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The life, death and regenerative ability of immature and mature rat retinal ganglion cells are influenced by their birthdate

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ABSTRACT

The extensive period of retinal ganglion cell (RGC) neurogenesis in the rat is associated with a protracted sequence of arrival of their axons into central targets such as the superior colliculus (SC) (Dallimore et al., 2002). Using *in utero* 5-bromo-2'-deoxyrudine (BrdU) injections to label early (embryonic day (E) 15) or late (E18 or E19) born RGCs, we now show that E15 RGCs with axons that enter the SC prenatally undergo programmed cell death earlier than late-born RGCs whose axons only reach the SC late in the first postnatal week. These late-born RGCs do not begin to die until postnatal day (P) 5/6. Removal of retrograde trophic support by P1 SC ablation initially only affects E15 RGCs; however by P5 death of late-born RGCs is increased, confirming that a switch to target dependency is delayed in this cohort. In a further experiment it was found that, following complete rostral SC transection at P2, the proportion of post-lesion axons originating from E19 RGCs was significantly greater than the proportion that normally makes up the retinotectal projection. Thus, even in neonatal brain, uninjured late-arriving axons are more likely to grow across a lesion site than injured axons undergoing regeneration. To study if birth date also affects regenerative potential in adulthood, autologous peripheral nerve (PN) was grafted onto the cut optic nerve in mature BrdU labelled rats. We found that, compared to E15 RGCs, a significantly greater proportion of late-born RGCs survived axotomy, but comparatively fewer of these surviving E19 RGCs regrew an axon into a graft. In summary, this research shows that the birthdate of RGCs significantly impacts on their subsequent life history and response to injury. Understanding how developing central nervous system (CNS) neurons acquire dependency on target-derived trophic support may lead to new strategies for enhancing survival and regeneration in adult CNS.

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Introduction

Neurons in the ganglion cell layer (GCL) are born in a central-to-peripheral gradient, with large cells generated before small cells at any particular location (Reese and Colello, 1992; Rapaport et al., 2004). Retinal ganglion cell (RGC) genesis begins at embryonic day (E) 13 in dorsocentral retina, spreading across the entire retina by E15. By E19 only smaller RGCs are born in the ventral periphery. Shortly after neurogenesis, RGCs send axons towards the optic nerve (ON) via the optic disc and first project axons to the contralateral superior colliculus (SC, tectum) at about E16.5 (Bunt et al., 1983). Axons of RGCs born on E16 have already grown into the SC by birth, whereas axons from RGCs that are born last (E19) take longer to reach central targets and only grow into the SC on postnatal (P) days 4–6 (Dallimore et al., 2002).

The phenomenon of programmed cell death (PCD) in the rat retina is well documented (Cowan et al., 1984). The number of RGC axons in the ON at birth far exceeds adult numbers (Potts et al., 1982; Sefton and Lam, 1984; Crespo et al., 1985) and there is extensive PCD of rat RGCs around the time of birth and in the first few postnatal days. PCD of retinotectally projecting RGCs is linked to a requirement for target-derived trophic factors (e.g. Carpenter et al., 1986; Sefton et al., 1987; Cui and Harvey, 1995; Ma et al., 1998; Spalding et al., 1998, 2004) and may also be associated with loss of neurons that make targeting errors within, for example, the SC (O'Leary et al., 1986). Consistent with the idea that developing RGCs acquire a dependency for target-derived factors, early postnatal removal of SC tissue during the period of maximal PCD results in a huge and rapid increase in RGC death (Harvey and Robertson, 1992).

The delay in the innervation of the SC by axons from late-born RGCs implies there must be a delay in their switching on a requirement for

Abbreviations: BrdU, 5-bromo-2'-deoxyrudine; CTB, cholera toxin B subunit; DAB, diaminobenzidine; DL, double-labelled; FG, fluorogold; GCL, ganglion cell layer; IC, inferior colliculus; ON, optic nerve; PCD, programmed cell death; PN, peripheral nerve; RGC, retinal ganglion cell; RT, room temperature; SC, superior colliculus; TL, triple-labelled; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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target-derived trophic support (Vogel and Davies, 1991). This in turn may mean that this cohort shows greater resistance to injury and greater regenerative potential. In the present study we employed a number of experimental approaches in neonatal and adult rats to address these various issues. First, to determine if the timing of PCD in RGCs varies dependent on birthdate and the time of the arrival of their axons in the SC, we used 5-bromo-2'-deoxyrudine (BrdU) to label RGC cohorts born on different embryonic days and then used terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) to quantify RGC death at different times after birth. In a second study, we examined whether removal of target-derived trophic support by ablation of the SC affects those RGCs that have not yet innervated their target, and how prolonged removal of trophic support affects RGC axonal innervation over the longer term.

