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Early postnatal development of the visual cortex in mice with retinal degeneration

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Abstract

This study characterizes the early postnatal development of the visual neocortex in C3H/HeNRj mice. These mice are homozygous for the Pde6b^{rd1} mutation, which causes retinal degeneration starting from postnatal day 7 (P7). To monitor the development of the visual cortex between P3 and P28 we used eight antigens known to be expressed at different developmental stages (*Nestin*, *tau3*, *β3- Tubulin*, *Calbindin*, *Doublecortin*, *MAP2*, *Parvalbumin* and *NeuN*).

Using semiquantitative analysis we traced the expression and localization of different developmental markers throughout the layers of the visual cortex. Cortical tissue sections corresponding to the first postnatal week (P3-P6) stained positively for *Nestin*, *tau3*, *β3-Tubulin* and *Calbindin*. These proteins are known to be involved in the migration of neural progenitor cells (NPCs) within the cortical plate.

At the time of eye-opening (P14), *Doublecortin*, *MAP2* and *NeuN,* markers for developing and maturing neurons involved in NPC differentiation are present. Between P9 and P21 *Nestin* and *Calbindin* disappear while *NeuN* and *Parvalbumin* expression increases in the course of visual neocortex development.

The findings of this study provide a snapshot of the dynamic changes in cortex formation during early postnatal development. So far, it is the first investigation on the postnatal development of the mouse visual cortex. Our results indicate that in C3H/HeNRj mice retinal degeneration during these early stages may not influence the maturation of the visual cortex. Until P28 in this mouse strain, the development of the visual neocortex is in accordance with data from other mice (C57BL/6) without retinal degeneration. Whether in older individuals of the C3H/HeNRj strain the visual neocortex will show signs of functional impairment has to be shown by future work.

1. Introduction

In this study, we traced the early postnatal development of the mouse visual cortex combining the expression of early, mid and late markers of neurogenesis. Such an investigation of the mouse visual cortex has not been done so far. The mouse strain investigated here is the C3H/HeNRj line with retinal degeneration, a strain commonly used in chronobiological studies due to its melatonin proficiency (Jilg et al. 2010, Pfeffer et al. 2017, Rawashdeh et al. 2016; Janvier Labs, Research Models C3H/HeNRj). These mice are close relatives of naturally occurring mice (*Mus musculus*; Hikishima et al. 2017). C3H/HeNRj mice carry the Pde6b^{rd1} mutation that is responsible for retinal degeneration (rd) leading to visual impairment (Carter-Dawson et al. 1978; Patz et al. 2004; Barabas et al. 2010). This degeneration process consists of two stages, the first of which affects the rods and is acute

between P0 and P17 (Barabas et al. 2010). The second stage extends over many months and affects the remaining cones. The mutation generates a defect in the ß-subunit of the photoreceptor cGMP phosphodiesterase 6 (PDE6-ß). This damage results in loss of function in the PDE complex by means of a cGMP, Na^+ and Ca^{2+} overload. Apoptosis starts at postnatal day P10, proceeded by an increase of cGMP content in the $Pde6b^{rd1}$ retina, days before signs of degeneration become apparent. It reaches its maximum around eye opening (P14) when rod degeneration is at its peak (Barabas et al. 2010). At P17, 2% of rods are left and 75% of cones persist, at P20 nearly all nuclei of photoreceptors have disappeared (Carter-Dawson et al. 1978) and the retina is rodless at P36. It is reported (Chen et al. 2006) that degeneration only affects the photoreceptor cells. Six weeks after birth, rd mice undergo a near-total vision loss (Chen et al. 2006).

In order to characterize the early postnatal structural organization of the visual cortex in C3H/HeNRj mice and to determine whether retina degeneration influences cortical development, we traced eight antigens between the postnatal stages P3 and P28. Ontogenetic studies about the early postnatal development of the visual cortex in mice are lacking and particularly the effect of retina degeneration on the maturation of the visual neocortex.

For analysis, antibodies against *Nestin*, *tau3*, *β3-Tubulin*, *Calbindin*, *Doublecortin*, *MAP2*, *Parvalbumin* and *NeuN* were used. All these antigens are proteins involved in the early postnatal development of the mouse neocortex (see below). *MAP2*, *Parvalbumin* and *NeuN* are those three of eight antigens applied here that persist throughout adulthood (for NeuN see P40; fig. 9). The parameters used to assess the postnatal maturation of neurons in the visual cortex included morphological analysis, the temporal dynamics in the antigen expression levels and their localization within the visual cortex. The results of this study in C3H rd mice show a similar maturation process as described previously in visually intact rodents.

2. Materials and Methods

In total we used 31 C3H/HeNRj rd mice distributed across seven postnatal developmental stages (P3, P6, P8, P14, P21, P28 and P40; table1, cf. p.5) with four mice per stage plus 3 rd mice for antibody testing. The experiments were performed in accordance with international standards as outlined under the German animal experimentation regulations (Rawashdeh et al. 2016).

As controls, we dissected and processed visual cortices of 12 week old C57BL/6 mice in the same way as in C3H/HeNRj mice. Immunohistological staining was done after the same protocol described here.

2.1 Specimen collection and cryoprotection

Brains of C3H/HeNRj mice were prepared using the standardized procedures described earlier (Rawashdeh et al. 2014, 2016). Briefly, mice of each postnatal stage investigated (P3-P28), were anesthetized with Ketamin/Xylazin 100mg/10mg/kg bodyweight and perfused transcardially with heparinized saline followed by fixative (4% paraformaldehyde solution in 0.1 M phosphate buffer [PB], pH 7.4). The brains were collected, postfixed for 48h in the same fixative, and cryoprotected using a two-step protocole (15% sucrose in 4% paraformaldehyde solution, followed by 30% sucrose in 4% paraformaldehyde solution). The brains were snap-frozen using methylbutane (by Sigma) at -25°C, and stored at -22°C.

2.2 Cryosectioning and identification of the visual cortex

Whole mouse brains were cut into 12µm thick coronal sections at -22°C using a cryostat (Leica® type). Brains corresponding to the postnatal stages P3, P6 and P8 were not removed from the calvarias, in order to provide structural support during sectioning.

Two consecutive sections each were collected on the same microslide in ascending order from the frontal to the occipital region of the telencephalic hemisphere.

For the localization of the visual cortex we relied on coordinates provided by Palomero-Gallagher and Zilles (2004), by Paxinos and Franklin (2012) “The Mouse Brain in Stereotaxic Coordinates” and Allen´s Mouse Brain Atlas – The Brain Explorer® 2 (2014). In coronal sections, the visual cortex starts rostrally at the vertical part of the fimbria hippocampi and reaches as far caudally as the posterior end of the telencephalic hemisphere. In all the mice analyzed by immunofluorescence, the complete visual cortex of both hemispheres was investigated. The total amount of microslides and brain sections, respectively, used for the analysis of the visual cortex in each ontogenetic stage, are given in Table 1.

Table 1: Total count of microslides (with two brain sections each) containing the visual cortex at each postnatal stage. Every eighth microslide in the same animal was used for the same antigen, and always both hemispheres were investigated. At the P40 stage we only sectioned the caudal part of the hemisphere, the rostral non-visual neocortex was excluded. Numbers in brackets mean the microslides containing the visual cortex used in this investigation.

