Section Editor

Developmental
Dr. John L. R. Rubenstein
LPPI Box F-0984
University of California at San Francisco
401 Parnassus Avenue
San Francisco
CA 94143-0984
USA

Vitamin D_3 and brain development

D Eyles [1,2], J Brown [1,2], A Mackay-Sim [3], J McGrath [1], F Feron [1,3]

1. Queensland Centre for Schizophrenia Research, Wolston Park Hospital, Wacol, Qld 4076 Australia
2. Department of Physiology and Pharmacology, University of Queensland, Qld 4072, Australia
3. Centre for Molecular Neurobiology, School of Biomolecular and Biomedical Science, Griffith University, Qld 4111 Australia

Address for correspondence
Darryl Eyles
Queensland Centre for Schizophrenia Research
Department of Physiology and Pharmacology
The University of Queensland
Brisbane, Qld 4072, Australia.
Email Eyles@mailbox.uq.edu.au
Fax 61 7 33651766
Phone 61 7 33652325

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Abbreviations:
$1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3; 25OH D_3 = 25-hydroxyvitamin D_3; VDR = vitamin D_3 receptor

Running title

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Abstract

Evidence for the presence of the vitamin D receptor in brain implies this vitamin may have some function in this organ. This study investigates whether vitamin D_3 acts during brain development. We demonstrate that rats born to vitamin D_3 deficient mothers had profound alterations in the brain at birth. The cortex was longer but not wider, the lateral ventricles were enlarged, the cortex was proportionally thinner and there was more cell proliferation throughout the brain. There were reductions in brain content of nerve growth factor and glial cell line-derived neurotrophic factor and reduced expression of $p75^{NTR}$, the low-affinity neurotrophin receptor.

Our findings would suggest that low maternal vitamin D_3 has important ramifications for the developing brain.

Key words: - Vitamin D; Brain development; Brain morphology; Neurotrophic factors, $p75^{NTR}$; Mitosis;

The links between vitamin D, calcium and bones have long been appreciated however in recent years an ever-widening range of actions has been described for this steroid hormone. Evidence now links vitamin D_3 with cell growth and differentiation, immune response and foetal development (Bouillon et al., 1995). Of particular interest is accumulating evidence for actions of vitamin D_3 in the brain (Garcion et al., 2002) and suggestions that early vitamin D_3 deficiency may be a risk factor for a number of disorders, including schizophrenia and multiple sclerosis (McGrath, 1999, 2001).

The vitamin D_3 receptor (VDR) is a member of the highly conserved nuclear receptor family that also includes the sex hormones, glucocorticoids, and retinoic acid (Lander et al., 2001). The VDR has been identified in the brains of rat (Stumpf et al., 1982; Stumpf and O'Brien, 1987; Prufer et al., 1999), hamster (Musiol et al., 1992) and human (Sutherland et al., 1992). Vitamin D_3 can be either ingested, or synthesized from the action of ultraviolet B radiation on a cholesterol metabolite found in the epidermis which results in the production of previtamin D. After two separate hydroxylations (first in the liver, then in the kidney) the most active form of the vitamin, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is produced. Both of these enzymes (vitamin D_3 25-hydroxylase and 25-hydroxyvitamin D_3-1α-hydroxylase) and the inactivating enzyme (vitamin D_3 24-hydroxylase) are present in brain, making it plausible that vitamin D_3 may act as an autocrine or paracrine factor in this organ (Miller and Portale, 2000; Garcion et al., 2002). Vitamin D_3 has many effects on adult brain tissue, *in vivo* and *in vitro*, including altering neurotrophin mRNA expression, neurotransmitter enzyme activity, and providing multiple levels of neuroprotection (Garcion et al., 2002). The VDR is present in the

developing rat brain and its sites of expression change during development prompting the speculation that vitamin D_3 may play a previously unrecognised role in brain development (Veenstra et al., 1998).

Vitamin D production is consistently associated with photoperiod duration, which in turn is influenced by latitude and season (Webb et al., 1988; Holick, 1995). The potential importance of an association between vitamin D and brain development takes on added weight in view of the surprisingly high levels of hypovitaminosis D in both developed and developing nations (Vieth and Carter, 2001).

Although the optimal levels of vitamin D_3 during pregnancy are poorly understood (Morley et al., 2002), low levels of vitamin D_3 during prenatal development are common. In the United States it is reported that, of women of the child-bearing ages 20 to 39, 12% had serum 25-hydroxyvitamin D_3 levels below the threshold defined for vitamin D_3 deficiency (15ng/ml) (Looker and Gunter, 1998). Similarly, serum vitamin D_3 insufficiency is common even in areas of abundant sunlight (McGrath et al., 2001b).

In this study, we investigated whether vitamin D_3 deficiency during gestation could alter brain development. Our results indicate that gestational vitamin D_3 deficiency has profound effects on the developing brain, including changes in volume, shape, cell proliferation and growth factor expression.

Experimental procedures

Maternal vitamin D_3 depletion

Female Sprague-Dawley rats were fed a prepared diet free of vitamin D_3 (Dyets Inc CA USA) but with normal calcium and phosphorous. Animals were housed under a 12-hour light/dark cycle using incandescent lighting free of ultraviolet radiation in the vitamin D_3 action spectrum (290-315 nm). After 6 weeks serum vitamin D_3 depletion was confirmed prior to mating using a commercial RIA (Diasorin MN USA) for 25-OH D_3, which provides the best overall indicator of vitamin D_3 status from both dietary and environmental exposures (Hollis, 1996). The resulting dams were housed under these conditions until the birth of the pups. Control animals were kept under standard lighting conditions and were supplied with standard rat chow containing vitamin D_3 (Dyets CA). Gestational times were normal in the deplete group. The animals did not appear to incur any suffering as a result of dietary manipulation and all efforts were made to minimise the number of breeding animals.

Because infant size may be inversely proportional to litter size only neonates from litters of 11 – 14 pups were selected for this study. Within 12 hours of birth 3 newborns of each sex were collected and birth weight recorded. The pups were killed by decapitation performed so that the brain stem was dissected in the same position for each animal. The brains were carefully removed, weighed and prepared for histology, protein or RNA analysis according to the protocols described below.

Blood was collected from neonates for 25-OH D_3 and calcium estimation. Vitamin D_3 levels were significantly greater in the control neonates 27.4 ± 9.6 compared with control maternal levels 14 ± 6 ng/ml ($P<0.001$, ANOVA n =14). Animals kept under vitamin D_3 deplete conditions were indeed severely depleted with levels of 1.8 ± 0.4 and 1.9 ± 1.3 ng/ml in the maternal animals and neonates respectively. Serum calcium levels in both controls (1.5 ± 0.4 mM) and deplete neonates (2.0 ± 0.6 mM) were somewhat lower than values previously reported for either maternal or postnatal rats (2.5 –2.0 mM) (Thomas and Forte, 1982; Lester et al., 1982; Brommage and DeLuca, 1984a; Brommage and DeLuca, 1984b). In any case vitamin D depletion combined with normal dietary calcium and phosphate intake did not lead to reduced serum Ca^{2+} in neonates, a finding in accordance with previous observations (Lester et al., 1982).

