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ARTICLE

Moderate ethanol consumption increases hippocampal cell proliferation and neurogenesis in the adult mouse

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Abstract

Alcoholism is a lifelong disease often associated with emotional disturbances and a high risk of relapse even years after detoxification. To explore if cell proliferation in the dentate gyrus of the hippocampus might be important for alcohol-induced brain adaptation, we analysed hippocampal neurogenesis and gliogenesis in adult C57Bl/6 mice that consumed moderate levels of ethanol (∼6 g/kg.d) in a two-bottle free-choice model during ∼10 wk. The mice developed a 53% preference for ethanol vs. water and displayed a blood ethanol concentration of 0.24‰ at the time of sacrifice. Bromo-deoxy-uridine (BrdU) was administered in different regimes to analyse proliferation, survival, cell distribution and differentiation of new cells in the dentate gyrus. Moderate ethanol consumption increased the proliferation of cells, which survived and developed a neural phenotype. Ethanol consumption did not induce apoptosis, neither did it change differentiation or the distribution patterns of the newly formed cells. The cell proliferation rate in the dentate gyrus returned to basal levels 3 d after ethanol withdrawal. We conclude that voluntary ethanol intake by mice can change the rate of cell proliferation in the dentate gyrus. These observations add to the emerging picture of dentate gyrus neurogenesis as a highly regulated process. Since there was no increase in apoptosis concomitant with the ethanol-induced increase in neurogenesis, it is possible that the new cells in the dentate gyrus may contribute to the long-lasting changes of brain function after ethanol consumption.

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Introduction

Chronic alcohol consumption results in a plethora of neurochemical adaptations in different brain regions, such as in the ventral tegmental area, nucleus accumbens and hippocampus (Fitzgerald and Nestler, 1995). These adaptations are reversible after detoxification and within a few months many aspects of brain neurochemistry are normalized (Nestler, 2001). However, chronic alcohol abuse also leads to a series of long-lasting behavioural consequences, such as memory loss, emotional disturbances and loss of control of alcohol intake.

The hippocampal formation is not only important for learning and memory but also for emotional processing and has a high capacity for synaptic and neurochemical adaptations (McNaughton, 1997). Therefore, the hippocampus is a potential target for studies of neuronal adaptations after chronic alcohol consumption. In rodents, the anterior hippocampus (for subdivisions see Moser and Moser, 1998) is important for spatial memory whereas the posterior hippocampus is more important for emotional processing such as expression of fear (Kjelstrup et al., 2002). Many post-mortem human brain studies describe a change in the size of the hippocampus in alcoholics, which is most likely the result of a loss of white matter and a decreased number of astrocytes (Harding et al., 1997; Jensen and Pakkenberg, 1993; Korbo, 1999). Another putative mechanism for structural rearrangement of the hippocampus after long-term ethanol consumption is the formation of new nerve cells. Neurogenesis in the adult brain occurs mainly in the subventricular zone and the

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subgranular zone of the dentate gyrus (Altman and Das, 1965; Doetsch et al., 1999; Eriksson et al., 1998; Johansson et al., 1999). Hippocampal neurogenesis in the adult brain has been shown in several models to be a highly regulated process. It appears that factors that can control affective states such as antidepressant treatments, running, steroid hormones and food deprivation all influence neurogenesis in the hippocampus (Gould et al., 1997; Lee et al., 2000; Malberg et al., 2000; van Praag et al., 1999). Recently, neurogenesis in the hippocampus was suggested to be a necessary component of antidepressant effects of pharmacotherapy in mice. Thus, when neurogenesis was blocked by irradiation over the hippocampus the antidepressant effect was lost (Santarelli et al., 2003).

Neurogenesis and cell proliferation have been analysed in several animal models of alcoholism. For example, binge ethanol administration for 1 and 4 d or ethanol administration via food during 6 wk, results in a decrease of neurogenesis in adult rats (Herrera et al., 2003; Nixon and Crews, 2002), while ethanol administrations for 9 d to postnatal rats has the opposite effect (Miller, 1995). Long-term self-administration of ethanol with repeated deprivation phases is a powerful model that has been used to characterize addiction, craving and relapse (Spanagel and Holter, 1999). The voluntary two-bottle free-choice ethanol consumption model on the other hand, has the advantage that ethanol intake is controlled more by the animal itself and that the model, therefore, is probably less stressful for the animal. This model could perhaps be compared to social alcohol intake at non-toxic doses in humans.

In the present study we have chosen to study singly housed animals in the two-bottle free-choice model to analyse the effects of a moderate voluntary ethanol consumption in adult mice on cell proliferation, survival, cell distribution and differentiation in the hippocampal formation. We administered the thymidine analogue bromo-deoxy-uridine (BrdU), which gets incorporated in the DNA during the cell cycle S-phase, to investigate alterations in the formation of new cells and neurogenesis. Specifically, three different BrdU administration protocols were used to investigate (i) the acute effects of ethanol on cell survival, (ii) the influence of 9 wk ethanol consumption on cell proliferation and (iii) how 11 wk of ethanol altered cell survival, cell distribution and neurogenesis. We hypothesized that non-toxic doses of ethanol might alter hippocampal neurogenesis, and thus, influence functions such as learning, memory and emotional processing.

