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ABSTRACT

Similar to neurons in the peripheral nervous system, immature CNS-derived retinal ganglion cells (RGCs) become dependent on target-derived neurotrophic support as their axons reach termination sites in the brain. To study the factors that influence this developmental transition we took advantage of the fact that rat RGCs are born, and target innervation occurs, over a protracted period of time. Early-born RGCs have axons in the superior colliculus (SC) by birth (P0) whereas axons from late-born RGCs do not innervate the SC until P4-P5. Birth dating RGCs using EdU allowed us to identify RGCs (i) with axons still growing towards targets, (ii) transitioning to target dependence, and (iii) entirely dependent on target-derived support. Using laser capture microdissection we isolated about 34,000 EdU^+ RGCs and analyzed transcript expression by custom qPCR array. Statistical analyses revealed a difference in gene expression profiles in actively growing RGCs compared to target dependent RGCs, as well as in transitional versus target dependent RGCs. Prior to innervation RGCs expressed high levels of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor receptor alpha but lower levels of neurexin 1 mRNA. Analysis also revealed greater expression of transcripts for signalling molecules such as MAPK, Akt, CREB and STAT. In a supporting *in vitro* study, purified birth-dated P1 RGCs were cultured for 24-48 hours with or without BDNF; lack of BDNF resulted in significant loss of early- but not late-born RGCs. In summary, we identified several important changes in RGC signalling that may form the basis for the switch from target independence to dependence.

Keywords: BDNF, development, retina, gene expression, CNTF receptor alpha, IGF-1

48 **Significance Statement**

49 During brain development many neurons die around the time that neural connections are
50 established. This cell loss is thought to be due to competition between neurons for limited
51 amounts of target-derived trophic support; responsive neurons receiving sufficient amounts of
52 such factors survive. But what factors sustain developing neurons prior to target innervation?
53 We took advantage of the fact that rat retinal ganglion cells (RGCs) are born, and target
54 innervation occurs, over a protracted time period. Using laser-capture microdissection of birth-
55 dated RGCs we compared gene expression in neurons prior to, during, and after innervation of
56 central targets. We identified numerous changes in RGC signalling that may form the basis for
57 the switch from target independence to dependence, from axonal elongation to
58 arborization/synaptogenesis.

59

INTRODUCTION

Programmed cell death (PCD) occurs throughout the developing nervous system and is a crucial step in the maturation of neural circuitry. For example, in the developing rat visual system PCD results in a loss of at least 50% of the maturing RGC population (Dreher et al., 1983; Perry et al., 1983; Crespo et al., 1985; Harvey and Robertson 1992). A key element in regulating the onset and distribution of PCD is the availability of neurotrophic factor support. In both the central and peripheral nervous system (CNS and PNS), removal or addition of neurotrophic factors respectively increases or decreases the amount of PCD (PNS: Levi-Montalcini & Angeletti 1968; Thoenen & Barde 1980; Hamburger & Yip, 1984; Oppenheim 1991; CNS: Cohen-Cory et al., 1996; Cui & Harvey 1995; Ma et al. 1998; Spalding et al., 1998; Spalding et al., 2005). Such findings are consistent with the neurotrophic hypothesis (Davies, 1996), which proposes that neurons with axons in target sites compete for limited amounts of target-derived neurotrophic factors, and only responsive neurons that receive sufficient amounts of these factors survive.

Developing neurons with axons en route to targets survive independent of support from target-derived trophic factors. However studies on cranial sensory neurons and lower motoneurons (Davies et al. 1987; Vogel & Davies 1991; Mettling et al., 1995; Enokido et al., 1999) suggest that there is a maturational switch to dependency on target-derived factors as axons grow into such targets. Additionally in the PNS, the further axons have to grow the longer it takes for their parent neurons to switch on their trophic dependency (Davies 1989; Vogel & Davies, 1991). Importantly, in PNS neurons, the transition to target dependency is delayed by the same amount of time *in vitro* as it would be *in vivo* (Davies et al. 1987; Davies, 1989; Vogel & Davies 1991), suggesting it is an intrinsic property of those neurons.