Stage	***Total no. of microslides***	***Visual Cortex microslides***
P3	*156*	*77-156 (79)*
P6	*121*	*58-121 (63)*
P8	*203*	*144-203 (59)*
P14	*180*	*116-180 (64)*
P21	*170*	*124-170 (46)*
P28	*170*	*118-170 (52)*
P40	*66*	*37-66 (29)*

2.3 Immunohistochemistry

The protocol used to immunohistochemically stain the brain sections was the same for all the antibodies listed in table 2. The brain sections of every 8th microslide from the visual cortex

of all ontogenetic stages (P3-P28, P40) were simultaneously stained by the same antibody. Briefly, the slides containing sections of the visual cortex were taken from the freezer (-22°C) and thawed to room temperature for 10min. The sections were rinsed with 0.01M phosphate-buffered saline (PBS; 2.85g Na_2HPO_4 x H_2O + 0.54g KH_2PO_4 +9g NaCl + in 1L distilled water, pH 7.4) For antigen retrieval, the tissue was transferred to a solution consisting of 18ml citric acid and 82ml sodium citrate in 900ml distilled water and heated in a microwave for 1min at 800W followed by 9min at 530W. The sections were then rinsed three times for 5 min in PBST (1L PBS with 3ml Triton X-100). Next, the tissue was blocked for 60min at room temperature (RT) using normal goat serum (Sigma) diluted in PBST (1:20), followed by over-night incubation in primary antibody (table 2) at +4°C. The sections were then rinsed three times for 5 min with PBST and incubated for 60 min at RT in the secondary antibody (goat-anti-rabbit Alexa-488 or goat-anti-mouse Alexa- 568) diluted (1:500) in PBST containing bovine serum albumin (BSA) 1:20 (by AppliChem Pancrea). Following incubation, the slides were rinsed three times in PBS for 5min each, coverslipped using the fluorescence mounting medium containing DAPI (4',6- Diamino-2-phenylindol, Dako) and stored at +4°C.

Table 2: Antibodies used in immunohistochemistry with company, article no. and dilution

Antibodies	*Company*	*Article no.*	*Dilution*
Nestin *(anti-mouse)*	Merck Millipore	MAB353	1:200
β3-Tubulin *(anti-mouse)*	Cell Signaling	4466	1:200
Doublecortin (*anti-rabbit*)	Cell Signaling	4604	1:200
Calbindin *(anti-rabbit)*	Swant	CB38	1:500
Parvalbumin *(anti-mouse)*	Swant	PV235	1:200
tau3 *(anti-mouse)*	Merck Millipore	05-803	1:200
NeuN *(anti-mouse)*	Merck Millipore	MAB377	1:200
MAP2 *(anti-rabbit)*	Cell Signaling	4542	1:200
Goat-anti-rabbit 488	Alexa	A-11034	1:500
Goat-anti-mouse 568	Alexa	A-11004	1:500

2.4 Imaging

For visualization of the immunoreacted sections we used a fluorescence microscope (Zeiss) equipped with the Axioskop 2 plus camera (SIP 73147 HAL 100) and the Axio Vision 4.8 software. For each antigen and brain section we monitored the appearance and disappearance of immunofluorescence signals, their number of specific signals and immunolabeling intensity, the shape of the labeled neurons and their location within the cortical plate.

For comparative purposes the settings of the microscope used for imaging the tissue was maintained constant throughout all the postnatal stages and antigens.

2.5 Evaluation of fluorescence signals

For the generation of data we applied a semiquantitative approach. The signals (somata, axons, dendrites) in every imaged section were screened, averaged, and given categories as to the number of signals and the intensity of signals (see Figures 5-7). In addition, we determined the location of the signals within the layers of the visual cortex. Neurons were distinguished from glia cells by means of their relatively larger size (perikaryon and large round nucleus) as well as by the conciseness of their nucleoli.

The numbers of specific signals (neurons) were counted in the sections of the four brains in one stage, e.g. P3 - *Nestin*, and then averaged. For counting we used the software ImageJ (by RSB) with the tool 'Multiple Spots.' Then the frequency of fluorescence signals was categorized (0-2 very low, 2-4 low, 4-6 moderate, 6-8 intermediate and 8-10 maximal, see figures 5-6). The values for NeuN are exceptionally high, they have special scaling in figure 6c.

For antigen intensity we averaged the signal intensities of each antigen (as in the 'number of signals', identified and categorized by two independent observers) in the sections of the four brains in one stage (e.g., P3). Antigen expression (e.g. *Nestin*) was categorized as: 1, very low; 2, low; 3, moderate; 4; intermediate; 5, maximal). For representation in figure 7 we always chose the highest category of intensity in all four brains of one stage.

For total immunoreactivity (see below) the signal numbers and intensities of antigen expression were added. This combination allowed approximate statements as to antigen expression throughout the postnatal stages of visual cortex development.

For topographical relationship of the immunoreacted material we used Nissl- stained sections of the visual cortex; they allow the allocation of labeled structures within the different layers of the cortical plate using morphological criteria of the neurons (fig. 8).

3. Results

The analysis of our results follows a three-step approach: (1) Number of immunofluorescence signals for postnatal stages P3 to P28, (2) Intensity of immunofluorescence signals and functional implications, and (3) The location of antigens in cortical layers and their morphology of labeled neurons. In figures 1-4 the investigated cortical area is shown for the adult mouse at different magnification, figures 5-7 present ontogenetic and semiquantitative immunohistological data. Figures 8 and 9 show highlights of numerical quantity and intensity, as well as the topography of signals for each antigen during the stages investigated.

The visual cortex of a C3H mouse in coronal view – histological cortex layers help to understand cortical development