Methods

Ethanol administration

Adult female C57Bl/6NIH mice (24.9 ± 2.9 g, aged ≈ 5 months, Charles River, Uppsala, Sweden) were caged individually, with lights completely on at 07:00 hours and completely off at 20:00 hours preceded by 1 h dawn and 1 h twilight respectively. The mice were subjected to a two-bottle free-choice model for ethanol consumption during 4 d, 9 wk or 11 wk. The mice in the ethanol groups had free access to one bottle containing 10% (v/v) ethanol and one bottle of tap water. The control groups were given two water bottles. The animals were sacrificed and dissected at 11:00 hours.

To analyse the acute effects of ethanol on cell survival in the dentate gyrus, animals were exposed to ethanol/water ($n=8$) or water/water ($n=8$) in the two-bottle free-choice model for 4 d. All animals were sacrificed on day 6 (Figure 1a).

The effects of ethanol intake on cell proliferation in the dentate gyrus was analysed by exposing one group of animals ($n=8$) to ethanol/water and one group of animals ($n=8$) to water/water in the two-bottle free-choice model for a 63-d period. Another group ($n=8$), the ethanol withdrawal group, was taken off ethanol at day 57. All animals were sacrificed at day 63 (Figure 2a).

To study survival, cell distribution and neurogenesis in the dentate gyrus an ethanol group ($n=9$) was given access to ethanol/water in the two-bottle free-choice model during the whole 77-d period of the experiment while a control group ($n=9$) consumed only water. The animals were sacrificed and dissected on day 77 (Figure 3a).

The weights of the bottles and the animals were recorded once a week, and the position of the water and ethanol bottles was changed at every occasion. The animals had free access to food. The experiments were approved by the local ethical committee for animal research in Stockholm.

BrdU labelling

Three experiments were performed using different regimes of administering BrdU. The thymidine analogue BrdU, which is incorporated during the S-phase of DNA synthesis, was administered to detect acute ethanol effects on cell survival (Figure 1a), the influence of 9 wk of ethanol intake on the number of dividing progenitor cells (Figure 2a) and to analyse the effects of 11 wk of ethanol intake on cell survival, cell distribution and neurogenesis (Figure 3a).

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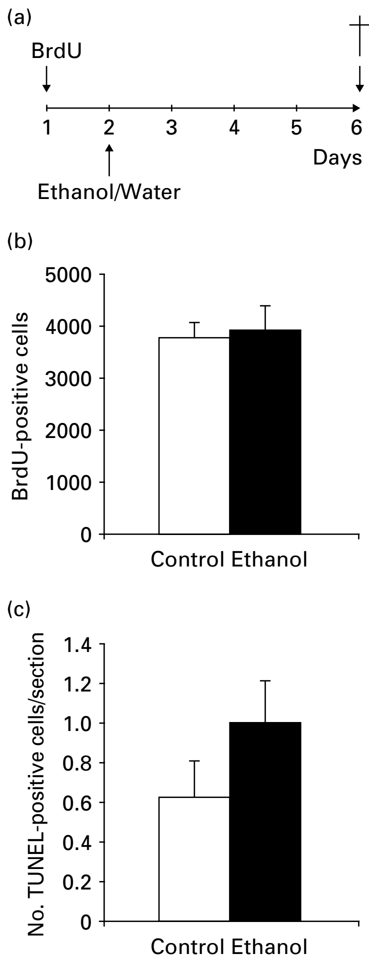

Figure 1. Acute ethanol experiment. (a) Animals were given the choice of drinking either ethanol or water in the two-bottle free-choice model during 4 d starting 1 d after BrdU injections. Control animals were given two bottles of water during the same period. All animals were sacrificed on day 6, indicated by a cross. (b) Four days of voluntary ethanol intake did not alter cell proliferation compared to animals that consumed water during the same period. There was, thus, no change in the number of BrdU-positive cells between the ethanol group and the control group. Values are mean ± S.E.M. counted in both hemispheres ($n=8$ animals/group). (c) Four days of ethanol consumption was not toxic to cells in the dentate gyrus. There was, thus, no difference in apoptosis (TUNEL-positive cells) between animals that had consumed ethanol/water for 4 d compared to animals that consumed water/water during the same period. Values are mean ± S.E.M. counted in both hemispheres (two sections/individual, $n=8$ animals/group).

