Retinocollicular synaptogenesis and synaptic transmission during formation of the visual map in the superior colliculus of the wallaby (*Macropus eugenii*)

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Abstract

Spontaneous retinal activity has been implicated in the development of the topographic map in the superior colliculus (SC) but a direct demonstration that it reaches the colliculus is lacking. Here we investigate when the retinocollicular projection is capable of transmitting information from the retina in a marsupial mammal, the wallaby (*Macropus eugenii*). The projection develops postnatally, allowing *in vivo* analysis throughout development. Quantification of retinocollicular synaptogenesis has been combined with electrophysiology of the development and characteristics of retinocollicular transmission, including *in vivo* and *in vitro* recording in the same animals. Prior to postnatal day (P) 12–14 *in vitro* recording detected only presynaptic activity in retinal axons in the colliculus, in response to stimulation of the optic nerve. Postsynaptic responses, comprising both *N*-methyl-D-aspartate (NMDA) and non-NMDA responses, were first detected *in vitro* at P12–14 and retinal synapses were identified. In contrast, postsynaptic responses to optic nerve stimulation could not be detected *in vivo* until P39, around the time that retinal axons begin arborizing. Around this age density and numbers of total synapses began increasing in the retinorecipient layers of the colliculus. By P55–64, the numbers of retinal synapses had increased significantly and density and numbers of retinal and total synapses continued to increase up to P94–99. During this time the map is undergoing refinement and degenerating axons and synapses were present. The discrepancy between *in vitro* and *in vivo* onset of functional connections raises the question of when retinal activity reaches collicular cells in the intact, unanaesthetized animal and this will require investigation.

Introduction

The spatially ordered projection of retinal ganglion cells onto their targets in the mammalian superior colliculus (SC) is widely used as a model system to study the development of neuronal connections, especially the formation of topographic maps. Development of the retinotopic map in the SC has been characterized anatomically in a number of species (Simon & O'Leary, 1992; Mark *et al.*, 1993; Chalupa *et al.*, 1996; Ding & Marotte, 1997; Chalupa & Snider, 1998). The initial retinal projection is only coarsely ordered and a precise map arises primarily through a remodelling process that includes the loss of aberrant branches and refinement of arbors. While recent investigations have concentrated on the molecular cues underlying the formation of such maps (see McLaughlin & O'Leary, 2005), studies of the formation of eye-specific connections in the brain indicate that neuronal activity arising in the retina might also play a role in the development of the map in the SC (Penn *et al.*, 1998; Huberman *et al.*, 2002; Huberman *et al.*, 2003) but see Cook *et al.* (1999); Crowley & Katz (1999, 2000). Indeed, pharmacological and genetic manipulations (Simon *et al.*, 1992; Grubb *et al.*, 2003; McLaughlin *et al.*, 2003; Chandrasekaran *et al.*, 2005; Mrsic-Flogel *et al.*, 2005) have

implicated spontaneous retinal activity in the process. However, for such activity, either permissive or instructive, to influence the development of the topographic map it must be transmitted to the SC and direct evidence for this is lacking. Few studies have examined the functional development of the retinocollicular projection especially in placental mammals (Molotchnikoff & Itaya, 1993; Itaya *et al.*, 1995; Lim & Ho, 1997) where the initial stages of axonal ingrowth occur prenatally, necessarily limiting such studies to *in vitro* preparations (Reece & Lim, 1998). Further, in the rat there is a discrepancy in the reported time of onset of synaptic transmission, which was found to occur prenatally *in vitro* (Reece & Lim, 1998) but postnatally *in vivo* (Lim & Ho, 1997).

In a marsupial mammal, the wallaby, development of the retinocollicular projection is entirely postnatal. The first retinal axons reach the SC a few days after birth (Ding & Marotte, 1996) and the stages in forming an ordered topographic map are well described (Mark *et al.*, 1993; Ding & Marotte, 1997). In this study we have investigated how activity arising in the retina might influence the formation of the topographic map in the SC, by examining the structural and functional development of the retinocollicular projection. Quantitative ultrastructure of retinocollicular synaptogenesis has been combined with an electrophysiological investigation of the onset and characteristics of retinocollicular transmission, including the testing of transmission in both *in vivo* and *in vitro* conditions in the same animals. This revealed that although the capacity for retinocol-

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lular transmission is present at early stages of development in the wallaby, it is not detectable *in vivo*.

