Experimental Neurology 250 (2013) 74–93

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Metabolic profiling of the mouse retina using amino acid signatures: Insight into developmental cell dispersion patterns☆

Jacqueline Chua [a,1], Lisa Nivison-Smith [b,1], Seong-Seng Tan [c], Michael Kalloniatis [a,b,c,d,*]

[a] Department of Optometry and Vision Science, University of Auckland, Auckland, New Zealand
[b] School of Optometry and Vision Science, University of New South Wales, Sydney, NSW, Australia
[c] Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Australia
[d] Centre for Eye Health, Sydney, Australia

ARTICLE INFO

Article history:
Received 11 July 2013
Revised 15 August 2013
Accepted 9 September 2013
Available online 13 September 2013

Keywords:
Retinal development
Amino acids
Pattern recognition
Cell dispersion
Immunocytochemistry

ABSTRACT

Pattern recognition has been used for the complete and statistically rigid classification of retinal neurons in vertebrates such as the adult cat, primate, rat and goldfish. Here, we label the mouse retina with antibodies against seven amino acids and use pattern recognition to characterize distinct retinal neurochemical cell classes based on their unique amino acid signatures. We followed the development of the cell classes in the X-inactivation transgenic mouse expressing the lacZ reporter gene on one X-chromosome. This mouse allows clonally related cells to be identified through differential β-galactosidase activity due to random X-chromosome inactivation. Pattern recognition analysis partitioned the retina into nine neuronal classes at birth, increasing to 19 classes at eye opening and 26 classes by adulthood. Emergence of new cell classes was partly attributed to new neuron types and partly to the splitting of classes from early ages from refinement of their amino acid profiles. All six GABAergic amacrine cell classes and most ganglion cell classes appeared by P7 whilst all the glycinergic amacrine cell classes did not appear till adulthood. Separable bipolar cell classes were not detected till eye opening. Photoreceptor cell classes were detected at P3 but inner and outer segments did not form separable classes until adulthood. More importantly, we show that cells which share common amino acid profiles also shared cell dispersion patterns. GABAergic amacrine cell classes with conventional and displaced counterparts transgressed clonal boundaries whereas GABAergic amacrine cell classes found exclusively in the inner nuclear layer and all glycinergic amacrine cell classes did not transgress. Ganglion cells displayed both dispersion patterns. This study provides a comprehensive neurochemical atlas of the developing mouse retina, tracking the amino acid levels within distinct neuronal populations and highlighting unique migratory patterns within subpopulations of inner retinal neurons.

Introduction

The vertebrate retina is composed of seven major cell types, generated in overlapping intervals during development. Ganglion cells, cone photoreceptors, amacrine cells and horizontal cells are generated prior to birth in the murine retina followed by rod photoreceptors, bipolar cells and glia after birth (Young, 1985a,b). Within these major cell groups, approximately 55–60 retinal cell subtypes have been revealed from the combination of different morphological, electrophysiological and neurochemical characteristics (Masland, 2001a,b). Classifying these cells using traditional univariate methods such as immunolabeling with cell markers is limited by an incomplete library of markers to specifically label each cell subtype.

Pattern recognition is a multivariate analysis that uses labeling patterns from a combination of antibodies on serial sections to identify different cell types. The immunoreactivity of serial sections using multiple antibodies forms a unique labeling pattern or "signature" and cells are classified into neurochemical groups based on common signatures. In the retina, the combination of amino acid antibodies has been used for this classification procedure (Marc et al., 1995) as every cell class contains a unique set of free floating amino acids to sustain cellular functions such as osmoregulation, intercellular signaling and protein synthesis (Werner and Chalupa, 2004). Examples of pattern recognition analysis using amino acids antibodies include the complete classification of the goldfish, primate and cat retinas (Kalloniatis et al., 1996; Marc et al., 1995, 1998b), visualization of excitatory signaling (Marc, 1999), classification of the entire ganglion cell layer (GCL) population

☆ Support: Health Research Council of New Zealand grant number 02/226; Retina Australia; a professorship funded by the Robert G. Leitl estate (MK). This work was supported in part by the National Health and Medical Research Council (NHMRC) and the Victorian Government through the Operational Infrastructure Scheme (SST) and the National Health and Medical Research Council (NHMRC) project grant #1009342 (MK).

* Corresponding author at: School of Optometry and Vision Science, University of New South Wales, Kensington, New South Wales, Australia. Fax: +61 2 8115 0799.
E-mail address: m.kalloniatis@unsw.edu.au (M. Kalloniatis).

[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.expneurol.2013.09.007

(Marc and Jones, 2002) and classification of the cortex (Hill et al., 2001). Pattern recognition is also applicable to the study of retinal disease and has been used for the visualization of retinal remodeling (Marc and Jones, 2003; Marc et al., 2003) and visualization of normal, ischemic and reperfused rat retina (Sun et al., 2007a,b).

Pattern recognition analysis has yet to be applied to the normal mouse retina at any developmental stage. Previous work however indicates that amino acid levels of the rat retina fluctuate during development and these changes reflect the maturation of neuronal function and metabolism (Fletcher and Kalloniatis, 1997). Thus, pattern recognition analysis of amino acid labeling has the potential to (1) classify and track neurochemical cell classes during development and (2) longitudinally examine and quantify amino acid levels within a known cell class over developmental time.