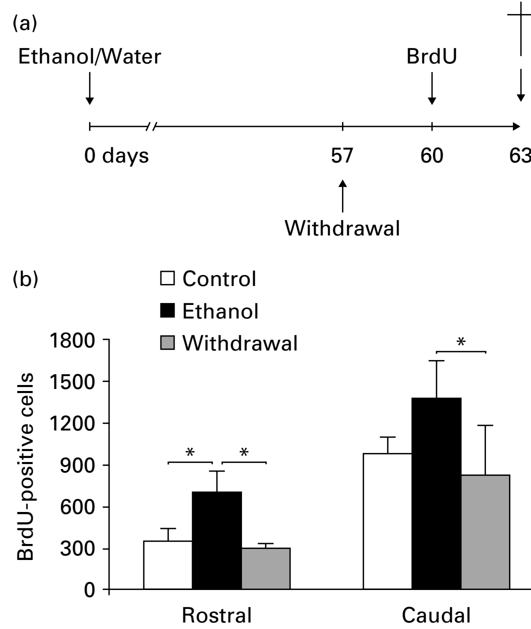

Figure 2. Cell proliferation experiment. (a) Animals in the ethanol group consumed ethanol/water during 63 d in the two-bottle free-choice model while the control group had access to water/water. The ethanol bottles were removed from the ethanol withdrawal group on day 57. BrdU was administered to all groups on day 60. All animals were sacrificed on day 63, indicated by a cross. (b) Voluntary moderate ethanol consumption increased cell proliferation in the dentate gyrus. The number of BrdU-positive cells increased in the rostral part of the dentate gyrus after moderate consumption of ethanol but there was no statistically significant increase in the caudal part. The cell proliferation decreased to control animal cell levels after 3 d of ethanol withdrawal in both the rostral and the caudal part of the dentate gyrus. Values are mean ± S.E.M. counted in both hemispheres ($n=8$ animals/group). * $p<0.05$.

In the acute ethanol experiment, BrdU [2×70 mg/kg i.p., 4 h apart (Sigma-Aldrich, Stockholm, Sweden), $n=8$/group] was administered on day 1 before the mice were given free-access to ethanol/water in the two-bottle free-choice model on day 2 and following 4 d (Figure 1a). The animals were sacrificed on day 6 and the brains frozen.

In the proliferation experiment animals were given BrdU (2×70 mg/kg i.p., 4 h apart, Sigma, $n=8$/group) 3 d before sacrifice. The ethanol withdrawal group was given BrdU (2×70 mg/kg i.p., 4 h apart, Sigma, $n=8$/group) after 3 d of ethanol withdrawal. This group was also sacrificed 3 d after the BrdU injections (Figure 2a).

In the survival, cell distribution and neurogenesis experiment the mice received BrdU (100 mg/kg, i.p., $n=9$/group) twice daily for 1 wk (Figure 3a). The animals were sacrificed 17 d after the first BrdU injection (Figure 3a).

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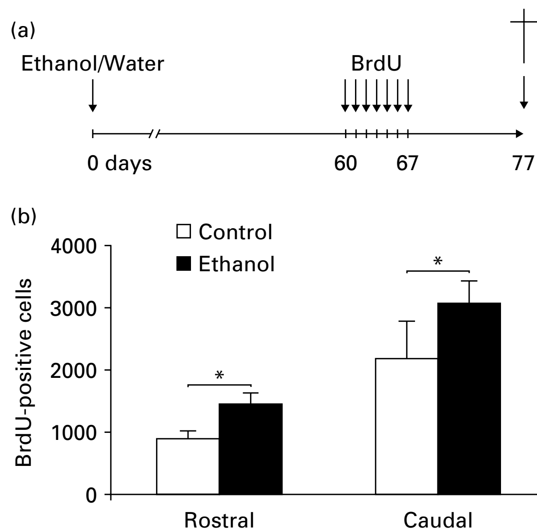

Figure 3. Survival, cell distribution and neurogenesis experiment. (a) Animals in the ethanol group consumed ethanol/water and animals in the control group consumed water/water in the two-bottle free-choice model during 77 d. BrdU was injected twice a day for 1 wk starting 17 d before the animals were sacrificed. All animals were sacrificed on day 77, indicated by a cross. (b) Cells in the dentate gyrus that are formed under the influence of ethanol survive for weeks. There was an increased number of BrdU-positive cells in the granule cell layer in both rostral and caudal parts of the dentate gyrus after moderate ethanol consumption. Values are mean ± S.E.M. counted in both hemispheres ($n = 9$ animals/group). * $p < 0.05$.

