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Passive avoidance training is correlated with decreased cell proliferation in the chick hippocampus

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Abstract

One-trial passive avoidance learning (PAL), where the aversive stimulus is the bitter-tasting substance methylanthranilate (MeA), affects neuronal and synaptic plasticity in learning-related areas of day-old domestic chicks (*Gallus domesticus*). Here, cell proliferation was examined in the chick forebrain by using 5-bromo-2-deoxyuridine (BrdU) at 24 h and 9 days after PAL. At 24 h post-BrdU injection, there was a significant reduction in labelling in MeA-trained chicks in both the dorsal hippocampus and area parahippocampalis, in comparison to controls. Moreover, double-immunofluorescence labelling for BrdU and the nuclear neuronal marker (NeuN) showed a reduction of neuronal cells in the dorsal hippocampus of the MeA-trained group compared with controls (35 and 49%, respectively). There was no difference in BrdU labelling in hippocampal regions between trained and control groups of chicks at 9 days post-BrdU injection; however, the number of BrdU-labelled cells was considerably lower than at 24 h post-BrdU injection, possibly due to migration of cells within the telencephalon rather than cell loss as apoptotic analyses at 24 h and 9 days post-BrdU injection did not demonstrate differences in cell death between treatment groups. Cortisol levels increased in the chick hippocampus of MeA-trained birds 20 min after PAL, suggesting the possibility of a stress-related mechanism of cell proliferation reduction in the hippocampus. In contrast to hippocampal areas, the olfactory bulb, an area strongly stimulated by the strong-smelling MeA, showed increased cell genesis in comparison to controls at both 24 h and 9 days post-training.

Introduction

The avian hippocampus (Hp) [and area parahippocampalis (APH)] plays a key role in spatial learning (Lee *et al.*, 1998; Regolin & Rose, 1999), food storing (Sherry *et al.*, 1989; Clayton & Krebs, 1994), homing (Bingman *et al.*, 1990) and one-trial passive avoidance learning (PAL) where chicks are trained to avoid pecking at a bead coated with a bitter-tasting substance, methylanthranilate (MeA) (Sandi *et al.* 1992; Nikolakopoulou *et al.*, 2006). It has been suggested that it shows homology to its mammalian counterpart based on neurochemical (Casini *et al.*, 1986) and embryological evidence (Kallen, 1962), although it lacks the cellular lamination which characterizes the mammalian Hp. The avian Hp can be divided into ventral (vHp) and dorsal (dHp) components based on immunocytochemical data (Erichsen *et al.*, 1991), tracing techniques (Szekely, 1999) and electrophysiological data (Siegel *et al.*, 2002) corresponding to Ammon's horn and the dentate gyrus (Szekely & Krebs, 1996), respectively, whereas the APH is similar to the subiculum (Casini *et al.*, 1986), although the boundaries are not clearly defined.

In domestic chicks avoidance learning has also been shown to affect parts of the limbic system such as the arcopallium (A) (Benowitz, 1972; Lowndes & Davies, 1994), a region that can be further divided into intermediate (AI) and dorsal (AD) parts. The AI is responsible for fear responses in birds (Goodman & Brown, 1966; Phillips & Youngren, 1971), unlike the AD which does not belong to the chick limbic system (Zeier & Karten, 1971; Davies *et al.*, 1997).

Additionally, the olfactory bulb (BO) has been suggested to be strongly linked to limbic structures participating in PAL (e.g. mesopallium and arcopallium) as training chicks with an odourless substance caused reduced avoidance compared with the strong-smelling MeA (Richard & Davies, 2000).

A recent study (Dermon *et al.*, 2002) has shown that one-trial PAL on the bitter-tasting substance MeA in chicks enhances cell proliferation in learning-related brain regions at 24 h and 9 days post-5-bromo-2-deoxyuridine (BrdU) injection. Neurogenesis has also been identified in the Hp of food-storing birds (Clayton & Krebs, 1994), after spatial learning (Patel *et al.*, 1997) and can be affected by seasonal changes (Barnea & Nottebohm, 1994), whereas in mammals it can be induced by exposure to an enriched environment (Nilsson *et al.*, 1999; van Praag *et al.*, 1999) and water maze training (Dobrossy *et al.*, 2003; Drapeau *et al.*, 2003). As limbic association areas are concerned with emotions and memory storage (Kluver & Bucy, 1997), we examined the effects of PAL in day-old domestic chicks on cell birth in Hp and structures of the limbic system including nucleus taeniae of amygdala (TnA) at 24 h and 9 days after BrdU injection. Our study indicates a significant reduction in newborn cells in dorsal and parahippocampal areas in MeA-trained chicks compared with control birds at 24 h post-BrdU injection but not at 9 days post-injection, although the number of BrdU-labelled cells is much lower overall in Hp at 9 days compared with 24 h post-injection.

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Materials and methods

Animal training and 5-bromo-2-deoxyuridine administration

Commercially obtained Ross Chunky eggs of the domestic chick *Gallus domesticus* were incubated and hatched in our own brooders. Chicks were maintained until they reached 18–22 h old and were then weighed and placed in pairs in aluminium pens, illuminated by red bulbs. Chicks were divided into three groups, one of which was the control (undisturbed) whereas the other two groups underwent passive avoidance training as described in Dermon *et al.* (2002). Pre-training was by presentation of a small white bead three times with an interval of 5 min between each presentation. Birds that successfully pecked at the training bead three times and the control group were then injected intraperitoneally with BrdU diluted in 0.9% NaCl solution (0.1 mg/g of body weight; Sigma, UK). At 30 min after injection chicks were presented with a 4-mm-diameter chrome bead coated with either water (water-trained group) or MeA (MeA-trained group), a very bitter substance that causes disgust responses. The MeA-trained chicks remembered the task and avoided a similar but dry bead at testing 24 h after the training whereas the water-trained animals pecked the dry bead. Chicks which failed to give the correct response to the task within 20 s and pecked at the dry bead although previously exposed to MeA (20% of the MeA group) were excluded from the study. Animals that remembered the task successfully were used for cell proliferation and migration studies and were killed 24 h or 9 days after training.

All experimental procedures were carried out under UK Home Office licence and were also in agreement with the European Communities directive (86/609/EEC) for the care and use of laboratory animals.

5-Bromo-2-deoxyuridine immunocytochemistry

Chicks were killed 24 h (control $n = 7$, water-trained $n = 8$, MeA-trained $n = 7$) or 9 days (control $n = 5$, water-trained $n = 5$, MeA-trained $n = 5$) post-BrdU injection. Animals were anaesthetized with sodium pentobarbitone (50 μL/g of body weight) and transcardially perfused with 0.9% NaCl including heparin followed by fixative solution [4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4]. The brains were removed and post-fixed overnight at 4 °C in 10% sucrose in 4% paraformaldehyde in 0.1 M PB. They were placed in 20% sucrose in 0.1 M PB overnight and then frozen using isopentane at −40 °C and stored at −80 °C.

The brains were cut in the coronal plane using a cryostat microtome (Leica, UK) and 50-μm-thick free-floating sections were collected in PB (0.1 M, pH 7.4). One set of sections in every 300 μm was used for immunocytochemistry.