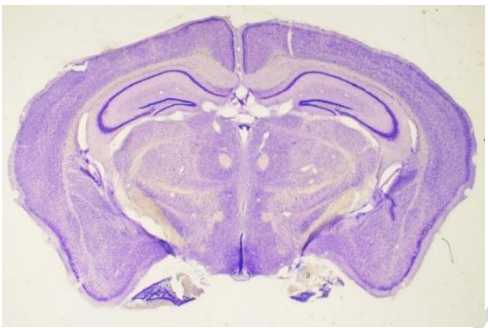


fig. 1

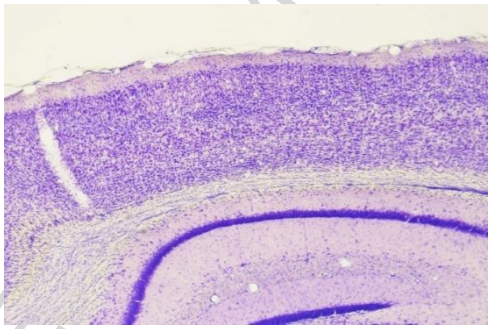


fig. 2

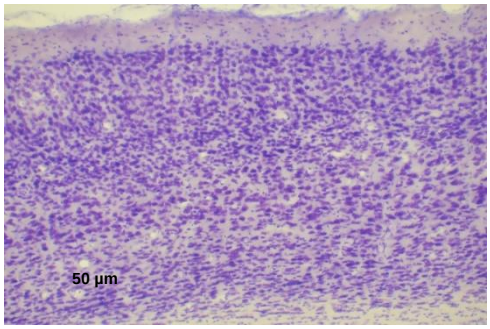
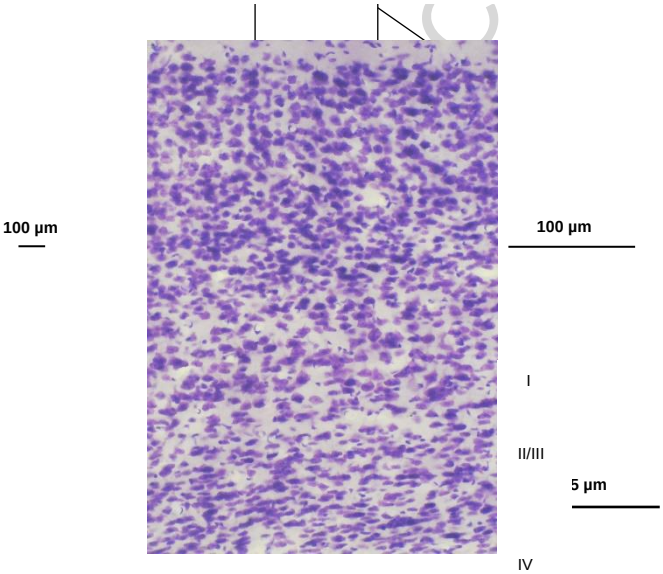


fig. 3



Figures 1-4: Visual cortex of the C3H mouse at different magnifications of a coronal brain section.
Fig. 1: whole brain section
Fig. 2: boxed detail of Fig. 1 with visual cortex.
Fig. 3: histology of visual cortex in overview.
Fig. 4: magnified part of visual area with typical layers I- VI.- Nissl stain.

3.1 Developmental changes in number of immunofluorescence signals for postnatal stages P3 to P28

To assess the development of the visual cortex in mice we mapped the temporal expression of developmental markers (Table 2, figs. 5 and 6) during the postnatal stages P3 to P28.

The visual cortex of postnatal stage P3 stained positive for five out of the eight developmental markers investigated: very low signal numbers for *Nestin*, *tau3, Parvalbumin* and low numbers for *β3-Tubulin* and *Doublecortin* (figs. 5 and 6).

While *Nestin* expression peaked at P6, all other markers found at P3 declined here except for *Calbindin* which appeared at this stage.

At postnatal stage P8, the neuronal markers *MAP2* and *Doublecortin* showed low to moderate signal numbers, respectively. *NeuN* was still at a low level. A very low expression of *Calbindin* was seen.

At P14 the developmental markers showed no or very low expressions. *NeuN* peaks as to signal number and remains the predominant marker of mature neurons in all later stages (fig. 6c). The expression of *MAP2* and *Parvalbumin* was very low but the latter antigen increased until P28.

P21 showed zero levels for *tau3, MAP2, Calbindin* and *β3-Tubulin*. *Doublecortin* signal number was very low and that of *Parvalbumin* rose to a moderate level (*NeuN* on a very high level, figs. 5 and 6c).

At postnatal stage P28, the markers specific for neuronal precursors and immature neurons (*Nestin, Doublecortin, tau3, β3-Tubulin* and *Calbindin)* have disappeared. *MAP2* was very low, *Parvalbumin* on an intermediate and *NeuN* on a high expression level (see fig. 6a-c).

The expression pattern of antigen signal numbers (fig. 5) in C3H/HeNRj mouse visual cortex development investigated in this paper is in correspondence with that in other rodents (Gilmore and Herrup, 1997; Takács et al. 2008; Wang and Mandelkov, 2016 and C57BL/6). This fact confirms the suitability and specifity of the antibodies used here.

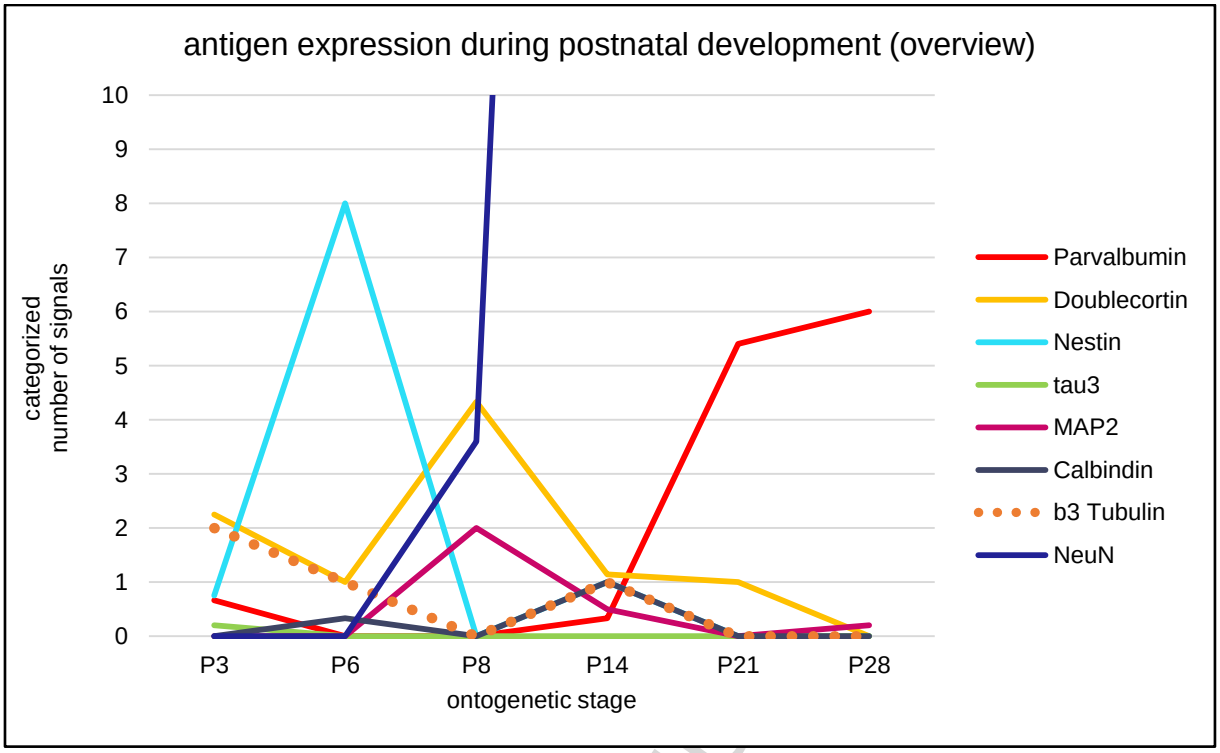
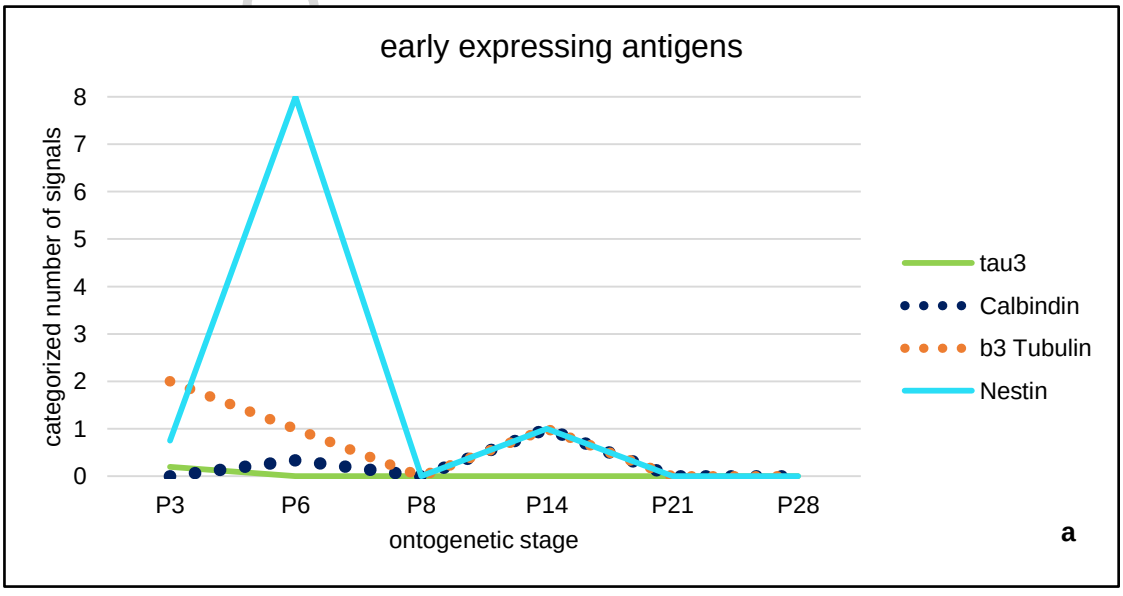


