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CEREBELLAR PARAMETERS IN DEVELOPING 15 DAY OLD RAT PUPS TREATED WITH PROPYLTHIOURACIL IN COMPARISON WITH 5 AND 24 DAY OLD
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ABSTRACT

Objective: **To investigate and quantify structural parameters in the developing cerebellum during hypothyroidism in pre and postnatal stages in 15 day old rat pups in comparison with 5 and 24 day old.**

Methods: **Propylthiouracil (PTU) was fed to rat dams during mating, pregnancy and nursing and their pups in drinking water. Consequently hypothyroxinemia was induced in the dams and the developing foeti during prenatal period and maintained in the dams and pups. The number of treated and control dams was five in each group. The treated and control pups were eight and eleven respectively. The whole cerebellum was dissected out and routinely processed for histological and morphometric analysis. Structural changes in cerebellum were estimated using "design based" stereological methods. The total volume of cerebellum, intracerebellar nuclei and cerebellar compartments were estimated using Cavalieri Principle. Numerical density of cells was estimated using the disector method and the total cell number was then calculated.**

Results: **In the 15 day pups there was significant reduction (P<0.05) in the mean volumes of cerebellum, internal granular layer, molecular layer, cerebellar cortex, mean ratio of the total volume of intracerebellar nuclei to the cerebellar volume and increased mean volume of external granular layer in treated pup group compared with control. The mean volumes of intracerebellar nuclei and white matter and the mean numerical densities and total numbers of neurons and Purkinje cells in intracerebellar nuclei and cerebellum respectively were nearly equal in control and treated groups. Significant increase (P<0.05) in the mean numerical density and total number of glial cells in treated pups compared with control was observed. There was significant decrease (P<0.05) in the mean neuron/neuroglia ratio in the intracerebellar nuclei, mean numerical density and mean total number of granule cells and reduction in the mean ratio of total number of granule/Purkinje cells in the treated group compared with control. The linear regression comparison for the total volume of the intracerebellar nuclei to total volume of the cerebellum in 5, 15 and 24 day control and treated pups and for the total number of glial cells on the total volume of intracerebellar nuclei in the same were significantly different (P<0.05). Total numbers of neurons and glial cells in the intracerebellar nuclei showed peak values in 15 day pups.**

Conclusion: **Thus PTU-induced hypothyroidism causes variation in quantitative structural parameters in developing cerebellum and disrupts progressive cellular developmental processes. Maintenance of normal T4 and T3 levels during growth and maturation of cerebellum is absolutely essential.**

INTRODUCTION

Hypothyroidism is a deficiency of thyroid hormones in the body. Treatment of pregnant rat dams and pups with Propylthiouracil induces hypothyroidism in the pups. Previous studies on propylthiouracil treated pups showed significant reduction (P<0.05) in mean body weights and reduced serum T4 and T3 in 5 and 24 day pups(1,2). Cerebellar parameters in the same, showed significant reduction (P<0.05) in the volumes of cerebellum and intracerebellar nuclei in treated pups compared with controls. Similarly the differences in the mean ratio of volumes of intracerebellar nuclei to cerebellar volume in the 5 and 24 day pups was not significant. The differences in the volumes of compartments in treated pups were significantly reduced (P<0.05) in 24 day pups compared with the control(1,2). In the estimation of numerical densities of neurons and glial cells in the intracerebellar nuclei in 5 and 24 day treated and control pups, the glial cells showed greater sensitivity to hypothyroidism in treated pups. On the other hand, the variation in the total number of neurons in the nuclei did not reach statistical significance. Analysis of the total numbers of cells in the cerebellar cortex in 24 day pups showed significant reduction (P<0.05) in the total number of Purkinje and granule cells respectively in treated pups compared with control(1,2). The ratio of the volume of intracerebellar nuclei to cerebellar volume decreased from 12% to 3.5% in the control and 14% to 4% in the treated group in 5 and 24 day pups respectively. The ratio of the total number of

neurons to neuroglia in the intracerebellar nuclei in 5 and 24 day pups was about a unit in both control and treated pups(1,2). Therefore it was important to study further the quantitative structural changes in cerebellum and intracerebellar nuclei in 15 day pups in order to understand clearly the developmental process during hypothyroidism using a rat model.

MATERIALS AND METHODS

Male and female Wistar Albino strain rats were purchased from commercial breeders, Harlan Olac (U.K). The condition of the animals, maintenance, body weights and experimental profile were as previously described(1,2). The treatment with propylthiouracil was continued during mating, pregnancy and after furrowing upto 15 days post paturation.