Sections from all experimental groups were processed simultaneously with the same solutions to avoid discrepancies due to processing. Sections were incubated for 2 h at 65 °C in 1 : 1 formamide/2× SSC, rinsed in 2× SSC for 5 min and then incubated in 2 N HCl at 45 °C for 30 min to denature DNA. The tissue was rinsed for 3 × 5 min and 1 × 10 min in 0.1 M PB and then transferred in 3% (v/v) hydrogen peroxide in 0.1 M PB (pH 7.4) for 10 min to block endogenous peroxidase. Washes in 0.1 M PB followed and the tissue was blocked with 1.5% (v/v) horse normal serum and 0.1% (v/v) Triton X-100 in 0.1 M PB for 20 min at room temperature (25°C). The sections were incubated overnight at 10 °C with a mouse anti-BrdU monoclonal antibody solution (Becton Dickinson, UK) diluted 1 : 100 in 0.1% Triton X-100 in 0.1 M PB, pH 7.4. The sections were washed in 0.1 M PB for 3 × 5 min and then incubated with a biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories, UK) diluted 1 : 200 in 0.1 M PB for 2 h at room temperature. The tissue was again washed in PB and then transferred to an avidin/biotin/peroxidase solution (Vector Laboratories, ABC kit, diluted 1 : 50 solution A and 1 : 50 solution B in 0.1 M PB, pH 7.4) for 1 h in the dark at room temperature. The tissue was rinsed successively in 0.1 M PB (pH 7.4) and 0.1 M Tris-buffered saline, pH 7.5. BrdU-containing cells were visualized by the polymerization of 0.05% diaminobenzidine (DAB kit, Sigma) in 0.01% hydrogen peroxide in Tris-buffered saline for 2 min at room temperature. The reaction was stopped by dipping the sections in Tris-buffered saline (0.1 M, pH 7.5) and they were mounted on gelatinated slides and left to dry. For microscopic observation, sections were dehydrated in graded series of ethanol, cleared with xylene and covered with mountant using Entellan rapid mounting media for microscopy (Merck, Germany). In order to control the occurrence of non-specific or cross-labelling, adjacent sections were incubated without primary or secondary antibody. No labelling was detected in any of the procedures.

Double-labelling immunofluorescence: 5-bromo-2-deoxyuridine and either nuclear neuronal marker or glial fibrillary acidic protein

To determine the nature of the newly generated cells (whether neuronal or glial) double-labelling immunofluorescence was carried out with labelling either by nuclear neuronal marker (NeuN), which labels mature neurones (Mullen *et al.*, 1992), or an antibody to glial fibrillary acidic protein (GFAP), which labels glial cells. Each of the steps of the first part of the reaction for the double-labelling studies, including the blocking step, was identical to those for BrdU immunohistochemistry as described above. Sections were incubated overnight at 10 °C with a rat anti-BrdU monoclonal antibody (Abcam, UK; diluted 1 : 100 in 0.1% Triton X-100 in 0.1 M PB). On the second day, after rinsing in 0.1 M PB, sections were incubated with Alexa Fluor 647 goat anti-rat IgG secondary antibody diluted 1 : 200 in 0.1 M PB for 2 h at room temperature to visualize BrdU-positive cells. The tissue was washed in 0.1 M PB for 3 × 10 min and then incubated in 2% goat normal serum, 0.1% Triton X-100 in 0.1 M PB and finally incubated for 48 h at 10 °C with antibodies to mouse anti-GFAP (diluted 1 : 500) or mouse anti-NeuN (diluted 1 : 1000) (all from Chemicon International), 0.1% Triton X-100 in 0.1 M PB, pH 7.4. The sections were rinsed in PB and incubated in an Alexa Fluor 488 goat anti-mouse IgG1 secondary antibody before washing and mounting on gelatinated slides. Slides were dried in the dark at room temperature and then coverslips were placed on the sections using fluoromount mountant (BDH, UK).

Apoptosis

Sections (20 μm thick) from the brains of the same animals used for cell proliferation studies were cut with a cryostat (Leica) and collected on gelatinated slides. One set of sections in every 120 μm was used for apoptotic studies. Apoptosis was examined via DNA fragmentation by the TUNEL assay (Gochuico *et al.*, 1997; Hara *et al.*, 1999) using an ApopTag peroxidase *in situ* apoptosis detection kit (Intergen). The reagents in the kit label the free 3′OH DNA termini with the use of terminal deoxynucleotidyl transferase, which catalyses the addition of nucleotide triphosphates to the free 3′OH ends of double- or single-stranded DNA.

Quantification

Cell profile counting was confined within the neuroanatomical borders of Hp, arcopallium, TnA and BO in addition to the ventricular zone adjacent to these areas. All slides were coded and all subsequent procedures were performed blind to training group. The total numbers

of BrdU-labelled cells were counted in the areas of interest in all sections (see cutting protocol) due to the small number of labelled cells and their distribution pattern. Images were captured using a DMX 1200 camera attached to an E800 microscope (both from Nikon). Analyses of data were performed using ANALYSIS software running on a Pentium IV personal computer. The number of labelled cells determined was divided by the volume of each brain region measured calculated by the method of Cavalieri (1635/1966; Wulfsohn *et al.*, 2004) (V_{area} = total area in $mm^2 \times 0.05$ mm $\times$ 6) and thus the number of BrdU-positive cells/mm^3 was calculated (cells per volume). For immunofluorescence analyses, studies were performed in sequential scanning mode to avoid cross-bleeding between channels and series of images along the z-axis, thus five z-stack images/hippocampal section (one optical section/1 μm) were captured using a Leica TCS confocal microscope from five serial sections of each brain (25 samples/area/brain) and all BrdU-positive cells in the dHp and vHp were counted and assessed for double labelling. Percentages for double labelling were determined by dividing the number of each type of double-labelled cells (ND) by the total number of BrdU-positive cells (NB) present in the specific brain area and then multiplying by 100 [% ND = (ND/NB) $\times$ 100].

Statistical analysis

All counted profile numbers were analysed using multiple-factor ANOVA and P-values < 0.05 were taken as statistically significant. Fisher LSD post-hoc analysis followed where appropriate.

Cortisol studies

Cortisol extraction was performed as previously described (Prasad *et al.*, 1986; Nozaki, 2001). Cortisol was determined as it is known to be a natural adrenal steroid in neonatal chicks (Kalliecharan & Hall, 1974) and its exogenous application suppressed mitotic activity in thyrofollicular cells (Ganguli *et al.*, 2001). Forty animals were trained simultaneously with those used for cell proliferation and apoptosis studies, decapitated (control n = 14, water n = 12, MeA n = 14) and the brains immediately removed. The Hp, arcopallium and medial striatum were dissected and frozen in isopentane. Because of the small tissue volume, the Hp could not be further divided into ventral and dorsal parts for cortisol studies. Samples were kept at −80° C until cortisol extraction. Tissue samples were homogenized in 5× their volume in phosphate-buffered saline (0.01 M, pH 7.3, containing 0.14 M NaCl). A volume (250 μL) of the homogenate was extracted twice with diethyl ether by strong vortexing for 1 min. The aqueous phase was frozen at −80 °C in isopentane and the ether layer was transferred to another tube. Combined extracts were dried in a water bath at 45 °C in an atmosphere of nitrogen. Extracts were resuspended in 250 μL phosphate-buffered saline containing gelatin (0.1%) and 100-μL aliquots (in duplicate) were used for radioimmunoassay following the protocol described in Nozaki (2001).

Extraction efficiencies were monitored for each sample by the addition of ^{3}H-cortisol to homogenates extracted in the same manner as samples that were used for radioimmunoassay. The average recovery for the samples was 92%.

