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ANATOMICAL CHANGES IN THE PRIMARY VISUAL CORTEX OF THE CONGENITALLY BLIND *CRX*−/− MOUSE

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Abstract—Mutations in the human cone-rod homeobox (*Crx*) gene are associated with retinal dystrophies such as Leber Congenital Amaurosis (LCA), characterized by complete or near complete absence of vision from birth. The photoreceptors of *Crx*−/− mice lack outer segments, and therefore cannot capture light signals through rods and cones, thus resulting in a lack of normal retinal ganglion cell activity from birth. Using specific antibodies to subsets of neurons and markers of activity, we examined the impact of this absence of sensory input on the development of the primary visual cortex (V1) in early postnatal *Crx*−/− mice, before wiring of the visual system is complete, and in adulthood. We revealed that *Crx*−/− mice did not exhibit gross anatomical differences in V1; however, they exhibited significantly fewer calcium-binding protein (parvalbumin and calbindin-D28k) expressing interneurons, as well as reduced nonphosphorylated neurofilament expression in V1. These results reveal that the *Crx* mutation and lack of light stimulation through the photoreceptor pathway regulate the development and phenotype of different neuronal populations in V1 but not its general morphology. We conclude, therefore, that photoreceptor-mediated visual input during development is crucial for the normal postnatal development and maturation of subsets of cortical neurons.

Key words: retinal dystrophy, photoreceptor, neurofilament, calcium-binding proteins, visual development, mutant.

The cone-rod homeobox transcription factor (*Crx*) plays a pivotal role in the morphological differentiation of both cone and rod photoreceptors, and is the earliest expressed photoreceptor marker in the retina (Chen et al., 1997; Furukawa et al., 1997). Mutation of the human *Crx* gene results in either congenital blindness or photoreceptor degeneration (Freund et al., 1997; Swain et al., 1997; Freund et al., 1998; Sohocki et al., 1998). The most severe form of inherited retinal blindness is Leber congenital amaurosis (LCA), which exhibits complete or near complete absence of vision from birth. Research into the molecular basis of LCA in the last 12 years has revealed the underlying disease genes, demonstrating that the *Crx* gene is involved in 70% of the cases (den Hollander et al., 2008).

Crx−/− mice exhibit a phenotype that closely reflects LCA. Indeed, electroretinograms reveal a complete lack of cone/rod response in the *Crx*−/− retina (Foxman et al., 1985). The photoreceptors of these mice lack outer segments, resulting in the complete absence of vision from birth (Furukawa et al., 1997). Degeneration of the outer retinal layers commences postnataly at 1 month of age and continues for 3–4 months, resulting in the complete ablation of the outer nuclear and plexiform layers. Despite marked degeneration of the outer retinal layers, the inner retina remains largely unaltered.

In the developing vertebrate retina, ganglion cells fire spontaneous bursts of action potentials long before the eye becomes exposed to sensory experience after birth. These early bursts are synchronized between neighbouring retinal ganglion cells (RGCs), yielding unique spatiotemporal patterns: “waves” of activity sweep across large retinal areas every few minutes. Both at retinal and extraretinal levels, these embryonic retinal waves are believed to guide the wiring of the visual system using Hebbian mechanisms of synaptic strengthening (Sernagor and Mehta, 2001). However in the *Crx*−/− mouse, RGC waves disappear earlier than normal, becoming replaced by large slow oscillations and strong bursting, persisting beyond the onset of degeneration (Adams et al., 2008), but still transmit through to the visual centers of the brain (Pignatelli et al., 2004; Morrow et al., 2005).