Three 15 day old pups were sampled randomly from each litter, weighed and anaesthetised by inhalation of ether. The pups were decapitated and blood from pups of common parenthood was pooled into one tube for serology. The head was skinned and the skull opened. The brain was dissected out and immersed in 10% phosphate buffered formalin. Each cerebellum was dissected from the brain stem by making a transverse incision through the cerebellar peduncles. The samples were processed as previously reported and embedded in historesin in plastic moulds(1,2). Each block was sectioned and the sections sampled in systematic random manner. 5 μm thick sections were stained in 0.01% Cresyl fast violet, while 40 μm thick sections for neuron and glial cell count in the intracerebellar nuclei were stained in 0.005% Cresyl fast violet. 40 μm thick sections for Purkinje cells count, were stained in 10% Giemsa at 50°C in an oven for 90 minutes, differentiated in acetic acid (4 drops acetic acid in 100 ml of distilled water) for 60 seconds. 10 μm thick sections for granule cells count were hydrolysed in 5N hydrochloric acid for 20 minutes at room temperature and stained with Schiff reagent. The whole process was done and each step carried out as previously described(1,2).

Volume estimation of cerebellum, intracerebellar nuclei and cerebellar compartments was done(1,2). The parafollicular lobes were not included in this study. The estimation of the mean cell count from each sample and for each cell type was efficiently performed using optical disectors(3-6). The number of slides analysed and the size of the unbiased test frame was very much influenced by the size and distribution of the cells under investigation. Therefore two sections were used for analysis of neurons and neuroglia in intracerebellar nuclei, six sections for Purkinje cells and two for granule cells. Uniform random fields were sampled in a systematic random manner from every section, the frame approaching the section in regular steps. The fields were analysed under an Olympus microscope connected to a video camera and television monitor(3,7). The statistical analysis of the results of each sample gave a coefficient of error of less than 10%(8). The numerical density of each cell type was estimated and the total cell number of cells for the corresponding cell type calculated as previously described(1,2).

Radio immunoassay: T4 and T3 serum levels in the pups were estimated from the pooled blood samples using radio immunoassay procedure (Immuno-diagnostic Systems, IDS, UK). The assay range for T4 was 25-300nMol/L and 0.3-9.0 nMol/L for T3 respectively.

Statistics: To test the null hypothesis, that there was no difference between pups from different treatment regimes, standard statistical parameters were calculated and the data was analysed by one factor analysis of variance (ANOVA)(1,2). The degree of variance between groups (classes), litters (dams) and individual pups was determined using a General Linear Model (GLM) procedure for unbalanced experimental groups. The effects of different treatment regimes on each cerebellar parameter were compared using Tukey's Studentized Range Test (TSRT) in combination with GLM at 5% level(9). Regression analysis was done on total volume of intracerebellar nuclei on the total volume of cerebellums total number of glial cells and neurons on the total volume of intracerebellar nuclei respectively in 5, 15 and 24 day pups.

RESULTS

Results of the mean body weight between the treated and control pups ranged from 26.70 ± 1.54 to 17.91 ± 0.51 gm in control and treated groups respectively. The difference was significant ($P<0.05$). The mean serum T4 and T3 concentrations ranged from 49.20 ± 14.69 to 33.27 ± 3.10 and 1.85 ± 0.16 to 1.37 ± 0.07 nMol/L. respectively for control and treated groups with no significant difference.

Volumes: Results of the volume estimates are shown in Table 1. The mean total volume of cerebellum (Vce) was reduced in the treated group compared with control. The difference was significant ($P<0.05$). The mean total volume of intracerebellar nuclei (Vnu) was nearly equal in both groups. The mean total volume of the white matter (Vwm) was nearly the same in both groups. The mean total volume of internal granular layer (Vig) and the molecular layer (Vml) were reduced in the treated group compared with control. The differences were significant ($P<0.05$). The volume of external granular layer was excluded from the estimate of the total volume of the molecular layer. The mean volume of the external granular layer (Veg) was larger in the treated pups than in the control. The difference was not significant. The mean total volume of the cerebellar cortex (Vc) was reduced while the ratio of the mean total volume of intracerebellar nuclei to cerebellum (Vnu/Vce) was increased in the treated group compared with control. The differences were significant ($R<0.05$).