A similar phenomenon may occur in the visual system. In chick RGCs, a switch from target independence to dependence has been reported (Rodriguez-Tebar et al., 1989). In rat, RGCs first appear at about embryonic day 13 (E13) and genesis continues until E19/E20 (Reese & Colello 1992; Rapaport et al. 2004). Soon after differentiation, RGCs begin to extend axons (Waid and McLoon, 1995), almost all of which project to the contralateral superior colliculus (SC) (Sefton et al., 2004). Axons of first-born RGCs reach the SC at about E16.5 (Lund and Bunt 1976; Bunt et al., 1983), a delay of 2-3 days, whereas axons of RGCs born on E18/E19 do not reach the SC until 4-6 days after birth, a delay of 8-9 days (Dallimore et al., 2002). Progressive innervation of the brain by different populations of RGCs as they mature has also recently been described in the mouse (Osterhout et al., 2014). In the rat, early-born RGCs begin PCD before birth whereas late-born RGCs only die between P4-P6 (Dallimore et al., 2010), thus a switch to target dependency should be revealed by differences in the time-course of PCD in early- versus late-born RGCs.

Previous studies have documented changes in gene expression in developing RGCs defined solely on the basis of the age of the animal from which the cells were obtained (e.g. Trimarchi et al., 2007; Wang et al., 2007; Moore et al., 2009). However, because of the prolonged neurogenesis of RGCs in rats, at any given pre- or postnatal age RGCs are at different stages of maturation, thereby constituting a heterogeneous population (Dallimore et al., 2002, 2010). Thus to more definitively characterize the factors that contribute to the switch to target dependency in maturing RGCs, at different times after birth we analyzed and compared gene expression in neonatal rat RGCs that had been identified and selected *purely on the basis of their day of neurogenesis*.

RGCs born on E15 or E18 were labelled with pulses of 5-ethynyl-2-deoxyuridine (EdU). RGCs were then investigated at different stages of target ingrowth: before axon ingrowth (E18 labelled

pups sacrificed at P0 or P1); during ingrowth (E18 labelled pups at P5); and after innervation (E15 labelled pups at P0). EdU positive ($^{+}$) RGCs were isolated from cryosections using single-cell laser capture microdissection (LCM) and RNA extracted for quantitative real-time PCR (qPCR). We selected genes that are either involved in trophic factor signalling or have been implicated in RGC survival and/or axonal outgrowth. Pathway specific discriminant analysis was used to compare gene expression profiles between RGCs with axons already in the SC and RGCs with axons still growing towards the SC and other major central target sites. Because the data from the LCM studies suggested changes in brain-derived neurotrophic factor (BDNF) signalling in maturing RGCs, we also compared *in vitro* the trophic requirements of purified P1 RGCs labelled on either E15 or E18 with 5-bromo-2'-deoxyuridine (BrdU).

MATERIAL AND METHODS

Animals.

Time-mated female Wistar rats (n=10) were used for EdU injections and gene expression studies. EdU was used for the *in vivo* qPCR experiments because the protocols for visualizing BrdU result in RNA degradation and an earlier pilot study found that it was not possible to capture BrdU labelled cells with LCM while maintaining RNA integrity. E15 or E18 (day after mating = E0) pregnant rats were anaesthetized with isofluorane (4% induction and 2% maintenance in 20% O_2/80% N_2O) and injected intraperitoneally (i.p.) with EdU (20 mg/kg maternal body weight) two times during the day (at 10am and 3pm) to ensure prolonged bioavailability (Zeng et al., 2010). Procedures were approved by institutional and government guidelines.

Tissue collection and processing.

Parturition occurred on E22/22.5 (day of birth = P0). P0, P1 or P5 pups were deeply anaesthetized with 0.2 mL i.p. pentobarbital sodium (Lethabarb, Virbac) and perfused

transcardially with 4% paraformaldehyde in diethylpyrocarbonate-treated 0.1M phosphate buffered saline (dPBS). Eyes were harvested immediately after fixation and the cornea and lens removed in dPBS, leaving the eye cup with retina attached. Retinas were post-fixed in 4% paraformaldehyde for 1 hour, immersed in 30% sucrose in dPBS for 1 hour and then into increasing concentrations of Jung Tissue Freezing Medium™ (Leica Microsystems) (25%, 50%, 75%, 100%; one hour immersion at each stage at 4°C), before being frozen. Eyecups were crysectioned at 10 µm and every second section mounted onto Menzel-Gläser SuperFrost® Plus glass slides (usually 8 retinal sections per slide), ensuring that the eyecup was positioned so that the central to peripheral retina was represented in every section. Instruments and cryostat surfaces were treated with RNase*Zap*® (Ambion) cleaning solution and 70% ethanol before use. Sections were stored at -80°C prior to immunohistochemistry and laser capture (see below).