Materials and methods

Animals

Wallaby pouch young (*Macropus eugenii*) were obtained from a breeding colony. Experiments were approved by the Animal Ethics Experimentation Committee of the Australian National University. Animals were either of known age (P30–38 and younger) or age was determined from a chart of head measurements of animals of known age ($n = 122$). A straight line regression of age on head length resulted in a table of predicted age as a function of head length with a standard error of 2 days.

Electron microscopy

Time points chosen were based on developmental milestones in the retinocollicular projection described in previous studies (Mark *et al.*, 1993; Ding & Marotte, 1996, 1997; Freeman *et al.*, 1997): Postnatal day (P)14 ($n = 3$) – optic axons cover most of the SC but are still very sparse; P28–30 ($n = 4$) – optic axons are distributed in coarse retinotopic order and are largely unbranched without terminal arbors; P38 ($n = 3$) – immediately prior to the onset of arborization; P42–44 ($n = 4$) – onset of arborization rostrally, concomitant with onset of synaptic activity there in response to optic nerve stimulation *in vivo*; P59–64 ($n = 4$) – axons from all retinal quadrants have arborized and many aberrant axons have disappeared; P94–99 ($n = 3$) – discrete terminal zones have decreased in size and aberrant axons are absent. Animals aged from P14–28 were anaesthetized by hypothermia while older animals were anaesthetized by intramuscular ketamine (Parke–Davis, 0.05–2 mg/100 g body weight) and xylazine (0.1–0.2 mg/100 g body weight). The retinal projection to the SC was labelled by an injection of 5% horseradish peroxidase conjugated to wheat germ agglutinin (WGA-HRP) in one eye. After 10–28 h survival, to allow for WGA-HRP transport, animals were deeply anaesthetized and perfused briefly with 0.9% sodium chloride followed by 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 for 30 min. The SC was dissected out and kept overnight at 4 °C in 0.1 M PB. After embedding in 4% agar, coronal sections through the SC were cut at a thickness of 100 µm with the aid of a vibratome into 0.1 M PB and reacted to visualize anterogradely transported WGA-HRP according to the cobalt-glucose oxidase diaminobenzidine method (Plummer & Behan, 1993). For light microscopy, alternate sections were mounted on slides, air-dried overnight, dehydrated and coverslipped. Every fourth section was processed for electron microscopy. Sections of the SC contralateral to the injected eye were divided into medial and lateral samples and postfixed in 1% osmium tetroxide in 0.1 M PB at pH 7.4 containing 1.5% potassium ferrocyanide for 2 h then dehydrated and embedded in Araldite. Ultrathin sections were cut and stained with lead citrate. Sections were examined in a Hitachi 7100 electron microscope. For the quantitative analysis a series of tracks at right angles to the pial surface were made through the entire depth of the retinorecipient layers of the SC (determined from adjacent sections mounted for light microscopy) at a magnification of 18 500 times. Three to four such tracks, of 5.9 µm in width, were made for both medial and lateral samples taken from the rostral one quarter of the SC in each animal. Every terminal making a synapse was photographed, the criterion for a synapse being a minimum of three synaptic vesicles adjacent to a postsynaptic membrane with a clear specialization. The

synaptic contact length of both unlabelled synaptic terminals and those containing WGA-HRP reaction product was measured at each age. For animals of age P14–P38 all synaptic contact lengths were measured. For older animals a minimum of 24–31 from each medial and each lateral sample were measured. The density of all labelled and unlabelled synapses was calculated. Mean numbers of both types of synapses per track through the retinorecipient layers were also calculated to take into account the increase in thickness of these layers during development. The total area of tissue examined in each animal ranged from 4307 to 7434 µm². Control tissue came from two animals, aged P90 and P100, which did not receive intravitreal injections of WGA-HRP but in which the tissue was processed as described above.

