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Postnatal development of NADPH-diaphorase expression in the visual cortex of the golden hamster**☆

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Abstract

Nitric oxide is an important neuromodulator in the brain and is involved in the development of visual system. But it is not clear how nitric oxide and nitric oxide synthase (NOS) are involved in the developing visual cortex of rodents. Thus we examined the expression of NOS activity in the postnatal developing visual cortex of the golden hamster by using histochemical technique for NADPH-diaphorase (NADPH-d). A heavily stained NADPH-d band was observed in the neuropil of the visual cortex. This NADPH-d band initially appeared in the cortical plate from the day of birth (P0) to postnatal day 4 (P4). From P7 to P21, this band was confined to area 17 and migrated to the deeper layers III–IV and V–VI before it eventually disappeared at P28. Such developmental trends of the band correlated well with the process of formation and establishment of the geniculo-cortical projection patterns. Thus, the areal specific development of the band suggests that NOS is closely related to the cortical differentiation and synaptic formation of the primary visual cortex. On the other hand, monocular eye enucleation on P1 could not alter the appearance of this NADPH-d positive band, indicating a non-activity dependant role of NOS. In addition, differences in the laminar distributions and developmental sequence between the heavily and lightly stained NADPH-d positive neurons during development suggest that they play different roles in the development.

Key Words: NADPH-diaphorase; nitric oxide synthase; postnatal development; visual cortex; area 17; golden hamster; neural regeneration

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INTRODUCTION

Nitric oxide (NO), a free radical gas, is a well-known neuromodulator in the brain^[1-12]. It plays multiple roles in the developing and mature brain, including synapse reconstruction and plasticity, neurotoxicity and neuronal death^[2-4, 7, 13-14].

Several studies indicate the role for nitric oxide synthase (NOS) in the development of visual system^[10, 15-18]. The presence of NOS coincides temporally with the formation of ipsilateral retinocollicular and retinogeniculate projections as well as the functional differentiation of primary visual cortex^[16-17]. Disruption of NO alters refinement in subcortical visual pathways, either in eNOS and nNOS (endothelial and neuronal isoform of NOS) knockout mice or after inhibition of NOS in the chick, rat and ferret^[16, 19-20], but does not affect ocular dominance formation in the primary visual cortex of the ferret and kitten^[21-23]. Thus NO has an effect on only some visual system pathways and only in certain species.

Though these reports suggest the role of NO in the visual pathway development, it is not clear how NO and NOS are involved in the developing visual cortex of rodents. Though there were reports showing that both the immunoreactivity and location of NOS positive neurons were altered in the developing rat visual cortex^[24-25], and there was a transient increase in both the level of enzyme and the activity of NOS in the developing visual cortex of the golden hamster^[17], no detailed description of the NOS distribution change in both neurons and neuropil was reported.

Thus, in the present study, we examined the expression of NOS and its reactivity patterns in visual cortical areas during development of golden hamster, by using histochemical technique for NADPH-d, a selective marker for NO^[26]. As a rodent, golden hamster has been established as an important experimental animal for the organization and development of the visual system^[27-30]. This species has a shorter gestation period (16 days) and is more immature at birth than rats and mice, thus facilitating the

investigation of the early developmental events of the visual system. Furthermore, our previous report^[31] has identified the existence of NADPH-d activity in a network of processes and special populations of neurons in the visual cortex of adult golden hamster, providing a good basis for studying the development of NADPH-d expression in visual cortex.

RESULTS

The neuropil of the visual cortical areas (17, 18a, and 18b) underwent a series of changes in NADPH-d reactivity during development from P0 to P60 (Figure 1). A continual heavily stained band of NADPH-d was found extending throughout the cortical plate (CP) of neonates (P0, P2 and P4) and this band became shorter and gradually reduced in size to reside in the upper layer of the visual cortex between P7 and P21 (Figure 1).