Table 1

A comparison of various brain volumes, between treated and untreated pups in 15 days old pups

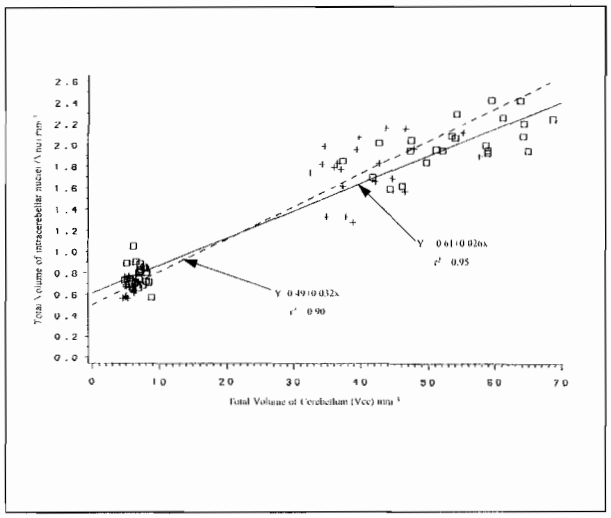
Parameter	Groups	
	Control Mean (SEM)	Treated Mean (SEM)
Sample	11	8
Vce. (mm^3)	46.69 (1.50)	37.11 (1.27)*
Vnu. (mm^3)	1.89 (0.06)	1.91 (0.06)
Vnu/Vce	0.04 (0.0008)	0.05 (0.0011)*
Vwm. (mm^3)	6.85 (0.22)	6.64 (0.23)
Vig. (mm^3)	21.13 (0.72)	17.90 (0.69)*
Veg. (mm^3)	4.37 (0.24)	5.10 (0.49)
Vml. (mm^3)	14.47 (0.64)	7.47 (0.47)*
Vc (mm^3)	39.92 (1.38)	30.47 (1.18)*

*Significantly different ($P<0.05$) compared with control
Key: Vce=Volume of cerebellum, Vnu=Volume of intracerebellar nuclei Vnu/Vce ratio, vwm=Volume of white matter, Vig=Volume of internal granular layer, Veg=Volume of external granular layer, Vml=Volume of molecular layer and Vc=Volume cortex

Regression comparison for the total volume of intracerebellar nuclei on the total volume of cerebellum in 5, 15 and 24 day pups showed significant difference (P<0.05) between treated and control groups (Figure 1).

Figure 1

Simple linear regression comparison of the total volume of the intracerebellar nuclei (Vnu) on the total volume of cerebellum (Vce) in 5, 15 and 24 day pups. + - - - - - + treated; □——□ control



Number estimates: Results of the cell counts are shown in Table 2. Comparison of the mean numerical density of neurons (Nvne) showed a small decrease of 3.5% in the treated group, while the mean total number of neurons (Nne) in the intracerebellar nuclei was decreased by 2.9%. These values are within the margin of statistical error. The mean numerical density of neuroglia (Nvgl) and the mean total number of glial cells (Ngl) were increased in the treated group compared with control and the differences were significant (P<0.05).

Table 2

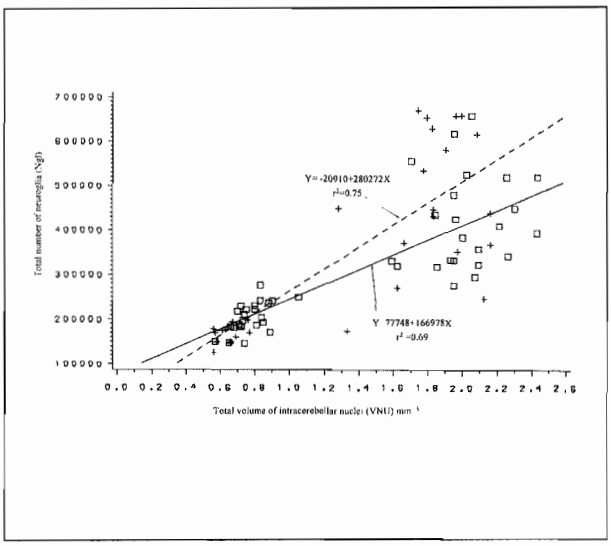
Mean values for numerical density (Nv) and total number (N) of neurons (ne), neuroglia (gl), Pukinje (pu) and granule (gr) cells estimated in the intracerebellar nuclei (nu) and cerebellar cortex for the control and treated pups in 15 day old pups

