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Comparing number and intensity of doublecortin-immunolabelled cells in the hippocampus and cerebellum in the postnatal mice brains, in different age groups

Zainab Zahid Saadoon
College of Medicine/University of Baghdad, Iraq – Baghdad

Abstract:

In the mammalian brain, adult neural stem cells reside in the sub- ventricular zone and sub granular zone (SGZ) of the hippocampus. Adult neurogenesis has been observed in all mammalian species including humans, and results in the formation of new neurons in the olfactory bulb and the dentate gyrus (DG) of the hippocampus. Adult hippocampal neurogenesis results in the formation of new neurons and is a process of brain plasticity involved in learning and memory. Doublecortin (DCX) is a microtubule associated protein that is critical for neuronal migration and the development of the cerebral cortex. In the adult, it is expressed in newborn neurons in the subventricular and subgranular zones but not in the mature neurons of the cerebral cortex. However, sections of the brain cerebellum of the early postnatal mice showed purkinji cells and cells in granular layer faintly immunoreactive for doublecortin antibody. Newborn neurons in the subgranular zone of the hippocampus and the purkinji cells that are immunoreactive for doublecortin showed gradual decrease in number and intensity with aging.

Aim of study: In this study we investigated the expression of doublecortin immunolabelled cells in both dentate gyrus of hippocampus of adult mice brain and in cerebellum of the first four days postnatal mice brains and correlated those expressions with successive age groups of both, by immunolabelling of doublecortin on series of paraffin sections.

Materials and Methods: adult mice and early postnatal mice brains used in the experiment to immunostain cells in the hippocampus and the cerebellum by antidoublecortin antibody on series of paraffin sections.

Results: we compared the number and intensity of doublecortin immunolabelled cells in the hippocampus across different age groups in the adult mice and in the cerebellum in first four days postnatal mice. We found that despite there was a decline in the number and intensity in hippocampus from early adulthood (8 weeks) to middle adulthood (4 months) still it's not very significant, however it became strong significant when we compared the early adulthood (8 weeks) and the late adulthood (6 months). In cerebellum there is a decline in the number and intensity from (0 days) to (2 days) postnatally, and it was significant, and it nearly disappeard in (4 days') age groups.

Keywords: doublecortin expression, hippocampus, cerebellum, age

INTRODUCTION

Contrary to was believed in many decades ago, that the brain is a quiescent organ; it is well known today that production of new neurons is not restricted to embryonic state, but extends throughout life in adult mammalian brains (Whitman and Greer, 2010) including humans (Eriksson, et al.(1998). The main of which originate in two main neurogenic niches in adult mammalian brains; subgranular zone (SGZ) of the dentate gyrus, subventricular zone SVZ of the lateral ventricle and both of which located in the forebrain (Oyarce and Nualart, 2014) and continue to produce new neurons throughout life (Altman J.1969). The proliferation of adult neural stem or progenitor cells is regulated by several extrinsic factors such as aging (Gebara et al, 2013). Doublecortin is a brain-specific gene, first identified in 1998, that is mutated in two human neuro-developmental disorders: X-linked lissencephaly "smooth brain" and double cortex syndrome (Gleeson et al., 1998). Mutations in this gene lead to a cell-autonomous defect in cortical neuronal migration (Gleeson et al., 1999). 'Double cortex syndrome' provides the commonly used shorter name for doublecortin, 'DCX' (Geoghegan and Carter, 2008). This gene encoded for doublecortin protein (DCX). The latter is a 40 kDa microtubule-associated protein (MAP) that is expressed in migrating neuroblasts during embryonic development and early neonatal period and retained in neurogenic areas in the adult brains (Francis et al., 1999; Couillard-Despres et al., 2005). In adult hippocampal neurogenesis DCX marks the period between the committed progenitor cell stages (type-2b/3) and the early postmitotic maturation stage and is absent from the radial-glia-like stem cells (type-1), the non-committed progenitor cells (type-2a) and the mature neurons (Couillard-Despres et al. 2005). When neurons are migrating, they demonstrate morphological alterations due to a variety of cytoskeletal changes within the soma and processes that lead to their subsequent movement. DCX appears to regulate the assembly and stabilization of microtubule actions in such a way that it co-localizes and co-assembles with them; and has a direct effect on microtubule polymerization (Gleeson et al., 1999). In the adult mouse brain, DCX is almost exclusively expressed by immature newborn neurons (differentiating and migrating) in restricted areas and is commonly used to distinguish immature neurons from mature ones, and to estimate neurogenic activity (Couillard-Despres et al., 2005). As such; "DCX" has recently been promulgated as a selective marker for this stage of neurogenesis in both the developing and the adult brain (Geoghegan and Carter, 2008). Doublecortin was found expressed extensively in those neurogenic regions dentate gyrus (the previous references) and also expressed in the developing cerebellum of mouse and in young adult's brains of mice while migrating and for a short time also after their settlement (Takács et al., 2008). Neurogenesis and neuronal migration of cells in the cerebellum continue into early postnatal life; migration of one class of cerebellar interneuron, unipolar brush cells (UBCs), may continue