Multiple retinas were allocated to individual groups, each group constituting a biological replicate that gave a distinct sample of microdissected RGCs for mRNA analysis. mRNA analysis experiments were carried out in two separate rounds. For an initial pilot LCM experiment we had estimated that 800 RGCs would yield about 8ng of total RNA (10pg per neuron); however when many genes failed to amplify during qPCR it became clear that we had insufficient RNA of necessary quality to carry out the desired analysis, thus a second more extensive experiment was performed to capture a significantly greater number of EdU^+ RGCs. The initial pilot experiment compared 5 replicate groups of E18 EdU RGCs with 5 replicate groups of E15 EdU RGCs, both at P0 (E18/P0 and E15/P0 label/sacrifice respectively). Five retinas, randomly selected from different litters, were pooled to form each replicate group (except for two E15 groups, one of which had 3 retinas and one had 4). The second round of laser capture compared E18 RGCs at P1 (E18/P1, 5 replicate groups) with E18 at P5 (E18/P5, 5 replicate groups) and E15 at P0 (E15/P0, 3 replicate groups). Each of these groups contained RGCs from 5 different rat pups. For textual clarity, from henceforward the E15/P0 RGCs will

be described as 'target-dependent RGCs', E18/P0 or P1 RGCs as 'growing RGCs', and E18/P5 RGCs as 'transitional RGCs'.

EdU and Brn3a immunohistochemistry.

EdU detection was performed using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen). Sections were permeabilized with 0.2% Triton X-100 (Progen Industries) in dPBS for 30 mins, washed with 3% protease-free bovine serum albumin (BSA; Sigma-Aldrich) in dPBS (2 x 5 mins), then incubated with 300 µL reaction cocktail prepared according to kit directions for 30 mins. In the first pilot run, cells were not double labelled with Brn3a to distinguish RGCs, because at P0 there are as yet no displaced amacrine cells in the ganglion cell layer (Perry, 1981). In the more extensive follow-up experiment we also wished to obtain laser captured E18 RGCs from P5 retinas, at a time when their axons are in the process of innervating the SC, however at this age potentially EdU^+ amacrine cells are now present in the ganglion cell layer. Thus for all groups in this second series we additionally immunostained retinal sections for Brn3a protein, an established marker for RGCs projecting to the SC (Nadal-Nicolás et al., 2009, 2012). Retinal sections were washed with dPBS (2 x 10 mins) and incubated overnight at 4°C with anti-Brn3a goat primary antibody (AB) (Santa Cruz SC-31984) 1:100 in antibody diluent (10% normal horse serum and 0.2% Triton X-100 in dPBS). After washes, sections were incubated in donkey anti-goat Cy3 AB (Jackson Immuno 705-166-147; 1:200 in AB diluent) for 2 hours.

Laser capture microdissection.

For both the pilot and second qPCR experiments, laser capture microdissection (LCM) was undertaken using a Carl Zeiss PALM CombiSystem. Retinal sections were viewed at 20x magnification; EdU^+ cells (first experiment) or $EdU^+/Brn3a^+$ RGCs (second experiment) were tagged using PALM Robo software and catapulted into 0.5 mL PCR tube cap containing 40 µL

proteinase K digest buffer (Qiagen). Only those cells that were heavily and evenly labelled for EdU (first experiment) or both EdU and Brn3a (second experiment) were selected. Focus and energy settings were adjusted for each new slide prior to loading the collection cap, to ensure a single cell was catapulted with each shot. After catapulting, sections were again checked to ensure single cells had been captured. Approximately 200 cells were dissected into each cap over a period of 1 hour before the cap was transferred to -80°C. In the initial experiments approximately 800 cells were collected for each of the five E18 replicate groups, and approximately 1000-1200 cells for each of the five E15 replicate groups, in which EdU^+ RGCs are more numerous. In the second LCM study, about 26,000 RGCs were captured, and each replicate of the ten E18 and three E15 groups contained up to 2000 $EdU^+/Brn3a^+$ RGCs. After LCM, microdissected cell samples were stored at -80°C until RNA extraction.

RNA extraction and reverse transcription.