Results

Cell proliferation studies

In short (24 h) and long (9 days) survival experiments, BrdU-positive cells were determined in the vHp and dHp, APH, AI and AD, nucleus taeniae of the amygdala (TnA) and BO after PAL at post-hatching day (P)2 and P9. The Hp and APH receive efferents from the AI (Casini *et al.*, 1986; Davies *et al.*, 1997), whereas the former demonstrates reciprocal connections to APH and TnA (Casini *et al.*, 1986; Atoji *et al.*, 2002). The BO sends afferents to the TnA (Reiner & Karten, 1985) and thus all of the areas of this study are interconnected, potentially affecting one another. The locations of these regions are shown in the schematic drawing of the coronal sections in Fig. 1. Cell proliferation and survival are regional and training specific. Digital images of BrdU labelling in the dHp and vHp are shown in Fig. 2A–E. There are obvious differences in labelling incidence between the dHp of control (Fig. 2A) and MeA-trained (Fig. 2B) birds with the latter demonstrating a lower level of BrdU cell labelling, although the vHp of control animals (Fig. 2C) does not show any differences in BrdU labelling in comparison to MeA-trained animals (Fig. 2D). In most cases, at 9 days after PAL there was reduced labelling in comparison to that at 24 h (Fig. 2E and F, illustration of BrdU labelling in the dHp 9 days after BrdU injection in control and MeA-trained animals).

Passive avoidance learning effects on 5-bromo-2-deoxyuridine labelling: quantification at 24 h post-training

Data for labelling levels in the hippocampal regions are shown in Fig. 3A and B. A three-way ANOVA showed statistical differences between the age of the animals ($F_{1,78}$ = 85.35, P < 0.0001), training group ($F_{2,78}$ = 4.245, P = 0.018), area of the brain (vHp, dHp or APH) ($F_{2,78}$ = 17.93, P < 0.0001), and the interaction between age (days after training) and training group (F = 10.7, P < 0.0001).

There were significantly fewer (46.5%) BrdU-labelled cells at 24 h post-training in the chick dHp (LSD post-hoc analysis, P = 0.0075) in the MeA-trained group in comparison to control animals. The vHp of the MeA-trained group showed an apparent reduced cell proliferation but this was not significant (P = 0.073).

The APH of water- and MeA-trained animals had considerably fewer labelled cells in contrast to controls (23.5% and 46%; P = 0.019 and P < 0.0001, respectively). Cell proliferation in TnA (Fig. 3C) was not affected by avoidance training at 24 h post-training.

There were no differences in BrdU labelling between the three chick groups in either the AI or AD (Fig. 3E); however, labelling was significantly higher in the AI compared with the AD of MeA chicks (P = 0.0046) (Fig. 3E).

In the BO (Fig. 3G) training significantly altered cell proliferation ($F_{2,24}$ = 10.33, P = 0.00058) with more BrdU cells in MeA-trained birds in comparison to either controls (> 48%) (P = 0.0087) or water-trained birds (40%) (P = 0.015).

Passive avoidance learning effects on the survival of 5-bromo-2-deoxyuridine-labelled cells: 9 days post-training

At 9 days post-training there were no significant differences in BrdU labelling in the Hp, TnA, AI and AD between the groups examined (Fig. 3B, D and F). Only the BO exhibited a significantly higher density of BrdU-positive cells in MeA-trained birds compared with the control (P = 0.013) and water (P = 0.009)-trained groups (Fig. 3H).

When comparisons were made between BrdU labelling at 9 days post-training vs. that at 24 h, there was reduced labelling in all areas of the Hp especially in animals of the control and water-trained groups (LSD post-hoc, vHp control P = 0.0004, vHp water P = 0.012, dHp P = 0.0005 and P = 0.01 correspondingly, APH P < 0.0001 and P = 0.00012). The reductions ranged from 63% in control birds, 70.5% in water-trained birds and 42% in MeA-trained birds in the vHp

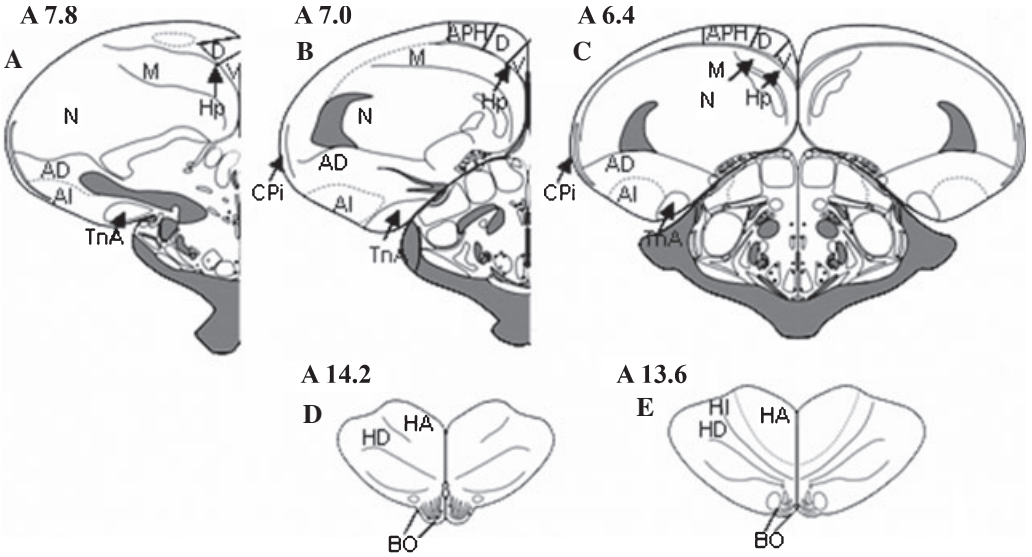

FIG. 1. Coronal sections of the chick brain representing different levels where 5-bromo-2-deoxyuridine-labelled cells were counted. (A–C) Location of the ventral (V) and dorsal (D) hippocampus (Hp), area parahippocampalis (APH), nucleus taeniae of amygdala (TnA), and intermediate and dorsal arcopallium (AI and AD, respectively). (D and E) Location of the olfactory bulb (BO) in the chick brain. M, mesopallium; N, nidopallium; CPi, piriform cortex; HA, apical part of the hyperpallium; HD, densocellular part of the hyperstriatum; HI, intercalated part of the hyperpallium.

with reductions in a similar range in dHp and APH in relation to short-term (24-h) experiments.

In TnA, there was also a significant reduction in BrdU labelling at 9 days after BrdU injection in the control and water-trained group with respect to these groups at 24 h post-training ($P = 0.001$ and $P = 0.0075$, respectively).

The biggest reductions were seen in the arcopallium (Fig. 3E and F). On average, comparing the means of the three groups, BrdU cell labelling at 9 days post-BrdU injection was only 20% of that at 24 h post-injection. In addition, three-way ANOVA conducted for TnA revealed differences which occurred due to the interaction of time after training and brain area ($F_{1,54} = 4.66$, $P = 0.036$). The data from all of the training groups demonstrated fewer BrdU-labelled cells at P9 in comparison to P2 in acropallium (LSD post-hoc test: control: AI $P < 0.0001$, AD $P = 0.00046$; water: AI $P < 0.0001$, AD $P = 0.00017$; MeA: AI $P < 0.0001$, AD $P = 0.0003$) (Fig. 3F).

The labelling reductions in the BO were also seen at 9 days compared with 24 h post-injection (three-way ANOVA for age, $F_{1,24} = 16.12$, $P = 0.00051$) in the water- and MeA-trained animals ($P = 0.015$ and $P = 0.024$, respectively).

Passive avoidance learning effects on type of proliferating cells: double-labelling immunofluorescence

Neuronal and glial cells were identified using antibodies to NeuN and GFAP, respectively. Double immunolabelling at 24 h and 9 days post-BrdU injection showed GFAP- and BrdU-labelled cells (Fig. 4A–C) and NeuN and BrdU in Fig. 4D–F. The percentages of double-labelled cells of either neuronal or glial type were unchanged between 24 h and 9 days post-training.