into adulthood (Manohara et al, 2011). Purkinji cells expressed doublecortin until early in 0 day postnatal in mice, as illustrated by (Gleeson et al., 1999). These cells gradually lost their expression. How those faint expression is lost with age, this is to be investigated in this work.

In this study we investigated the number of positive expression of doublecortin immunolabelled cells and the intensity of this positivity in both dentate gyrus of hippocampus of adult mice brains and cerebellum of 0 day, 2 days and 4 days postnatal mice brains and correlated those expressions with age by immunolabelling of doublecortin on series of paraffin sections.

MATERIALS AND METHODS

For the hippocampus study, fifty apparently healthy adult mice (Micromys minutus) weighing 30-40 gm. were collected from the Animal House of the National Centre of Researches and Drug Monitoring/Baghdad. Breeding had been done in well aerated cages; given *ad libitum* access to food and water. Mice were grouped in three age groups: 8 weeks, 4 months, 6 months' group. For the cerebellum study, another fifty healthy postnatal first week mice grouped into three age groups: 0 days, 2 days, and 4 days. All mice were anesthetized by Ketamine (75 mg/kg body weight) intraperitoneally by insulin syringe and waited until fully anesthetized, then perfused intracardially with 4% paraformaldehyde [Selfine-India] prepared in Phosphate Buffered Saline PBS [Fluka-GERMANY]. The Calvaria dissected and brain harvested *en block*. The harvested brain was sliced sagittaly and trimmed and fixed for 20 hours in the same fixative used for the perfusion. Tissue slices were washed from fixative with running tab water for 5 minutes, and then dehydrated with ascending graded conc. of ethanol alcohol [Scharlau-Spain] and clearing in chloroform [Riedel-de Haen-Germany]. Brain slices were impregnated with molten paraffin at 61-63°C [Scharlau-Spain], melting point (56-58°C) then embedded in the same paraffin. Sectioning had been done with Microtome [Histoline laboratories MRS-3500-Italy]. Sections thicknesses were 5 µm. Sections were mounted on charged slides [AFCO-China]. Deparaffinization had been done by Xylene [Scharlau-Spain] and rehydration with descended grades of ethanol, washed with tab water for 2-3 min then the slides were immersed in Harris hematoxylin staining by Harris hematoxylin [SYRBIO-S.A.R] for 2 min washed with running tab water for 5 min then immersed in eosin [SYRBIOS. A.R] for 40 seconds, then dehydrated in ascending concentration of ethanol alcohol. After dehydration, tissues were cleared in xylene, one change for 1 min and finally they were mounted with DPX [HISTOFLUID-GERMANY] and cover slipped. For the immunohistochemical staining, marker used is Anti-Doublecortin antibody (ab18723) [ABCAM –UK] Rabbit polyclonal to Doublecortin. Detection kit (including the secondary antibody and chromogen) was [Expose Rabbit Specific Detection Kit (ab80437) [Abcam-UK]]. The secondary antibody was goat anti-rabbit polyclonal antibody “readymade”. The primary antibody was diluted to 1:1000 by Phosphate Buffered Saline PBS [Fluka-GERMANY]. Incubation of the primary antibody with the tissue was for 2 hours and secondary antibody for 1 hour. All incubated at 30°C. Application of DAB had been done in dark for five minutes and counterstaining was done by Harris hematoxylin for 1 minutes.