fig. 5: Overview of antigen expression during the postnatal period investigated here (P3- P28). The numerical incidence of antigen signals was attributed to a series of semiquantitative levels (0-2 very low, 2-4 low, 4-6 moderate, 6-8 intermediate and 8-10 maximal). For better understanding of the results, this figure is divided into three subfigures in the following (figs. 6a-c). Note the different scales in the figs. 5, 6a and b versus 6c as well as the overlap of Nestin, Calbindin and β3-Tubulin between P8 – P28.



β3- Tubulin

c

9

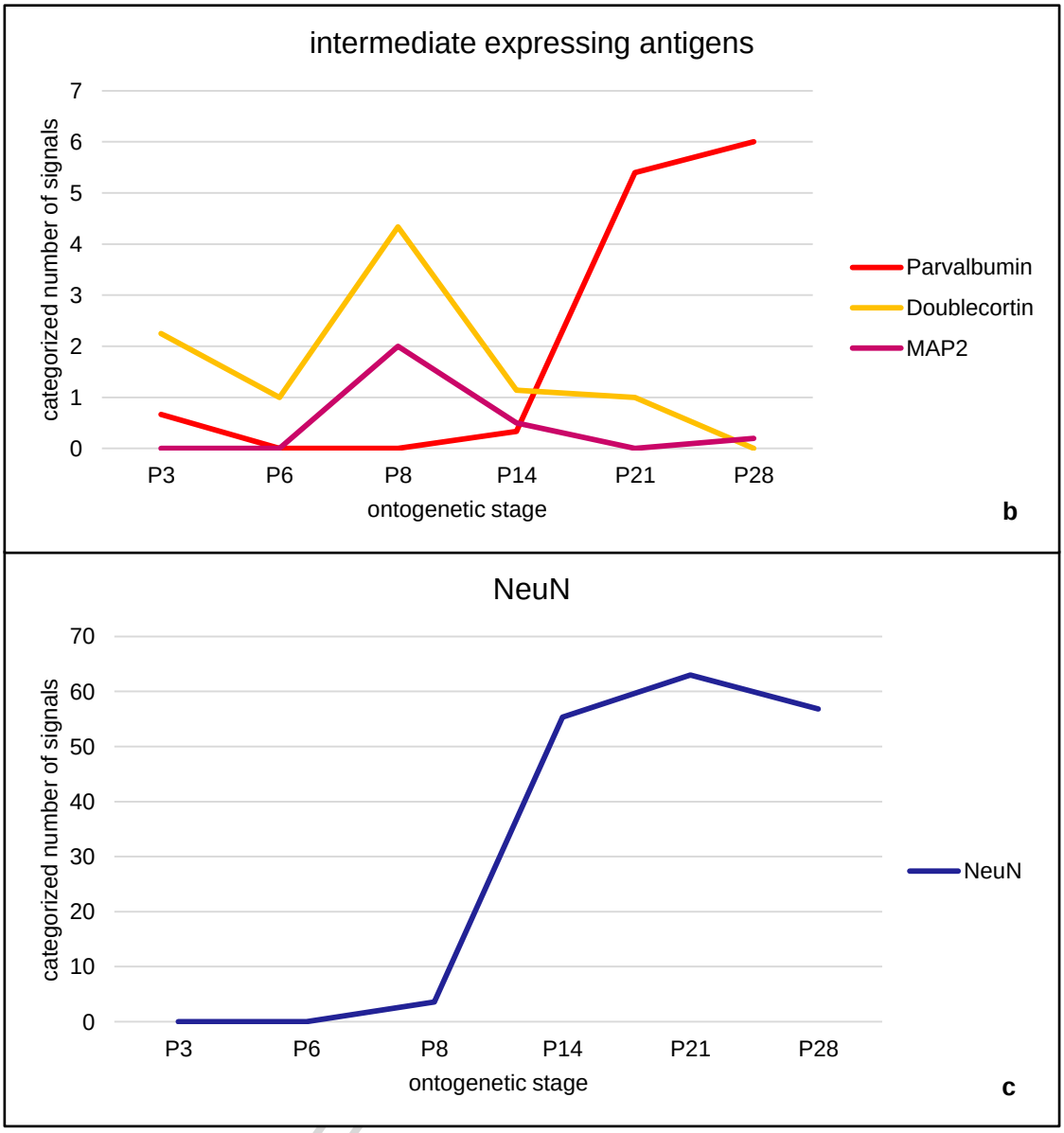


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fig. 6: Detailed presentation of the antigens with numbers of signals within the period investigated. Antigens with an embryonic character present early after birth are shown in (a), those with an intermediate number of fluorescent signals characteristic for the 'turning point' in (b), and NeuN as a marker for mature neurons in (c) persists from P8 onward at a high expression level. Scaling of the Y-axis is different in figs. 6c because of the very high amount of antigen NeuN presented here. Note the labeling of fig. 6a was slightly changed with respect to the survey fig. 5.

3.2 Intensity of immunofluorescence signals and functional implications

3.2.1 General data

The intensity of signals for each single antigen along the ontogenetic stages investigated was assigned to five semiquantitative levels (very low to maximal; see fig. 7). Early signals in the postnatal ontogenesis of mice were *Nestin, tau3, β3-Tubulin, Calbindin, Doublecortin and Parvalbumin*. Signals appearing in later stages were *MAP2* and *NeuN*. Signal number and signal intensity, however, are not strictly correlated (figs. 5-7). Citations in text refer to papers on rodents.