Histology and immunohistochemistry

Once removed and weighed, brains were fixed for two hours in freshly prepared 4% paraformaldehyde/phosphate buffered saline (PBS, pH 7.2), thoroughly rinsed, cryoprotected in 0.32M sucrose, embedded with cryostat mounting medium and frozen. Sections were cut on a IEC cryo-microtome (8 um thick), thaw-mounted on pre-coated slides (Superfrost Plus, Lomb scientific) and stored at –80^{o}C until use. Sections were stained in a series of 1 in 15 for nissl substance using cresyl violet. For immunohistochemical investigations single sections were selected at the same medial coronal plane. Individual antigens were visualised as follows. Endogenous peroxidase activity was quenched by incubating sections in 0.3% fresh H_2O_2 in either PBS (when the antigen was located on the outer surface of the membrane) or pure methanol (when the

antigen was cytoplasmic, nuclear or located on the inner surface of the membrane) for 30 minutes. Non-specific staining was blocked by incubation for 1 hr with non-immune serum, appropriate for the secondary antibody, at a dilution of 1:10 in PBS containing 2% bovine serum albumin and 5% non fat dry milk. Sections were then incubated for 1 hr with primary antibodies diluted in the same blocking solution. Polyclonal anti-trk A,B and C antibodies (Santa Cruz, CA), polyclonal anti-vitamin D_3 receptor (Santa Cruz,CA), monoclonal anti-proliferating cell nuclear antigen (PCNA) (DAKO, CA) and monoclonal anti-p75 antibody (mc 192, Neubody, Flinders University, Australia) were used at the concentrations of 2 µg/ml, 2 µg/ml, 8 µg/ml and 10 µg/ml respectively. Negative controls were performed by omitting the primary antibody or by a 2 hour pre-incubation of the primary antibody with a five fold increased concentration of the corresponding blocking peptide. Sections were washed in PBS, incubated for 1 hr at room temperature with the appropriate biotinylated secondary antibody (diluted 1:200 in PBS containing 0.5% BSA), washed in PBS and incubated for 1 hr with an avidin-biotin-horseradish peroxidase complex (1:50, Vector labs, Burlingame, CA), rinsed and finally incubated for 5 min in Tris-HCl (0.05 M, pH 7.6) containing the chromogen diaminobenzidine (DAB, 0.05%) and H_2O_2 (0.004%). Slides were dehydrated and mounted in Depex. Photographs were taken on an Olympus microscope with a digital camera (Apogee Instruments) and Optimas software.

Brain Morphology

Comparisons of brain and body weights were made on pups from a median litter size (n = 11). In the breeding program a total of nine litters of this size were produced from both

deplete and control groups. Two males and two females were used in this study from each litter making a total of 36 pups from each group. All morphological changes reported occurred independent of sex. Brain region area, length and width measurements were made from digitised section images (x20) that were analysed using NIH image software. Cortical and ventricle volume estimations were made from randomising a series (1 in 15) of sections through the whole structure under consideration. Volume estimations were then made using Cavalieri's principal

$$V = \sum APt$$

where V is the volume of the structure; $\sum A$ is the sum of the cross-sectional areas; P is the inverse of the sample fraction ; and t is the section width (Gundersen and Jensen, 1987).

Other morphological observations were made on single sections using well-defined landmarks. Hippocampal, brain width and 3rd ventricle area measurements were made on a series of three single sections at a level consistent with the anterior portion of the thalamus, the widening of the optic chiasm and immediately posterior to the decussation of the corpus callosum. Cortical depth and callosum thickness measurements were made on a series of three single forebrain sections at the level of the decussation of the anterior commissure.

Apoptosis, mitosis and cell density counting

For these studies, sections from 10 control and 10 deplete neonates with an equal number of males and females were used. For each animal, three brain sections were assessed. On

each section, four specific regions from both hemispheres were examined:- In this study we limited our examinations to 4 brain regions. The hypothalamus was chosen due to its central role in endocrine function in the brain. The cingulate gyrus was selected as a representative cortical region. The dentate gyrus was chosen as a recognised site for post-natal neurogenisis (Altman and Das, 1965). The basal ganglia/amygdala was investigated due to the abundant presence of VDR's in this region in the rat embryo (Veenstra et al., 1998).

To establish the presence of apoptotic nuclei sections were dried and incubated in the dark for 30 minutes in PBS solution containing 1 μM of bisbenzimide (Hoechst Blue 33258, Sigma). To establish whether a cell was undergoing mitosis, 3 sections from the immediately posterior slide were immunohistochemically stained for PCNA. Sections were viewed (800X) using an immersed lens and cells with strong immunochemical staining for PCNA or obvious nuclear fragmentation (Figure 2) were counted from digitised images. The total number of cells could also be determined in these sections as hoechst blue stains all cells and in the case of the PCNA stained cells, phase contrast images could be counted.

Cell numbers were estimated in each section using the method of Abercrombie (Abercrombie, 1946).

$$N_C = N_A \ (T)/(T+D)$$

where N_C is the corrected number of nuclei whose centres lie in the plane of the section, N_A is the actual number of neuronal profiles counted in the area of the section, T is section

thickness (8 uM) and *D* is the average diameter of the cell nuclei in each section. Calculation of N_C is subject to error due to overestimation of *D* when the smallest nuclear segments within the section are missed (Weibel, 1980). In the present use this error should be similar in control and deplete groups since the nuclear profiles were of similar ovoid shape in all sections. Furthermore, there were no significant differences between the average minimum nuclear profile areas in the control and deplete groups (compared by t-tests for each of the brain regions). The corrected numbers of cell nuclei represent the numbers whose centres lie in the plane of the section.

Digistised images were assessed using NIH image software. Quantitation was performed blind to group status by two independent observers. Inter-rater reliability was > 90? %. Each count was expressed as a percentage relative to total cell number and cell density was expressed per unit of surface (e.g. % apoptotic cells/mm^2).