For statistics, Aperio imagescope (Leica) was used for images analysis regarding the number and intensity of the immunolabelled cells. Anova method and (t test) for comparison between 3 different age groups in regards to the number and intensity of immunolabelled cells with antidoublecortin antibody. Comparison of these parameters between every pair of study groups by post hoc test.

RESULTS

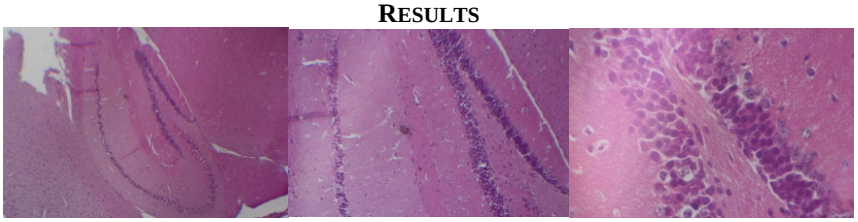

Figure (1) Dentate gyrus of the 8 weeks' group, 6 months and 8 months' group by H and E demonstrated no difference (the above images of the 8 weeks group)

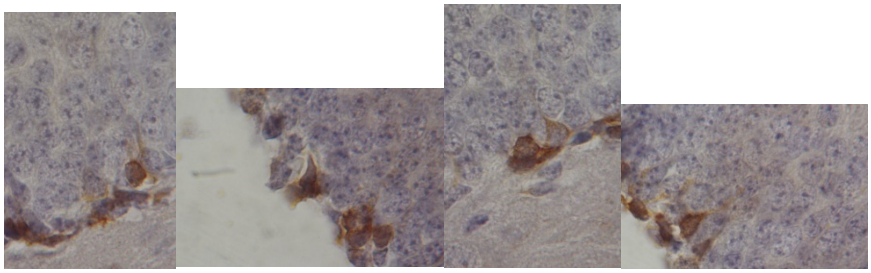

Figure (2) Dentate gyrus of the 8 weeks' group by immunohistochemistry using antidoublecortin antibody staining the neuroblasts in the subgranular zone with intense staining

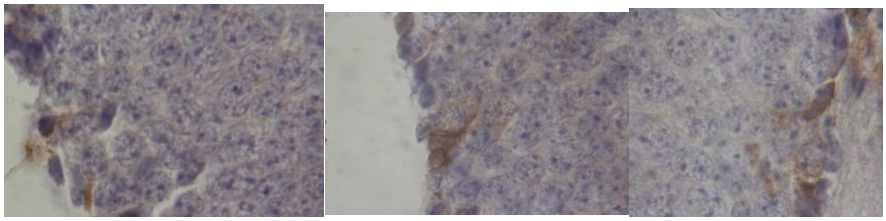

Figure (3) Dentate gyrus of the 4 months group by immunohistochemistry using antidoublecortin antibody demonstrated faint staining weaker than the younger age group and less abundant immunostained cells in the subgranular zone