However, when the proportion of BrdU- to NeuN-labelled cells and that of BrdU- to GFAP-labelled cells was examined in the dHp and vHp (Fig. 5A–H), differences were observed in the percentages of cells coexpressing NeuN and BrdU in the vHp and dHp between the control and MeA-trained group. In the vHp, the MeA-trained group showed significantly more double-labelled cells for NeuN/BrdU (46.3%) in comparison to controls (31.8%, $P = 0.03$) (Fig. 5A). In contrast, the dHp of the MeA-trained group showed fewer NeuN/BrdU cells (34.7%) in comparison to controls (49%, $P = 0.04$) (Fig. 5B). Additionally, in control animals there were significantly more NeuN/BrdU-positive cells in the dHp in comparison to the vHp ($P = 0.013$) (Fig. 5A and B). The percentage of BrdU/NeuN cells did not change between training groups at P9 (Fig. 5C and D), whereas it appeared similar to that of P2 (vHp: 36.4% control, 37% water-trained, 43% MeA; dHp: 34.7% control, 43% water-trained, 39.5% MeA, respectively). BrdU/GFAP labelling remained stable between P2 and P9, whereas no differences were apparent between the training groups (Fig. 5E–H). Confocal microscope images showed clearly that BrdU-labelled cells and NeuN and GFAP labelling identified the separate classes of cells.

Passive avoidance learning effects on apoptosis

Apoptosis was examined in the vHp, dHp and APH at two different post-hatching stages (P2 and P9). TUNEL-positive cells appeared small and round; usually a single cell was highlighted but sometimes two cells were visible in close proximity. In Fig. 6A single cells are shown in the dHp and vHp, respectively, whereas in Fig. 6B two TUNEL-labelled cells are closely located in the dHp of control and MeA-trained animals, respectively. Quantitative data for apoptotic cells following PAL at 24 h and 9 days post-training are shown in Fig. 7A and B. In general, apoptosis was higher at 24 h in the three regions examined, declining by 9 days post-training, although there were differences in the number of apoptotic cells between the three groups (MeA, water and control). Three-way ANOVA showed significant differences with chick age after training ($F_{2,48} = 4.019$, $P = 0.024$).

Post-hoc comparisons in the 24-h group showed that the dHp of the water-trained group presented more apoptotic cells in comparison to the dHp of the control and MeA-trained group ($P = 0.0037$ and $P = 0.0016$, respectively, Fig. 7A). Also, regional specific apoptosis was found in the vHp of the MeA-trained group at 24 h where there

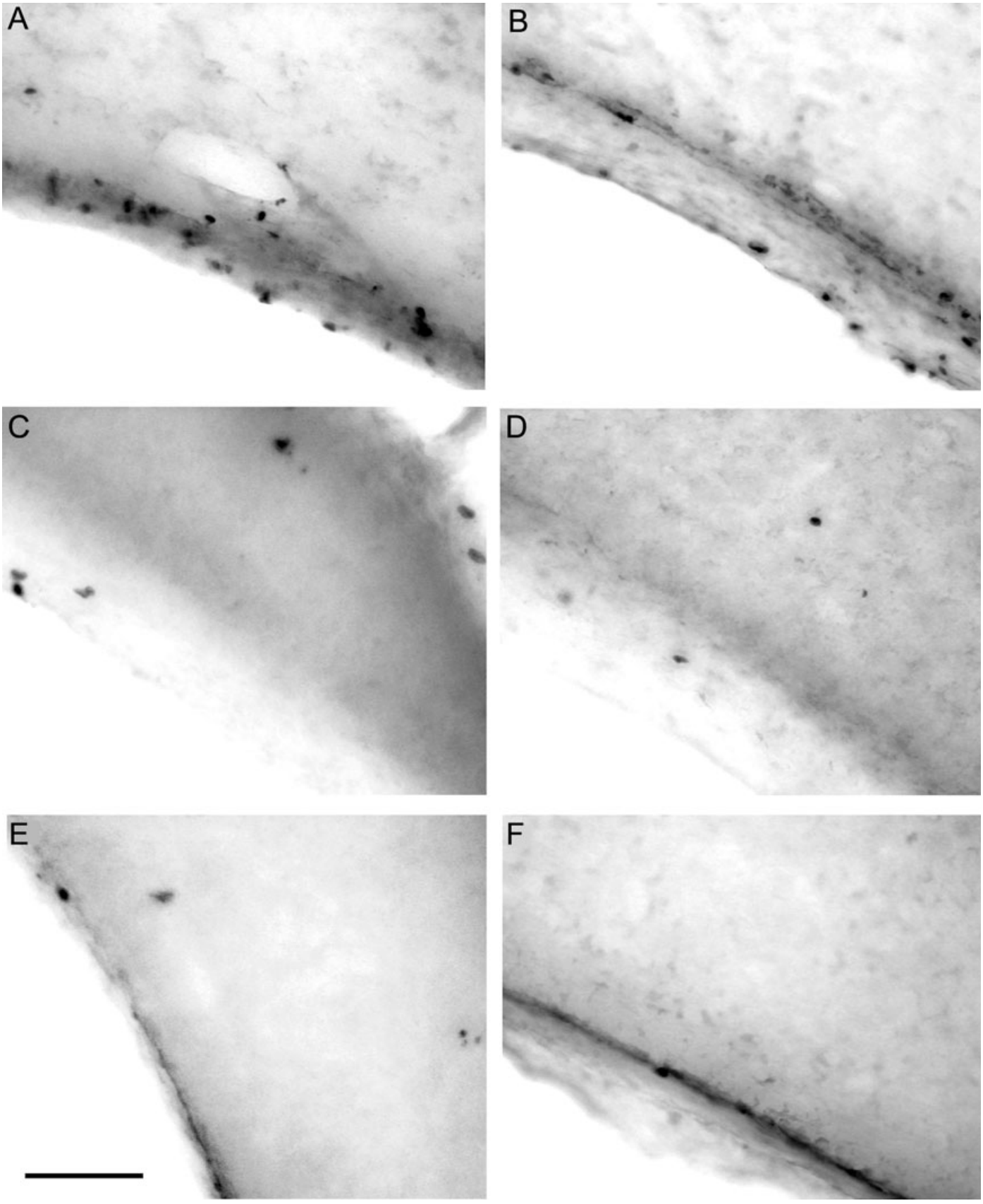

FIG. 2. Dorsal hippocampus of control (A) and methylanthranilate (MeA)-trained (B) 2-day-old chicks. Ventral hippocampus of a control (C) and an MeA-trained (D) animal. Images of 5-bromo-2-deoxyuridine-positive cells in the chick ventral hippocampus of control (E) and MeA-trained (F) animals 9 days post-training. Scale bar, 50 μm.