Nestin is known as an embryonic marker (data sheet MAB353, Millipore). In the postnatal period investigated, it was detected at the beginning (moderate intensity) and continues at very low levels. This protein is an intermediate filament of the cytoskeleton and expressed in neuronal progenitor cells, particularly during the G1-S phase of the cell cycle, when neuronal progenitor cells elongate their fibers. *Nestin* production is upregulated as to signal numbers (figs. 5 and 6a: P6).

Tau3 expression, generally, was present at very low levels of intensity. For the most part it is seen in early postnatal stages (P3) as a special marker during the migration of neuronal progenitor cells (NPCs). Tau proteins are primarily active in the distal portion of axons where they provide microtubule stabilization but also flexibility. Thus, it is essential for axonal growth and maturation in the cerebral cortex (Takei et al. 2000). Here, it is also found in dendrites and dendritic spines and may be involved in synaptic plasticity (Wang and Mandelkov 2016). In the mouse brains investigated, the regulatory function of *tau3* was seen at P6 (not shown in figs. 5 and 6a): a phosphorylation of *tau3* characterizes the endpoint of its existence and the end of cell migration (fig. 7).

β3-Tubulin is expressed in late embryonic and early postnatal stages. As shown in figure 5, the number of signals decreased from P3 to P8 but then increased at stage P14, a possible evidence for a turning point in neural development. Its signal intensity, however, was very low (P14, fig. 7). *β3-Tubulin* is an early marker of NPC differentiation and final migration in the visual cortex of rodents (see Gilmore and Herrup (1997).

Calbindin expression in mice appears first on embryonal day E19 (see Shamley et al. 1992, for rodents), and it persists during postnatal development until P21 (see figs. 5, 6a). In our ontogenetic series, the number of signals of *Calbindin* immunoreaction increased slightly from P8 to P14 (Alcántara et al. 1993); signal intensity, however, was very low (fig. 7). In rodents after birth, *Calbindin* characterizes nonpyramidal neurons of all cortical layers except the molecular layer.

Doublecortin signal numbers were low to very low in the first postnatal week and moderate around P8 (figs. 5 and 6b). Signal intensity was very low but rose to a moderate level at P8. In the third postnatal week (fig. 5: P14 - P21), i.e. with the onset of terminal differentiation and the appearance of *NeuN* (Takács et al. 2008, Brown et al. 2003), *Doublecortin* signal numbers were also very low and signal intensity was low to moderate (below). Like other microtubule-associated proteins (*tau3*, *β3-Tubulin*), *Doublecortin* stabilizes, bundles and organizes microtubules and thus directs the migration of neuronal progenitor cells (NPCs)

from the ventricular zone into the cortical plate (Bai et al. 2003, Friocourt et al. 2007). NPCs express *Doublecortin* at P3 while dividing, and neuronal daughter cells continue to do so during the following 2-3 weeks until they start differentiation into mature neurons. The increase of *Doublecortin* number and intensity of signals was accompanied by the appearance and increase of *NeuN* (see figs. 5 -7, below).

MAP2 is microtubule-associated and plays a lifelong role in the differentiation of both immature and mature cortical neurons. Signals appeared at P8 spontaneously and persisted at lower numbers and intermediate to low intensities until P28 and later (figs. 5 and 6, 7). *MAP2* is known as an obligatory marker for neurons like *NeuN* (data sheet *MAP2*, CellSignaling). *MAP2* signals are correlated with the terminal differentiation of dendrites (dendritic shape), in association with microtubules, and is thus an indication for synaptic plasticity. In this respect it is possible that, also in the mouse, during differentiation several forms of *MAP2* (*MAP2c*, *MAP2a* and *MAP2b*) are successively involved in the design of neuron-specific processes (Izant and McIntosh 1980).

In the rat brain *Parvalbumin* has been shown to completely co-localise with *Calbindin* (Alcantara et al., 1993; DeLecea et al., 1995; figs. 5-7). Our C3H rd mice show the same situation. In rat and C3H mice, both proteins are related to monitor Calcium-related processes in interneurons of the mouse visual cortex. While the neurons down-regulate *Calbindin* expression in signal number (at very low signal intensity) (in rodents: Alcantara et al., 1996; Cauli et al., 1997), both the signal number and intensity of *Parvalbumin* rise and the protein persists throughout life, thus substituting *Calbindin. Parvalbumin* illustrates the progress of NPCs in early postnatal development, but not as specific as *Calbindin* does (for immature nonpyramidal neurons).

NeuN expression appears in postnatal development when neuronal progenitor cells (NPCs) withdraw from the cell cycle and thus with the initiation of terminal differentiation of the neurons. It persists until the adult stage (Mullen et al. 1992). In our mice, *NeuN* signals were detected first at stage P8, at a low signal number and very low signal intensity (figs. 5-7). The signal number rises strongly until P14 and stays very high at least until P28 (fig. 6c). Its signal intensity proceeds at a low level. *NeuN* is a marker for mature neurons and, together with *MAP2*, can reliably detect all kind of nerve cells (Sahara et al. 2012). In our study we used *NeuN* to focus on postnatal processes of development in the later stages considered here. In P28 the number of *NeuN* signals slightly dropped (figure 6c) but was reported to stay at a high level until P60 in long-term cortex cultures of other mice (Lesuisse and Martin 2002).

As a whole, the ontogenetic timeline presented here is in complete accordance with data in the literature concerning functional implications of these proteins during the development of the visual cortex. Our results suggest a normal cortex formation in C3H/HeNRj mice as seen in other rodents and expected for other mice.

3.2.2 How total immunoreactivity helps to estimate the impact of antigens

The combination of the two parameters (number and intensity of fluorescence signals) may be useful for further in-depth analysis of early postnatal processes in the mouse visual cortex: together these parameters show the relevance of these proteins during cortical ontogenesis.

Early appearing antigens like *Nestin, tau3*, *β3-Tubulin* and *Calbindin* are important for the migration and maturation of neurons in the visual cortex. Later in development, *MAP2*, *Parvalbumin* and *NeuN* promote further maturation and differentiation of neurons and persist throughout life.

Within this pattern of antigen expression, stage P8 looks like a turning point (figs. 5- 7): about this time *Nestin* declines to very low signal numbers and intensity levels. In contrast, *Doublecortin* and *MAP2* have peaks in signal number and are relatively high in signal intensity whereas *NeuN* and *Parvalbumin* start a maximal (NeuN) or a protracted moderate to intermediate rise in signal number (*Parvalbumin)*. Such dramatic changes in antigen expression at this stage obviously reflect fundamental developmental processes that take place in the period investigated here and indicate a two-step development. Further topographical parameters will supplement these insights.