Parameters	Groups	
	Control Mean (SEM)	Treated Mean (SEM)
Sample size	11	8
Nvne./mm³	205000 (7000)	197000 (11000)
Nne.	386000 (15000)	375000 (15000)
Nvgl./mm³	248000 (18000)	321000 (20000)*
Ngl.	466000 (35000)	609000 (31000)*
Nne/Ngl.	0.86 (0.05)	0.62 (0.03)*
Nvpu/mm³	18000 (1000)	19000 (800)
Npu.	377000 (24000)	324000 (15000)
Nvgrx10³/mm³	6216 (245)	4853 (235)*
Ngrx10³	131811 (8121)	86044 (2905)*
Ngr/Npu	358 (21)	262 (21)*

*Significantly different (P<0.05) compared with control

Figure 2

Simple linear regression comparison of the total number of glial cells on the total volume of the intracerebellar nuclei in 5, 15 and 24 day pups. + ————+ treated; □————□ control



The mean neuron to neuroglia ratio (Nne/Ngl) in the treated group was reduced in the treated group compared with control and the difference was significant (P<0.05). The mean total numbers of neurons and glial cells respectively showed peak values in 15 day treated and control pups.

The mean numerical density of Purkinje cells (Nvpu) was increased by 5.4%, while the mean total number of Purkinje cells (Npu) was reduced by 14.2% in the treated group compared with control, but the variation did not reach statistical significance. The mean numerical density (Nvgr) and the mean total number (Ngr) of granule cells were reduced in the treated group compared with control. The differences were significant (P<0.05). The mean ratio of total number of granule to Purkinje cells (Ngr/Npu) was reduced in the treated group compared with control and the difference was significant (P<0.05).

Regression comparison for the total number of glial cells (Figure 2) on the total volume of intracerebellar nuclei in 5, 15 and 24 showed significant difference (P<0.05) between the treated and control pups.

DISCUSSION

The 5, 15 and 24 day studies have shown that the total volume of cerebellum is significantly reduced (P<0.05) in all treated groups. Apparently the mean volume of cerebellum in treated pups is severely affected due to cumulative reduction in numbers of different cell types and the growth of neuropil as indicated by reduced volumes of compartments(2, 10,11). Regression comparison of the total volume of intracerebellar nuclei on the total volume of cerebellum in 5, 15 and 24 day control and treated pups

(Figure 1) showed the volume of intracerebellar nuclei is dependent on the volume of cerebellum (r = 0.95 and 0.90 respectively). The difference between the regression lines was significant (P<0.05). As the volume of cerebellum increased between five and 24 days, the ratio of the volume of intracerebellar nuclei to the volume of cerebellum decreased showing that this is an important parameter especially in comparative investigations on the volume of cerebellar cortex. On the other hand the mean total volume of intracerebellar nuclei in 15 day treated pups was equal to the control. This probably was due to the presence of a large number of glial cells in and around the nuclei compared with control pups (Table 2).

The mean volume of the white matter in 15 day pups, (which included the volume of the intracerebellar nuclei) was equal in both treated and control pups (Table 1) showing that the white matter was not affected in the treated group in 15 day pups as compared to 24 day pups whereby the difference in the total volume was significant (P<0.05) compared with control (2).

The internal granular layer which includes the internal granular layer proper and Purkinje cell layer expands rapidly after the first postnatal week as the granule cells migrate from the external granular layer(12-14). The difference in reduction of the mean volume of internal granular layer in the 15 day treated group was significant compared with control (P<0.05) (Table 2); similar to that in 24 day pups(2). Hypothyroidism has slowing effects on the migration of the granule cells(10,15).

The difference in the reduction of the mean volume of the molecular layer was significant (P<0.05) in the treated pups compared with control (Table 1). The value in the treated group is 66% of the value in the control suggesting that growth in cell processes was severely reduced. The increase in volume of the molecular layer between 15 and 24 days in control and treated groups was 7.67 mm^3 and 3.04 mm^3 respectively.

The volume of cerebellar cortex depends on the volume of its compartments and the total number of cells within. The results for both 15 and 24 day pups showed significant difference (p<0.05) in the reduction of the mean volume of the cerebellar cortex in the treated groups compared with controls. The volume increase between 15 and 24 days in controls and treated groups was 10.02 mm^3 and 4.29 mm^3 respectively.