Immunohistochemistry

Serial coronal 30-μm sections were cut on a cryostat through the entire hippocampus starting at −0.94 mm from Bregma and ending at −3.88 mm from Bregma (Franklin and Paxinos, 1997). Every tenth section was processed for BrdU immunohistochemistry. Sections were post-fixed in 4% formaldehyde in PBS for 10 min, rinsed in PBS followed by incubation in 2 M HCl for 30 min at 37 °C to increase exposure of BrdU epitopes to the primary antibody. After washing in PBS, sections were blocked in a blocking solution (10% goat serum in 0.1% Tween in PBS) overnight at 4 °C. Sections were then incubated for 90 min at room temperature with the primary antibody against BrdU (rat anti-BrdU, Harlan Seralab, Leics., UK, 1:100) diluted in the blocking solution, followed by 3 × 30 min 0.1% Tween PBS rinses. Sections were next incubated with biotinylated goat anti-rat biotin (Vector Laboratories, Burlingame, CA, USA, 1:200) in the blocking solution for 60 min at room temperature. After another series of rinses (0.1% Tween PBS, 90 min), avidin-biotin (Vector Laboratories) was administered for 40 min followed by rinses in PBS alone for 1 h, and visualization using 3,3′-diaminobenzidine (DAB) (Sigma). The sections were counterstained with haematoxylin (Vector Laboratories) and mounted.

To establish the relative distribution of newly formed cells in the dentate gyrus, we arbitrarily divided the granule cell layer into (i) an inner layer (two cells into the hilus and two cells into granule cell layer), (ii) a middle layer and (iii) an outer layer (one cell into the granule cell layer and one cell out in the molecular cell layer) (Figure 4c).

For immunofluorescence double labelling of BrdU/NeuN and BrdU/S100β, where NeuN is a marker for neurons and S100β a marker for glial cells, the same protocol as described above was used, adding one antibody at a time. Primary antibodies used for immunofluorescence were: NeuN (mouse anti-NeuN, Chemicon, Hants., UK, 1:1500) and S100β (rabbit anti-S100β, Swant, Bellinzona, Switzerland, 1:100). Secondary antibodies were: FITC (donkey anti-rat FITC, Jackson, West Grove, PA, USA, 1:200), Cy3 (donkey anti-mouse Cy3, Jackson, 1:800 or donkey anti-rabbit Cy3, Jackson, 1:400).

Co-localizations of BrdU/NeuN and BrdU/S100β were detected on two sections/animal [−2.06 mm from Bregma, −2.30 mm from Bregma (Franklin and Paxinos, 1997)] where all cells on one side were investigated for co-localization using a number-weighted sample of BrdU-immunoreactive cells and confocal microscopy (LSM 510 META, Zeiss, Göttingen, Germany). Images were digitally processed for contrast (Adobe Photoshop, Mountain View, CA, USA).

TUNEL staining

Detection of apoptotic cells was made on two sections from every individual [−2.06 mm from Bregma, −2.30 mm from Bregma (Franklin and Paxinos, 1997)] using an in-situ TUNEL cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, sections were fixed in 4% formaldehyde for 20 min followed by incubation in a blocking solution (0.3% H_2O_2 in methanol) for 30 min. The sections were then incubated in a permeabilization solution (0.1% Triton, 0.1% sodium citrate) at room temperature for 30 min followed by the TUNEL reaction for 1 h at 37 °C.

Stereology

The 'optical fractionator' was used to count BrdU-immunoreactive cells in the dentate gyrus (West and

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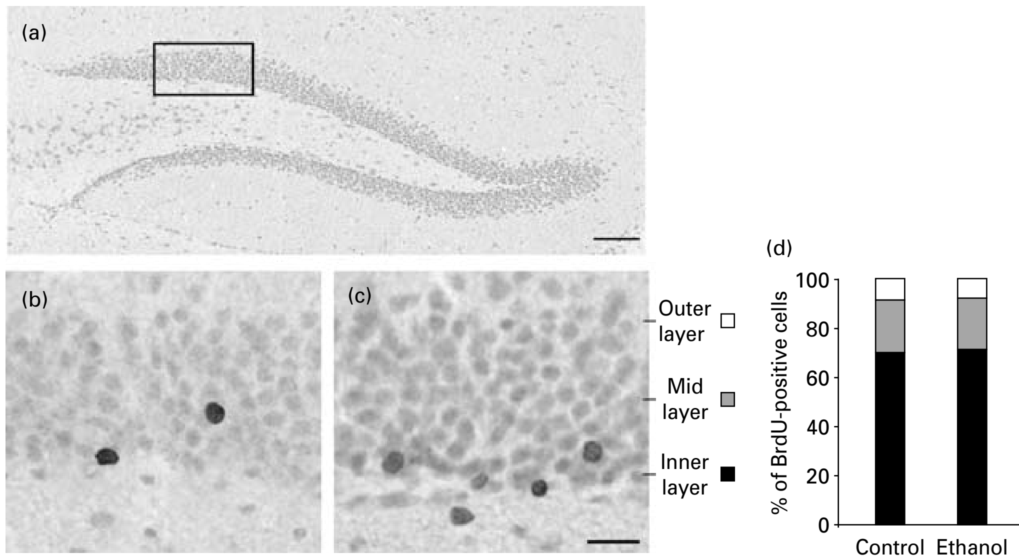