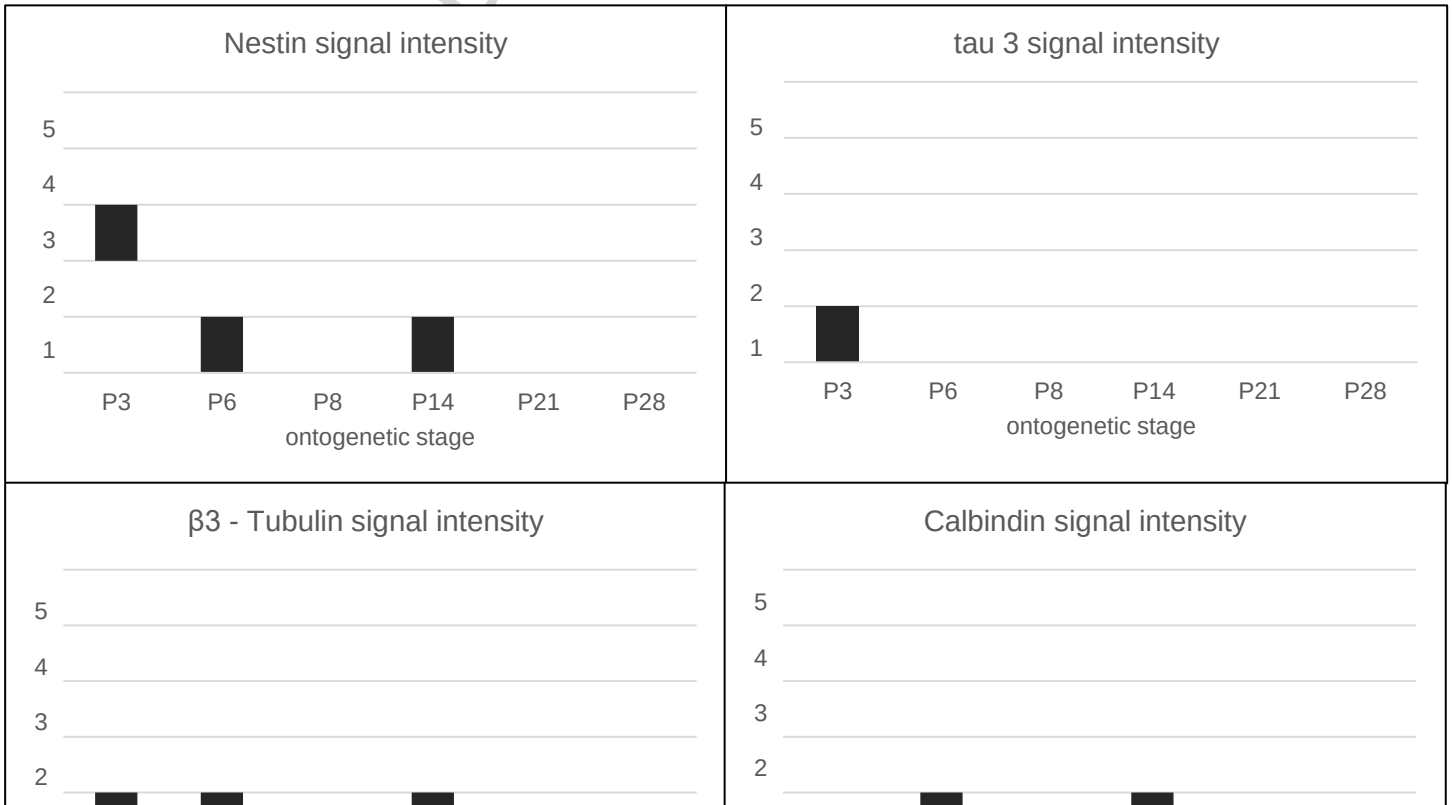


fig. 7: Signal intensity for each antigen during the time period investigated (ontogenetic stages). Intensities of antigens can be: 1 very low, 2 low, 3 moderate, 4 intermediate and 5 maximal. This graph shows the progress in maturation and differentiation of neurons by means of the appearance, disappearance, and signal intensity of each antigen.

3.2.3 The location of antigens in cortical layers and the morphology of neurons

Another point of our investigation is to relate the number and intensity of signals (figs. 5, 6, 7) to their location in the developing visual cortex of C3H/HeNRj mice including neuron morphology (fig. 8). In routine (Nissl) sections of the mouse brain (figs. 1-4) the visual area was determined by means of the mouse brain atlas of Paxinos and Franklin (2012) and the highest expression of antigen fluorescence found was assigned to the respective cortical layers in the routine sections (figs. 4 and 8) [citations indicate corresponding papers in the literature]. Figure 8 presents details of the relevant areas showing maximal neuron counts and labeling intensity for each antigen throughout the ontogenetic stages studied.

Nestin with moderate numbers of signals at stage P6 and a moderate signal intensity at stage P3 (and even lower intensities in later stages) showed a predominant labeling of circular to triangular somata of layer IV (fig. 8).

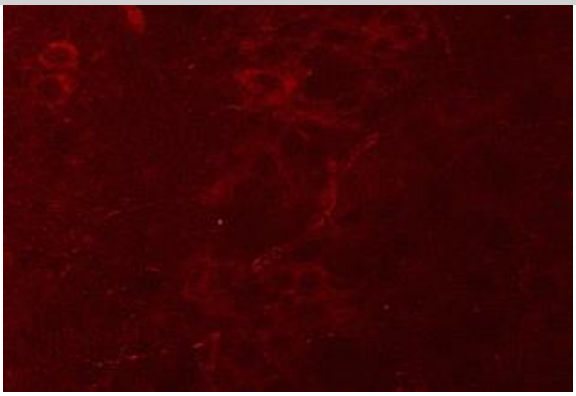
Tau3 signals were predominantly localized in layer IV of P3, with very low numbers and intensity.

β3-Tubulin combined its highest number of signals (low) at stage P3 with a generally very low signal intensity in layer VI (Rakic 1988), which in the adult animal comprised multiform and spindle-shaped cells.

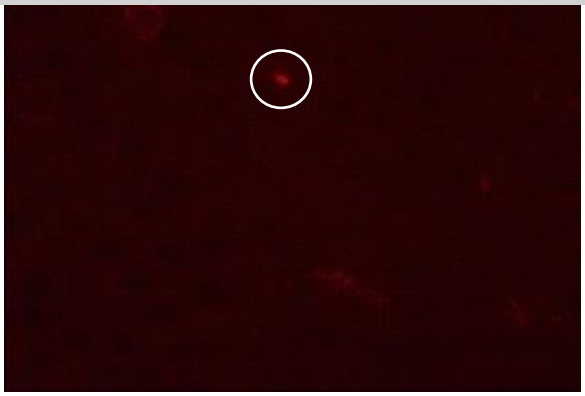
Calbindin had its highest signal number (very low) at stage P14 and very low intensities in layers II/III in neurons with two short processes at both ends of the ovoid perikaryon (fig. 8).

Doublecortin and *MAP2* (figs. 8) had their highest number of signals in stage P8, with the highest intensity of immunoreactivity (moderate and intermediate, respectively) found in cortical layers II/III. At this cortical level, small immunoreactive cone-shaped somata of pyramidal cells and granule cells were found (see below, Takàcs et al. 2008).

