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Expression of nicotinamide adenine dinucleotide phosphate-diaphorase in the retina of postnatal golden hamsters deprived of light stimulation

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Abstract

Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase (NADPH-d) expressing neurons in the retina of golden hamsters have been identified to be a subset of amacrine cells that provide a major source of Nitric Oxide (NO) in retina. This subset of amacrine cells in mouse retina was recently proved to contain the circadian clock gene *Per1* (D.Q. Zhang, T. Zhou, G.X. Ruan, D.G. McMahon, Circadian rhythm of Period 1 clock gene expression in NOS amacrine cells of the mouse retina, Brain Res., 1050 (2005) 101–109). However, it remains unknown whether these clock-related NADPH-d amacrine cells can be regulated by light stimulation and thus synchronized to ambient day/night cycle. A previous study has reported that NADPH-d expressing amacrine cells in postnatal hamsters exhibited a surge after eye-opening (D. Tay, Y.C. Diao, Y.M. Xiao, K.F. So, Postnatal development of nicotinamide adenine dinucleotide phosphate-diaphorase-positive neurons in the retina of the golden hamster, J. Comp. Neurol., 446 (2002) 342–348) suggesting a possible effect of light on the NADPH-d amacrine cells. In order to further reveal the relationship between NADPH-d amacrine cells and light stimulation, the present study focuses on the changes of the expression of NADPH-d in the retina of postnatal hamsters reared in completely deprived light conditions. Prior to eye opening, P12 hamster pups were subjected to either bilateral eyelid suturing or dark rearing. On P28 a subgroup of light deprived hamsters was returned to lighting conditions and the expression of NADPH-d activities in the retina was assessed. In hamsters reared in the 12:12 light-dark cycle, the number of NADPH-d amacrine cells in the ganglion cell layer (GCL) increased right after eye-opening and reached the adult level gradually. However, hamsters subjected to both bilateral eyelid suturing and dark rearing, the number of NADPH-d amacrine cells in GCL was maintained at a low level but increased again upon returning to the 12:12 light-dark condition. In contrast, the number of NADPH-d expressing amacrine cells in the inner nuclear layer (INL) remained low and unaltered regardless of the lighting environment. This study demonstrates that there are two subpopulations of NADPH-d expressing amacrine cells with respect to different locations in the retina of hamsters. Different from those in INL, the NADPH-d amacrine cells in GCL of postnatal hamsters are dependent on the lighting environment implicating that these clock-related amacrine cells and the production of NO might be under a modulation of light stimulation.

Keywords: NADPH-diaphorase; Amacrine cell; Nitric oxide synthase; Visual deprivation; Hamsters

Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is an oxidative enzyme that transfers electrons from NADPH to an electron acceptor such as Nitro Blue Tetrazolium, resulting in the formation of blue insoluble formazans, as revealed by the NADPH-d histochemistry. The NADPH-d histochemistry has been used to demonstrate the presence of a NO-producing enzyme, the Nitric Oxide Synthase (NOS). NOS converts L-arginine into Nitric Oxide (NO) and citrulline using NADPH as an electron donor. NO, a labile free radical and an important neurotransmitter, plays a variety of roles in the physiology and pathology of mammalian retina. The expression of NADPH-d in retina has been investigated in several different mammalian species (See [6] for a review), such as cats [16,17], rabbits [10,12], guinea pigs [3], rats [9,13], and hamsters [8,15]. In all animals studied, the expression of NADPH-d or NOS is mainly located in inner retina, specifically the amacrine cells in inner nuclear layer (INL) and displaced amacrine cells in ganglion cell layer (GCL).

These NADPH-d or NOS expressing amacrine cells provide the primary source of NO in inner retina, and the probable tar-

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gets of NO are bipolar cells, ganglion cells and other amacrine cells. It has been shown that NO modulates the cyclic guanosine monophosphate (cGMP) channel in target cells and thus is involved in cellular physiological activities. However, their exact roles in visual function remain unclear. It has been found that during the postnatal development of hamsters, the expression of NADPH-d amacrine cells exhibits a significant increase after the opening of eyes (P14, postnatal day 14) suggesting that the expression of NADPH-d amacrine cells might be related to light stimulus [15]. Recently, Zhang et al. proved that NOS expressing amacrine cells contain the clock gene *Per1*, indicating that NOS expressing amacrine cells might function as an intra-retinal circadian clock [19]. However, whether and how light stimulation modulates the NOS expressing cells remains to be investigated, and the differences with respect to their locations in INL or GCL are yet to be studied.

In this present study, we are reporting that NADPH-d amacrine cells in inner retina can be grouped into two functional different subtypes and we observed significant changes in the number of NADPH-d expressing amacrine cells of hamster pups reared in different lighting environment and its expression of one subtype is regulated by light stimulation.