Protein analysis

Brains were homogenised using glass potter apparatus in 1 ml of cold 0.32M sucrose containing a protease inhibitor cocktail (Boehringer Mannheim) and 1mM phenylmethylsulfonyl fluoride, and were stored frozen. The protein concentration of homogenates was estimated using a commercial Bradford assay (Biorad CA). Total brain vitamin D_3 receptor density was qualitatively assessed by western blot using the same polyclonal antibody used for immunohistochemistry. Brain neurofilament and glial fibiliary acidic protein (GFAP) content was assessed by western blot using antibodies from Sigma Mo. and DAKO (Denmark) respectively. Free levels of nerve growth factor,

(NGF); Glial derived neurotrophic factor, (GDNF); Brain-derived neurotrophic factor (BDNF); and neurotrophins 3 and 4 (NT-3, NT-4), were established using commercial ELISAs (Promega WI). Specific protein concentrations were expressed relative to total protein concentration.

Reverse transcription-PCR of neurotrophic factors and their receptors

Brains were homogenised in Solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH7.0; 0.5% sarcosyl, 0.1M 2-mercaptoethanol). Total RNA was isolated as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Superscript Choice System (Life Technologies) was used for reverse transcription of 5 μg of total RNA from 10 control animals and 10 vitamin D_3 depleted animals. PCR conditions were optimised by varying $MgCl_2$ concentration and cycle number to determine linear amplification ranges for each primer pair. PCR products were identified by size and confirmed by DNA sequencing. Results are expressed as the optical density of PCR product relative to the optical density of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistics

All comparisons were conducted blind to treatment. Most statistical comparisons between those animals raised under vitamin D_3 normal and deplete conditions were by ANOVA. Where just a single variable was compared between two groups, significance was assessed by an unpaired t test. When comparing immunohistochemical response $p75^{ntr}$, comparisons were made by coding and randomising sections followed by visual

assessment by 2 raters. Predefined levels for positive (dark staining) and negative responses (light staining) were established. Greater than 95% agreement was achieved in the assessment between raters. This descriptive data was assessed using Fisher's exact test. In all analyses $P < 0.05$ represented the level of significance.

Results

Vitamin D_3 depletion changed the shape and size of the brain

We initially investigated whether vitamin D_3 depletion altered any gross morphological features of brain and discovered several substantial changes (Table I). Vitamin D-depleted pups were heavier than the control animals and their brains were heavier but the ratio of brain to body weight was not significantly different (Table I). Vitamin D-depleted pups had hemispheres that were larger and longer but not wider leading to a larger length/width ratio (Table I). The larger, longer cortex was associated with a larger lateral ventricle volume (Fig 1*A*,B). The other ventricular structures were unaltered. Lateral ventricles in the depleted group were double that of the controls even when corrected for the increased hemispheric volume (Fig 1*C*,*D*). We next investigated whether the larger ventricle volumes were achieved through a reduction in the sizes of structures surrounding them. To this end we measured the width of the corpus callosum, the cross-sectional area of the hippocampus and the thickness of the adjacent neocortex. In an absolute sense these structures were similar in treated and control groups, but when normalised for whole-brain cross-sectional area the neocortex was shown to be thinner in the vitamin D_3 deficient animals (Table I).

Table 1 about here

Figure 1 about here

Vitamin D_3 depletion increased the number of mitotic cells

We investigated whether the increased size of the brain was due to an increase in rate of cell proliferation or a decrease in the rate of cell death. The balance between cell birth and death regulates the number of neurons and glia in the brain and contributes to overall brain volume. Cells with characteristic staining for mitosis and apoptosis are shown in figure 2.

Figure 2 about here

Cell proliferation was higher in vitamin D-depleted pups compared to controls (Figure 3A). The percentage of cells in mitosis was quantified in four areas: the dentate gyrus of the hippocampus, the hypothalamus, the basal ganglia/amygdala, and the cingulate gyrus of the cortex. Vitamin D_3-depleted pups had twice the number of cells that were mitotic in the first three of these regions but not in the cingulate gyrus (Figure 3A). The percentage cells immunopositive for PCNA in these regions varied between 0.5 and 0.1 %. These counts were made only at a single time-point - at birth - and hence represent an incomplete analysis of cell proliferation in the developing brain. For example, cell proliferation has usually finished in the cortex at birth so that changes in cell proliferation in the cingulate gyrus may have been missed. In contrast, cell proliferation in the dentate gyrus continues into adulthood (Eriksson et al., 1998).

Analysis of cell death in the brain did not reveal any statistically significant differences between vitamin D-depleted pups and controls, although there was a slight decrease in apoptotic cell numbers in all brain regions studied (Figure 3B). Vitamin D_3 depletion did not alter cell density in any brain region examined (Figure 3C). These cell counts of

mitotic cells, apoptotic cells and cell densities represent the number of cells in two-dimensional sections of the regions studied and are therefore comparable with each other. Thus in the absence of a change in cell density, we conclude that vitamin D_3 depletion leads to an increased proportion of cells in mitosis without a balancing increase in the proportion of cells in apoptosis. These observations are consistent with an overall growth in the size of the brain as we observed (Table I).

Figure 3 about here

Vitamin D_3 depletion decreased expression of NGF and GDNF

Given the role of vitamin D_3 in promoting NGF and GDNF expression in cell lines, we investigated the effect of vitamin D_3 depletion on these and other neurotrophins. Growth factor protein levels were quantified in whole brain extracts using a commercial ELISA. Vitamin D_3 depletion decreased free NGF and GDNF levels by 17 and 25% respectively, compared to controls. (t-test $P < 0.05$ n=14) (Figures 4A and B). The protein expression levels of the other three neurotrophins (BDNF, NT-3 and NT-4) were not affected by vitamin D_3 depletion (data not shown). Vitamin D_3 depletion did not affect the expression of the mRNA for any of these growth factors, when assayed using semi-quantitative RT-PCR (Figure 4C).

Figure 4 about here

Vitamin D_3 depletion decreased expression of $p75^{NTR}$ but not other neurotrophin receptors

We investigated whether vitamin D_3 depletion would alter the expression of neurotrophin receptors throughout the brain. We observed an obvious decrease in $p75^{NTR}$ expression. In controls $p75^{NTR}$ immunoreactivity was most striking in the stria terminalis. Staining was also present in the basal ganglia/amygdala, lateral hypothalamus, optic tract and cortex. In the neonatal cortex trk A and $p75^{NTR}$ immunoreactivity appeared to be present in distinct bands. Trk A was restricted to the outer supragranular layer (data not shown) and $p75^{NTR}$ appeared restricted to the inner infagranular portion. In all regions $p75^{NTR}$ immunoreactivity was drastically reduced in the vitamin D-depleted pups ($P<0.001$, Fisher's exact, n =12) (Figure 5). Vitamin D_3 depletion did not affect the expression of mRNA for trkA, trkB or trkC the neurotrophin high affinity receptors. However in parallel with the greatly diminished immunohistochemical expression of $p75^{NTR}$, there was a 30% decrease in expression of $p75^{NTR}$ mRNA in whole brain extracts (ANOVA, $P<0.01$, n=10, Figure 4C).