Electrophysiology

For *in vitro* studies of whole collicular preparations 39 pouch young, aged from P4 to P36 were anaesthetized by hypothermia and decapitated. The head was quickly transferred to a dissecting bath superfused with chilled (below 10 °C) isotonic sucrose solution with the following composition: sucrose, 266 mM; KCl, 3 mM; CaCl₂, 2 mM; MgCl₂, 1 mM; NaHCO₃, 17 mM; D-glucose, 12 mM; saturated with 95% O₂, 5% CO₂. After immersion a craniotomy was carried out to expose the brain. The brain was then gently lifted to reveal the optic nerves, which were cut as close to the eye as possible. The brains were split longitudinally along the midline except at the optic chiasma, where both optic nerves were left attached to the side used for recording. The cerebral cortex was removed. After the dissection and a rest period of approximately 1 h, the preparation was transferred to the recording chamber. The preparation was laid on its medial side and secured with nylon threads onto the Sylgard base of the chamber. The chamber was superfused with artificial cerebrospinal fluid (ACSF) with the following composition: NaCl, 124 mM; KCl, 5 mM; CaCl₂, 2 mM; MgSO₄, 2 mM; NaHCO₃, 26 mM; NaH₂PO₄, 1.25 mM; D-glucose, 11 mM; saturated with 95% O₂ and 5% CO₂, and bath temperature was maintained at 29 °C.

For *in vitro* collicular slices ten pouch young, aged from P40 to P142 were anaesthetized as above, both colliculi were removed and placed in chilled ACSF saturated with 95% O₂ and 5% CO₂, trimmed and mounted for sectioning. Sagittal slices, 400-µm thick, were cut on a vibratome in chilled isotonic sucrose solution saturated with 95% O₂ and 5% CO₂. Prior to recording they were transferred to ACSF saturated with 95% O₂ and 5% CO₂ at 37 °C for 30 min and then held at room temperature for at least 60 min. Recording was at 30 °C.

In vivo experiments were performed on six pouch young wallabies of either sex, aged from P25 to P49. The pouch young were anaesthetized with intraperitoneal injection of ethyl carbamate (Urethane, Fluka AG, 20% w/v) at a dose of 3 mg/100 g body weight. Additional anaesthesia was given 2–4 times at one-quarter of the original dose every half hour and thereafter when needed. The animal was placed in an artificial pouch consisting of a flexible polythene bag surrounded by a water jacket kept at 37 °C. The head, held in a holder mounted on a ball joint, was fixed in a standard position with the palpebral fissure horizontal, as measured by a sighting spirit level. The electrocardiogram was recorded continuously and monitored on a chart recorder and loudspeaker. This served as an indicator of the physiological condition and level of anaesthesia of the animal. A drill hole approximately 1 mm in diameter was made in the skull directly above the SC. The dura was pierced and the recording electrode was advanced by a piezoelectric stepper (Burleigh Instruments) so angled that the electrode penetrated the colliculus perpen-

dicular to the collicular surface. At the end of the *in vivo* experiment whole collicular preparations from each animal were set up for *in vitro* recordings as described above.

Recording electrodes for both *in vitro* and *in vivo* experiments were made of glass capillary filled with either 2 M sodium acetate or 1 M sodium chloride and had a resistance of 1 M Ω or less. Optic nerve stimulation (whole colliculus preparations) or optic tract stimulation (collicular slice preparations) was delivered by a glass coated bipolar tungsten electrode. Square pulses (0.02–0.2 ms duration, 1–80 V) were used at a frequency of 1–60/s for *in vitro* experiments. For the *in vivo* study, the eyelid and cornea were cut horizontally and the lens and most of the vitreous humour removed. The stimulating electrode was placed on the contralateral optic nerve head under visual guidance. The optic nerve was stimulated with square pulses using similar parameters to those used in the *in vitro* experiments. The stimulus strength was kept below that which would produce any movement of the eye or circumorbital tissues. Optic nerve-evoked potentials recorded in the superior colliculus were amplified using a Neurolog AC amplifier (Neomedix Systems) and band pass filtered at 0.2–2000 Hz for *in vivo* experiments. An Axoclamp amplifier (Axon Instruments) was used for *in vitro* preparations and the signal was low pass filtered (DC to 2000 Hz). The signal was digitized at 10 kHz, averaged and stored using a personal computer. A calibration pulse of 100 μ V was sometimes given before the nerve stimulus.