RNA extraction was performed using the Qiagen RNeasy FFPE kit and RNA was converted to cDNA and preamplified for qPCR using the Qiagen RT^2 PreAMP cDNA synthesis kit. In the initial qPCR experiment, due to the smaller than expected amount of RNA that was collected, RNA samples could not be quantified to ensure there was equal RNA input amounts in the reverse transcription reaction for each group, thus qPCR data were normalized only to the geometric mean of the reference genes PPIA and RPL10A (Becker et al., 2010). These genes showed a close pattern of expression changes across the groups. In the follow-up LCM study, in which a greater number of RGCs was captured, the concentration and integrity of RNA could be quantified using the Agilent RNA 6000 Pico Kit run on an Agilent 2100 Bioanalyzer prior to housekeeper normalization in qPCR; 1 ng total RNA from each sample was reverse transcribed. While there was still RNA degradation (RNA Integrity Numbers < 8.0) due to fixation and the unavoidable and extensive tissue processing prior to laser capture, the amount of RNA was sufficient to carry out cDNA synthesis and qPCR. In both the pilot and second follow-up

experiment, cDNA was preamplified using the Qiagen RT^2 PreAMP cDNA synthesis kit for 8 cycles.

qPCR. qPCR was performed using Custom Qiagen RT^2 Profiler PCR Arrays and Qiagen RT^2 SYBR Green ROX FAST Mastermix. Custom PCR arrays contained primers for 90 genes of interest (Table 1), as well as 3 housekeeping genes and 3 controls to verify PCR performance. A Corbett Rotor-Gene 6000 cycler was programmed with the following cycling settings: 95°C for 10 mins, followed by 60 cycles of 95°C for 15 secs and 60°C for 30 secs. Fluorescence threshold was fixed within the exponential phase of amplification and was identical for each run. A melting program (0.5°C stepwise from 60ºC to 95°C) was run after cycling and samples with multiple or abnormal melt peaks or those that crossed threshold after cycle 45 were excluded from analysis.

Statistical analysis.

In both the first and second qPCR rounds, expression of each gene between conditions was compared using the ΔCt method (Livak & Schmittgen, 2001; Pfaffl, 2001; Schmittgen & Livak, 2008). In both rounds, two reference genes (PPIA and RPL10a) were used to normalize expression across groups; the third (HPRT) was not used due to excessive variance. For each gene of interest, comparisons of gene expression across the conditions were performed: growing vs target-dependent RGCs, growing vs transitional RGCs, and transitional versus target-dependent RGCs. For each pairwise comparison, a fold change value was calculated to indicate relative gene expression level and a Student's *t* test was performed. A p value <0.05 was considered statistically significant, and only fold changes greater than ±2 were considered. Data are presented as mean fold change.

Discriminant Analysis.

To further assess changes in gene expression between groups in the second LCM study, we used discriminant analysis, a multivariate statistical technique used for differentiating groups using multiple quantitative variables. Using JMP software, we calculated two canonical scores which represented all of the variability of the dataset. The canonical scores were then compared between groups to identify significant differences using ANOVA. Differences in individual gene expression were then determined using ANOVA and Tukey Post Hoc tests.

A first pass analysis included expression profiles of all 45 genes that were successfully amplified to determine whether there were significant differences between the groups of RGCs at different developmental stages. Having established differences between the groups in this global analysis, we then performed a more focussed analysis to look for differential activation of individual pathways at the different developmental stages. We examined genes implicated in selected pathways related to BDNF signalling as follows: downstream of TrkB via PLCg (BDNF, CAMK1, CAMk2d, CAMk2g, CREB, PLCg1) and downstream of TrkB via Ras (BDNF, CREB, MAP2k2, MAPk1, MAPk3). We also examined pathways implicated in neuronal survival and axon elongation: Akt signalling (Akt1, Akt3, Bax, BDNF, GSK3B) and JAK-STAT signalling (CNTFRa, JAK2, STAT1, STAT3).

In vitro analysis of trophic dependence.