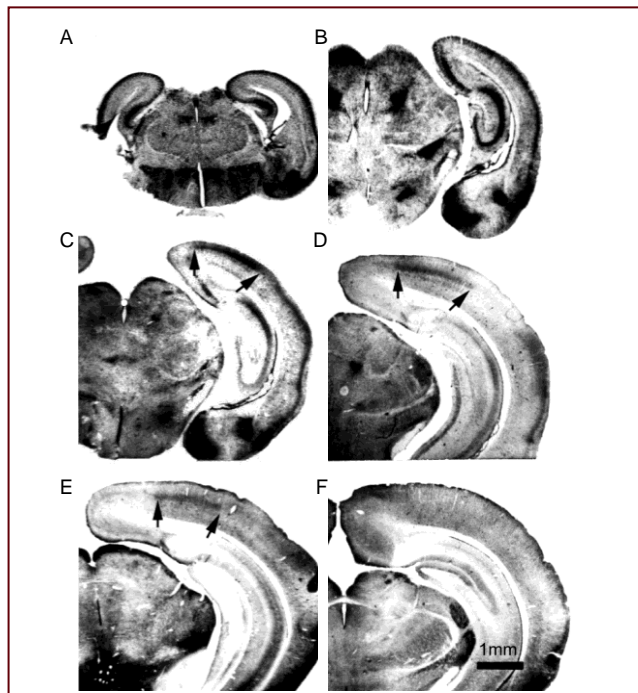


Figure 1 Coronal sections of the golden hamster brains showing the development of NADPH-d positive bands in the visual cortices. The bands at P0 (A) and P4 (B) were continuous and no area specificity was found in the visual cortices. At P7 (C), P14 (D) and P21 (E) the bands showed area specificity and located within area 17 of the visual cortex. At P28 (F), the band disappeared. [Arrows show the borders of 17/18b or 17/18a.](#)

Compared to the adjacent Nissl stained sections, the NADPH-d band corresponded to the boundaries of area 17/18b medially and area 17/18a laterally. As the band diminished in size, it moved progressively from the superficial region of the cortex to a deeper stratum (Figure 2). The band was composed mainly of strong NADPH-d positive structures in the interstitial space including some neurons and numerous neuronal processes. This band was basically comprised of two layers; the outer one was thick and heavily stained and

located in the inferior part of CP (P7) or layer III-IV (P14-P21) while the inner layer was thin and lightly stained, located in layer V-VI (Figure 2). However, when the animal grew to P21, the band became indistinct and the two layers were no longer discernible. Nevertheless, the medial and lateral borders remained coincide with the boundaries of 17/18b and 17/18a respectively. At P28 and thereafter this band vanished and only those NADPH-d positive neurons and their numerous processes remained.

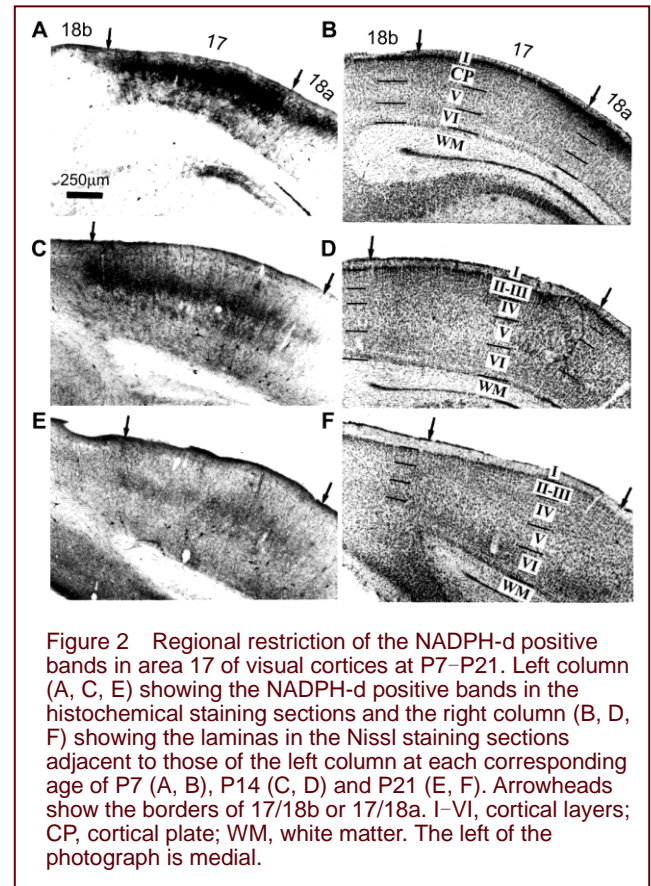


Figure 2 Regional restriction of the NADPH-d positive bands in area 17 of visual cortices at P7-P21. Left column (A, C, E) showing the NADPH-d positive bands in the histochemical staining sections and the right column (B, D, F) showing the laminas in the Nissl staining sections adjacent to those of the left column at each corresponding age of P7 (A, B), P14 (C, D) and P21 (E, F). Arrowheads show the borders of 17/18b or 17/18a. I-VI, cortical layers; CP, cortical plate; WM, white matter. The left of the photograph is medial.