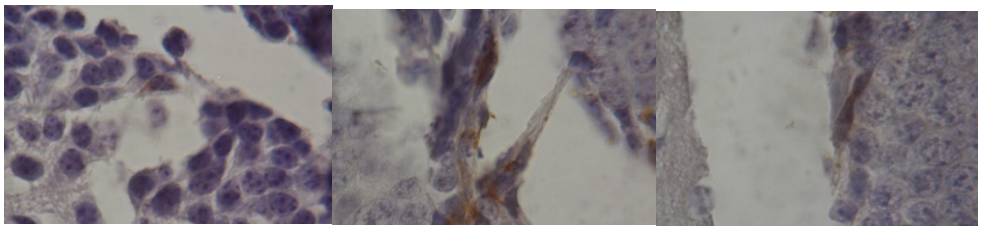

Figure (4) Dentate gyrus of 6 months' age group

Table (1): Comparison between three study groups of hippocampus by ANOVA

Parameter	G1 N=11 Mean±SD	G2 N=11 Mean±SD	G3 N=10 Mean±SD	P value
NP ($x10^5$)	83.64±35.14	49.66±2.86	31.41±5.87	<0.001
IP ($x10^5$)	11022.71±4305.22	6520.64±397.83	4237.31±965.63	<0.001

NP number of positives
IP intensity of positives
G1 is the group of 8 weeks age group
G2 is the group of 4 months age group
G3 is the group of 6 months age group

Table (2): Comparison of different parameters between every pair of study groups of hippocampus by post hoc test

Dependent Variable	1st group	2nd group	*P* value
NP ($x10^5$)	G1	G2	0.002
	G1	G3	<0.001
	G2	G3	0.132
IP ($x10^5$)	G1	G2	0.001
	G1	G3	<0.001
	G2	G3	0.127

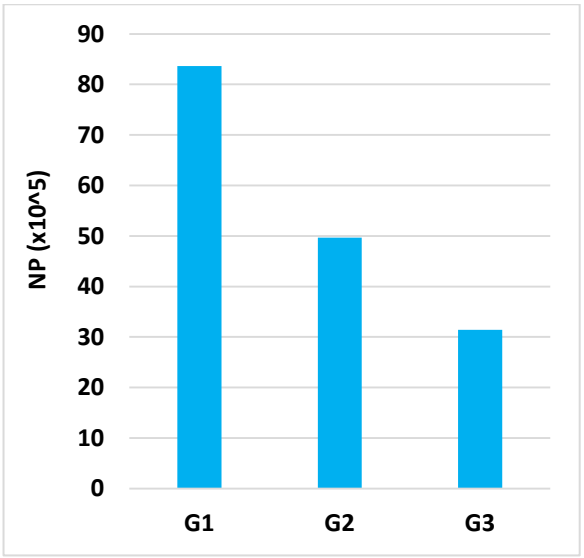

Chart (1): No. of positive cells in the dentate gyrus in the three study groups

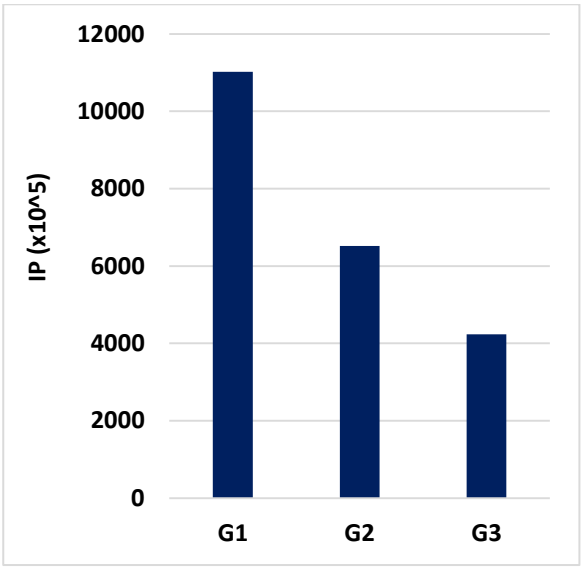

Chart (2): No. of total intensity of positive cells in the dentate gyrus in the three study groups