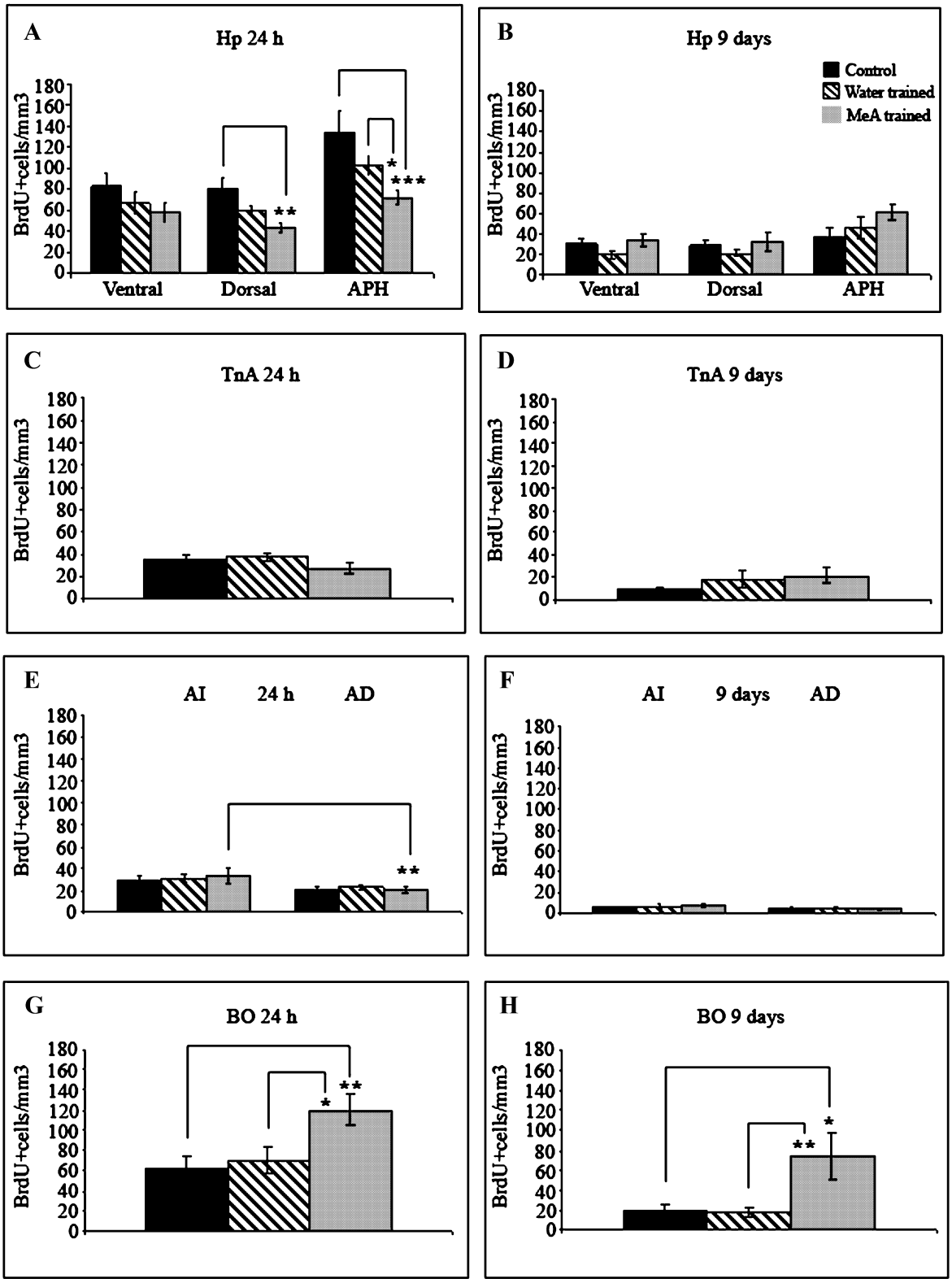

FIG. 3. Quantitative analyses to estimate the number of 5-bromo-2-deoxyuridine (BrdU)-labelled cells/mm^3. (A) The dorsal hippocampus (Hp) of the control animals shows more BrdU-positive cells in relation to the methylanthranilate (MeA)-trained group ($P = 0.0075$). In the area parahippocampalis (APH) the water- and MeA-trained groups show fewer BrdU-positive cells in comparison to controls ($P = 0.019$ and $P < 0.0001$, respectively). The MeA-trained group shows a further reduction in cell proliferation in relation to the water-trained group ($P = 0.017$), however, at post-hatching day P9 (B) no differences are apparent between the training groups. (C and D) Cell proliferation in the nucleus taeniae of amygdala (TnA) is not affected by training. (E) The MeA-trained group shows more BrdU-labelled cells in the intermediate arcopallium (AI) in relation to the dorsal arcopallium (AD) ($P = 0.0037$) and no changes in the number of BrdU-positive cells are observed at P9 (F) in AI or AD. (G and H) The olfactory bulb (BO) of the MeA-trained animals has more BrdU-positive cells in relation to control and water-trained groups at 24 h ($P = 0.0087$ and $P = 0.015$) and 9 days post-BrdU injection ($P = 0.013$ and $P = 0.009$). Vertical bars indicate SEM. *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0001$.