Pharmacological agents used during *in vitro* recording, bicuculline methiodide (+/-)-2-amino-5-phosphovaleric acid (APV) and 6,7-dinitroquinoxaline-2,3-dione (DNQX), were all purchased from Research Biochemicals International. They were prepared as 1000 $\times$ stock solutions and added directly to the ACSF. DNQX was prepared using dimethylsulfoxide (DMSO). In two control experiments the same concentration of DMSO added to the bath had no effect on the optic nerve-evoked potential recorded in the colliculus. For recording of activity in the optic nerve in response to stimulation in the SC a tight fitting suction electrode was used.

Results

Anatomy

HRP reaction product appeared as small electron dense profiles of variable shape in axons and axon terminals (Plummer & Behan, 1993; Fig. 1B–E; see also Fig. S1 in Supplementary material). They were not seen in structures that could be identified as dendrites, cell bodies or glia, suggesting that there was no transneuronal transport of WGA-HRP in this situation. Such profiles were absent in controls that did not receive an intravitreal injection of WGA-HRP prior to histochemical processing.

The mean synaptic contact length of synapses in medial and lateral samples, both labelled and unlabelled, did not differ significantly at each age group (two-sided, unpaired *t*-test, data not shown), meaning that synaptic densities and numbers could be validly compared between these samples at each age. The only significant difference found was in unlabelled synapses at P29. Density (numbers/100 μ m²) was significantly higher ($P = 0.003$) laterally (1.15 ± 0.11) than medially (0.47 ± 0.09) as was the number of synapses/column (14.41 ± 1.41 laterally, 6.36 ± 1.21 medially; $P = 0.005$). Results from medial and lateral samples have been combined in Fig. 2. Mean synaptic contact lengths of labelled and unlabelled synapses did not differ significantly over age (ANOVA, data not shown), allowing densities and numbers to be validly compared. Inputs from interneurons, deeper collicular layers and brainstem may contribute to unlabelled synapses.

At P14 the superficial retinorecipient region of the SC had large extracellular spaces (Fig. 1A), a feature of immature SC (Cooper & Rakic, 1983; Warton & McCart, 1989; Correa-Gillieron & Cavalcante, 1999). Terminals making synaptic contact were extremely rare, especially labelled terminals (Fig. 1B). Synapses often appeared to be made between axonal growth cones of irregular shape containing flocculent material (Fig. 1A) and dendritic growth cones with similar characteristics (Skoff & Hamburger, 1974) (Fig. 1C). Labelled axons, sometimes containing vesicles, were seen more frequently (Fig. 1A). At P28–30 and P38 extracellular space remained prominent and bundles of unmyelinated axons, some containing reaction product, increased in the superficial layers (Fig. 1D) but synapses remained low in number and density (Fig. 2). A labelled growth cone just beneath the pial surface is seen in Fig. 1D making a synapse behind the tip. Rare labelled axosomatic synapses were also seen (Fig. 1E). At P42–44 there was a significant increase in total numbers of synapses compared to the previous age (Fig. 2D; ANOVA, $P = 0.02$) although labelled synapses (Supplementary material, Fig. S1, A) showed no significant changes (Fig. 2A and B). Extracellular space was also reduced. The most striking feature of the neuropil at P55–64 was the presence of numerous degenerating profiles, including unlabelled (Fig. 1F) and labelled (Fig. 1G) synapses and axons (Fig. 1H). Despite this there were significant increases in both density (Fig. 2C, ANOVA, $P = 0.03$) and numbers (Fig. 2D, ANOVA, $P = 0.005$) of total synapses over that seen at P42–44. While labelled synapses had increased (Fig. 2A and B), this was not significantly different compared to the P42–44 data. However, a significant increase in numbers of labelled synapses was detected over the period from P14 to P55–64 (Fig. 2B, ANOVA, $P = 0.04$). By P94–99 there were further large increases in the density (Fig. 2A and C) and numbers (Fig. 2B and D) of labelled and total synapses (ANOVA; labelled synapses, density $P = 0.02$; numbers $P = 0.003$; total synapses, density $P = 0.004$; numbers $P = 0.003$). Overall, the increases in these measures over the period P29 and P94–99 were extremely significant (ANOVA, $P < 0.0001$). Degeneration was still prominent at P94–99, and included neuronal profiles containing vesicles and synapses and dendrites (Supplementary material, Fig. S1, B and C, respectively). Extracellular space had decreased further.