In those animals whose right eye was enucleated at P1 and survived for 13 days, the NADPH-d positive band in visual area 17 could still be observed both in the contralateral and in the ipsilateral visual cortex with no obvious morphological distinctions from those observed in the normal (Figure 3). In those animals whose corpus callosum were transected on one side of the brain at P5 and survived till P14, NADPH-d positive bands in the contralateral visual cortex were present and similar to those observed in the age matched normal animals (data not shown).

Two types of NADPH-d positive neurons were observed in the brain sections of all ages studied. A population of heavily NADPH-d stained neurons with dark somata and long discernible dendrites were found scattering amongst the lightly stained neurons with fewer dendrites as described previously^[31]. These two types of neurons displayed different distribution pattern and developmental sequence.

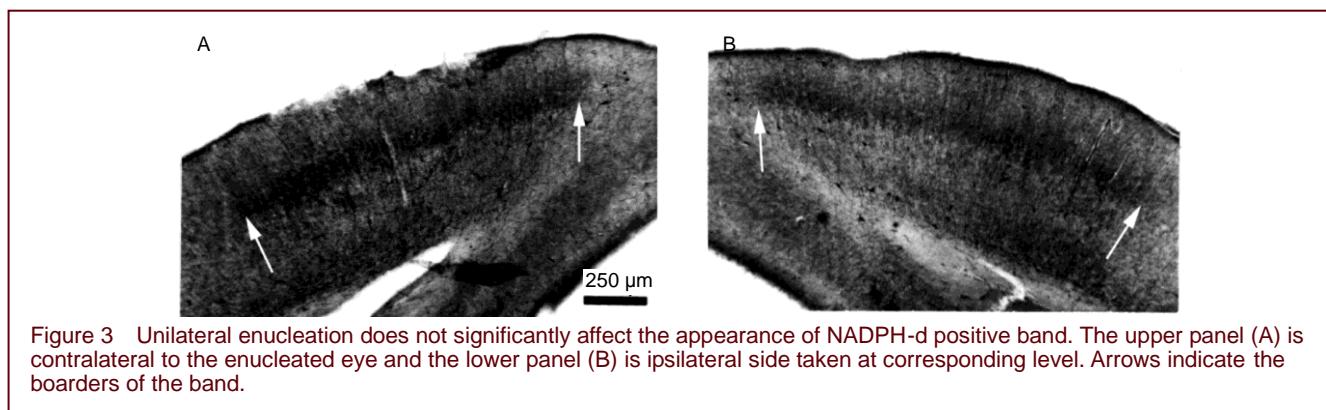


Figure 3 Unilateral enucleation does not significantly affect the appearance of NADPH-d positive band. The upper panel (A) is contralateral to the enucleated eye and the lower panel (B) is ipsilateral side taken at corresponding level. Arrows indicate the borders of the band.

The occurrence and distribution of those heavily stained neurons (arrow in Figure 4A) were more evenly spread throughout development extending from P2 ($-72/\text{mm}^2$) to adulthood ($-34/\text{mm}^2$) with the peak of about $87/\text{mm}^2$ neurons per animal at P4 and P7 (Figure 4C). The percentage distribution of heavily stained neurons in the visual lamina was highest in layer VI followed by layer V in all ages studied (Figure 4B). The number of NADPH-d positive neurons in the white matter (WM) was relatively low before P7 thence it increased to attain adult level at P14 (Figure 4B). The estimated total number of the heavily stained NADPH-d positive neurons in layers V, VI and WM of the visual cortex accounted for about 90% of neurons counted in this category.