Figure 4. Distribution of newly formed BrdU-positive cells [dark areas in (b) and (c)] in the granule cell layer of the dentate gyrus. Panel (a) illustrates a low magnification image of the dentate gyrus in one hemisphere of the mouse brain. The boxed area illustrates from which region the representative high magnification images in (b) and (c) were obtained (scale bar 100 μm). Note that some of the new cells (dark areas) have migrated into deeper granule cell layers in both control animals (b) and in animals with access to ethanol (c) (scale bar 200 μm). BrdU-positive cells were counted in three defined layers: (i) the inner layer, (ii) the mid layer and (iii) the outer layer. (d) The relative distribution of BrdU-positive cells in the granule cell layer of the dentate gyrus in animals that were given BrdU during 1 wk. BrdU-positive cells have the same distribution in ethanol and control animals, with most of the cells in the inner layer, some cells in the mid layer and scattered cells in the outer layer. Values are means counted in both hemispheres ($n = 9$ animals).

Gundersen, 1990; West et al., 1991). Briefly, every tenth section was systematically sampled [section sampling fraction (s.s.f.) = 1/10] after randomly selecting the first section within the first interval. An unbiased counting frame with known area was superimposed on the field of view by appropriate software (Stereologer™, SPA Inc., VA, USA). Counting frames were systematically distributed with known x and y steps throughout the marked region from a random starting point. The area of the counting frame relative to the area associated with the x and y steps gives the second fraction [area sampling fraction (a.s.f.)]. The height of the optical dissector relative to the thickness of the section results in the third fraction [height (h)/thickness (h)]. The total number of neurons is given by

$$N_{\text{total}} = \Sigma Q - \cdot \frac{1}{\text{s.s.f.}} \cdot \frac{1}{\text{a.s.f.}} \cdot \frac{t}{h},$$

where ΣQ is the number of neurons counted in the dissectors. Optical fractionator estimates are free of assumptions about cellular shape and size and are unaffected by tissue shrinkage. The dentate gyrus including an area exceeding the subgranular zone by two cell diameters and an area exceeding the molecular layer by one cell diameter was manually outlined using a 10× lens. Cell counts were performed with a 60× lens (numerical aperture = 1.4). The rostral and caudal half of the systematic random sample of sections were defined as the rostral or caudal part of the dentate gyrus. The separation between the rostral and caudal part was made at −2.41 mm from Bregma (Franklin and Paxinos, 1997).

Blood alcohol concentration

To determine blood ethanol concentrations, trunk blood was taken when the animals were sacrificed and collected in heparinized tubes. The tubes were centrifuged at 3200 rpm for 10 min and serum was assayed for ethanol using the NAD:NADH enzyme spectrophotometric method (Sigma-Aldrich).

Statistical analyses

Because of large variations in cell counts from the cell proliferation experiment and from the survival, cell distribution and neurogenesis experiment, non-parametric statistical analyses were used. Kruskal–Wallis analysis of variance (ANOVA; Statistica,

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StatSoft, Tulsa, OK, USA) was thus applied to treat data from cell counts. This statistical method was also used to treat data from the TUNEL-staining experiment. Student's *t* test was applied to treat cell counting data from the acute ethanol experiment.

Results

Four days ethanol consumption has no effect on cell survival in the dentate gyrus

To analyse whether moderate voluntary ethanol consumption in adult female C57BL/6 mice has any acute toxic and/or cell proliferation effects in the granule cell layer of the dentate gyrus, singly housed mice were given ethanol/water in the two-bottle free-choice model for ethanol consumption for 4 d after receiving BrdU (Figure 1a). The mice had an average ethanol preference of 37% and consumed 4.3 ± 0.67 g ethanol/kg.d.

No changes in the number of BrdU-labelled cells were detected with this regime of BrdU and ethanol administration (Figure 1b). Nor did we detect a change in the number of cells displaying free DNA ends indicative of apoptosis in the dentate gyrus using TUNEL staining (Figure 1c).

Moderate ethanol consumption increases cell proliferation in the dentate gyrus

To investigate if ethanol can influence cell proliferation, singly housed adult female C57Bl/6 mice, ~5 months old at the start of the experiment, were given a free choice of drinking either 10% ethanol or water in the two-bottle free-choice paradigm during 63 d (Figure 2a). The mean blood ethanol concentration was 24 mg/dl at the time of sacrifice (11:00 hours). The mean weight of the animals was 24.9 ± 2.9 g at the start of the experiment and 29.6 ± 3.8 g at the time of sacrifice. There was no difference in body weight between the different groups at any time of the experiment. The mice increased their ethanol consumption during the first 2 wk after which they levelled off at an average intake of 6.05 ± 0.93 g ethanol/kg . d, which corresponds to a 53% preference for ethanol.

