Int. J. Devl Neuroscience 33 (2014) 62–80

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

Amino acid signatures in the developing mouse retina☆

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ARTICLE INFO

Article history:
Received 22 November 2013
Accepted 13 December 2013

Keywords:
Retina development
Amino acids
Glutamate
GABA
Glycine
Glutamine
Aspartate
Alanine
Taurine
Immunocytochemistry

ABSTRACT

This study characterizes the developmental patterns of seven key amino acids: glutamate, γ-amino-butyric acid (GABA), glycine, glutamine, aspartate, alanine and taurine in the mouse retina. We analyze amino acids in specific bipolar, amacrine and ganglion cell sub-populations (i.e. GABAergic vs. glycinergic amacrine cells) and anatomically distinct regions of photoreceptors and Müller cells (i.e. cell bodies vs. endfeet) by extracting data from previously described pattern recognition analysis. Pattern recognition statistically classifies all cells in the retina based on their neurochemical profile and surpasses the previous limitations of anatomical and morphological identification of cells in the immature retina. We found that the GABA and glycine cellular content reached adult-like levels in most neurons before glutamate. The metabolic amino acids glutamine, aspartate and alanine also reached maturity in most retinal cells before eye opening. When the overall amino acid profiles were considered for each cell group, ganglion cells and GABAergic amacrine cells matured first, followed by glycinergic amacrine cells and finally bipolar cells. Photoreceptor cell bodies reached adult-like amino acid profiles at P7 whilst Müller cells acquired typical amino acid profiles in their cell bodies at P7 and in their endfeet by P14. We further compared the amino acid profiles of the C57Bl/6J mouse with the transgenic X-inactivation mouse carrying the lacZ gene on the X chromosome and validated this animal model for the study of normal retinal development. This study provides valuable insight into normal retinal neurochemical maturation and metabolism and benchmark amino acid values for comparison with retinal disease, particularly those which occur during development.

1. Introduction

Amino acids play essential roles in retinal maturation, function, survival and neurotransmission. Glutamate is present in almost all retinal cells and is the major excitatory neurotransmitter (Ehinger et al., 1988; Fletcher and Kalloniatis, 1996; Kalloniatis et al., 1996; Marc et al., 1990, 1995; Massey, 1990; Wässle and Boycott, 1991). GABA and glycine are the major inhibitory neurotransmitters present in the lateral elements of the retina including horizontal and amacrine cells (Marc, 1989; Massey and Redburn, 1987; Mosinger et al., 1986; Pourcho, 1996; Yazulla, 1986). Other amino acids play roles in retinal metabolism and osmoregulation (Bender, 1985; Tsacopoulos et al., 1998). Glutamine is the predominant precursor of glutamate (Pow and Crook, 1996; Pow et al., 1994) and is present in virtually all neurons (Ishikawa et al., 1996; Kalloniatis et al., 1994). Aspartate and alanine are also metabolic precursors, but are found at a low levels across the retina due to their rapid conversion to other metabolites (Erecinska and Silver, 1990). Taurine is found in high levels in photoreceptors, bipolar cells and Müller cells and is likely to be used for cellular osmoregulation and neuroprotection (Kalloniatis et al., 1996; Marc et al., 1990, 1995; Pasantes-Morales et al., 1986; Pow et al., 1994).

The location of retinal amino acids, their synthesizing, and their degradation enzymes vary considerably in disease states including ischemia (Napper et al., 2001; Neal et al., 1994; Osborne and Herrera, 1994; Sun et al., 2007a), retinal detachment (de Souza et al., 2012; Marc et al., 1998b; Sherry and Townes-Anderson, 2000), retinal degeneration (Fletcher and Kalloniatis, 1996, 1997; Gibson et al., 2013; Okada et al., 2000) retinopathy of prematurity (Downie et al., 2007, 2010), light damage (Oraedu et al., 1980; Wasowicz et al.,

☆ 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).

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http://dx.doi.org/10.1016/j.ijdevneu.2013.12.005

2002), diabetes (Vilchis and Salceda, 1996) and surgical intervention (de Souza et al., 2013). However, many ocular diseases occur during development, meaning disease related changes to amino acid levels can be confounded by those associated with normal retinal maturation. Gibson et al. (2013) illustrated this in a rodent model of inherited retinal degeneration where amino acid neurochemistry was similar between normal and degenerative retinae before eye opening but changed dramatically once rod degeneration had initiated. Thus, in order to use amino acid profiles as a measure retinal disease, it is essential that the normal development of retinal amino acids is fully elucidated.

The development of glutamate, GABA, glycine and glutamine has been quantified in the mouse retina using immunohistochemistry and biochemical assays (Gibson et al., 2013; Orr et al., 1976). Quantification however, is mostly limited in large indiscriminant populations, i.e. all bipolar cells or all ganglion cells. Thus subtle changes in amino acids between retinal sub-populations (i.e. cone and rod bipolar cells) are likely to be missed. Traditional methodology of cell classification is limited for developing tissue as the anatomical or morphologically characteristics of cells may not yet be established. However, in a previous study, we showed that pattern recognition can rigidly classify all cells of the developing mouse retina into unique "theme classes" (Chua et al., 2013). Pattern recognition uses the overlapping immunoreactivity patterns of amino acids to create unique signatures for each cell class and segregates them accordingly with a less than 1% classification error (Marc et al., 1995). As pattern recognition classifies cells regardless of cellular location or morphology, this method is particularly advantageous in analysis of immature tissue as specific cell populations can be tracked even if they do not reach their mature retinal location or morphology until adulthood (Kalloniatis et al., 2013).