On the other hand, the lightly stained NADPH-d positive neurons (arrow head in Figure 4A), were more numerous than the heavily stained neurons with the ratio of about 100 to 1 (Figure 4C). This class of neurons was more abundant during early postnatal days from P2 to P7 with the peak at P4, and the total number greatly decreased thereafter. They were distributed in two clusters within the visual cortex; one resided in the CP and the other in laminas V, VI and WM (Figure 4B). They were found in relatively high percentages in layer V and VI between P4 and P14. However, from P21 to P60, their distribution shifted more towards the superficial layer (I–III) and the percentage of these lightly stained NADPH-d positive neurons increased significantly in the WM at P60. The cell diameter and its change during development also differed between heavily stained NADPH-d positive neurons and lightly stained neurons. The size of the lightly stained NADPH-d positive neurons was significantly smaller than the heavily stained neurons over the entire period of investigation (Figure 4D, *t*-test for corresponding age, $P < 0.0001$). Furthermore, the growth pattern of the two classes of NADPH-d positive neurons was different: there was a slightly steeper initial increase in the size for the heavily stained neurons compared to the gradual and continuous insignificant increase in the lightly stained population (Figure 4D).

Thus it is evident that these two types of NADPH-d positive neurons exhibited distinct characteristics in their

distribution pattern, developmental sequence and soma size.

DISCUSSION

In the present study we found a NADPH-d positive band during the development of golden hamster cortex. This band corresponds to the entire cortical visual area 17 extending from the border of area 17/18a laterally to area 17/18b medially from P7 to P21. Though a band-like distribution of NOS positive neurons was also reported in the developing visual cortex of rat^[24] from P7 until adulthood, there was no description of its location. In our previous study of NADPH-d activity in the visual cortex of adult golden hamster^[31], we didn't find the NADPH-d positive band either. Thus, our present result is the first to report the areal characteristic of the NADPH-d positive band in the rodent cortex. Observations of NOS expressing specific pattern was also reported in the superior colliculus and ventral lateral geniculate nucleus of the rat during development^[32], suggesting that NOS-specific pattern was a common occurrence exhibited in the developing visual system of rodents.

Our results support that NO plays an important role in the synaptic formation of geniculocortical projection and functional areal differentiation during visual cortex development. The timeline of NADPH-d band is consistent with the reported transient expression and activity increase of NOS in the developing visual cortex of golden hamsters^[17], further supporting the role of NOS. The extents and location of this NADPH-d positive band (1.9 ± 0.2 mm at P7 and 2.3 ± 0.1 mm at P14) correspond well to those from the geniculocortical projection (1.8 ± 0.2 mm at P6 and 2.2 ± 0.2 mm at P12) reported by Krug *et al.*^[30]. It is further suggested that the presence of this NADPH-d positive band closely follows the formation of the geniculocortical projection of the golden hamster^[30], and with the disappearance of the band at P28 the process of synaptogenesis and the maturation of area 17 has been accomplished. The above evidence suggests that there is a close relationship between the NADPH-d positive band and the geniculocortical topographic projection.