To label proliferating cells, all animals were administered BrdU 3 d before sacrifice (Figure 2a). The hippocampus can functionally be divided into a rostral and a caudal part. We, therefore, counted BrdU-immunoreactive cells along the rostral–caudal axis of the dentate gyrus. Moderate ethanol consumption during 63 d increased cell proliferation in the rostral part of the dentate gyrus ($p < 0.05$, Figure 2b), while there was no statistically significant change in the caudal part.

To analyse whether access to ethanol had any long-lasting effects on cell proliferation, an ethanol withdrawal group was taken off ethanol 3 d before BrdU administration (Figure 2a). No signs of withdrawal symptoms, e.g. seizures, were observed. Cell proliferation was lower in the ethanol withdrawal group in both the rostral and the caudal part of the dentate gyrus compared to animals with unrestricted access to ethanol ($p < 0.05$, Figure 2b). The cell proliferation in mice that had consumed ethanol for 57 d and then experienced 3 d of ethanol withdrawal, had returned to levels seen in control mice.

Cells formed under the influence of ethanol survive for weeks

To elucidate the fate of the increased number of cells formed during ethanol exposure, animals were exposed to the two-bottle free-choice paradigm for ethanol consumption for 77 d and sacrificed 17 d after the first BrdU injection (Figure 3a). Interestingly, there was an increase of newly formed cells that had survived both in rostral and caudal parts of the dentate gyrus in the ethanol group ($p < 0.05$, Figure 3b). This increase was of the same magnitude as the increase in cell proliferation. Thus, the new cells formed during ethanol intake survive to the same extent as cells in the control group. TUNEL staining was performed on representative sections to detect if moderate ethanol consumption would induce DNA fragmentation, indicative of apoptosis in the hippocampus. However, there were no indications of increased DNA fragmentation in the ethanol group (data not shown). The effect of moderate ethanol consumption was, thus, to increase cell proliferation without impairing cell survival.

Ethanol consumption does not change the distribution pattern of newly proliferated cells

Typically, newly formed cells in the subgranular zone of the dentate gyrus migrate into the granule cell layer and differentiate into neuronal or glial phenotypes. To characterize the distribution of the new cells in the ethanol group, we mapped the distribution of the BrdU-positive cells in the granule cell layer 17 d after the first BrdU injection. The granule cell layer was divided into three parts (see Methods section). Ethanol did not change the distribution pattern of the newly formed cells compared with the control group (Figure 4a–d). Thus, most of the BrdU cells were encountered in the inner layer. Few cells were found in the middle

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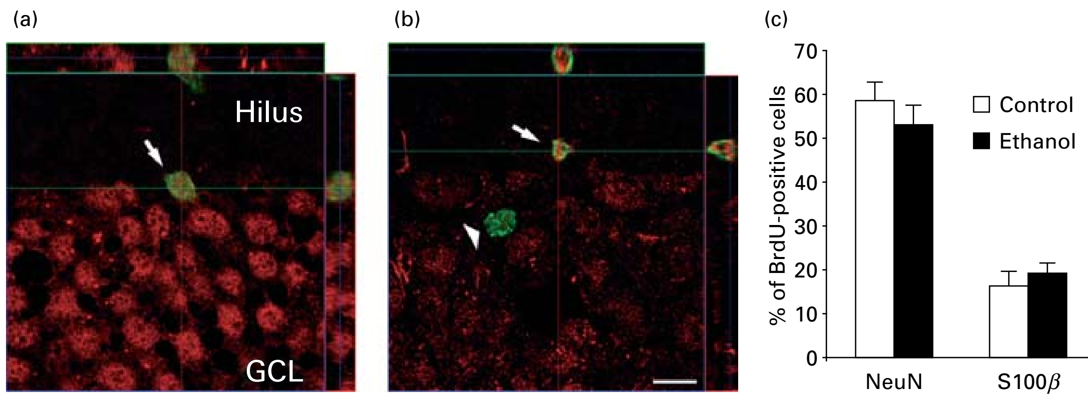

Figure 5. Phenotypic characterization of newly formed cells in the granule cell layer of the dentate gyrus. Panel (a) illustrates neurons in the granule cell layer (GCL) while panel (b) illustrates glial cells also in the granule cell layer of the dentate gyrus. NeuN-immunoreactivity (neuron, red) is detected in a BrdU-immunoreactive cell (green) located in the subgranular zone of the dentate gyrus (a, arrow). S100β-immunoreactivity (glial cell, red) is detected in a BrdU-immunoreactive cell (green) also located in the subgranular zone of the dentate gyrus (b, arrow). The arrowhead illustrates a BrdU-positive cell lacking S100β-immunoreactivity (b) (scale bar 10 μm). From the scanned volume, ~0.3-μm-thick image planes were chosen as indicated by the thin lines, to visualize co-localizations. (c) Percentage BrdU-positive cells expressing either NeuN- or S100β-immunoreactivity. No differences in differentiation are seen between the ethanol and control groups.

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layer and scattered cells in the outer layer (Figure 4a–d).

