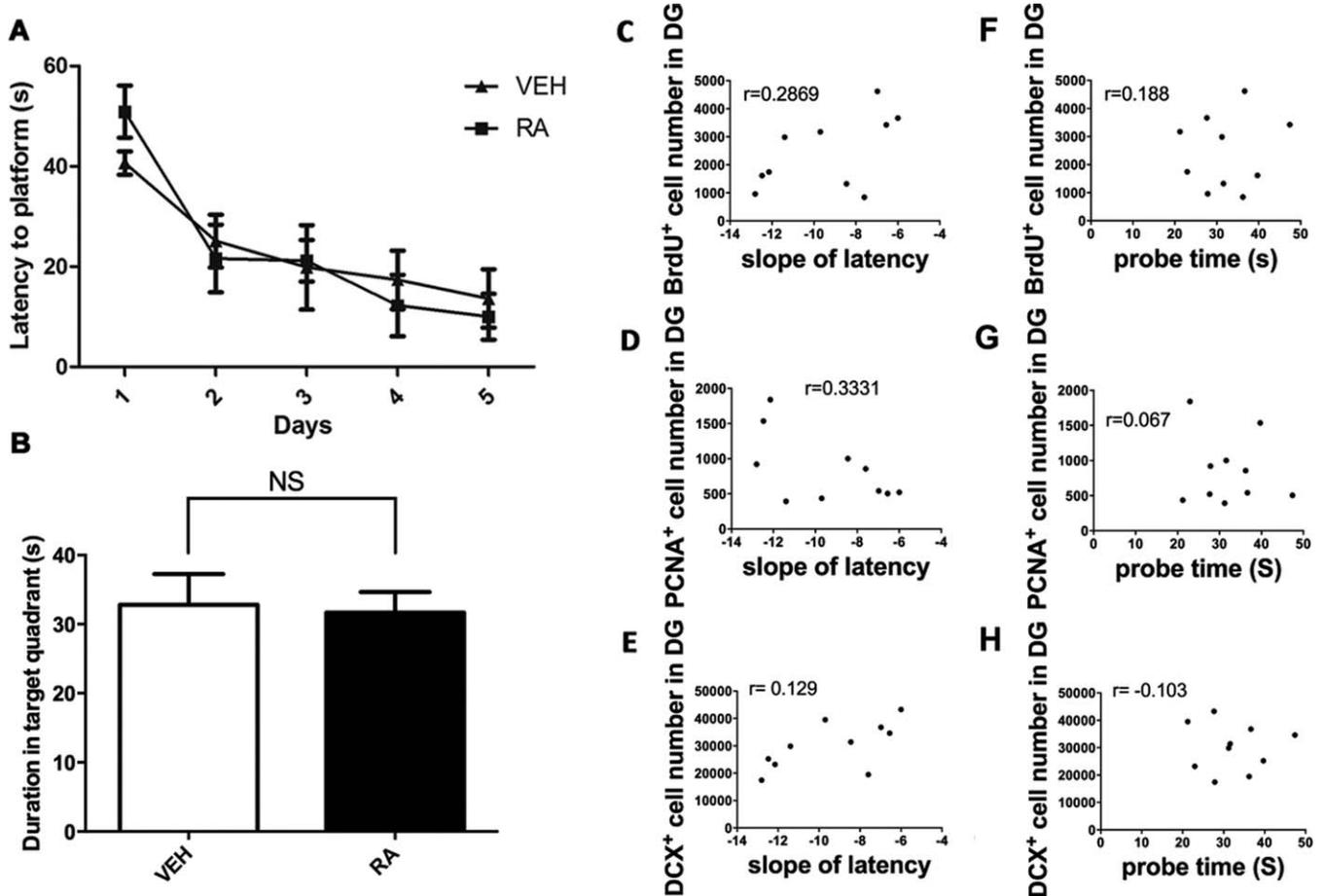
FIGURE 6. Effects of chronic all-trans retinoic acid (RA) treatment on the performance of spatial learning and memory task in the Morris water maze (MWM) test. (A): Latency to the platform was compared between chronic vehicle (VEH) vs. RA group across days 1–5 in the MWM acquisition trials. No significant difference was found between the two groups ($P = 0.917$). Data are presented as mean ± SEM ($n=6$ animals per group). (B): No significant difference was found between duration in target quadrant compared between chronic VEH vs. RA group during the probe trial of the MWM test ($P = 0.833$). Data are presented as mean-± SEM ($n=6$ animals per group). (C): No significant correlation between BrdU[+] cell number in dentate gyrus (DG) and the corresponding slope factor of latency curve in the MWM acquisition test was revealed ($n=10$; correlation coefficient $r=0.287$, $P =0.111$). (D): No significant correlation between PCNA[+] cell number in DG and corresponding slope factor of latency curve was found ($n=10$; correlation coefficient $r=0.333$, $P =0.081$).