Figure 5 about here

Vitamin D_3 depletion did not affect expression of the VDR or the ratio of neurons:glia

We investigated whether vitamin D_3 depletion would regulate the expression of its nuclear receptor. There was no difference between vitamin D-depleted pups and controls in the expression of the vitamin D_3 receptor throughout the brain (data not shown). This was confirmed using Western analysis of whole brain extracts (Figure 6). As an indication of the relative changes in neurons and glia in whole brain we investigated the ratio of neurofilament protein (as a marker of neurons) and GFAP (as a marker of

astrocytes) relative to total brain protein in extracts. These ratios were not affected by vitamin D_3 depletion (data not shown).

Figure 6 about here

DISCUSSION

We show here significant changes in brain development induced by vitamin D_3 deficiency *in utero*. Vitamin D_3 deficiency affected brain gross morphology, cellular proliferation and growth factor signaling. These changes were substantial: a 30% increase in hemisphere length, a 200% increase in lateral ventricle volume, a doubling of the mitotic rate in certain brain regions and decreases of 17%, 25% and 30% in the expression of NGF, GDNF and $p75^{NTR}$, respectively.

Rat pups were bigger at birth in the deplete group. Their brains were also proportionally larger (Table I). This increase in brain size was reflected in longer, but not wider, corticies implying a distortion in the way the cortex was formed during embryogenisis rather than simply an increase in overall brain size. It is unlikely that these shape changes are a consequence of vitamin-D related alteration in skull bone growth. The neurocranium derives from intramembranous ossification which is driven to a large extent by the shape and size of the underlying brain (Moss et al., 1987).

There was a significant increase in the proportion of proliferating cells throughout the brain in vitamin D_3 depleted neonates. There was also less apoptosis in all regions,

although this was not statistically significant. Although it is not possible to model the population dynamics of cell proliferation and survival from analysis of a single time point, these observations are consistent with the overall increase in brain volume in the deplete group. We do not know which cell types contribute preferentially to the larger brain seen in the vitamin D_3 deficient animals. However a qualitative analysis of whole brain content of GFAP (an indicator of astrocyte number) and neurofilament (an indicator of neuron number) by western blot revealed no gross differences between control and depleted brains (data not shown). From this analysis the larger brains would be consistent with both more neuronal and glial matter, either in cell numbers or volume or both.

In other tissues, vitamin D_3 is antiproliferative e.g. heart (O'Connell et al., 1997); gut (Menard et al., 1995); kidney (Weinreich et al., 1996) and promotes apoptosis in a variety of malignant cells e.g. glioma (Naveilhan et al., 1994; Baudet et al., 1998; Canova et al., 1998); breast cancer (Mathiasen et al., 1999); colon cancer (Vandewalle et al., 1995). Our findings of increased proliferation and decreased apoptosis in the brain indicate that vitamin D_3 may have similar effects on the cell cycle of neuronal and glial precursors as it does on other proliferating cells. For example, 1,25-dihydroxyvitamin D_3 down-regulates cyclins, proteins that govern transition points through the cell cycle (Laud et al., 1997), and up-regulates the proto-oncogene c-*myc* (Baudet et al., 1998).

Vitamin D_3 may also affect neuronal and glial population dynamics because it directly regulates the expression of several neurotrophic factors. Vitamin D_3 upregulates protein levels of both NGF (Wion et al., 1991; Neveu et al., 1994a; Neveu et al., 1994b; Musiol

and Feldman, 1997) and GDNF (Naveilhan et al., 1996b) in vitro and in the brain (Saporito and Carswell, 1995). The trophic actions of NGF on cholinergic neurons in the basal forebrain and GDNF on basal ganglia dopaminergic neurons are well known but less attention has been devoted to their role in development of other parts of the brain. The amount of NGF does not rise to its maximum level until two weeks after birth (Ip et al., 2001). Therefore a reduction in its levels may be more significant at later developmental stages. GDNF is expressed in a time- and region-specific manner throughout the brain during development and is an important regulatory agent in this organ (Ikeda et al., 1999). In peripheral neurons GDNF acts as a chemoattractant and may modulate neuronal migration (Young et al., 2001). The apparent down-regulation of NGF and GDNF protein compared with mRNA may reflect the poorer quantitative power of RT-PCR compared with ELISA. Alternately this may even reflect post-transcriptional regulation similar to that observed between vitamin D and the down-regulation of Tropoelastin (Pierce et al., 1992).

It is tempting to correlate the reported changes in cell proliferation and cell death with the reduction in neurotrophins and neurotrophic receptors observed here. However vitamin D_3 is clearly not the only regulator of the expression of these neurotrophic factors (Carswell et al., 1992; Saporito et al., 1993) nor the only regulator of cellular differentiation. Additionally other neurotrophins may also affect these processes yet have different trajectories during development. Therefore to make such a conclusion at this stage would appear preliminary.

The reductions in NGF and GDNF content in brain correlated with a striking reduction in both the expression of $p75^{NTR}$ mRNA and its immunohistochemical response. Vitamin D_3 responsive elements are present in the promoter region of the $p75^{NTR}$ gene (Naveilhan et al., 1996a). Vitamin D_3 also regulates $p75^{NTR}$ expression in glioma cells, (Naveilhan et al., 1996a; Baas et al., 2000). In the present study we examined $p75^{NTR}$ immunohistochemically in medial sections from brain regions where this receptor is known to be prominently expressed i.e. stria terminalis, the basal ganglia/amygdala, lateral hypothalamus and the infagranular portion of cortical plate (Yan and Johnson, 1988). In these regions vitamin D_3 depletion virtually abolished $p75^{NTR}$ immunoreactivity. The localisation of $p75^{NTR}$ in these regions overlaps considerably with the distribution of the VDR in the brain (Veenstra et al., 1998). The stria terminalis has consistently been shown to contain the greatest density of uptake sites for 1,25-dihydroxyvitamin D_3 in rat brain (Stumpf et al., 1982; Stumpf and O'Brien, 1987). The embryonic basal ganglia and hypothalamus also express VDRs (Veenstra et al., 1998; Prufer et al., 1999). The overlap in the distribution of $p75^{NTR}$ and VDR in the developing brain together with the reduction in mRNA and immunohistochemical expression of $p75^{NTR}$ in vitamin D_3 deficient neonates leads us to conclude that vitamin D_3 is a potent regulator of $p75^{NTR}$ in the developing brain.