Ethanol consumption increases neurogenesis and gliogenesis in the dentate gyrus

Phenotypic characterization of the newly formed cells was carried out using NeuN-immunoreactivity as a marker for neurons and S100β-immunoreactivity as a marker for glial cells. Approximately 60% of BrdU-immunoreactive cells in both the ethanol and control groups were NeuN-immunoreactive while ~20% of the cells in both groups displayed S100β-immunoreactivity (Figure 5a–c). The remaining cells did not express either of the two markers. There was no difference between the ethanol and control groups in relative proportions of BrdU co-localization with NeuN or S100β. Thus, ethanol increased the number of newly proliferated cells that survived and expressed a neuronal marker, as well as those that expressed a glial marker.

Discussion

In the present study we investigated if voluntary ethanol consumption in adult mice would influence cell proliferation, neurogenesis or apoptosis in the dentate gyrus. A key factor in our experiments was that adult mice had free access to one water bottle and one bottle of ethanol during the whole experiment and were, therefore, able to choose and regulate ethanol intake at will. The mice did not reach neurotoxic ethanol dosing. The blood alcohol concentration was 0.24‰ (24 mg/dl) at the time of sacrifice (11:00 hours) after ~10 wk of ethanol consumption, which corresponds to a level of alcohol commonly encountered in humans under controlled social conditions (Eckardt et al., 1998).

Hypothetically, increased cell proliferation and neurogenesis after ethanol consumption could be a compensatory mechanism in response to ethanol-induced hippocampal cell losses (Pawlak et al., 2002; Zharkovsky et al., 2003). For example, it is possible that ethanol has an acute neurotoxic effect and that new cells that are formed just before ethanol exposure are vulnerable to ethanol-induced damage. To test this hypothesis the thymidine analogue BrdU, which is incorporated into the DNA during the S-phase of cell division, was administered the day before mice were given access to ethanol during 4 d. Interestingly, there was no difference in the number of BrdU-labelled cells in dentate gyrus when comparing the group of animals with access to ethanol with the control group. Nor could we detect a significant number of apoptopic cells in the dentate gyrus by TUNEL staining in any of the groups. Thus, moderate ethanol consumption during 4 d is not neurogeneic, neither is it toxic for cells in the dentate gyrus in our model of voluntary ethanol consumption for singly housed adult female C57BL/6 mice.

Having ruled out an acute neurotoxic effect, we analysed if ~10 wk of ethanol consumption could effect cell proliferation, survival, differentiation or cell

distribution. BrdU was used to label the dividing progenitor cells, first by using a cell proliferation protocol, to determine the number of newly formed cells a short period after BrdU administration and second using a protocol to detect cell survival, cell distribution and neurogenesis, in which labelled cells are counted at longer times after BrdU administration. Cell proliferation takes place in the subgranular zone of the dentate gyrus. The cells then migrate into the granule cell layer and differentiate into glial cells and neurons that send out axons via the mossy fibre pathway to CA3 and dendrites into the molecular layer (Duman et al., 2001; Gage, 2000; Gross, 2000; Kempermann et al., 2003; van Praag et al., 2002).

Around 10 wk of access to ethanol in the two-bottle free-choice model for ethanol consumption increased the number of BrdU-labelled cells in the granule cell layer of the dentate gyrus. Hypothetically, this increased number of cells could be a compensatory effect in response to putative accumulating cytotoxic effects of 10 wk of ethanol consumption. However, we could not detect a significant number of TUNEL-stained cells in the dentate gyrus even after 10 wk of ethanol consumption, indicating that there was no significant apoptosis in our model for voluntary ethanol consumption. Toxic effects of ethanol would also most likely result in an increase of the relative proportion of newly formed glial cells vs. nerve cells. This did not happen. Toxic effects of long-term ethanol consumption might also be more subtle, such as influencing the distribution of newly formed cells. However, we could not detect any effect of long-term ethanol intake in the two-bottle free-choice model on the distribution pattern of the newly formed cells within the depth of the dentate granule cell layer.

Binge and forced ethanol administration to animals, which results in toxic doses of ethanol, causes neurodegeneration and decreased neurogenesis in the hippocampus (Herrera et al., 2003; Nixon and Crews, 2002; Zharkovsky et al., 2003). However, neurodegeneration in the hippocampus has not been found in unbiased stereology analysis of human post-mortem brain tissue from alcoholics. Such studies have demonstrated a decrease in hippocampal size due to a reduction of white matter and numbers of astrocytes, but not in a reduction of neurons (Harding et al., 1997; Jensen and Pakkenberg, 1993; Korbo, 1999). Evidence is thus lacking for nerve cell losses in the hippocampus of alcoholics. Voluntary free-choice models of ethanol consumption in rodents lead to non-toxic hippocampal ethanol concentrations and are, thus, perhaps more relevant than different forced ethanol administration models for studying the effects of ethanol on hippocampal plasticity.