Electrophysiology – *in vitro* recording

A diagram of the *in vitro* preparation is shown in Fig. 3A. At P4 an optic nerve-evoked potential of small amplitude (20–80 μ V) was detected only in the superficial layer of the rostralateral region of the SC (Fig. 3A, position a). The response attenuated more caudally (Fig. 3A, position b) and no response was observed beyond one-quarter of the rostrocaudal extent of the colliculus (Fig. 3A, position c). This is consistent with a previous histological study (Ding & Marotte, 1996), showing that retinal afferents have just arrived in the rostralateral region of the SC at this age. At P9 in the rostral SC (Fig. 3B) a response characterized by two negative peaks was observed when the stimulation frequency was kept below 0.03 Hz. At approximately 1 Hz this waveform changed to a single peak response with a reduction in amplitude (Fig. 3B, last trace), suggesting that some axons are not capable of following the higher stimulation rates at this age. The rate used in all subsequent experiments was standardized to once every 30 s (0.03 Hz). At P4–9 the response attenuated when the recording electrode penetrated into the deeper layers of the colliculus and no reversal of the waveform was observed. Bath application of glutamate antagonists had no effect on the response (data not shown). These findings indicate that the responses recorded

FIG. 1. (A) Low power view within the retinorecipient layers of the superior colliculus in an animal on postnatal day 14. Extracellular space is prominent (asterisks) and few synapses are present (arrowhead). The axon terminal of the one shown contains floccular material (f) characteristic of a growth cone. Numerous unmyelinated axons are present. Retinal axons (arrows), including preterminal axons containing vesicles, can be identified by the presence of electron dense reaction product. Scale bar, 1 μ m. (B and C) Examples of labelled retinal synapses on P14. Arrows indicate the electron dense reaction product and arrowheads indicate the synaptic contact. The postsynaptic profile in C contains floccular material characteristic of a dendritic growth cone. Scale bars, 0.5 μ m. (D) Low power view at the pial surface (p) in an animal on P38. Numerous unmyelinated axons are present. Some can be identified as retinal (arrows) by the presence of electron dense reaction product. An arrowhead indicates a putative synapse made by a retinal axon proximal to its growth cone (g). e, glial endfoot. Scale bar, 1 μ m. (E) Axosomatic synapse (arrow) made by a retinal terminal in the retinorecipient layer of the SC in an animal on P38. Electron-dense reaction product in the terminal is indicated by an arrowhead. Scale bar, 1 μ m. (F–H) Degenerating profiles in the retinorecipient layers of the SC in an animal on P64. (F) Degenerating synapse characterized by increased electron density in the axon terminal. The arrow indicates the synaptic contact. (G) Degenerating retinal synapse (arrow), identified by horseradish peroxidase reaction product (arrowhead), containing electron dense debris. (H) Degenerating axon. Scale bars, 0.5 μ m.

in the more superficial layers of the SC originated from presynaptic volleys in optic axons.

At P12–14 a negative going potential could be recorded at the surface in the rostrolateral half of the SC in response to optic nerve stimulation and it extended to the rostral three-quarters by P20. This mirrors the pattern of ingrowth of retinal axons (Ding & Marotte, 1997). Within the rostral quarter of the SC the waveform of the evoked potentials was more complex than seen previously. The response changed from a negative wave on the surface to a positive wave of slightly longer latency at 400 μ m (Supplementary material, Fig. S2), indicating that it is arising from the synaptic current of postsynaptic cells (Freeman *et al.*, 1997). To confirm that the response included a