Visual experience has been clearly demonstrated to play an important role in the patterning of the visual centers of the brain (e.g. Wiesel and Hubel, 1963; Hubel and Wiesel, 1970). Ongoing development of visual system areas, especially with respect to the maturation of the neuronal circuitry, is highly dependent on organized retinal activity (Chapman, 2000). At the level of the lateral geniculate nucleus (LGN), for example, patterned retinal activity is required for the formation of eye-specific regions, and this input remains important for the maintenance of areal segregation (Chapman, 2000; Zhou et al., 2003; Demas et al., 2006). However, little is known about whether early retinal activity shapes the development and maturation of cellular subtypes in the visual cortex, although it has been demonstrated that such activity mediates binocular competition important for shaping receptive fields in V1 (Huberman, 2006). Furthermore, studies in the mouse during the precritical period have demonstrated that V1 is susceptible to ex-

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Abbreviations: Crx, cone-rod homeobox gene; LCA, Leber congenital amaurosis; LGN, lateral geniculate nucleus; NeuN, neuronal nuclei specific protein; NNF, nonphosphorylated neurofilament; P, postnatal day; PB, phosphate buffer; PBS, phosphate buffer saline; PFA, paraformaldehyde; RGC, retinal ganglion cell; S1, primary somatosensory cortex; V1, primary visual cortex; wt, wild-type.

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perience dependent competitive modification, in which it was observed that refinement of ipsilateral eye retinotopy is delayed by contralateral deprivation (eyelid closure) but enhanced if the contralateral eye is enucleated. These differences were most apparent at postnatal day 15 (around eye opening), well before the start of the critical period, thus indicating that experience-dependent binocular plasticity is unlinked with the critical period and commences much earlier than originally thought (Smith and Trachtenberg, 2007).

In this present study we address whether deletion of the mouse cone-rod homeobox (*Crx*) gene results in changes in the cytoarchitecture and neuronal phenotype in the visual centers in the brain. By examining specific subsets of interneurons and pyramidal cells, which are generally associated with particular epochs during and after development (Lasek, 1981; Celio, 1990; Hendrickson et al., 1991), we have spatiotemporally profiled the distribution of specific classes of neurons in both the early postnatal (P10; prior to onset of retinal degeneration) and adult (following retinal degeneration) *Crx*−/− mice and compared with wild-type (wt) mice.

EXPERIMENTAL PROCEDURES

Animals and visual stimulation paradigm

The brains of cone-rod homeobox mutant (*Crx*−/−) mice and wild-type (wt) C57BL/6 littermates were collected at postnatal day (P) 10 and at 4–5 months (adult). Animals were maintained on a standard laboratory diet and water *ad libitum*. All animal procedures were conducted under the UK Home Office, Animals (Scientific procedures) Act 1986 ensuring the minimum number of animals were used and experiments were conducted in the most humane manner.

In order to qualify functional activity in the visual centers of the brain, we examined the expression of Fos, the protein product of the immediate early gene *c-fos*, which is rapidly induced following neuronal activation (Morgan and Curran, 1991; Chaudhuri, 1997; Greferath et al., 2004). Animals were placed in a dark room for 16 h prior to the visual stimulus paradigm (protocol adapted from van der Gucht et al., 2005). Following this conditioning period, mice were exposed to ambient light in the laboratory, and additionally to a 500 W light for 1 h before perfusion. To ensure Fos protein expression was only upregulated in the visual cortex, music was kept playing overnight and environmental enrichment (tubes and toys) was provided in the cages. This ensured continuous activation of the auditory and somatosensory cortices, and thus only basal levels of Fos protein expression in these sensory cortical regions would be observed at the time of perfusion.

Tissue processing

Animals were subsequently anaesthetized with an overdose of pentobarbitone sodium (80 mg/kg i.p.) and perfused with 0.1 M heparinized phosphate buffer (PB; pH 7.2, warmed to 37 °C), followed by 4% paraformaldehyde (PFA) in 0.1 M PB. The cerebral tissues were then postfixed for 24 h in 4% PFA containing 10% sucrose at 4 °C, followed by cryoprotection with 30% sucrose in PB. Brains were then slowly frozen on dry ice before being stored at −80 °C. Subsequently, five series of adjacent coronal sections (30 μm thick) (either from the primary visual (V1) or somatosensory cortex (S1)) were cut with a cryostat and stored at −20 °C in a cryoprotective solution (50% 0.05 M PB, 30% ethylene glycol, 20% glycerol) before immunohistochemical analysis (within 14 days). Series cut for Nissl-substance staining were stored in 0.1 M phosphate-buffered saline (PBS), and subsequently processed with 0.1% Cresyl Violet (MP Biomedicals, OH, USA). This histological stain enabled the demarcation of layers and areal boundaries of the cortex.