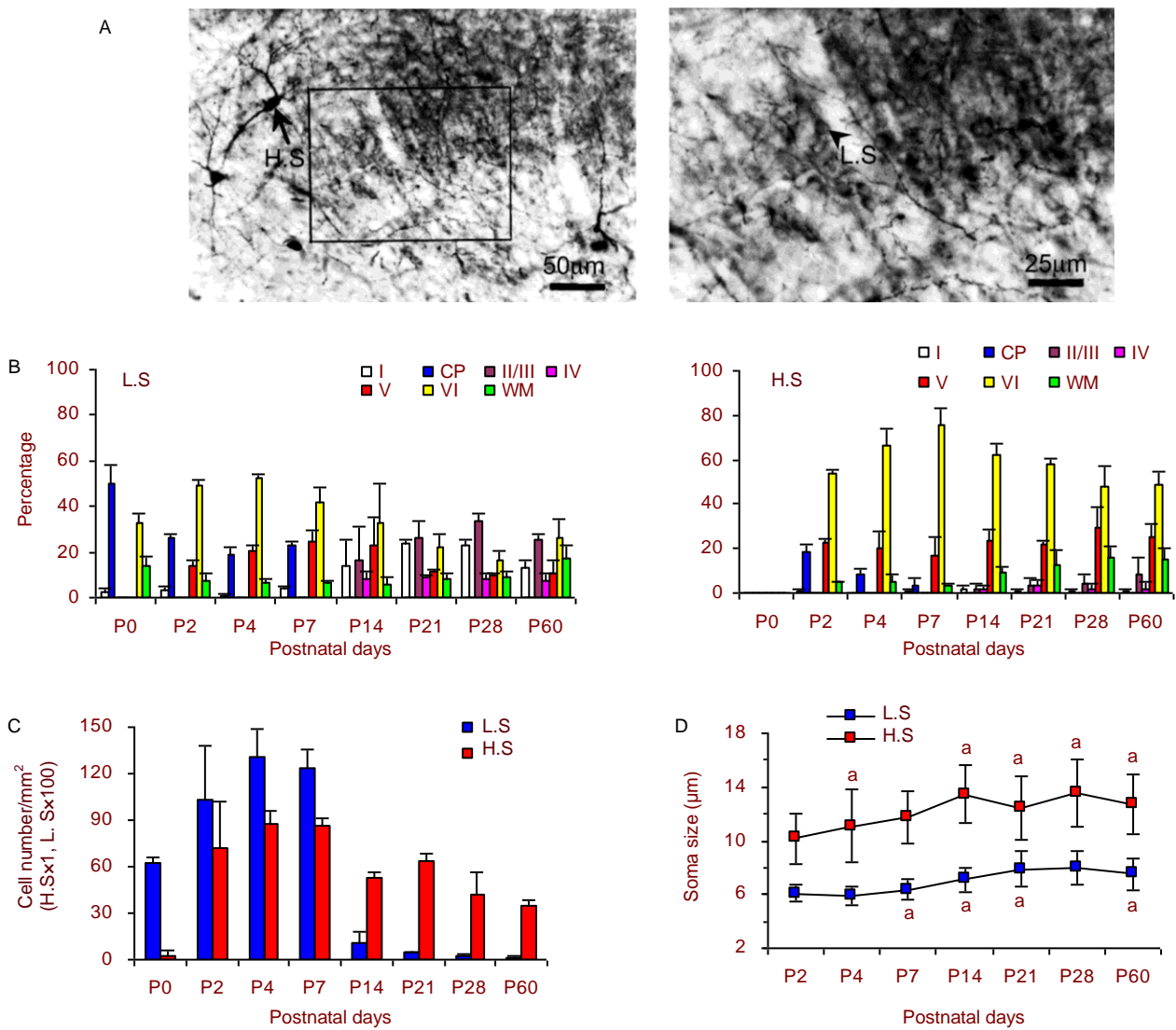


Figure 4 Heavily and lightly stained NADPH-d neurons exhibit different lamina distribution, developmental sequence and soma size. (A) The coronal section of the brain at P14 showing the heavily stained neurons (H.S, arrow) and lightly stained neurons (L.S, arrowhead). The area enclosed by the rectangle in the left is enlarged and shown in the right. (B) The lamina distribution of lightly stained neurons and heavily stained neurons during development. The ordinate represents the percentage of positive neurons of each layer to the total number of positive neurons in all layers including the white matter. Each value represents an average of positive neurons from three animals at each age counted in five coronal sections for each brain. CP: Cortical plate; I-VI: cortical layers; WM: white matter. (C) The density of cells in all cortical layers at different ages. Note that the number of lightly stained neurons is almost 100 times more than that of heavily stained ones. (D) The changes of average cell diameter with age. Asterisk (a) denotes a significant difference between the current diameter and that of earlier age ($P < 0.05$). Error bar: Standard deviation.

Several studies have addressed the possible physiological and pathological roles of NO in the visual system. Studies carried out in neonatal and adult rats demonstrated that eye enucleation does not change the expression of nNOS, but affects its distribution within neurons^[33-34]. In contrast, monocular enucleation in young rats^[35] and ocular deprivation in monkeys^[36] appeared to generate a down-regulation of NOS in both SC and DLG. On the other hand, an upregulation of nNOS protein level was found in both the SC and the DLG of adult rats^[37] and in adult chicks after retinal lesions^[38]. In light of those conflicting results, our results demonstrate that deafferentation of the corpus callosal inputs and monocular enucleation of eye cannot

effectively alter the expression of NADPH-d positive band in the visual cortex of golden hamster, indicating that the expression of NOS is not activity-dependent in cortex. Whether it is regulated by genetic control needs further investigation. In our previous morphological study^[31] of neurons expressing NADPH-d in the visual cortex of adult golden hamsters, we have identified the two types neurons with different expression of NADPH-d-the darkly and lightly stained neurons, and described their different soma size and lamina distribution (which is consistent with our current result on P60), suggesting their different functions. In the present study, on the basis of those differences existed at each age corresponding

developmental age, and the distinct developing tendencies of distribution changes of the cell size and lamina, we proposed that heavily and lightly stained neurons played different role in the development, supporting the opinion that they belong functionally to different neurons^[31]. The different calbindin expression in these two types of neurons^[39] may attribute to their functional difference in the development. With both calbindin and GABA expression and much larger in number than heavily stained neurons, the NADPH-d lightly stained neurons may play a more significant role in the intra-cortical neuronal activation than heavily stained neurons with little calbindin expression.