postsynaptic component, glutamate blockers were used in two preparations from animals aged P12 and P14 (Fig. 3C and D). Removal of bath Mg^{++} revealed a slow potential (Fig. 3C, second trace) which could be blocked by the addition of APV, a *N*-methyl-D-aspartate (NMDA) channel antagonist (Fig. 3C, third trace). The fast component (arrow in Fig. 3C) was blocked by the non-NMDA glutamate blocker, DNQX (Fig. 3C, fourth trace). The remaining waveform was presumably generated by presynaptic volleys in optic axons. DNQX had no effect on axonal conduction along the optic nerve (Fig. 3D). These findings suggest that optic axons have formed functional synapses in the superficial layers of the rostral SC as early as P12–14.

FIG. 2. (A) Mean density (number/100 μm^2) of labelled, retinal synapses in the SC at different ages. (B) Mean numbers of labelled, retinal synapses calculated from counts in tracks 5.9- μm wide through the retinorecipient layers of the SC at different ages. (C) Mean density (number/100 μm^2) of all synapses in the retinorecipient layers of the SC at different ages. (D) Mean numbers of all synapses calculated from counts in tracks 5.9- μm wide through the retinorecipient layers of the SC at different ages. Error bars show the standard error of the mean.

By P26–36 ($n = 3$) an evoked response, reversing with depth, could be recorded from the entire SC and glutamate blockers had a similar effect to that described at P12–14. Thus by P26 optic nerve axons are capable of mediating synaptic activity across the entire SC.

Although a detailed study was not made in older animals, both NMDA (responses blocked by APV) and non-NMDA responses (blocked by DNQX) contributed to the glutamatergic postsynaptic responses at P59–60 and at P140–142, just after eye opening. In the latter age group non-NMDA responses dominated the response.

Comparison of *in vitro* and *in vivo* recording

Current source density analysis of evoked potentials in response to optic nerve stimulation *in vivo* did not detect synaptic activity until P45 (Freeman *et al.*, 1997), in contrast to our present results *in vitro*, where it was demonstrated at P12–14. To test for *in vitro* vs. *in vivo* differences and to minimize the variability between animals, *in vivo* recordings were made in the SC in six animals aged P25 to P49, followed by *in vitro* recordings in the same animals. In four animals aged younger than P39, there was no reversal of the polarity of the optic nerve-evoked response recorded *in vivo*. A negative-going waveform was recorded at the surface and this attenuated when the electrode was advanced deeper into the SC, indicating presynaptic activity only. However, a clear polarity reversal appeared *in vitro*. Figure 4 illustrates responses in an animal aged P32. Only a negative waveform was recorded *in vivo*, while *in vitro* the

FIG. 3. (A) Left hand panel shows a diagram of a lateral view of the *in vitro* preparation. Rostral is to the left. The recordings obtained from positions a, b and c in the colliculus of an animal on P4, after stimulation of the optic nerve are shown in the corresponding panels on the right. As the recording electrode was moved caudally the response decreased (b) and then disappeared (c). (B) *In vitro* recordings from the rostral colliculus of an animal on P9 in response to stimulation of the optic nerve at different frequencies of stimulation. With increasing frequency the waveform changed to a single peak and attenuated. (C) Effects of APV and DNQX on the response in the rostral quarter of the colliculus to stimulation of the optic nerve in an *in vitro* preparation from an animal at P14. Each trace is an average of ten sweeps at a frequency of stimulation of 0.03 Hz. Apart from the responses shown in the first and last traces 10 μM bicuculline was present in the ACSF to block any local inhibition. The control trace shows the response in ACSF at a depth of 300 μm from the surface. In magnesium-free ACSF (second trace) a slow potential was present, which was blocked by 50 μM APV (third trace). The fast component of the response (arrow) was unaffected but was blocked by the addition of 2 μM DNQX (fourth trace). The residual response presumably represents the presynaptic volley in optic axons (see D). Responses returned to control levels in magnesium-free ACSF (fifth trace) and normal ACSF (sixth trace). (D) Control experiment showing that DNQX had no effect on optic nerve conduction. The control trace shows the response in the optic nerve to stimulation in the colliculus. With the addition of DNQX (2 μM) there was no change in the response.