All the animals used in this study were Syrian golden hamsters (*Mesocricetus auratus*) of various ages ranging from early postnatal day 8 (P8) to young adult at 8 postnatal weeks (P8w) obtained from the Laboratory Animal Unit of the University of Hong Kong, and maintained in standard laboratory conditions. The animals were randomly divided into three treatment groups: (i) control group (Ctrl) in which hamsters were kept in the standard laboratory conditions with a 12 h:12 h light-dark cycle; (ii) dark-reared group (DR) in which hamsters were maintained in total darkness from postnatal day 12 (P12), prior to eye-opening, until the day of sacrifice. A dim red light was used for a brief period to feed the hamsters and change the beddings because hamsters are not sensitive to dim red light [2,5]. On P4w, half of the hamsters reared in the complete darkness were reintroduced to the light controlled room (RL^{DR}) with a 12 h:12 h light-dark cycle for 1–4 weeks before they were sacrificed and the other half remained in the dark room until sacrifice at predetermined time; (iii) bilateral eyelid suture group (BS) whose eyelids were sutured bilaterally 2 days before the eye opening at P12 under sodium pentobarbital (Nembutal, 50 mg/kg body weight i.p.) anesthesia. The eyes of the BS group were inspected twice a day for any damage to the suture. Once found, the suture was repaired immediately; however, if damage was too extensive, the animal was removed from further experimentation. All the eyelid-sutured hamsters were reared in a light-controlled room, with a fixed lighting schedule similar to that for animals in the Ctrl group. At P4w, the sutures of some of the hamsters in the BS group were removed to allow these animals (RL^{BS}) conventional 12:12 light–dark cycle until they were sacrificed. The remaining hamsters still having their eyelids sutured were kept until they were sacrificed at predetermined time points.

In the Ctrl group, three animals from age P8, P12, P16, P3w, P4w, P5w, P6w, P7w and P8w (adult) were sampled at each time point. In the DR group and BS group, three animals each from P16, P3w, P4w, P5w, P6w, P7w and P8w (adult) were studied. Animals re-exposed to 12:12 light–dark cycle from both RL^{DR} and RL^{BS} groups were sacrificed at P5w, P6w, P7w and P8w. The data from RL^{DR} and RL^{BS} groups were similar and were referred to as the RL group for analysis. All procedures carried out in these experiments were conformed to the Animals Ordinance (Cap. 340) issued by the Department of Health of the Government of the Hong Kong Special Administrative Region and adhered to the experimental protocol reviewed and approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research (CULATR 833-03).

The golden hamsters were anesthetized with an overdose of sodium pentobarbitone (Nembutal; 100 mg/kg body weight i.p.) prior to sacrifice. Both the left and right eyes were enucleated and the cornea and lens were removed before fixing them in 2% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) at room temperature for half an hour. The whole retina was separated from the vitreous body and pigmented epithelium. Four incomplete radial cuts were made in superior, inferior, temporal and nasal parts of the retina such that the four retinal quadrants were connected to each other near the optic nerve head. The retinas were post-fixed in the same fixative for 1 h and then transferred to 0.1 M phosphate buffer (PB; pH 7.4) at 4 °C until they were processed for NADPH-d histochemistry. The NADPH-d reaction was performed as described by Lau et al. [8]. In brief, the retinas were incubated in 0.1M PB containing 0.3% Triton X-100, 0.1 mg/ml nitro blue tetrazolium (NBT), and 1 mg/ml β-NADPH at 37 °C for 60–90 min (all chemicals were obtained from Sigma). After thorough rinsing in 0.1 M PB, both the left and right retinas were flat mounted onto chromealum-coated slides and coverslipped with aqueous mounting medium (DAKO).

In addition, four retinas from normal adult hamsters were processed for paraffin embedding after NADPH-d histochemistry staining, and 10 μm transverse sections were cut with a rotatory microtome. The serial sections were mounted on glass slides, dewaxed, dehydrated and coverslipped with Permount.

To estimate the number of NADPH-d expressing neurons in both GCL and INL of the whole-mount retinas, we used the same counting method as that described by Lau et al. [8]. A series of counting areas, each covering $100 \times 100\ \mu m^2$ and arranged radially with 0.2 mm (P8–P16), 0.25 mm (P3w–P4w) or 0.3 mm (P5w-P8w) intervals from the optic disc (OD), were selected in each of the four quadrants of the retina. The total number of sampled sites for the counting of NADPH-d neurons in GCL and INL ranged from 45 to 64 covering about 1.5% of the retinal area of the each of the three age groups of the animals. The counting of the NADPH-d neurons was made visually under a Nikon microscope with a 40 × objective and 10× eyepieces equipped with a square micrometer. Different focusing depths were adjusted to distinguish the NADPH-d neurons located in GCL from those distributed in INL. The data from all the sampled areas of each eye were pooled and the mean densities of NADPH-d neurons in the GCL and INL were calculated separately. The area of the flat-mounted retinas was measured using a PC-based image-analyzing system (Stereo-investigator) attached to the Nikon microscope. The number of NADPH-d neurons in GCL and INL of each retina was then calculated by multiplying the mean