In the final series of studies we examined whether early- and late-born RGCs differ in their regenerative capacity after injury. Lesioned adult mammalian CNS axons do not exhibit significant spontaneous regrowth; however damage to immature mammalian CNS pathways is generally associated with greater plasticity and new growth across a lesion site (Schneider et al., 1985; Martin et al., 1994; Nicholls and Saunders, 1996). Even a small delay in the time of injury can significantly alter the potential for growth in neonatal brains. After complete SC transection in P2 rats there is some growth of RGC axons distal to the lesion but by P6 no such post-lesion growth is seen (Tan and Harvey, 1997). In adult CNS, experimental interventions are required to prevent the degeneration of injured neurons and promote regeneration. In the visual system, one such model is to use autologous peripheral nerve (PN) transplanted onto the cut ON. The PN graft provides a permissive environment through which surviving RGC axons can grow long distances (Bray and Aguayo, 1989; Thanos, 1997; Dezawa and Adachi-Usami, 2000; Harvey et al., 2006; Berry et al., 2008). We therefore used the neonatal SC transection and adult PN graft approaches to determine how the timing of RGC neurogenesis influences the way RGCs respond to both neonatal and adult insults, and thus whether birthdate has any bearing on the ability of RGCs to survive injury and successfully regenerate an axon.

Materials and methods

Animals

Wistar rat pups from time-mated pregnant rats were used in all experiments. Day of mating was designated as E0 and the day of birth defined as P0. All subsequent time points refer to these initial days. Surgical procedures were approved by the Animal Experimentation Ethics Committee (University of Western Australia) and conformed to National Health & Medical Research Council guidelines.

Bromodeoxyuridine injections

BrdU is a thymidine analogue that labels dividing cells in the S-phase of mitosis. Time-mated pregnant rats at E15, E18 or E19 were anaesthetized with halothane (5% in 80% N_2O/20%O_2) and injected intraperitoneally (IP) with 0.5 mL of 10 mg/mL BrdU (Sigma, St Louis, MO, USA) in 0.007 N NaOH in sterile saline (per 100 g maternal body weight). BrdU has been shown to have a temporary bioavailability (Boswald et al., 1990; Takahashi et al., 1992; Hayes and Nowakowski, 2000), therefore administration of BrdU was given at 3 different time points during the day—9 am, 1 pm and 5 pm, in order to label a sufficient number of cells undergoing proliferation over a prolonged period relating to a specific gestational day. Parturation routinely occurred at E22/E22.5. Most subsequent surgery and analysis was carried out on E15 or E19 labelled rats.

PCD experiments—fluorogold (FG) retrograde labelling

Because of the progressive arrival of displaced amacrine cells in the GCL of rats after P0 (Perry and Walker, 1980; Perry et al., 1983), in pups P1 and older, RGCs were identified by retrogradely labelling them with FG. Pups in the P0 age group did not require FG injections because at this age displaced amacrines have not yet reached the GCL. P1 to P5 pups were anaesthetized with ether and a small bone flap was made to expose the SC. Multiple injections of 0.1 µl of a 4% aqueous suspension of fluorogold (FG, Fluorochrome Inc., Denver, CO, USA) were made into the left SC via a glass micropipette. Injections were made across the entire extent of the SC in order to label as many RGCs as possible. Because the retina projects to upper SC layers, care was taken to ensure that the tip of the micropipette remained superficial. Following the injections, gel foam was placed on the SC to ensure that any excess FG would remain on the SC surface, the bone flap was replaced and skin sutured. Pups were left on a heat pad until recovery and then returned to their mothers. Twenty-four hours later, pups were deeply anaesthetized with 0.05–0.1 mL sodium pentobarbitone (IP) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Experimental eyes were removed and a small cut was made in the nasal half of the retina to ensure correct orientation was maintained throughout processing. Some animals from these litters were used as controls to determine if any RGC death was exacerbated by the multiple FG injection procedure in the SC. These rat pups did not receive any FG injections but were processed in exactly the same way as experimental animals, including BrdU processing (as outlined below).

Superior colliculus ablation experiments

At P1 or P6, rat pups were anaesthetized with ether, a bone flap made and the left SC was entirely removed using gentle suction. Gelfoam (UpJohn, Kalamaxoo, MI, USA) was placed in the resultant SC cavity. From previous work (Harvey and Robertson, 1992; Cui and Harvey, 1995) it has been shown that RGC death following SC ablation is maximal about 24 h later. In some pups cell death was therefore assessed at 24 h but other pups were allowed to survive for 96 h post SC removal. At these times, rats were anaesthetized, perfused, and retinas prepared for processing as described earlier. Control animals in the ablation experiments were taken from the PCD experimental data. Animals were processed at the same time points and all subsequent processing was the same.

P2 lesion experiments—transection of the SC

At P2, 52 rats received complete transection lesions of the left rostral SC (Gan and Harvey, 1986; Tan and Harvey, 1997). Pups were anaesthetized using ether, a small bone flap was made over the left SC and the superior brachium and rostral part of the SC was completely transected from the lateral edge to the midline using a specially designed microblade. To ensure lesions were complete the cut was carefully extended medially and laterally using microscissors. Care was taken to minimize bleeding and not to damage SC caudal to the transection. Once the lesion had been made and haemostasis was achieved, the bone flap was replaced and the skin sutured. Pups were returned to their mothers after recovery.

P2 lesion experiments—FG injections and cholera toxin subunit B (CTB) eye injections

At either P18 or P19, pups with a P2 SC transection received injections of the retrograde tracer FG distal to the lesion site. Animals were anaesthetized with 2,2,2-tribromoethanol (Sigma; 200 mg/kg body weight, IP) and a bone flap was made to expose the left posterior cerebral hemisphere. A small part of the hemisphere was aspirated to

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