In conclusion, our result is the first to report the areal characteristic of the NADPH-d positive band in the rodent cortex, which is specifically confined to area 17 between P7 and P21. The close relationship between the NADPH-d positive band and the geniculo-cortical topographic projection suggest NO's role in the formation of the projection.

MATERIALS AND METHODS

A total of 30 golden hamsters (*Mesocricetus auratus*) with ages ranging from day of birth (P0) to two-month (P60) old adults were used in this study. The animals were divided into a larger normal developmental group comprising of 24 animals; three each of P0, P2, P4, P7, P14, P21, P28 and P60 and an experimental group comprising of 3 animals which were subjected to unilateral right eye enucleation at P1 and another 3 animals underwent corpus callosum transection at P5. All procedures carried out in these experiments were conformed to the *Animals (Control of Experiment) Ordinance (Cap. 340)* issued by the Department of Health, the Government of the Hong Kong Special Administrative Region and the experimental protocol approved by the University of Hong Kong Committee on the Use of Life Animals in Teaching and Research. For the immunostaining, animals were anesthetized with an overdose of sodium pentobarbitone (100 mg/kg body weight *via* intraperitoneal injection) and perfused transcardially with 0.85% saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH7.4). The caudal third of the brain containing the visual cortex was cut at 40 μ m with a cryostat microtome. Two series of alternate sections were collected for NADPH-d histochemical reaction as described previously^[18] and for cresyl violet (0.25%) staining as control for identifying cortical lamina and the boundaries of visual cortex area.

For eye enucleation, the skin around the right eye of P1 pups was incised after deep ether anesthesia, and then the entire eyeball was removed. The operated animals were kept in an incubator till fully recovery before they were returned to their mother. These animals were allowed to survive till P14 before they were humanely killed under an overdose of sodium pentobarbitone

(100 mg/kg body weight *via* intraperitoneal injection). For corpus callosum transection, the skin on top of the skull of P5 animals was incised to expose the cranium after anesthesia. A window extending from bregma to lambda anteroposteriorly and 1.0 to 5.0 mm mediolaterally was opened to expose the cerebral cortex. The cerebral cortex overlying the corpus callosum was removed by aspiration and the corpus callosum was then transected with a surgical blade in a posteroanterior direction. The wound was filled with gelform (UpJohn, USA) before the piece of cranial bone was replaced and the skin sutured. The animals were revived in an incubator before returning to their mother. These animals were allowed to survive till P14.

As our previous report^[27], two types of NADPH-d positive neurons were observed in the visual cortex, according to the density of the enzyme reaction product. The first type consisted of neurons with heavily stained soma and long dendrites (Figure 4A, arrow). The second type consisted of neurons with lightly stained soma but no visible processes (Figure 4B, arrow head).

NADPH-d positive neurons in the visual cortex were counted under an Olympus microscope. The visual area (for P2–P4) or area 17/18a (for P7–P60) was scanned tangentially from the cortical surface through to the white matter (WM) for the NADPH-d expressing neurons. Alternate sections of the brain were selected for counting of neurons and averages of three animals of each age were then calculated. The maximum diameter of the stained NADPH-d neurons in the visual area for each animal was randomly measured with computer-based image analyzing system (Neurolucida) and histograms of the distribution of lamina and cell diameter were computed. In this study, cytoarchitectonic features based on the study on the development of geniculate-cortical projections in hamsters^[30] were used to define the borders between area 17 and area 18a or 18b. As in other rodents, layer IV (or CP before layer IV formed) in area 17 was wider and had a higher cell density compared to 18a or 18b^[40-41].

Author contributions: Ying Xu was responsible for the main experiment conducting and manuscript writing. Yuemei Xiao and Yuncheng Diao were responsible for the experiment concept and design. Kwok-Fai so provided technology and information support. Ying Xu and Kwok-Fai So were responsible for the funding.

Conflicts of interest: None declared.

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Ethical approval: This experiment was approved by the University of Hong Kong Committee on the Use of Life Animals in Teaching and Research.

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