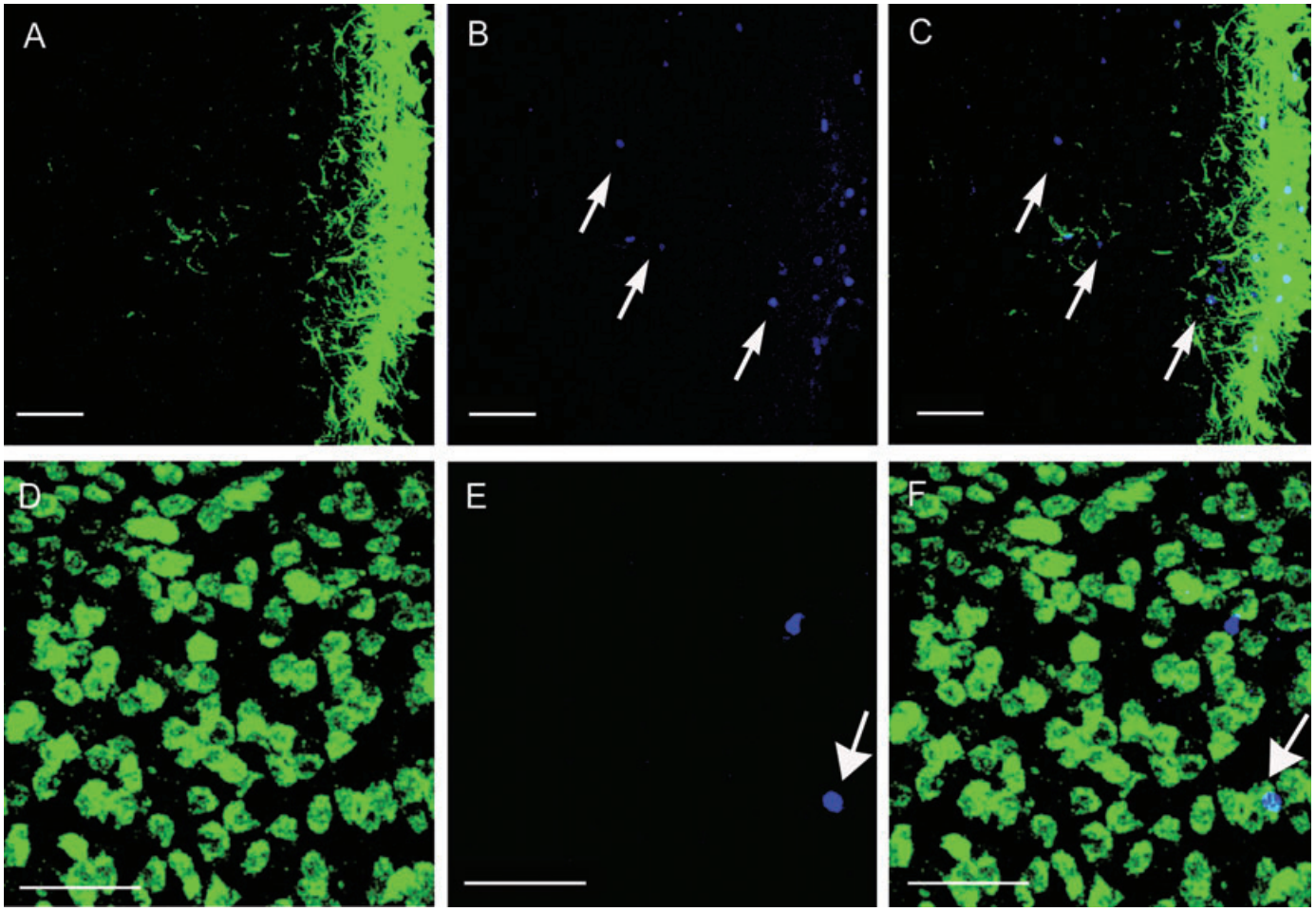

FIG. 4. Confocal images of 5-bromo-2-deoxyuridine (BrdU)-labelled cells (blue) and nuclear neuronal marker (NeuN)- or glial fibrillary acidic protein (GFAP)-positive cells in the chick hippocampus. (A–C) Confocal micrograph (merged image of 30 1-μm optical sections) of cells double-labelled for BrdU and GFAP (green) at the dorsal hippocampus (dHp) close to the pial zone at 24 h after BrdU injection. Cells that migrate to the inner parts of the brain are also visible (not double-labelled, arrows). Most of the BrdU-positive cells do not demonstrate double labelling for GFAP phenotype. (D–F) Stacks of confocal planes (30) of a cell double-labelled for BrdU and NeuN (thick arrow) in the dHp of the control group at 24 h post-BrdU injection. The BrdU-positive cell at the top of the image is not double labelled for BrdU and NeuN. Scale bar, 50 μm.

were more apoptotic cells in comparison to the dHp and APH of the same group ($P = 0.022$ and $P = 0.035$) (Fig. 7A).

By 9 days post-training, the dHp of the MeA-trained group had fewer apoptotic cells than in the vHp ($P = 0.028$) (Fig. 7B).

Passive avoidance learning effects on cortisol levels in the chick arcopallium, striatum mediale and hippocampus at 20 min after training

Data of cortisol (ng/g per brain area) at 20 min after training are presented in Fig. 8. Three-way ANOVA indicated significant statistical differences for the area ($F_{2,9} = 6.47$, $P = 0.018$) of study and the training group ($F_{2,9} = 18.38$, $P = 0.0007$). The levels of cortisol in the MeA-trained group in the arcopallium at 20 min after training were lower (39% decrease, $P = 0.0037$) in comparison with the Hp. However, in the medial striatum of MeA-trained chicks there was a 46% and 49.5% increase in comparison to the control and water-trained groups ($P = 0.017$ and $P = 0.021$, respectively) In the Hp, the MeA-trained group showed increased cortisol levels in comparison with the control (91% increase, $P = 0.013$) and water-trained birds (85.5% increase, $P = 0.016$).

Discussion

Our data clearly demonstrate a region-specific reduction in cell proliferation at 24 h post-BrdU injection in the dHp of MeA-trained chicks in comparison to untrained controls. This finding contrasts with previous data on cell proliferation in the intermediate medial mesopallium (IMM) after avoidance learning (Dermon *et al.*, 2002). One possible reason for this is that cells in the Hp may undergo a higher rate of apoptosis in MeA-trained chicks in comparison to control birds than in other regions of the chick brain such as the IMM. However, apoptotic studies at 24 h after BrdU administration did not reveal any differences in the number of apoptotic cells between control and MeA-trained groups although it is possible that apoptotic death shows a transient peak at an earlier time point, which is not present at 24 h after training. It is noteworthy that at P2 the water-trained group showed increased apoptosis although no reduced cell proliferation.

In contrast to the dHp, in the vHp there was no obvious decrease in cell proliferation in the MeA-trained group, indicating that the two subdivisions of the Hp react differently to PAL, supporting the idea of possible functional differences between the two regions which was suggested by earlier studies after ischaemia (Horner *et al.*, 1998) and PAL (Nikolakopoulou *et al.*, 2006).