Nine time-mated female Wistar rats were injected i.p. at E15 (n=2) or E18 (n=7) with BrdU, 50mg/kg maternal body weight, 3 times during the day (at 9am, 1pm and 5pm). At P1, pups were euthanized with sodium pentobarbital (Lethabarb) and eyes were removed. Retinas were dissected from eyes and pooled into dPBS, then dissociated using the MACS neural dissociation kit (Miltenyi Biotec) according to manufacturer's instructions. RGCs were isolated using the MACS RGC Isolation kit (Miltenyi Biotec), following standard depletion and selection

protocols. Cells were resuspended in defined serum free growth medium (Ullian et al., 2004; modified from Bottenstein and Sato, 1979). Neurobasal media contained B27 supplement, triiodo-thyronine, transferrin, progesterone, sodium selenite, n-acetyl cysteine (Sigma-Aldrich), l-glutamine (2mM), sodium pyruvate (1mM) (Life Technologies), insulin (5ug/ml), and forskolin (10um, Sigma), with or without BDNF (50ng/ml, Peprotech). RGCs were seeded onto poly-d-lysine (PDL, 70kDa, 10ug/ml, Sigma) and mouse type-1 laminin (Sigma) coated 8 well culture slides (Falcon, BD).

Purified RGC populations were fixed after 1 or 2 days in culture (analogous to P2 and P3 *in vivo*). For immunostaining, cells were fixed with 4% paraformaldehyde, washed, and double labelled with anti-β-III tubulin antibody (TUJ1 clone, rabbit, Covance, PRB-435P, 1:4,000) with goat anti rabbit Cy3 secondary (Jackson, 111166047, 1:400), both for 30 mins at room temperature, followed by 30 mins 2M HCl treatment for 30 mins at 37ºC. Cells were then incubated overnight with BrdU antibody (mouse, Roche, 11170376001, 1:100) in diluent containing 4% normal goat serum (NGS), 3% BSA and 0.3% Triton X-100, washed, and incubated for 2 hours at room temperature with goat anti mouse FITC secondary antibody (Cappel 55521, 1:100). Slides were coverslipped in Dako Fluorescent mounting medium.

Data were obtained from 4 separate culture preparations of purified E15 RGCs, and 5 preparations of purified E18 RGCs. Two wells were counted for each culture, for each condition (plus or minus BDNF, 24 or 48 hours survival). Slides were examined by light and multi-channel fluorescent microscopy using an Olympus BX50 microscope equipped with a motorized stage (x,y,z), and a digital colour top mounted camera (Q-imaging), and linked to a computer with Stereo-Investigator software (V10.0, MBF Bioscience). Each well of the culture slide was outlined at its border using a 4x objective (bright field) using the contour mapping feature in the

Stereo-Investigator software. The sampling area consisted of 100 uniform but random counting frames (550um by 330um) which were calculated using the Systematic Random Sampling layout feature (total area sampled about 18mm^2, approximately 25% of the surface area). All βIII-tubulin$^+$ cells within each frame were counted using fluorescent light (TRITC filter), and classified according to either the presence or absence of prominent βIII-tubulin$^+$ processes. Incorporation of BrdU into RGC nuclei was classified subjectively by a single operator and an assessment made of the nuclear area containing BrdU (either no BrdU, less than 50% of nuclei area expressing BrdU, or more than 50%). Each βIII-tubulin$^+$ RGC was therefore counted and classified based on the presence of absence of processes and BrdU content within the nucleus. Results were tallied by the software, and exported to Microsoft Excel for further analysis. Data were quantified from 4 E15 and 5 E18 cultures (two wells counted for each culture, for each condition).

To examine BDNF expression in late-born RGCs, in two additional purified RGC cultures from litters labelled at E18 with BrdU, RGCs were maintained for 24 or 48 hours in Neurobasal media without recombinant BDNF. At 24 or 48 hours, cultures were fixed in 4% paraformaldehyde, and after blocking (10% NGS, 0.2% Triton X-100 for 30 mins) cultures were immunostained with antibodies to BDNF (rabbit, BiosensisR-172-20 or Santa Cruz – sc546) or β-III tubulin (Covance PRB-435P, 1:400) in blocking buffer for 1 hour followed by incubation in secondary antibodies (1 hour, anti-rabbit Cy3). Cultures were then reacted for BrdU as described above and coverslipped.

Anti-BDNF antibody specificity.