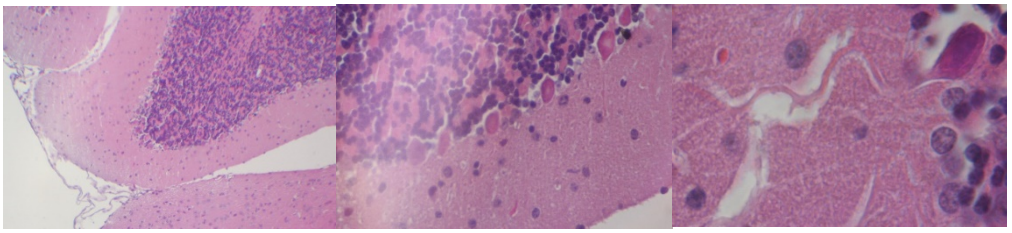

Figure (5) Cerebellum of all groups demonstrated no difference by H and E regarding the three layers of the cerebellar cortex, note purkinji cells extend their dendrits through the molecular layers with extensive arborizations (the above images of the 4 days group)

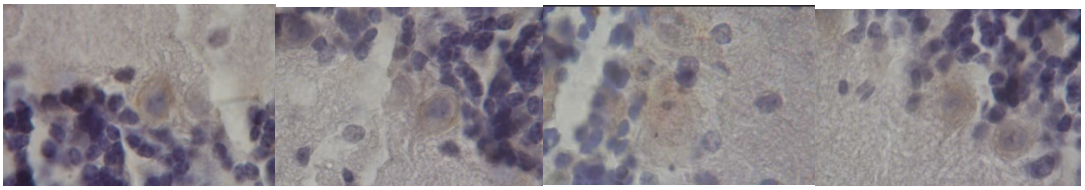

Figure (6) Cerebellum of the 0 day age group by immunohistochemistry using antidoublecortin antibody, note the immunostained Purkinji cells

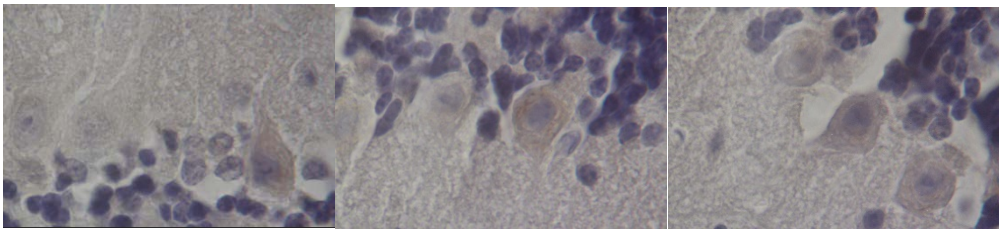

Figure (7) Cerebellum of the 2 days' age group by immunohistochemistry using antidoublecortin antibody, note the Purkinji cells faintly stained

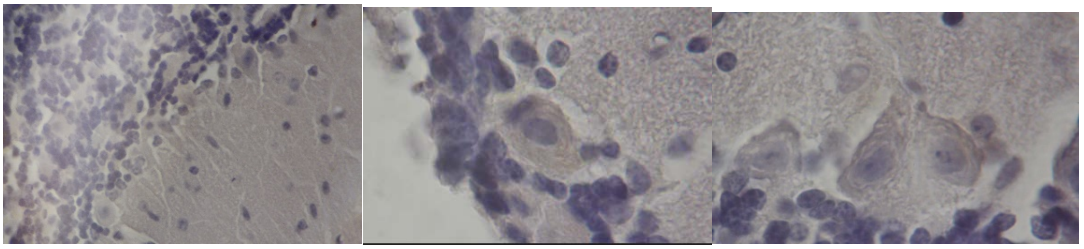

Figure (8) Cerebellum of 4 days' group

Table (3): Comparison between three study groups of cerebellum by ANOVA,

Parameter	G1 N=16 Mean±SD	G2 N=16 Mean±SD	G3 N=17 Mean±SD	P value
NP ($x10^5$)	31.18±11.0	23.24±3.17	16.06±2.33	<0.001
IP ($x10^5$)	4295.38±1437.89	3303.49±339.72	2253.34±414.73	<0.001