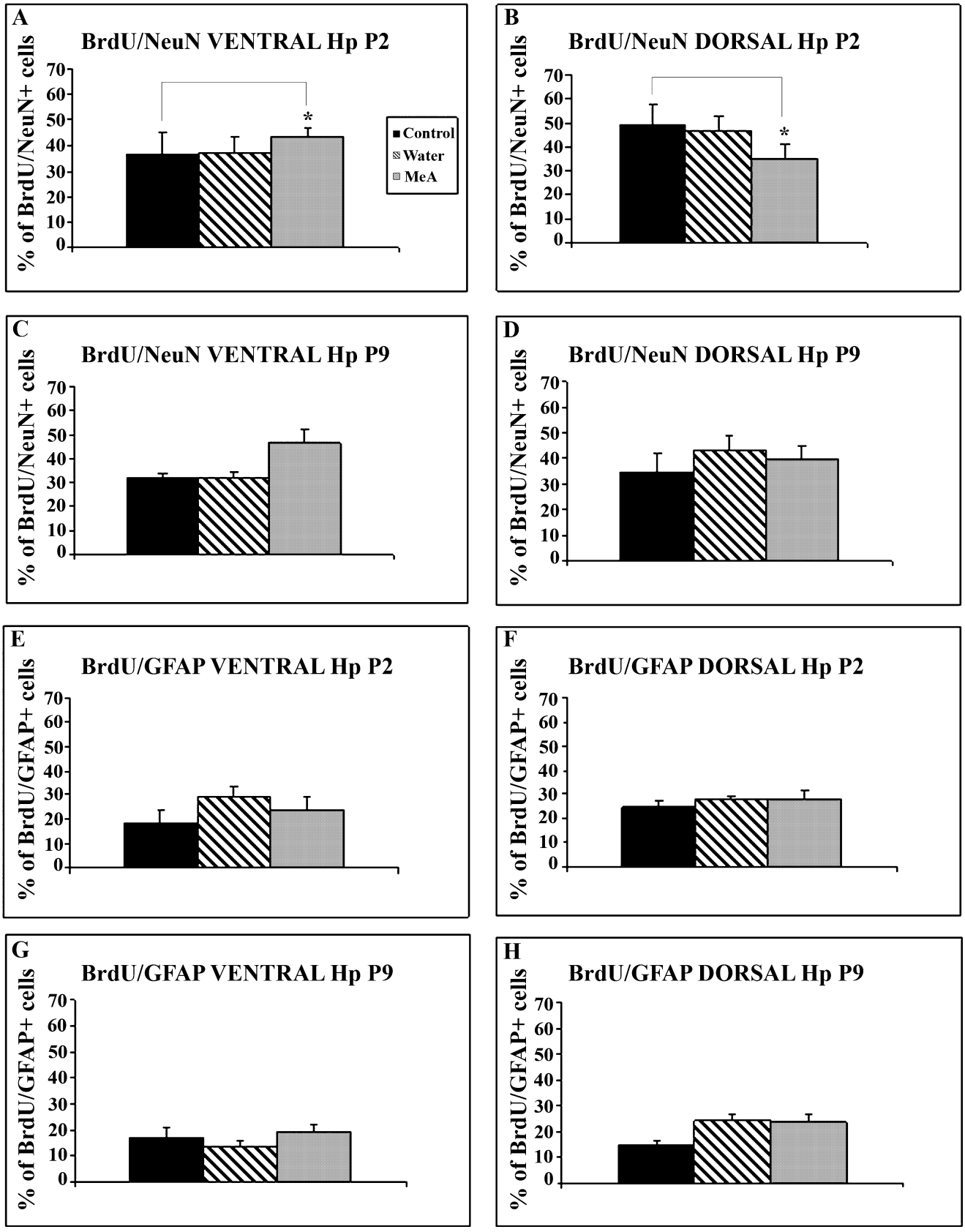

FIG. 5. Means of percentages of cells double-labelled for 5-bromo-2-deoxyuridine (BrdU) and nuclear neuronal marker (NeuN) or glial fibrillary acidic protein (GFAP). (A) In the ventral hippocampus (Hp) the methylanthranilate (MeA)-trained group shows more cells double-labelled for BrdU/NeuN in relation to controls ($P = 0.03$) and indicates that the ventral Hp shows less colocalization for BrdU and NeuN in control animals in relation to the dorsal Hp (dHp). (B) The MeA-trained group has less BrdU/NeuN cells in the dHp in relation to controls ($P = 0.04$). No differences exist between post-hatching day P9 training groups for BrdU/NeuN cells (C, D), whereas GFAP/BrdU cells show no discrepancies between groups or testing time points (E–H). Vertical bars indicate SEM. *$P \leq 0.05$.

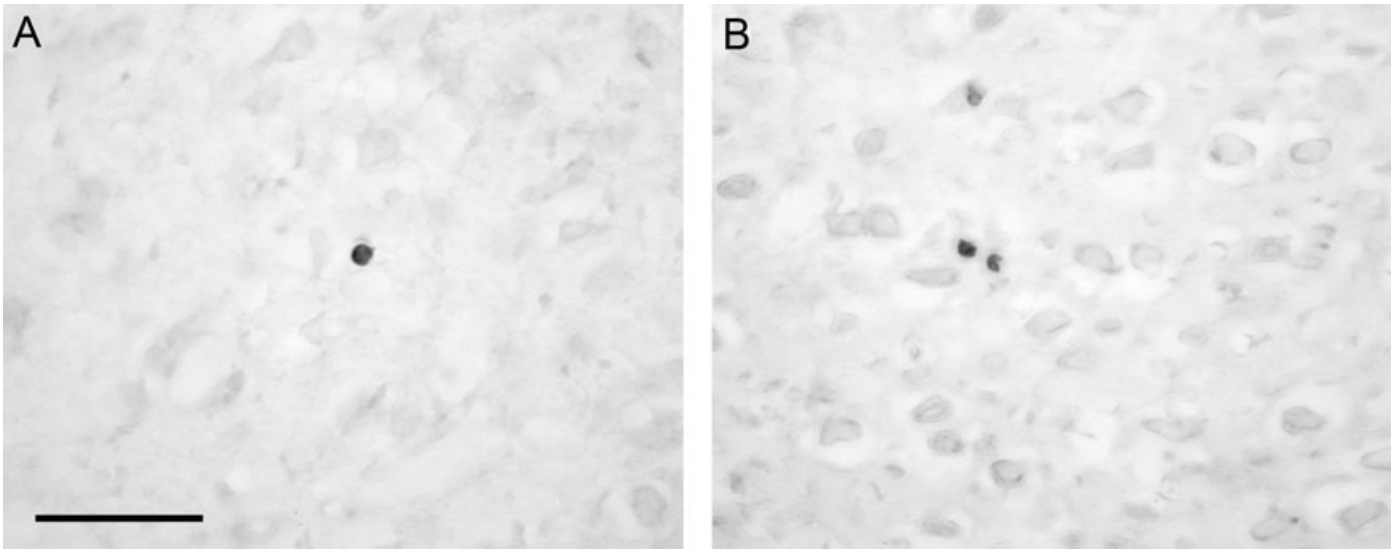

FIG. 6. TUNEL-labelled cells in control (A) and methylanthranilate-treated (B) animals 2 days post-training. Scale bar, 50 μm.

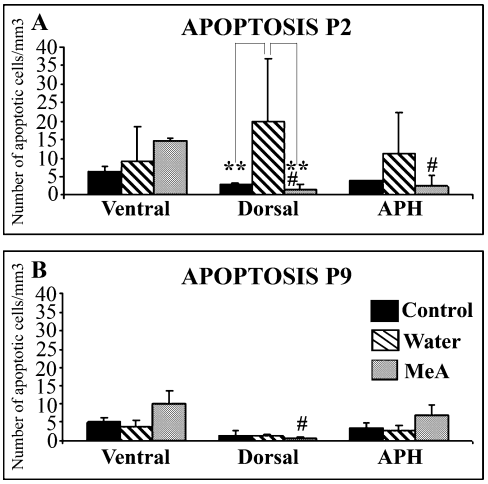

FIG. 7. TUNEL experiments were carried out at post-hatching day P2 and P9. Data are presented as means of apoptotic cells/mm^3 in the chick hippocampus. (A) In the dorsal hippocampus (dHp) the water-trained group shows more apoptotic cells in relation to control ($P = 0.0037$) and methylanthranilate (MeA)-trained groups ($P = 0.0016$). In the same graph we show (#) the increased cell death of the ventral hippocampus (vHp) of the MeA-trained group in relation to the dHp ($P = 0.022$) and area parahippocampalis (APH) ($P = 0.035$). (B) At P9 the dHp of the MeA-trained group shows fewer apoptotic cells in relation to vHp ($P = 0.028$). No differences exist between training groups at this age. Vertical bars indicate SEM. $^{**}P \leq 0.005$.

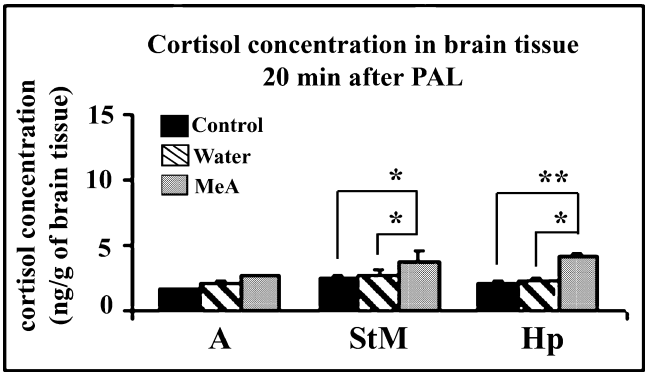

FIG. 8. Group mean values showing the concentration of cortisol in ng/g of brain tissue in the arcopallium, striatum mediale and hippocampus (Hp) of control, water-trained and methylanthranilate (MeA)-trained group (developmental control $n = 12$, water-trained $n = 14$, MeA-trained $n = 14$). At 20 min after training, in the StM and Hp the MeA-trained group shows significantly higher cortisol levels in relation to control and water-trained groups. Columns indicate means of data and vertical bars indicate SEM. A, arcopallium; PAL, passive avoidance learning. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$.

In the APH, there was also a significant reduction in cell proliferation in both the MeA-trained and water-trained groups compared with controls. It may therefore be that the presentation of a novel object (bead) in itself causes alterations in cell proliferation. Interestingly, auditory filial imprinting causes spine density reduction in the chick dorsocaudal nidopallium (Bock & Braun, 1999) and mediorostral nidopallium/mesopallium (Bock & Braun, 1998). Therefore, learning does not necessarily cause a synaptic increase; a decrease may occur as a natural result of neuronal activation and remodelling (Goda & Davis, 2003). Notably, recent studies in mice (Ehninger & Kempermann, 2006) have shown that water maze training causes hippocampal neurogenesis reduction due to the combination of stress and learning emphasizing in particular that this type of training does not provide a strong learning stimulus by itself to provoke neurogenesis enhancement.