waveform reversed in polarity in the deeper layers of the SC, indicating functional retinocollicular transmission. Addition of 20% urethane *in vitro* at this age, approximately 100 times the dose *in vivo*, did not block synaptic transmission (Supplementary material, Fig. S3). This argues against a direct effect of anaesthetic on retinocollicular transmission *in vivo*. In the remaining two animals aged P39 and P49 the polarity of the response reversed with depth *in vivo*, indicating postsynaptic activity. Under *in vitro* conditions, a similar reversal was

FIG. 4. Responses at different depths in the colliculus to stimulation of the optic nerve in an animal on P32. Each trace is an average of five sweeps at a frequency of stimulation of 0.03 Hz. Responses recorded *in vivo* show a non-reversing wave form with depth indicative of presynaptic activity only, representing the response of optic axons. *In vitro* there was a clear reversal of the potential with depth, indicative of a postsynaptic response.

observed between 600 and 750 μm below the surface of the SC, close to the recording site *in vivo* (data not shown).

Discussion

This study in the wallaby has shown that optic axons, *in vitro*, are capable of conducting impulses from the time they reach the SC. Postsynaptic responses first appear *in vitro* at P12–14 and optic synapses are present at this time. However, *in vivo* postsynaptic responses are first evident much later, at P39, about the time that synapses begin to increase in number in the retinorecipient layers of the SC. By P55–64 identified optic synapses have increased significantly and continue to increase up to P94–99, during the period when the retinocollicular map is refining and degenerating profiles are common.

Development of retinal synapses and map formation

In the initial stages of formation of the retinotopic map in the SC, largely unbranched retinal axons, tipped with growth cones are distributed in coarse retinotopic order (Ding & Marotte, 1997). The present study shows that these axons, including growth cones, are already making synapses. In contrast, in the mouse (Sachs *et al.*, 1986) synapses were not detected prior to axonal arborization. In the wallaby axons begin arborizing at P41–45 and in their retinotopically correct positions (Ding & Marotte, 1997). Both retinal and total synaptic density and numbers increase from around this time up to the oldest age examined. Concomitant with this, the topographic map is undergoing refinement with elimination of aberrant axons and a decrease in size of terminal zones (Ding & Marotte, 1997). Refinement is achieved, at least in part, by degeneration of both axons and axon terminals as shown by their presence in the SC during this time. The degeneration coincides with the death of retinal ganglion cells (Spira & Marotte, 1989).

NMDA and non-NMDA components comprise the retinocollicular response

The glutamatergic excitatory response comprised both NMDA and non-NMDA components from its onset. These components have also

been described in the retinocollicular pathway of the rat at the time of onset of synaptic transmission (Reece & Lim, 1998) and also later in development (Isa *et al.*, 1998). They are also a feature of glutamatergic transmission in early postnatal mouse colliculus (Jüttner *et al.*, 2001). NMDA receptors are involved in activity-dependent refinement of developing projections (Constantine-Paton *et al.*, 1990) and antagonists disrupt the removal of aberrant retinal axons in the rat colliculus (Simon *et al.*, 1992). In the wallaby they have the potential to mediate activity-dependent effects both during the early stage of coarse retinotopy and during the refinement of the projection after terminal zones form. However, any effect of retinal activity during the early stage may be precluded, as retinocollicular synapses may not be transmitting neural activity *in vivo* (see below). It is possible that the synapses may be mediating trophic effects at this stage. For example, brain-derived neurotrophic factor is expressed in the visual system of the wallaby (Marotte *et al.*, 2004), as it is in other species and it has multiple roles in visual development (Cohen-Cory & Lom, 2004).