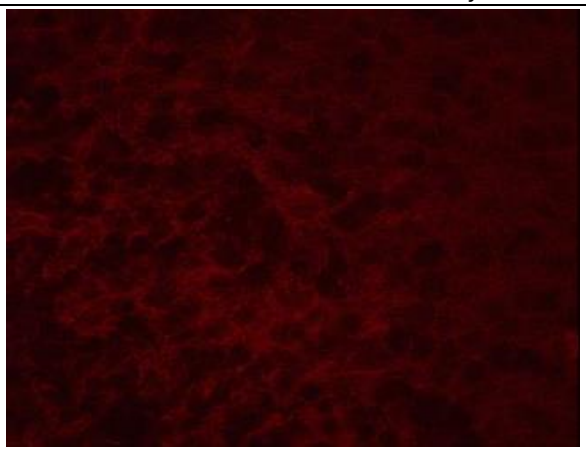
Parvalbumin and *NeuN* had their highest number of signals (moderate and maximally high, respectively) at stage P21/P28 in cortical layers II/III and IV, respectively (figs. 5-8). Such signals occurred in many small granule cells and interneurons (high neuron density) (Mètin et al. 1988). Their highest signal intensity was found at P21 for Parvalbumin (intermediate) and at P14 - P28 for NeuN (low).



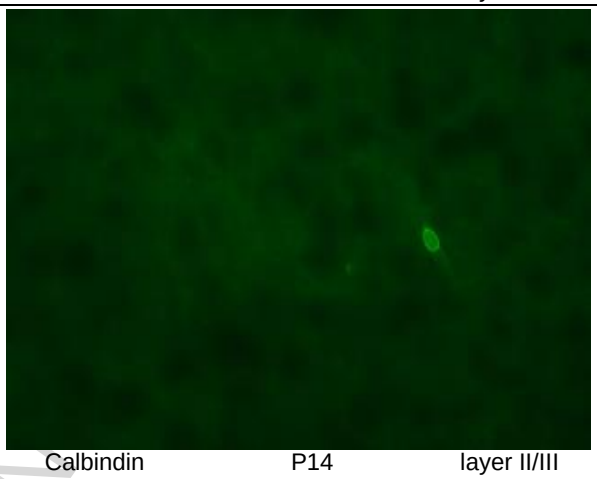
Nestin P6 layer IV



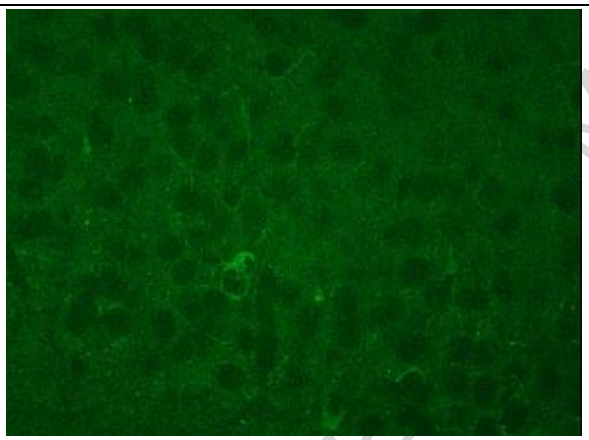
tau3 P3 layer IV



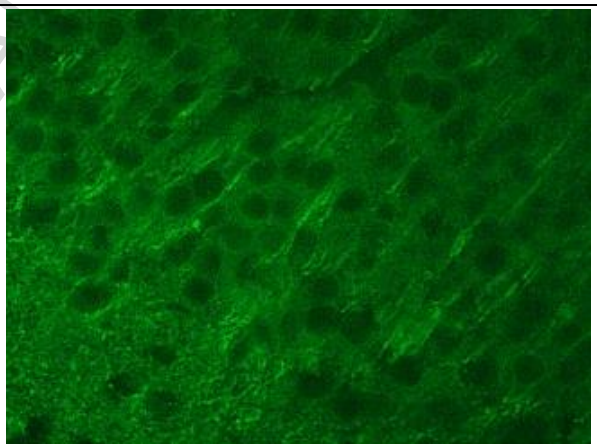
β3- Tubulin P3 layer VI



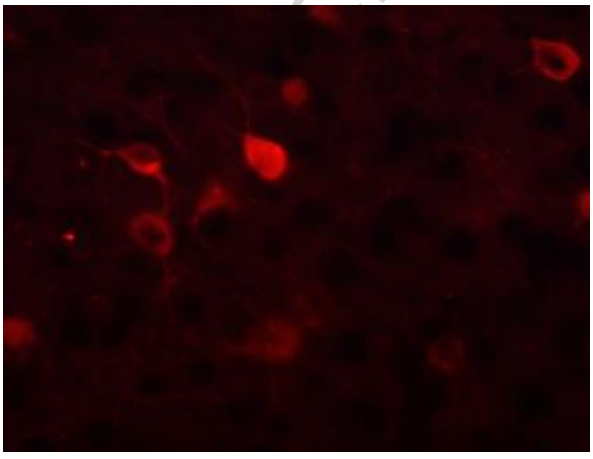
Calbindin P14 layer II/III



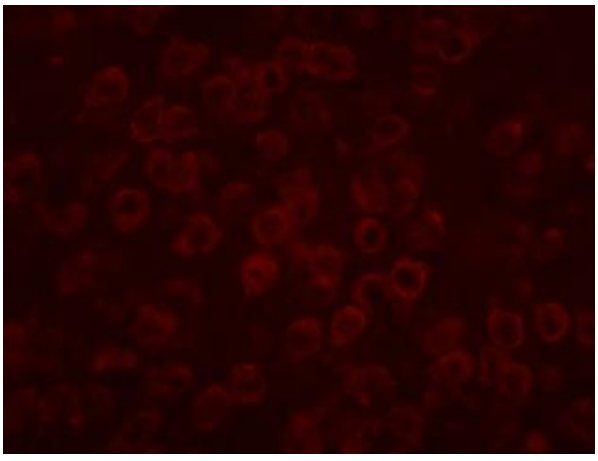
Doublecortin P8 layer II/III



MAP2 P8 layer II/III



Parvalbumin P28 layer II/III



NeuN P28 layer IV

fig. 8: Highlights of numerical quantity, intensity and the topography of signals for each antigen during all stages investigated. As seen in fig. 7, the highest intensity can be localized at one specific age. The peak of detectable signals together with the highest intensity at a specific age (figs. 5 and 6) can be localized in several layers in the neocortex investigated (supported by figs. 1-4). The combination of these parameters allows a summative evaluation of antigen expression during the development of the visual neocortex. Circle in tau3/P3/layer IV marks artifact.