The consequences of reduced $p75^{NTR}$ expression in the developing brain may be considerable. At later developmental stages $p75^{NTR}$ is a proapoptotic signal to cells when coexisting trk expression is absent (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Chao et al., 1998; Friedman, 2000). The finding that trk A and $p75^{NTR}$ distribution are

discrete in the cortex suggests that other cells in the developing brain may be subject to trk-independent neurotrophin signaling via $p75^{NTR}$. As such signaling is likely to be proapoptotic the reduction of $p75^{NTR}$ in vitamin D_3 deficient neonates would reduce cell death, consistent with our observations.

VDR density is upregulated by 1,25-dihydroxyvitamin D_3 in a number of tissues (Darwish and DeLuca, 1993). The mechanism is believed to be via ligand-induced stabilization of the receptor rather than increased synthesis (Wiese et al., 1992). In the absence of the steroid therefore we hypothesised that the VDR might be subject to greater degradation. Paradoxically, depletion of vitamin D did not alter the expression of the VDR across the neonatal brain. The regulation of the VDR may therefore be more complicated than previously thought. A more quantitative comparison of VDR in the brain from animals depleted of vitamin D_3 has not yet been done.

Vitamin D_3 and brain development

Although we have demonstrated that low prenatal vitamin D_3 alters brain development in the neonate, the long term consequences associated with these changes remain unclear. We speculate that the altered morphology, the increased rate of mitosis, and the disruption in neurotrophic systems, may deflect the normal trajectory of brain development and involution across the life span. In this report we have concentrated on the effects of low maternal vitamin D on brain development in utero. Given that we show maternal deprivation of vitamin D affects the foetal brain at the gross morphological,

cellular and molecular levels, studies examining the impact of hypovitaminosis D on postnatal brain development and functioning now appears warranted. Future studies will need to examine if less severe maternal hypovitaminosis D in the rat is also associated with altered brain development in the neonate. In addition, it would be of interest to explore if there were critical periods during pregnancy associated with increased vulnerability to hypovitaminosis D. This is of course difficult to study in a maternal rat given the long half-life of this steroid (2-3 weeks) and its short gestational period (22 days). Such a study would be more appropriate in an animal with a longer gestational period such as a guinea pig (68 days).

Our findings contribute to the growing recognition of the important role of steroids in brain development and adult brain function (Melcangi and Panzica, 2001; Puia and Belelli, 2001). Steroids such as pregnanolone promote microtubulin polymerisation (Murakami et al., 2000) and myelin synthesis (Garcia-Segura and Wise, 2000), and dehydroepiandrosterone induces neurite extension (Compagnone and Mellon, 1998). Confirmatory studies showing abnormal brain development in vitamin D related knock-out models have not yet been published. Studies in mice that have had the receptor for vitamin D or the enzyme responsible for its synthesis, CYP27B1, knocked out have not reported obvious brain defects. We therefore await studies that focus their efforts in this direction. Nevertheless, the weight of experimental data presented here supports the hypothesis that vitamin D_3 is involved in brain development (McGrath et al., 2001a).

The idea that maternal vitamin D_3 insufficiency may induce long-lasting changes in foetal brain development is supported by studies on other steroid hormones. Nutritional interventions have created prolonged or permanent effects on other agents that are mediated via nuclear receptors such as the glucocorticoids and thyroid hormones (Dauncey et al., 2001). These changes, in turn, can profoundly influence normal development. Additionally deficiencies in other nutrients have been linked with abnormalities in brain development (i.e. folate and neural tube closure, iodine and cretinism) (Brown et al., 1996).

Although the public health implications for low prenatal vitamin D_3 in the CNS are not understood this first study provides the first biological evidence that low maternal vitamin D could impair brain development.

Figure Legends

Fig 1. Ventricular enlargement in the brains of vitamin D_3 deplete neonates

Representative examples of control (A) and vitamin D_3 deplete (B) brain sections, Bar = 1mm.

(C) Lateral ventricular volumes. * ($t = 2.47$, $df = 11$, $P = 0.011$)

(D) Ventricular volumes as a ratio of hemisphere volume * ($t = 1.93$, $df = 11$, $P=0.033$).

Bar graphs indicate means ± SEM.

Fig 2.

(A) Mitotic cells exhibiting characteristic immunohistochemical staining with anti-PCNA antibody (arrow), nonmitotic cell (arrowhead).

(B) Apoptotic cells exhibiting characteristic fragmented nuclei (arrow) and non-apoptotic cells (arrowhead) stained with bisbenzimide.

Fig 3

(A) Vitamin D_3 depletion increased the percentage of cells undergoing mitosis in the dentate, * ($P<0.001$); hypothalamus, * ($P<0.05$) and basal ganglia/amygdala, * ($P<0.05$), but not the cingulate, (ANOVA, Bar graphs indicate means ± SEM, n=10).

(B) Vitamin D_3 depletion appeared to decrease the percentage of cells undergoing apoptosis in all four brain regions investigated but this was not significant.

(C) Brain cell densities in vitamin D_3 deplete and control animals (cells/ mm^2).

Vitamin D_3 depletion did not alter cell densities in any brain region investigated.

Fig 4

(A) Vitamin D_3 depletion decreased the amount of NGF protein in the neonatal brain, ng/g protein (n=14 ± SEM) * ($P < 0.015$ unpaired t-test).

(B) Vitamin D_3 depletion decreased the amount of GDNF protein in the neonatal brain, ng/g protein (n=14 ± SEM) * ($P < 0.01$ unpaired t-test).

(C) Semi-quantitative RT-PCR data showing expression of neurotrophic factors and selective neurotrophin receptors is unaltered by maternal vitamin D_3 depletion. The non-selective neurotrophin receptor $p75^{ntr}$ was the only factor reduced 30% (n=10 ± SEM) * ($P<0.01$ ANOVA). Results are expressed as the optical density of PCR product relative to the optical density of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Fig 5

(A). $p75^{NTR}$ immunoreactivity was prominent in the infagranular portion of the cortex in control animals, (1); basal ganglia/amygdala, (2); lateral hypothalamus, (3); and the stria terminalis, (4).

(B) Vitamin D_3 depletion appeared to drastically down-regulate $p75^{NTR}$ immunoreactivity. Sections were coded, randomised and assessed visually ($P<0.001$ Fisher's exact test, n=12). Bars = 1mm.

Fig 6

(A) Western blot showing no decrease in the Vitamin D_3 receptor protein (VDR) in deplete (lanes 5-8), compared with control animals (lanes 1-4).

(B) Semi-quantitation of VDR by densitometry.

Bar graphs indicate means ± SEM (n=14).