Immunohistochemistry

All sections were subjected to standard immunohistochemical protocols, which included appropriate negative and positive controls (see Bourne and Rosa, 2006; Bourne et al., 2007; Sia and Bourne, 2008). Sections were removed from the cryoprotectant and washed in 0.1 M PBS (2×20 min). Non-specific protein binding at the secondary antibody stage was prevented by pre-blocking with 5% normal goat serum or 5% normal rabbit serum in PBS-Triton-X for 1 h. Sections were then incubated in respective primary antibodies overnight at 4 °C. Primary antibodies used to detect specific neuronal cell populations were as follows; mouse anti-neuronal nuclei specific protein(NeuN) (1:500, Chemicon International, Temecula, CA, USA), rabbit anti-parvalbumin (1:3000, Swant, Switzerland), mouse anti-calbindin-D28k (1:10,000, Swant, Switzerland), mouse anti-SMI-32 (1:2000, Covance Research Products, Berkeley, CA, USA), rabbit anti-c-Fos (1:15,000, Calbiochem, Germany), mouse anti-synaptophysin (1:1000, Chemicon International, Temecula, CA, USA) at 4 °C for 16 h. This was followed by incubation with either of the following secondary antibodies for 2 h at room temperature: biotinylated rabbit anti-mouse immunoglobulin (1:500, Dako, Glostrup, Denmark) or HRP goat anti-rabbit (1:250, Zymed, CA, USA). Sections were then processed with streptavidin–biotin horseradish peroxidase, followed by streptavidin-biotinylated HRP (1:200; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) for 1 h. After a further 2×10 min washes in 0.1 M PBS, immunoreactivity (IR) was visualized with the use of metal-enhanced diaminobenzidine (DAB) substrate kit (Immunopure®, Pierce Biotechnology Inc., Rockford, IL, USA, with reaction being stopped after 1–2 min). All sections were then mounted, dehydrated in graded alcohols, defatted in xylene and coverslipped with DPX (BDH, Poole, Dorset, UK). For double immunofluorescence, sections were incubated with a primary antibody followed by either goat anti-rabbit Alexa 488 (1:600; molecular probes) or goat anti-mouse Alexa 594 (1:800; molecular probes) secondary antibody for 1 h at room temperature. Sections were washed 3×20 min with 0.1 M PBS, then coverslipped with Fluoromount (Dako).

Qualitative and quantitative analyses

Demarcation of V1 was achieved on the basis of previous studies of nonphosphorylated neurofilament (NNF) immunolabeling in the mouse (Van der Gucht et al., 2007; van Brussel et al., 2009). In the present study, analyses was performed on a region of V1 at approximately −4.00 mm from Bregma, in the more lateral half towards the border with V2PL (Van der Gucht et al., 2007), which consists of the binocular zone (van Brussel et al., 2009).

Sections were examined for brightfield using a Zeiss Axioplan imaging microscope. Low- (10x)—and high-power (40x) photomicrographs (1300×1030 dpi) were taken on a Zeiss Axiocam digital camera connected to Axiovision software (v. 4.4; Zeiss). Images were cropped and sized using Adobe Photoshop CS and montages were generated in Illustrator CS. Fluorescence images of Alexa Fluor® 488 (red) and Alexa Fluor® 594 (green) labeled sections were acquired and digitized separately. Images were also obtained using a Leica SP5 5 channel confocal microscope, equipped with a HCX PL APO 63×/1.40 objective, running LAS AF software (Leica MicroSystems, Manheim, Germany).

For cellular quantification, sections of *Crx*−/− and wt mice immunostained for Fos, NeuN, parvalbumin and calbindin-D28k were analysed on an Olympus microscope and analyzed with