Onset of transmission *in vivo* is later than *in vitro*

There was a difference of approximately a month between the onset of retinocollicular transmission, detected *in vitro* (P12) and *in vivo* (P39). The onset of transmission *in vivo* is around the time that retinal axons begin arborizing in the SC (Mark *et al.*, 1993; Ding & Marotte, 1997) and is well before the possibility of light-evoked responses. In the wallaby photoreceptor outer segments do not begin to form until P85 (Spira & Marotte, 1989) and eye opening is around P140. Furthermore, we find that the onset *in vivo* is not accompanied by either the initiation of, or a significant increase in retinocollicular synaptogenesis. In the rat SC a similar difference between *in vitro* (at E20; Reece & Lim, 1998) and *in vivo* (at P2; Lim & Ho, 1997) onsets has been reported and in both the rat (Simon & O'Leary, 1992) and wallaby the *in vivo* onset is close to the time that axons begin arborizing in the SC.

There are a number of possible reasons for the difference in onset between the two conditions. Anaesthetic and temperature would be likely candidates but the anaesthetic used *in vivo* did not suppress synaptic transmission *in vitro* and the lower temperature *in vitro* compared to *in vivo* would be more likely to detrimentally affect rather than enhance potentials. We consider firstly, that the *in vitro* preparation may not reflect the real *in vivo* physiology. *In vitro*, inputs to the SC have been removed or disrupted, which may normally inhibit retinal responses, although cortical input is not one. Cortical axons do not reach the SC until P71 (Sheng *et al.*, 1990) and are not functional until around eye opening (Freeman *et al.*, 1997). Secondly, an increase of up to 60% in synapse numbers has been reported in slice preparations compared to perfusion fixed hippocampus (Kirov *et al.*, 1999). The suggestion that this is in response to the loss of synaptic activity when the slices are first removed from the rest of the brain is supported by experiments in hippocampal slices in which transmission was blocked (Petrak *et al.*, 2005). If a similar increase in synaptogenesis occurred in the present experiments *in vitro* this may have increased postsynaptic responses to a detectable level. Thirdly, a suppressive mechanism may exist *in vivo* to inhibit the onset of electrical activity. There is a frequency-dependent gamma-aminobutyric acid (GABA) mediated attenuation of conduction in the optic nerve of the developing rat (Sakatani *et al.*, 1991; Lim & Ho, 1998) and wallaby (Lim, 1997). This may act to prevent the transmission of excessive activity from the retina in the early postnatal period when local inhibitory mechanisms are poorly developed in the SC (Warton *et al.*, 1990; Shi *et al.*, 1997). *In vitro*, GABA may leach out and improve conduction in the optic nerve (Reece & Lim, 1998).

Implications for the role of activity in formation of the map

A number of recent studies have emphasized a role for spontaneous retinal activity in the development of coarse retinotopy in the SC (Mrsic-Flogel *et al.*, 2005) and in the refinement of the map in the lateral geniculate nucleus (Grubb *et al.*, 2003) and the SC (Chandrasekaran *et al.*, 2005). However, the only study to demonstrate that such activity reaches the visual centre under investigation was an *in vitro* study of the retinogeniculate pathway in the mouse (Mooney *et al.*, 1996). The present study shows that activity arising in the retina has the potential to influence the formation of the topographic map over almost its entire development but the later onset of synaptic transmission *in vivo* suggests that it is only during the period of arborization and refinement that activity may reach the SC. We also observed attenuation of optic nerve responses at frequencies considerably less than those that spontaneous retinal activity can reach (Galli & Maffei, 1988). Further, spontaneous activity in the rat SC during refinement of the projection was unaffected by blocking transmission in the optic nerve (Itaya *et al.*, 1995). All these findings indicate that investigation of the developing retinocollicular pathway in an unanaesthetized, intact animal will be necessary to determine exactly when retinal activity reaches the SC. This would be feasible in the wallaby, with its protracted postnatal development of the retinocollicular pathway.

Supplementary material

The following supplementary material may be found on

<http://www.blackwell-synergy.com>

Fig. S1. Retinal synapse and degenerating profiles in the SC.

Fig. S2. Evoked responses in the SC *in vitro* on P12.

Fig. S3. Effect of urethane on the evoked response in the SC *in vitro* on P32.

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Abbreviations

ACSF, artificial cerebrospinal fluid; APV, (+/-)-2-amino-5-phosphovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; P, postnatal day; PB, phosphate buffer; SC, superior colliculus; WGA-HRP, horseradish peroxidase conjugated to wheat germ agglutinin.

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