(E): No significant correlation was present between DCX[+] cell number in DG and corresponding slope factor of latency curve ($n=10$; correlation coefficient $r=0.129$, $P=0.308$). (F): No significant correlation between BrdU[+] cell number in dentate gyrus (DG) and corresponding probe time (duration in the target quadrant in the probe trial) spent in the MWM probe test was revealed ($n=10$; correlation coefficient $r=0.188$, $P =0.603$). (G): No significant correlation between PCNA[+] cell number in DG and corresponding probe time spent in the MWM probe test was found ($n=10$; correlation coefficient $r=0.067$, $P =0.855$). (H): There was no significant correlation present between DCX[+] cell number in DG and corresponding probe time spent in the MWM probe test ($n=10$; correlation coefficient $r=-0.103$, $P =0.777$). Note that both the number of BrdU[+], PCNA[+], and DCX^{+} cells and the individual corresponding slope factor of latency curve or probe time are based on pooled data from the two treatment groups.

role, consistent with earlier studies (Heine et al., 2004; Steiner et al., 2004), that together make it less likely that RA effects involve astroglia.

Related papers by Bonnet et al. (2008) and Jacobs et al. (2006) have reported reductions in adult neurogenesis in vitamin A-deprived animals, which could be restored by RA treatment (Jacobs et al., 2006; Bonnet et al., 2008). Our current study differs in that we studied the effect of exogenous, and direct ICV RA administration on neurogenesis in intact, non-deprived animals. Clearly, RA is an essential factor within the

stem cell niche of the hippocampal SGZ (McCaffery et al., 2003; McCaffery et al., 2006); but given that endogenous RA is necessary to regulate hippocampal neuronal birth and differentiation, the prolonged deprivation in vitamin A-depleted animals in e.g. the Bonnet study may have induced indirect (peripheral) changes, or affected (compensatory) retinoid-mediated molecular events, e.g. on neurotrophin receptor expression. Together with the differences in temporal design, dose and way of RA application (ICV vs. ip), this may have altered RA homeostasis and neurogenesis in the brains of these

animals, which could explain some of the discrepancies with our current results (McCaffery et al., 2003; Jacobs et al., 2006; McCaffery et al., 2006; Schug et al., 2007; Bonnet et al., 2008).

In contrast to the lack of correlation between neurogenesis measures and hippocampal learning, we found clear correlations with sucrose preference, a measure for (an-)hedonia, which is a core depressive-like behavioral phenotype in rodents (Treadway and Zald, 2011). The current strong correlation of sucrose preference scores with neurogenesis measures after chronic RA exposure suggests that reductions in neurogenesis are paralleled by and strongly linked to the depressive symptoms. Such reductions are considered important in mood disorders (Vogel, 2000; Kempermann and Kronenberg, 2003; Boldrini et al., 2009; Lucassen et al., 2010; Snyder et al., 2011; Eisch and Petrik, 2012; Lucassen et al., 2013; Lucassen et al., 2014), and are consistent with the reported loss of sucrose preference in neurogenesis-deficient and NCAM-deficient mice (Aonurm-Helm et al., 2008; Snyder et al., 2011; Wang et al., 2015), with studies showing that increasing hippocampal neurogenesis is sufficient to reduce depressive-like behavior (Veena et al., 2009; Hill et al., 2015) and other correlative studies (Zhang et al., 2010; Wang et al., 2015), and with reductions found in cytogenesis in several animal models for depression and in hippocampal tissue of major depressive patients (Boldrini et al., 2009; Lucassen et al., 2010; Lucassen et al., 2014). Whereas neurogenesis and their specific inputs and projections have been implicated in antidepressive action, mood and HPA axis regulation (Opendak and Gould, 2011; Snyder et al., 2011; Hill et al., 2015; Drew et al., 2015), it remains to be studied how exactly such reductions can relate to this particular type of depressive-like behavior.