The fact that, in the water-trained group, there were significantly more BrdU-labelled cells compared with MeA-trained animals suggests the possibility that methyl anthranilate invokes stress responses and indeed our data showed cortisol elevation after avoidance learning. As cortisol is known to be elevated after social stress (Mohamed & Hanson, 1980) and induces cell proliferation reduction in chicks (Ganguli *et al.*, 2001) whilst causing song impairments in songbirds (Miller, 2003), it seems reasonable to suggest the possibility that stress might be causing effects on cortisol levels following PAL. In chicks, cortisol levels have not been measured previously after avoidance learning; however, corticosterone, which is very similar in form and function and is the main glucocorticoid in the chicken bursa and thymus (Lechner *et al.*, 2001),

has been shown by Sandi & Rose (1997) to be significantly higher in the plasma of MeA-trained chicks than in controls. Indeed, plasma corticosterone has been suggested to show levels in control chickens (1.2 ± 0.8 ng/mL) (Dehnhard *et al.*, 2003) similar to those for cortisol demonstrated in this study in tissue (1.7–2.27 ng/g tissue). Corticosterone is associated with stress in the rat Hp (de Kloet, 2000) and the effects of this stress may be expressed in terms of a reduced proliferative rate (Gould *et al.*, 1998; Gould & Tanapat, 1999), especially in the dentate gyrus, which is equivalent to the dHp of chicks (Erichsen *et al.*, 1991; Siegel *et al.*, 2002). The rise in levels of cortisol after avoidance learning could negatively influence mitotic activity, as occurs in the thyroid gland (Ganguli *et al.*, 2001). As it has been documented that the dHp is equivalent to the dentate gyrus (Erichsen *et al.*, 1991; Siegel *et al.*, 2002), it might therefore be expected that it would be affected by stress more severely than its ventral counterpart, exhibiting cell proliferation/neurogenesis reduction. Our cortisol studies indicate elevated cortisol levels at both the striatum mediale (StM) and Hp; however, the cell proliferation pattern differs between the two areas studied. It should be noted, however, that due to technical difficulties in sample preparation the dHp and vHp were analysed together; therefore, we cannot exclude the possibility that, if studied separately, the dHp might have demonstrated much higher cortisol levels that could explain the variability between StM and Hp cell genesis.

Here, our double-labelling studies with BrdU and NeuN showed reduced NeuN-positive cells in the dHp with respect to control animals at P2. This finding might suggest that stress in the chick brain reduces neurogenesis, whereas it does not affect the number of glial cells (GFAP studies). In addition to the data presented in this study, recent studies from our laboratory (Nikolakopoulou *et al.*, 2006) demonstrate a significant reduction in synaptic density in the dHp but not vHp of MeA-trained chicks. Thus, it may be suggested that, as in mammals, synaptic (Magarinos & McEwen, 1995; Sandi *et al.*, 2003; Stewart *et al.*, 2005) and neuronal (Tanapat *et al.*, 1998, 2001) decreases are a possible outcome of stressful experiences. However, before we can be confident of the precise nature of the effect of stress in cell proliferation in the chick Hp following avoidance training, more data would be required on both the time scale of cortisol elevations following training and its levels in subregions of the Hp.

Cell proliferation is enhanced in the AI in comparison to the AD of the MeA-trained group. As AI responds to fear, it would also probably be activated by high stress and learning in a similar way to the mammalian amygdala (Akirav *et al.*, 2001). The effects of passive avoidance training may be expressed differently in the arcopallium and Hp, and consequently differential responses to stress and fear following the avoidance training experience should be expected. The AD, in contrast, is not affected probably because it does not belong to the limbic system. Future double-labelling studies could clarify if there are any disparities in neurogenesis between MeA-trained and control groups in the AI.

No differences occurred in BrdU cell labelling in the TnA between the different groups. The BO of the MeA-trained animals shows significantly elevated cell proliferation with regard to controls and water-trained animals. It is known that chicks react to smell (Wenzel & Sieck, 1972; McKeegan, 2002) so an increase in cell proliferation may not be unexpected. Chicks can smell MeA and react aversively to its odour (Marples & Roper, 1997) as observed from the latencies to peck the bead dipped in MeA. Therefore, it has been suggested that MeA may play a role in stimulating memory consolidation (Burne & Rogers, 1997). Other bitter but odourless substances (e.g. denatonium benzoate) do not appear to be strong aversive stimuli for chicks (Marples & Roper, 1997) and, as a consequence, have a limited ability to stimulate long-term memory after PAL. This may imply that PAL is, in part, an olfaction task linking BO with the limbic system (Richard & Davies, 2000). Interestingly, the only area in which the differences in cell proliferation between training groups are preserved at 9 days after BrdU injection is the BO. The significant increase of BrdU-labelled cells in the MeA group is conserved in the long survival experiments, even though there is a significant reduction in the number of BrdU-labelled cells between 24 h and 9 days. Indeed, at 9 days post-BrdU injection all of the areas examined show a reduced number of labelled cells in comparison with animals killed 24 h after BrdU injection. The explanation for this is unclear. The likelihood that cell migration is responsible has yet to be proven. However, previous studies have shown a decrease in the number of new cells between 1 and 2 weeks after BrdU injection (Cameron *et al.*, 1993; Gould *et al.*, 1999) in the adult rat Hp, which is attributed most probably to cell death. Interestingly, at 9 days post-training there are no differences between the training groups in the Hp, indicating that the effects of stress, if we assume that it is the cause of cell proliferation reduction, have no long-term effects and thus no cell genesis differences were apparent.

In contrast to the rat dentate gyrus where newborn cells require 3 weeks to mature (Cameron *et al.*, 1993; Kuhn *et al.*, 1996), in the chick Hp we found cells double-labelled for NeuN and BrdU only 24 h after bromodeoxyuridine injection, signifying primitive maturity relative to mammals possibly due to a shorter life span. However, none of the cells which resided on the Hp ventricular zone coexpressed NeuN and BrdU, even at 9 days after birth unlike studies in the IMM and StM (Dermon *et al.*, 2002). One explanation for this finding could be that the newborn cell maturation time is area dependent or that there is variability due to different experimental approaches. These cells are perhaps still immature neurones as they were not labelled for GFAP. It is notable that 2-day-old animals have a less developed glial fibre network in comparison to 9-day-old animals, which agrees with previous studies (Kalman *et al.*, 1998).

In summary, the present data have demonstrated clearly that at 24 h following BrdU injection there is a significant training-induced reduction in cell proliferation in the dHp and APH and at 9 days post-injection there is a decrease in BrdU cell labelling in all areas of the Hp compared with those at 24 h. Increases in BrdU cell counts occur in the BO. The cell reductions do not appear to be due to apoptosis but may be due to migration of cells to other areas of the telencephalon. The reduction of cell proliferation in the dHp appears to be correlated with an increase in cortisol levels, indicating the possibility that stress may have an effect on the avoidance training process.

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Abbreviations

AD, dorsal arcopallium; AI, intermediate arcopallium; APH, area parahippocampalis; BO, olfactory bulb; BrdU, 5-bromo-2-deoxyuridine; dHp, dorsal hippocampus; GFAP, glial fibrillary acidic protein; Hp, hippocampus; MeA, methylanthranilate; NeuN, nuclear neuronal marker; P, post-hatching day; PAL, passive avoidance learning; PB, phosphate buffer; TnA, nucleus taeniae of amygdala; vHp, ventral hippocampus.

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