In addition to neurochemical maturation, spatial positioning of neurons into regular arrays or mosaics is also essential to the formation of functional circuits in the central nervous system (Tan and Breen, 1993; Wässle and Boycott, 1991). Tangential displacement of migrating retinal neurons is proposed as a mechanism for the spatial positioning of retinal mosaics where neurons disperse sideways to accommodate the new cells to preserve regular cell spacing (Galli-Resta et al., 1997; Wässle and Riemann, 1978). Reese et al. (1995) showed that cone photoreceptors, horizontal cells, some amacrine cells and some ganglion cells are tangentially displaced in the mouse retina. However, further characterization of displaced retinal cells has only been conducted using a limited set of cell markers (Acosta et al., 2008; Reese and Tan, 1998). Pattern recognition can overcome this limitation by providing a complete map of retinal subclasses and thus in combination with cell dispersion analysis, the migration pattern of all neuronal cell classes can be followed. Furthermore, as radial and tangential cell dispersion is driven by functional imperatives related to circuitry, pattern recognition can indicate if amino acid signatures are correlated to modes of cell dispersion.

This study has two aims. Firstly we classify cell populations according to their amino acid signature patterns during the development of the mouse retina using pattern recognition and secondly, we correlate these amino acid signature patterns with their modes of radial and tangential dispersion patterns.

Material and methods

Ethics statement

All experimental protocols were approved by the Howard Florey Institute Animal Ethics Committee and conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Ed.).

Animals

Retinae were obtained from female X-inactivation transgenic mice expressing the *lacZ* gene on a single X-chromosome. In hemizygous female mice, random X-inactivation which occurs prior to the formation of the optic vesicle results in transgene expression in approximately 50% of the retinal progenitors (Tan et al., 1993). Mature retinal neurons inherit the X-active status of their parent cells so that in the adult retina, labeled and unlabeled clones can be used to trace lineage relationships and spatial positioning (Reese and Tan, 1998; Reese et al., 1995, 1999).

Details of the transgenic mouse line have been previously described (Tan and Breen, 1993; Tan et al., 1993, 1995). Briefly, hemizygous X-inactivation transgenic female mice were generated from mating of C57Bl/6 control males and homozygous females carrying the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG) lacZ reporter gene on the X-chromosome. Mice were maintained on a 12 h light/dark cycle and had access to standard mouse chow and water ad libitum. Mice were used at the ages of P1, P3, P7, P14, and adult (n = 4 for each age group).

Tissue collection

Adult mice were killed by cervical dislocation and neonatal mice (younger than P8) were placed on gauze saturated with ice-cold saline and euthanized. The eyes were enucleated and the anterior structures removed to create an eyecup preparation. Eyecups were immersion fixed in 2% (w/v) paraformaldehyde, 1% (w/v) glutaraldehyde in 0.1 M phosphate buffer.

β-Galactosidase detection

Detection of β-galactosidase was modified from methods described by Tan et al. (1998). Briefly, fixed eyecups were stained for 48 h at room temperature in 0.1 M phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Nonidet P-40, 5 mM $K_3[Fe(CN_6)]$, 5 mM $K_4[Fe(CN_6)]$ and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside with agitation. Cells with β-galactosidase activity were detected by their blue reaction product. Tissue were dehydrated and embedded in epoxy resin composed of a 2:1 ratio of dodecenyl succinic anhydride and Eponate 812 (ProSciTech, Queensland, Australia) followed by the addition of 2,4,6-tris (dimethylaminomethyl) phenol (ProSciTech, Queensland, Australia) at a 1:100 dilution (Fletcher and Kalloniatis, 1996; Kalloniatis and Fletcher, 1993; Kalloniatis et al., 1996; Sun et al., 2007b; Tan et al., 1998). Samples were selected from the central third of the retina, cut in either the radial or the tangential planes, stacked and re-embedded in resin. The resin embedded tissue was serially sectioned (200 nm) followed by a 2 μm section used to visualize β-galactosidase activity.

Immunocytochemistry and antibody characteristics

Post-embedding immunocytochemistry was performed as described previously (Kalloniatis and Fletcher, 1993; Marc et al., 1990, 1995; Nivison-Smith et al., 2013; Sun et al., 2007a,b; Tan et al., 1998). Serial 200 nm sections were incubated in primary antibodies donated by Dr Robert Marc, University of Utah, licensed through Signature Immunologics (Salt Lake City, UT) and commercially available through Chemicon (Billerica, MA) and Abcam (Cambridge, MA). Dilutions and specificity of antibodies are described in Table 1. The primary IgGs were detected with goat-anti-rabbit secondary IgGs coated with a 1 nm gold particle (British BioCell International, Cardiff, UK: Kalloniatis and Fletcher, 1993; Marc et al., 1990). All samples were processed and visualized in an identical manner.

Pattern recognition analysis

Fig. 1 provides a flow chart of the qualitative analysis and pattern recognition analysis performed in this study modified from Hill et al. (2001), Kalloniatis et al. (1996) and Marc et al. (1995). Serial sections labeled for each of the seven amino acids were aligned with one another using image registration (Fig. 1, *image registration*) described by Wolberg and Mangasarian (1990). Briefly, a master image and uncorrected image were selected and 50–100 control points (i.e. anatomical landmarks such as blood vessels, cell bodies) were overlaid between the images with extreme precision using a mathematical model which computes a warping transformation via a first order polynomial function (GCPworks software, PCI Geomatica, Richmond Hill, Ontario, Canada).

Registered micrographs were inverted using a logical NOT operation (Adobe Photoshop CS, Version 8.0 for Windows, Adobe Systems, U.S.A.) so that a pixel value of 0 indicated low immunoreactivity and a maximum value of 255 indicated high immunoreactivity (Fig. 1, *pixel conversion*). Amino acid images could be analyzed without pattern