4. NeuN - a special player throughout postnatal life

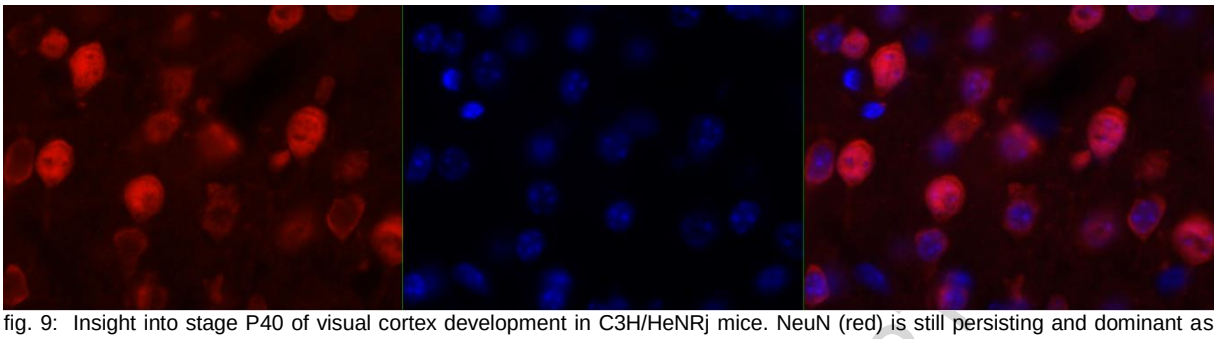


fig. 9: Insight into stage P40 of visual cortex development in C3H/HeNRj mice. NeuN (red) is still persisting and dominant as described above. It is a 'special player' in the group of antigens investigated here, because of its existence throughout most of postnatal life. Most of the other antigens seen before have decreased while NeuN, as a marker for mature neurons, is strong as never before. This suggests that the processes described in this study have come to an end until P40. Left: Alexa 568, middle: DAPI, right: combination.

As a final point of our study we show *NeuN* in the visual cortex of a 6 week old (P40) adult C3H/HeNRj mouse. The persistence until P40 and the strong expression shows a special role of this antigen – the processes described before (migration, maturation and differentiation of neuroblasts) have come to an end and markers for neuron maturity like NeuN are predominant. At this ontogenetic stage, the photoreceptors of the retina have already degenerated. The pattern of antigen expression seen here is very similar to that in other rodents and does not indicate any defect in structural development of the visual cortex in C3H/HeNRj mice.

5. Discussion

The aim of this study was to investigate the development of the visual neocortex during the postnatal stages P3 to P28 of C3H/HeNRj rd mice with retinal degeneration. Parameters were i) the appearance, number, progression, intensity and disappearance of antigen signal expression and ii) the morphological and topographical characterization of labeled neurons (shape of the perikaryon and cell processes and their location within the cortical plate). This is the first time overall that the early postnatal period of visual cortex formation in mice is investigated.

With respect to their appearance, we can divide the developmental markers investigated into two groups: First, those expressed at early stages after birth (P3-P6: *Nestin, tau3*, *β3-Tubulin* and *Calbindin,* see figs. 5 and 6a) are responsible for the migration of neuronal progenitor cells (NPCs) to the emerging visual cortical plate and their final integration (Bai et al. 2003; Fricourt et al. 2007). Second, antigens expressed from P8 to P14 and beyond (*Doublecortin*, *MAP2*, *Parvalbumin* and *NeuN,* see figs. 5, 6b and 6c) are involved in neuron maturation of

rodent visual cortex. Thus, stage P8 seems to mark a 'turning point' between two phases of visual cortex development.

In other words: The change from one group of antigens to the other is interpreted as the beginning of NPC differentiation at stage P8 (Sahara et al. 2012). The migration of NPCs is completed when the differentiation and maturation starts at P8.

All these processes reported here in C3H/HeNRj rd mice (see Results) reflect the situation found in the literature on visual cortex development in other rodents with an intact retina. They suggest that no obvious deviations from the general plan of rodent cortical development occur in the C3H/HeNRj mouse (Alcantara et al. 1996; Cauli et al. 1997; Sahara et al. 2012). This is remarkable because – during this period of time − the origin of the visual pathway in C3H/HeNRj mice is successively affected by the degeneration of photoreceptor cells in the retina. Controls from the visual cortex of the adult (96 days old) C56BL/6 mouse with no visual impairment show the same antigen expression of the ontogenetic markers (*MAP2*, *Parvalbumin* and *NeuN*) as seen here in the 40-day-old C3H/HeNRj mouse which becomes blind. Obviously, there is no difference between the two strains with respect to the antigens investigated during the early postnatal development of the visual cortex. This may mean that the visual cortex in C3H/HeNRj mice is semi-independent from the afferent visual pathway.

Electrical stimulation of the degenerated retina in 6-8 week old C3H rd mice with near-total vision loss (Chen et al. 2006) evoked electrically elicited responses (EER) in the visual cortex of these animals as in normal mice. These EER experiments seem to indicate a physically complete visual pathway in "rd" mice as in normal mice. Light exposure of the retina, however, generated no detectable signals in primary visual cortex of these C3H/HeNRj rd mice (Chen et al. 2006), in contrast to the situation in normal (visually non-impaired) mice. This could mean that all parts of the visual system in "rd" mice exist with the exception of the photoreceptors in the retina which in healthy mice supply the visual cortex with adequate physiological signals (Chen et al. 2006).

CONCLUSION

Although the selection of antigens for this study of the visual cortex in C3H/HeNRj rd mice is limited, it gives good insight into the immunohistochemistry of cortical migration, maturation and differentiation – based on the detailed chronology of development displayed here and in agreement with the relevant literature on rodents with intact photoreceptors in the retina.

Important ontogenetic phenomena such as simultaneous peaks in number and intensity of immunohistological antigen signals around P8 (*NeuN, Doublecortin, MAP2*) and the succession of *Nestin/NeuN* and *Calbindin/Parvalbumin* indicate functional transitions in the cortical development of these mice about the time of eye-opening.

Concluding from our data we could not find any evidence that retinal degeneration influences the development of the visual cortex in the early postnatal period of C3H/HeNRj rd mice. At eye opening (P14), when the apoptosis of photoreceptors is at its peak, and when the antigens investigated in the visual cortex show a maximal diversity in activity, the loss of visual input obviously does not interfere with neuronal cell maturation in the postnatal cortical plate of the investigated “rd” mice. This is also true for later postnatal stages and may indicate a certain morphogenetic independence of the visual cortex during ontogenesis and evolution. Recently, however, the voxel- based quantitative assessment of the cortical gray substance in different mouse strains including C3H/HeNRj mice revealed a smaller volume of the visual cortex of 119-day-old mice of this rd strain (Hikishima et al. 2017).
Further studies may show whether the visual cortex of C3H/HeNRj mice suffers structural or functional deficits at older age or whether collateral input from other sensory modalities and brain regions in some way can help maintain or modify parts of the visual cortical area.

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Highlights

- For the first time we investigated the postnatal development of the visual cortex in mice, particularly in C3H/HeNRj mice with retinal degeneration.
- Most interestingly, the C3H/HeNRj mice do not differ substantially in immunohistochemical development of the visual cortex from visually non-impaired mice (C57BL/6).
- We do not know whether old age animals of C3H/HeNRj mice show morphological and physiological changes from the normal situation. We can only suspect that the visual cortex in old age animals will show deficits in function.
- Perhaps retinal input is necessary for the development of a functional visual cortex. However, it may be that other cortical areas and modalities replace the lacking visual input and by this help to maintain the 'visual' cortex.
- However, Hiskishima et al. 2017 showed that in these C3H/HeNRj mice the volume of the visual cortex of 119 days old mice is smaller than in other mouse strains.