The mean numerical density and mean total number of neurons in the intracerebellar nuclei in 15 day pups was equal in both treated and control groups. Thus the level of hypothyroidism did not affect their proliferation. On the other hand the difference in the mean numerical density and the mean total number of glial cells in the intracerebellar nuclei respectively showed significant increase (P<0.05) in the treated pups compared with control.

Glial cell proliferation in the rat increases in the second postnatal week and the beginning of myelination is observed around this time(14,17). Therefore the increase observed in the treated pups could be due to retarded glial cells migration. The mean neuron to neuroglia cell ratio was decreased and significant (P<0.05) in the treated pups compared with control. If a close relationship in numbers of glial cells and neurons is maintained during development of the cerebellum, the gliosis in treated group showed that glial cells are more susceptible to hypothyroidism in developing brain. This is consistent with previous observations(1,2,18). Different types of glial cells are found in the cerebellum and probably the cell types proliferate, differentiate and migrate at different times. Thus the distribution pattern and localization of the different glial cell types may be distorted in the treated pups. The total number of neurons and neuroglia in 5, 15 and 24 days showed peak counts in 15 day pups and subsequent reductions in 24 day pups. If this reduction is due to apoptosis(19) then any functional improvement on removal of the constraint once mature cell numbers have been achieved can only be due to regenerative process within the remaining cells especially on the part of the neurons. Regression comparison for the total number of neurons on the total volume of intracerebellar nuclei in 5, 15 and 24 day pups did not reach statistical significance. On the other hand regression comparison for the total number of glial cell on the volume of intracerebellar nuclei in 5, 15 and 24 day control and treated pups (Figure 2) showed significant difference in the regression lines (P<0.05) (r = 0.67 and 0.75 respectively).

The mean numerical density of Purkinje cells in the 15 day pups was nearly equal in treated and control pups. The mean total Purkinje cell numbers in treated group showed a mean reduction of >50,000 (14.2%) suggesting that the numbers of large neurons were not affected by fifteenth day. However, there was a 50% mean reduction in the total number of Purkinje cells between 15 and 24 days in both control and treated pups [(377(24); 324 (15) and 250(15); 174(7))10^3].

The volume of the external granular layer in 15 day pups was increased in the treated group compared with control. The total granule cell count in the internal granular layer in the treated pups was 65% of the count in the control (Table 2). Reduced rate in cell proliferation and migration from the external granular layer and retarded increase in the internal granular layer in hypothyroid pups has been reported(15,16,20). The mean numerical density and the mean total number of granule cells respectively in 15 day pups showed significant reduction (P<0.05) in the treated group compared with control. Thus a serious deficit existed both in packing density and absolute cell numbers in the treated group.

Completion of cell migration is followed presumably by growth of dendrites and the establishment of synaptic connections with mossy fibres and Golgi cells. It is clear that failure in glial cells proliferation and subsequent migration to their mature positions coupled with failure to produce adequate amounts of cytoplasm in other cell types including granule cells will negatively affect these processes(15,21,22). Thus the molecular layer probably lacks the full complement of either dendrites or parallel fibres or both in hypothyroid pups. However the total

number of granule cells continued to increase up to 24 days in both control and treated pups(2).

The difference in the mean ratio of granule to Purkinje cells, showed significant reduction ($P<0.05$) in the treated group compared with control. Comparison data show that there are optimal cell interaction rates between different neuronal types at 5, 15 and 24 days(1,2,23). The significant reduction in the mean total value in 15 day pups suggests that an optimal ratio of granule cells to mossy fibres Golgi cells, Purkinje and stellate cells will be highly constrained. Previous authors have reported a delay in the appearance of cerebellar glomeruli and abnormal development because of delayed migration of granule cells(16). Poor development of granule cells will incapacitate the ability of cerebellum to respond to messages from sensory receptors and the pyramidal system. The pups in the treated group will not have the full complement of interacting cell units as compared to the control despite the fact that they may have a stable neuronal circuit(2,23). Thus the total electrical activity and energy will be reduced in the treated pups.

The implication of these results is that the developing nervous system in the cerebellum is established on a strictly balanced ratio of repeating interacting units for stable neuronal circuits which depends on maintenance of normal T4 and T3 levels during growth and maturation of cerebellum. It is clear that hypothyroidism causes deficiencies in structural parameters and disrupts predictable cellular interaction patterns in the developing cerebellum. The 15 day results show the transition point where the process of cell multiplication in different cortical cells shifts to cell maturation in the rat.

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