Interestingly, voluntary moderate ethanol consumption for ∼10 wk appears to increase cell proliferation. The newly formed cells survive, have the same cell distribution pattern and differentiate as cells do in control groups. The consequence of the increased cell proliferation and the apparently normal survival, differentiation and cell distribution pattern of newly formed cells would ultimately lead to a net increase of both neurons and glial cells. To test whether the ethanol-induced increase of cell proliferation is a stable adaptation or if there is a need for continuous ethanol intake in order to maintain the high cell proliferation, we stopped ethanol for 3 d before BrdU administration in a group of mice, thus inducing a situation of ethanol withdrawal. After 3 d without ethanol, cell proliferation had already returned to basal levels, which suggests that the increased cell formation is directly driven by the ethanol intake, rather than being an ethanol-induced permanent change of cell proliferation rates.

The functional consequence of increased cell proliferation, neurogenesis and gliogenesis in the dentate gyrus in our model of moderate ethanol intake remains to be elucidated. Recently, it was described that new cells formed in the subgranular zone do indeed migrate into deeper granule cell layers of the dentate gyrus. There they differentiate into neurons and glial cells. The neurons send out axons along the mossy fibre pathway to the pyramidal cell layer of CA3, establish synaptic contacts and display electrophysiological characteristics of neurons (Hastings and Gould, 1999; Kempermann et al., 2003; Markakis and Gage, 1999; van Praag et al., 2002). Although we have not demonstrated that new cells in our particular model do indeed integrate in functional neuronal networks, the fact that all parameters analysed, such as differentiation, survival and cell distribution appear normal, suggests that the additional neurons formed in the ethanol group are functional.

It is possible that conditions with positive emotional impact might be coupled with an increase in cell proliferation while down periods might be associated with a decrease in cell proliferation (Gould et al., 1997). In the present study, female mice were investigated because they tend to consume more ethanol (Middaugh et al., 1999). They were also kept one to a cage because group-housed animals do not consume much ethanol (Yanai and Ginsburg, 1976). Singly housing rodents, including C57BL/6 mice, is in itself stressful and it is, therefore, possible that such stress is also associated with depression and anxiety (E. Åberg,

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A. Kuzmin, S.-O. Ögren, L. Olson, S. Brené, unpublished observations; Ferrari et al., 1998; Palanza et al., 2001). Depression is associated with decreased hippocampal size in humans and low rate of cell proliferation in the rat hippocampus (A. Bjørnebekk, A. Mathé, S. Brené, unpublished observations; Bremner et al., 2000; Gould et al., 1997; Sheline, 2000). It is, thus, possible that ethanol had anxiolytic effects in the present experiments (Colombo et al., 1995; Spanagel et al., 1995). thereby counteracting a stress-induced depression of neurogenesis. Such presumably anxiolytic or antidepressant effects on cell proliferation have been noted for antidepressant treatments (Malberg et al., 2000; Santarelli et al., 2003). The ethanol-mediated increase of cell proliferation was no longer seen in the group of animals that had experienced 3 d of alcohol withdrawal prior to BrdU administration. Interestingly, the rate of cell proliferation can thus be changed within a matter of days and ethanol is one factor that can cause such changes.

The hippocampus is important for learning and memory and interacts with both mesolimbic pathways and the amygdala, areas important for reward and emotional processing (Koob et al., 1998; Pikkarainen et al., 1999; Robbins and Everitt, 2002). Our findings show the largest increase of newly formed cells in the rostral part of the dentate gyrus and because of large variations, not significant in the caudal part. Nevertheless, the survival pattern of the newly formed cells from both the rostral and the caudal part, is the same in the whole dentate gyrus. These cells that survive also display a normal cell distribution pattern and they also differentiate normally. Functionally, the rostral part of hippocampus is important for spatial learning whereas the caudal part is more involved in emotional processing (Kjelstrup et al., 2002; Moser and Moser, 1998). New nerve cells that are formed under the influence of alcohol in both rostral and caudal parts of the dentate gyrus could, therefore, based on localization, become part of neuronal networks involved in both spatial learning and emotional processing. In fact, associative long-term potentiation can be induced more easily in young newly generated granule cells than in mature neurons in the hippocampus (Schmidt-Hieber et al., 2004). All these data together support the idea that the new cells that are formed under the influence of ethanol become incorporated normally in the network of the hippocampus and could, thus, be involved in normal hippocampal functions.

We report here that long-term voluntary moderate ethanol consumption in mice increases cell proliferation and neurogenesis in the dentate gyrus of the hippocampus. We hypothesize that the new nerve cells formed under the influence of voluntary moderate alcohol consumption could integrate into neuronal networks that may have a role in learning, memory and emotional processing. Alterations of cell proliferation and neurogenesis in the dentate gyrus might, thus, constitute one mechanism underlying long-term adaptive changes after ethanol consumption.

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Statement of Interest

None.

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