This study aims to follow the development of major neurotransmitters: glutamate, GABA and glycine and metabolically significant amino acids: glutamine, aspartate, alanine and taurine in specific populations and sub-populations of retinal cells. We do this by first extracting the unique neurochemical signatures associated with each theme class determined by pattern recognition in our previous study (Chua et al., 2013). We use these signatures to isolate specific cell groups in the developing retina before determining their amino acid content as a function of age. Since theme class nomenclature is arbitrarily assigned based on the primary amino acid present, we regroup theme classes into commonly used cell groups before analysis. For example, for the GABA signature, theme classes **Γ1–Γ6** where collectively analyzed as GABAergic amacrine cells (Chua et al., 2013). This served to permit easy comparison between this work and previous amino acid analysis in other mouse models. We apply this analysis to the transgenic X-inactivation mouse which contains the *lacZ* gene for β-galactosidase on the X chromosome, and allows retinal cells to be tracked by differential β-galactosidase activity due to random inactivation of the transgene (Reese et al., 1995, 1999; Tan and Breen, 1993; Tan et al., 1995). We compare the amino acid profiles of the transgenic X-inactivation mouse with the normal C57Bl/6J mouse and validate the transgenic model for the study of amino acid retinal development.

2. Materials and methods

All the quantitative amino acid data presented in this manuscript were derived from data available from Chua et al. (2013). For ease of referencing, we include below the methodology in obtaining the amino acid labeling pattern as well as an explanation on how data was extracted from the Chua et al. (2013) and Gibson et al. (2013) studies.

2.1. 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).

2.2. Animals and tissue collection

Transgenic hemizygous female mice carrying the lacZ gene in the maternal X chromosome (Tan and Breen, 1993; Tan et al., 1993) were used at P1, 3, 7, 14, and adult (P90). Dissection and retinal incubations were performed under constant fluorescent room lighting of ~200–300 lux. The eyes were enucleated and the anterior structures removed creating an eyecup preparation. Eyecups were immersion fixed in 2% paraformaldehyde and 1% glutaraldehyde for 1 h, dehydrated and placed in resin (ProSciTech, Queensland, Australia). Samples were selected from the central third of the retina for all experiments ($n = 4$ mice for each age group). Mice retinae were cut in the radial planes, stacked and re-embedded in resin. The resin embedded tissue was serially sectioned (200 nm) using an ultramicrotome.

2.3. Immunocytochemistry and antibody characteristics

Post-embedding immunocytochemistry was performed as described previously (Chua et al., 2013; Kalloniatis and Fletcher, 1993; Marc et al., 1990, 2005; Sun et al., 2007a, 2007b). Briefly, serial sections were incubated with the primary antibodies described in Table 1. Antibodies were produced as bovine serum albumin-micromolecule immunogen by standard hapten coupling methods, raised in rabbits and confirmed to be selective for protein glutaraldehyde-linked micromolecule antigens using dot immunoassays (Marc et al., 1990, 1995; Matute and Streit, 1986). Primary antibodies were detected with goat-anti-rabbit secondary antibodies coated with a 1 nm gold particle (British BioCell International, Cardiff, UK). Labeling patterns in this study matched previous work using the C57Bl/6J mouse strain (Gibson et al., 2013).

Tissue was viewed on a Leica DMR light microscope (Leica Microsystems Ltd., Heerbrugg, Germany) and images acquired in 8-bit format corresponding to greyscale images with digital brightness values from 0 to 255 (Fig. 1A). Prior to image capture, light settings were adjusted so image data fell into the mid-range of pixel values. No contrast or brightness adjustments were made prior to image analysis. All samples that underwent quantitative analysis were processed, probed and visualized in an identical manner.

2.4. Pattern recognition analysis

Data for pattern recognition of all cells in the developing mouse retina was extracted from Chua et al. (2013) and briefly described in Fig. 1. Aligned retina images labeled for one of seven amino acids (Fig. 1A) were inverted with a logical NOT operation so that high immunoreactivity displayed a high pixel value over a range of 0–255 (Fig. 1B). Pixels were plotted in 7-dimensional space and using a clustering algorithm, separable theme classes were extracted based on clusters of pixels with common values among the seven amino acids (Jones et al., 2003; Kalloniatis et al., 1996; Marc and Cameron, 2001; Marc and Jones, 2002; Marc et al., 1995; Sun et al., 2007a, 2007b). Theme classes were viewed from the color coded theme map and the cellular identities of theme classes were assessed by correlating amino acid content (derived from cells within these theme maps) with Nissl-stained sections as well as using morphology and retinal location (Fig. 1C; Hill et al., 2001;