Figure Captions

Fig.1. Vitamin D depletion induces ventricular enlargement

Fig.2. Characteristic mitotic and apoptotic cells

Fig.3. Vitamin D_3 depletion increases mitosis in the brain

Fig.4. The effect of vitamin D_3 depletion on neurotrophic factors in the brain

Fig.5. Distribution of $p75^{NTR}$ in a medial rat brain section

Fig.6. Vitamin D_3 depletion does not alter the levels of its own receptor

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Table 1. Effect of vitamin D_3 depletion on gross and regional brain morphology

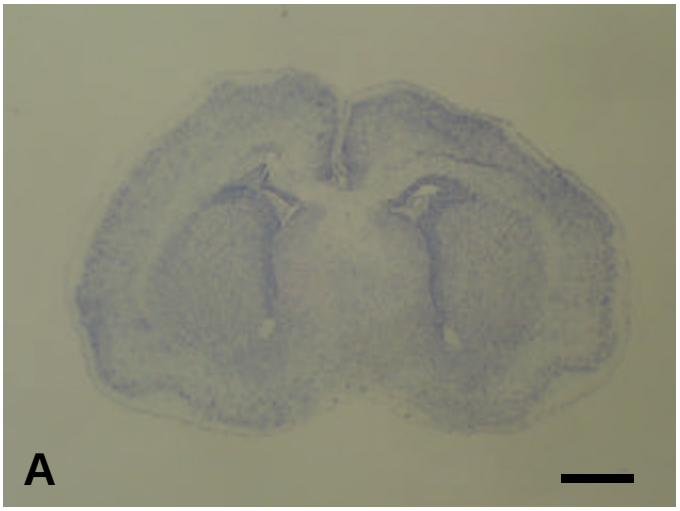
Parameter	Control n = 12	Vitamin D_3 deplete n = 12
Body weight g	5.59 ± 0.11	6.24 ± 0.12, ***
Brain weight g	0.26 ± 0.005	0.29 ± 0.003, ***
Brain weight as a ratio of body weight	4.09 ± 0.05 x10E-3	4.19 ± 0.05 x10E-3
Hemisphere length mm	4.99 ± 0.23	6.33 ± 0.29, **
Hemisphere width mm	7.40 ± 0.27	7.74 ± 0.26
Hemisphere length/width	0.50 ± 0.06	0.66 ± 0.05 *
Combined cortical hemisphere volume (mm^3)	86.61 ± 7.73	128.8 ± 6.26 ***
Total lateral ventricle volume (mm^3)	0.84 ± 0.17	2.41 ± 0.56 **
Total lateral ventricle volume as a % of hemisphere volume	0.44 ± 0.07	0.96 ± 0.24 *
3^{rd} ventricle area as a % of brain cross-sectional area	0.4 ± 0.07	0.42 ± 0.06
Hippocampal cross-sectional area as a % of brain cross-sectional area	2.59 ± 0.12	2.59 ± 0.10
Cortical mantle thickness as a ratio of brain cross-sectional area	3.46 ± 0.36 x10E-1	2.58 ± 0.09 x10E-1 **
Corpus callosum width mm	0.37 ± 0.09	0.37 ± 0.06

(mean ± standard error)

* P<0.05 compared with control

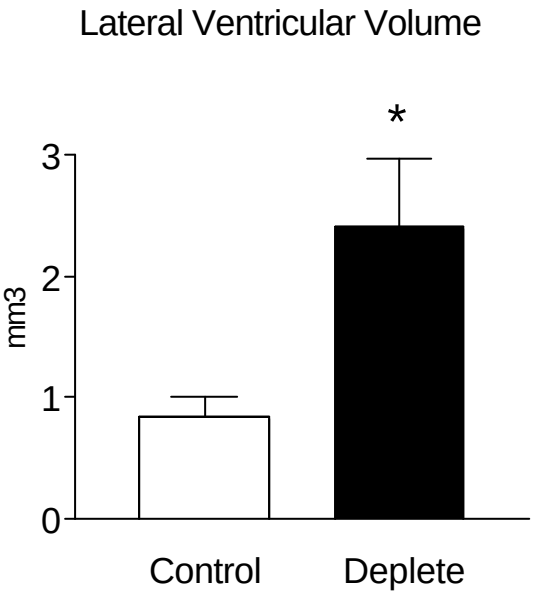
** P<0.01 compared with control

*** P<0.001 compared with control

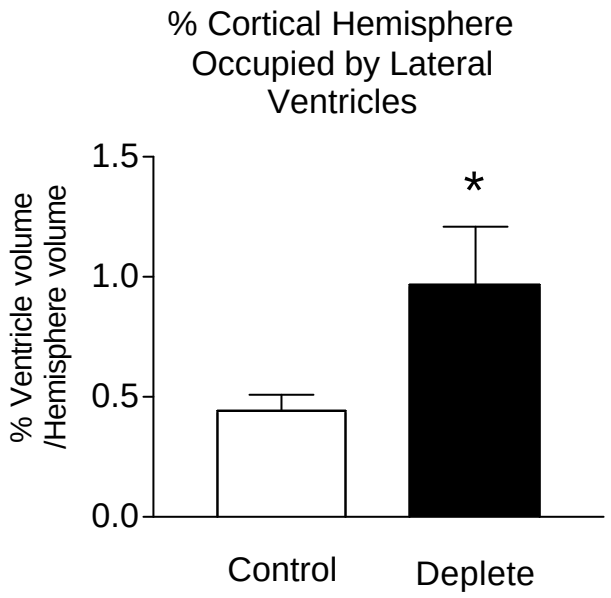

A


B

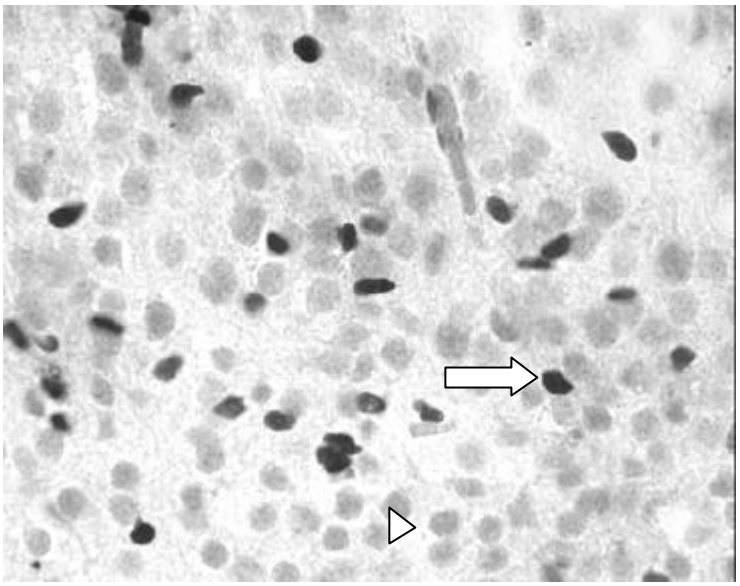
C


Lateral Ventricular Volume
mm3
3
2
1
0
*
Control
Deplete

D


% Cortical Hemisphere Occupied by Lateral Ventricles
% Ventricle volume /Hemisphere volume
1.5
1.0
0.5
0.0
*
Control
Deplete