Intact neurogenesis appears to be required for the efficacy of antidepressants (Santarelli et al., 2003). However, ablation of neurogenesis per se often does not induce a depressive phenotype (Santarelli et al., 2003; Henn and Vollmayr, 2004; Petrik et al., 2012). Mood and anxiety disorders are highly heterogeneous and a single theory cannot fully capture the complexity of their pathology (Miller and Hen, 2015). In the current study, although suppressed neurogenesis is identified as an important correlate and potential mechanism by which RA exerts its effect on mood regulation, it does not necessarily suggest a unique causative role for neurogenesis (Lucassen et al., 2013; Schoenfeld and Cameron, 2015) and indirect effects of RA on the monoaminergic system could not be excluded (Samad et al., 1997; Valdenaire et al., 1998; O'Reilly et al., 2007; O'Reilly et al., 2008). Whether RA-induced impairment in neurogenesis alone is thus entirely sufficient to actually cause depression in humans, or whether it rather may represent an initial risk factor, that further depends on additional factors or stressors awaits future studies (Lucassen et al., 2013; Lucassen et al., 2015; Miller and Hen, 2015).

Using our current experimental design, we did not detect any deficits in spatial learning or memory after RA treatment in the water maze task, and neither was a correlation present with hippocampal neurogenesis measures. While several studies have indicated neurogenesis to be important for learning and memory as often determined using a water maze test (Snyder et al., 2005; Dupret et al., 2007; Kee et al., 2007; Dupret et al., 2008; Koehl and Abrous, 2011; Abrous and Wojtowicz, 2015; Tronel et al., 2015), such correlations depend on the animal strain, sex, age, difficulty and type of task and the temporal design of the study (Greenough et al., 1999; van Praag et al., 1999; Shors et al., 2002; Rhodes et al., 2003; van Praag et al., 2005; Leuner et al., 2006; Waddell et al., 2011). For example, strain-dependent differences in, or reductions of, neurogenesis do not always correlate with spatial learning or memory performance in rats, whereas effects on mood or anxiety-related changes may still occur (McEwen, 1999; Fuchs and Gould, 2000; Shors et al., 2002; Leuner et al., 2006; Wojtowicz et al., 2008; Jaholkowski et al., 2009; Revest et al., 2009; Groves et al., 2013; Martinez-Canabal et al., 2013; Trinchero et al., 2015).

While we had shown before that GR dysregulation is involved in the HPA activation by RA (Hu et al., 2013), RA effects are presumed to be largely mediated by RARs. Despite a relatively low affinity for the RARs, RA is quite efficient at inducing transactivation (Idres et al., 2002). Although we did not do an extensive characterization, since RA is supposed to primarily mediate its effects through binding to the RAR-α (Idres et al., 2002; Crandall et al., 2004), the current upregulation of RAR-α protein expression we found in the hippocampus after chronic RA treatment indicates not only that the drug targets the hippocampus, but also suggests a possible activation of downstream signaling pathway. How this activation then results in the current hippocampal structural alterations remains elusive. One possibility is that downstream effectors of RAR signaling are involved in the exit of neural progenitors from the cell cycle. In support, RARs have recently been implicated as putative cell cycle regulators in developing neurons (Watanabe et al., 1999; Gallo et al., 2002; Mao et al., 2011; Janesick et al., 2015), but the further detail is still poorly studied.

## CONCLUSION

Increased retinoid levels form a risk factor for depressive symptoms and mood disorders. Here, we show that chronic RA treatment robustly suppressed adult hippocampal neurogenesis and induced depressive-like behavior, without altering spatial memory performance. Moreover, neurogenesis changes correlated closely with depressive-like behavior. Our findings suggest that impairments in neurogenesis may contribute to retinoid-associated depressive symptoms. They highlight a role for retinoid in neuropsychiatric disorders and provide a framework to develop possible intervention strategies.

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