NP number of positives
IP intensity of positives
G1 group of 0 day age group of cerebellum
G2 group of 2 days age group of cerebellum
G3 group of 4 days age group of cerebellum

Table (4): Comparison of different parameters between every pair of study groups of cerebellum by post hoc test

Dependent Variable	1st group	2nd group	*P* value
NP ($x10^5$)	G1	G2	0.004
	G1	G3	<0.001
	G2	G3	0.009
IP ($x10^5$)	G1	G2	0.007
	G1	G3	<0.001
	G2	G3	0.004

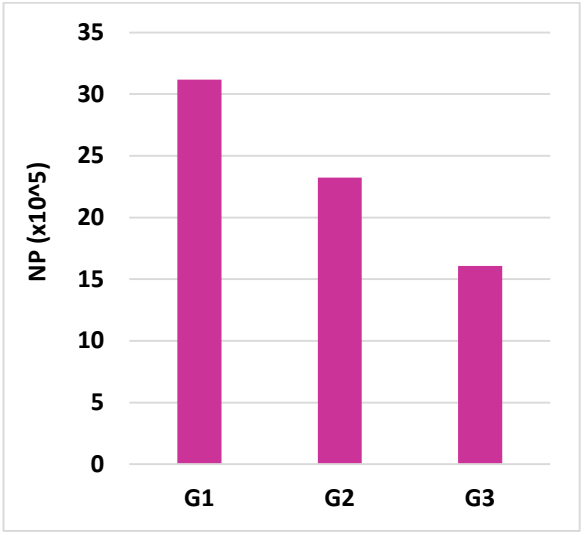

Chart (3): No. of positive purkinji cells in the cerebellum in the three study groups

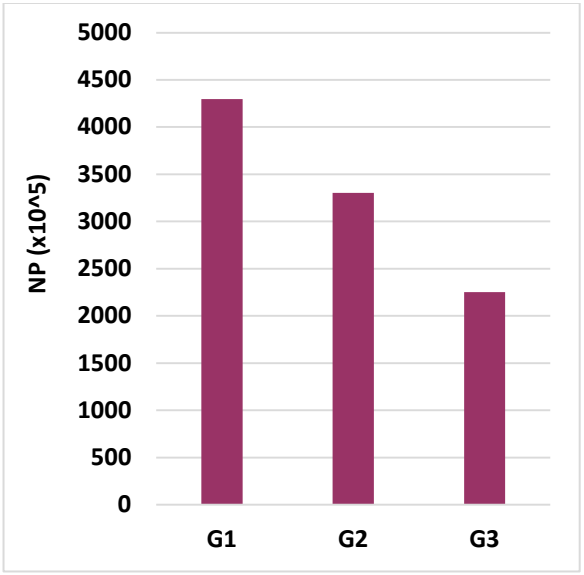

Chart (4): No. of total intensity of positive purkinji cells in the cerebellum in the three study groups