A



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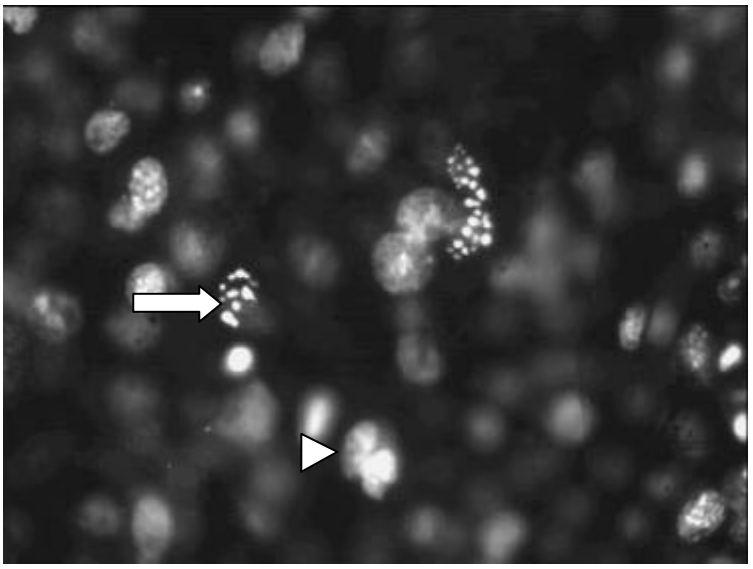

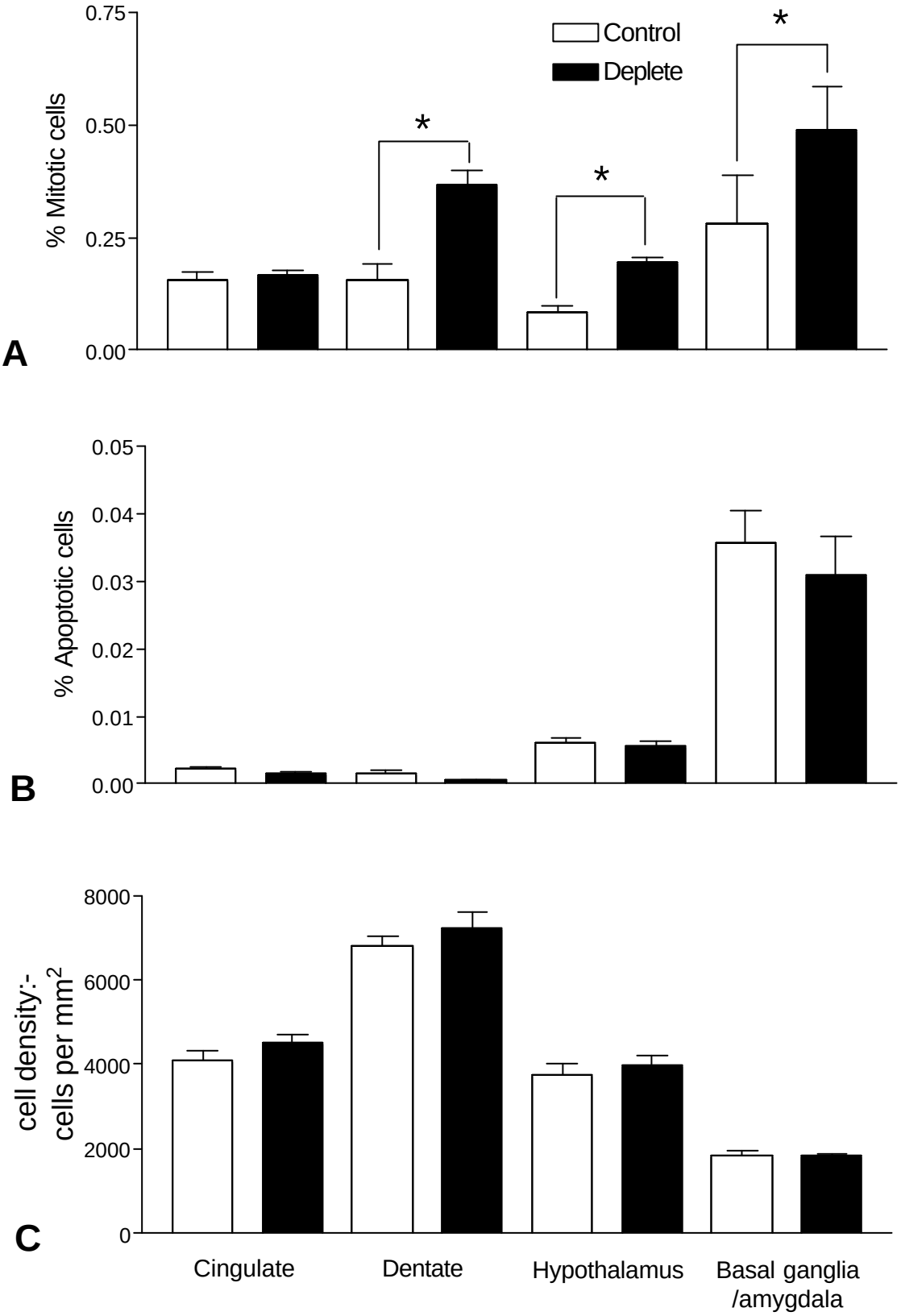
A
% Mitotic cells
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0.50
0.25
0.00
Control
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*
*
*
B
% Apoptotic cells
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0.04
0.03
0.02
0.01
0.00
C
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cells per mm2
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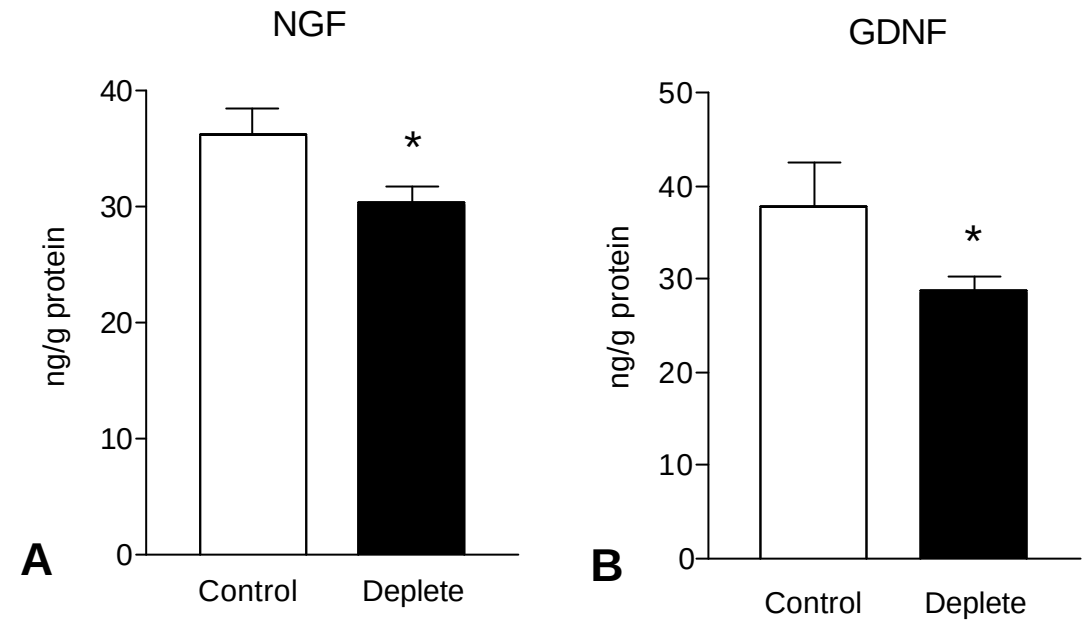
6000
4000
2000
0
Cingulate
Dentate
Hypothalamus
Basal ganglia
/amygdala


NGF
GDNF
ng/g protein
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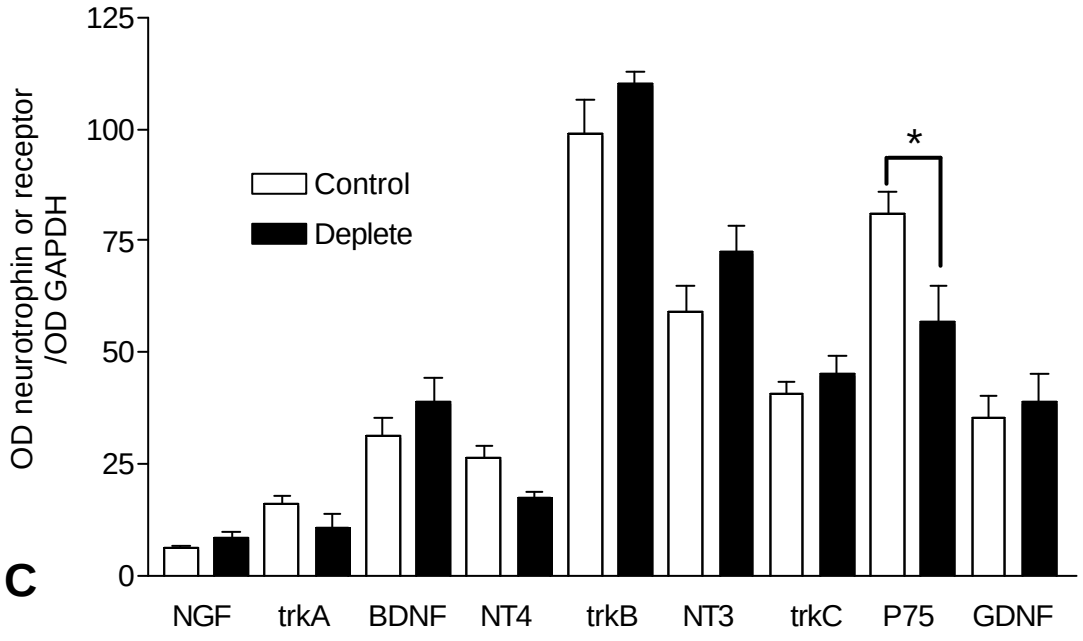
OD neurotrophin or receptor /OD GAPDH
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100
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P75
GDNF
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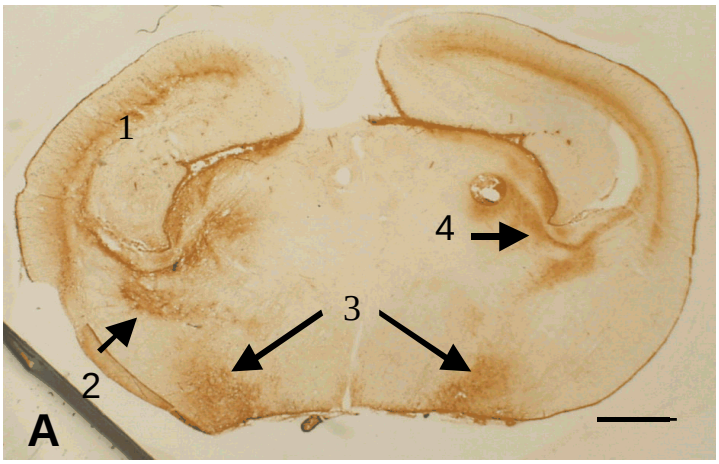
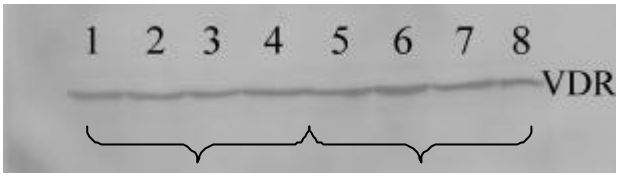


Control **Deplete**

A



Control Deplete

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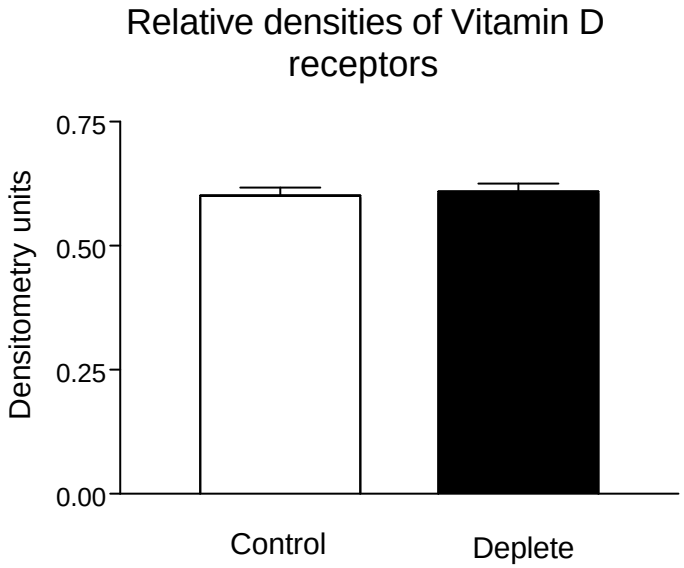

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