DISCUSSION

The neurogenic regions characterized by the presence of neural precursor cells,
able to generate neurons, and a permissive microenvironment, the niche, together forming one functional unit (Klempin et al. 2011). Doublecortin is clearly an interesting marker molecule to study neuronal differentiation of newly generated cells in neurogenic regions of the adult brain. In some cases, DCX expression alone has been taken as indication of adult neurogenesis (Zhang et al. 2010) (Zhang and jiao 2015). In the course of adult hippocampal neurogenesis transient DCX expression characterizes migration and links the neuronal precursor cell stage with a post mitotic immature stage (Plumpe et al. 2006), Thus DCX plays a role for hippocampal lamination (Corbo et al. 2002). In the cerebellum, DCX expressed by purkinji cells as late as the first day's post-natal period (Gleeson et al., 1999) and by granular cells and other migrating cells as late as the adulthood (Manohar et al., 2012) (Takács J et al., 2008) and indicate the ongoing migration of the immature neurons. In this research, DCX-positive (DCX+) cells are found in the dentate gyrus migrating into the inner third of the granule cell layer where they enter a second postmitotic phase subsequently differentiating into immature neurons (Ming and Song, 2011). As the cells transition from migrating neuroblast to postmitotic immature neuron, they undergo a morphological shift to cells with a rounded soma and processes of various lengths and complexities (Costa et al.,2011). And Upon maturation into dentate granule neurons these cells extend both axonal and dendritic projections to integrate into the functioning hippocampal circuitry (Toni N. and Schinder A. 2016). In this research, as illustrated by the previous reference and by (Klempin et al 2011), images of the hippocampus clearly illustrated these neuronal extensions upon entering the inner third of the granular zone immunostained by doublecortin, obviously at the stage of migration before being mature neuron, as doublecortin exclusively confined to this stage of neurogenesis (Couillard-Despres, et al. 2005). We investigated different age groups of adult mice using doublecortin (DCX) immunohistochemistry (IHC) for neuroblasts DCX-positive cells in the subgranular zone of the dentate gyrus, we found that doublecortin immunolabelled cells number and intensity in dentate gyrus decreased with age, this goes with (Knoth et al., 2010). We found that there is strong significant decrease between group1 and group3 and also between group1 and group2 but lesser significant, the first age group is 8 weeks, the second age group is 4 months and the third age group is 6 months. Steadily decline of neurogenesis in the hippocampus during aging goes with (Kuhn et al., 1996), However, the latter study took older age groups and use other markers. Sections of the brain cerebellum of the 0 day postnatally mice showed purkinji cells immunoreactive for doublecortin antibody as illustrated by (Gleeson et al., 1999) and doublecortin immunoreactive cells in the granular layer as illustrated by (Manohar et al 2012). Three successive ages of the cerebellum in the 1st week postnatally was immunostained for doublecortin antibody and quantified the number of positives and the intensity of those positives of purkinji cells and compared in 3 differrent age groups; the first age group is 0 day, the second is 2 days, and the third group is 4 days' age group. In the cerebellum age groups there was faint expression of doublecortin in first two days postnataly, this goes with (Manohar et al., 2012), but with continuous decline represented by significant decline between group 1 and group 3 and to lesser extent between group 1 and 2, this was similar to (Takács J et al., 2008), however we compared the level of expression in purkinji cells in first four days postnatally, whereas the latter compared the expression in the granular layer in different age groups. Our study suggested that areas of adult and early postnatal period of brain maintain DCX expression not only while migrating but for a short time also after their settlement as

explained by (Takács J et al., 2008), reflecting a role of DCX in adult neuronal plasticity in addition to a developmental role in migration as illustrated by (Manohar et al., 2012). The expression of doublecortin has been linked to structural plasticity and morphological changes associated with migration, axonal guidance and dendrite sprouting (Klempin et al 2011).

CONCLUSION

Our data indicates that while DCX represents a dividing progenitor population in the neurogenic niche of the hippocampus in adulthood it also labels a non-dividing neurons with immature traits in the non-neurogenic niche in the cerebellum in the early postnatal period. In this contest we compared the number and intensity of doublecortin immunolabelled neuroblasts in the hippocampus across different age groups in the adult mice and in the cerebellum we compared the doublecortin immunolabelled purkinji cells in first four days postnatal mice. We found that despite there is a decline in the number and intensity in hippocampus from early adulthood (8 weeks) to middle adulthood (4 months) still it's not so significant, however it became very significant when we compared the early adulthood 8 weeks and the late adulthood 6 months. In cerebellum there was significant decline in the number and intensity from early postnatal (0 days) to 2 days, however it nearly disappeared when we close to 4 days' groups.

DCX signifies transient neuronal lineage commitment together with migration and neural structural plasticity in the adult hippocampal niche, and postnatal cerebellum. this is necessary for rapid adaptation to environmental changes, and indicate continuous plasticity in early life and may be throughout life in hippocampus (extended age group study required). This may be through continuous adaptation or continuous learning.

Disclosure statement

There is no conflict of interests to declare.

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