To confirm that both the BDNF antibodies bound specifically to BDNF we carried out an absorption control test using recombinant BDNF protein (Human BDNF, Peprotech). First,

316 BDNF protein was eluted in 2x Laemmili Buffer (250 mM Tris, 10% (v/v) glycerol, 4% (w/v)
317 SDS, 2% (v/v) β-mercaptoethanol, 0.005% (w/v) bromophenol blue (Sigma-Aldrich)), in double
318 deionized water (DDW, pH 6.8), separated by SDS-PAGE (Mini-PROTEAN TGX Stain-Free
319 Precast Gels; Bio-Rad) and transferred to nitrocellulose membrane (Trans-Blot Turbo Mini
320 Nitrocellulose Transfer Pack, Bio-Rad). Recombinant proteins were detected by Western blot
321 using 3% (w/v) skim milk powder in Tris buffered saline containing Tween (TBS-T; 100 mM
322 Tris, 154 mM NaCl, 0.1% (v/v) Tween 20, in DDW pH 7.5) blocking buffer, antibodies to
323 BDNF (rabbit, Biosensis R-172-20, 1:500 or Santa Cruz – sc 546, 1:500) and α-rabbit-HRP
324 secondary antibody (1:10,000 dilution; Pierce Scientific). Proteins were visualized by
325 chemiluminescence (Immun-Star; Bio-Rad, USA) using the Chemi-Doc system (Bio-Rad,
326 USA). In the absorption control, the anti-BDNF antibody (1 µg, rabbit, Biosensis R-172-20) was
327 incubated with 10ug of BDNF diluted in 100 µl blocking agent and incubated overnight at 4°C.
328 The pre-absorbed primary antibody was then used to immunostain purified RGC cultures using
329 the protocol described above.

330

331 **RESULTS**

332 qPCR of laser captured RGCs before, during and after target innervation.

333 In retinas used for LCM, EdU^+ cells were seen in the ganglion cell layer (GCL) and other layers
334 of the retina. In E15 injected EdU groups, EdU^+ cells in the GCL were found in all regions of
335 the retina, while in E18 EdU groups there were far fewer double-labelled (DL) $EdU^+/Brn3a^+$
336 neurons. These RGCs were mostly located in the periphery, consistent with previous reports
337 (Reese & Colello, 1992; Dallimore et al., 2002). LCM using the PALM DuoFlex Combi system
338 successfully catapulted individual cells from retinal sections. Figure 1 shows retinal sections
339 stained for EdU and Brn3a, with DL cells tagged before and after laser catapulting.

Two separate LCM studies were carried out. In the initial pilot experiment we captured almost 9,000 RGCs, giving at least 800 RGCs per replicate group; however with this number of RGCs RNA and cDNA yields were below the sensitivity of the NanoDrop and Agilent Bioanalyzer 6000 Nano Kits. We nonetheless proceeded to qPCR analysis of the entire cDNA stock obtained in this first experiment. Several genes exhibited at least a 4-fold change in mRNA expression levels between target-dependent and growing RGCs, although due to failure of amplification in some samples none of these changes reached statistical significance. Genes encoding BDNF, NFATc3, Adcyap1, EphA4 and STAT3 all appeared to be up-regulated in growing RGCs compared to target-dependent RGCs, whereas ApoE and NFATc4 were down-regulated. Importantly, BDNF transcript levels exhibited by far the greatest reduction between growing and target-dependent RGCs, consistent with PCR data from the second LCM study.

The initial LCM work was needed to establish optimal protocols for identifying gene changes in individually isolated postnatal RGCs. Based on this first study, we next used LCM to accumulate approximately three times as many RGCs (25,871 in total). In this second experiment only $Brn3a^{+}$, EdU^{+} RGCs were selected for capture. Up to 2,000 RGCs were used in each replicate qPCR group. In the second laser capture experiment, of the initial 90 genes selected for amplification (Table 1), 45 genes were consistently amplified in sufficient groups to allow for statistical comparison. These were: Akt1, Akt3, ApoE, Bax, Bcl2, BDNF, CAMK1, CAMK2d, CAMK2g, Cdc42, CNTFRa, CREB1, DSCAM, EphA4, Fos, GHR, GSK3B, HAND1, IGF-1R, Il6R, IL6st, JAK1, KLF4, KLF7, MAP2k2, MAPK1, MAPK3, MAPK8, MAPK9, mTOR, NFATc3, NFATc4, Nrxn1, Ntrk2, Optn, PLCg1, POU4F1, PTEN, Rac1, RhoA, Rps6ka2, Sort1, STAT1, STAT3, Stk24 (Fig. 2A).

Growing RGCs showed altered expression of a significant number of genes when compared to either target-dependent or transitional RGCs. The 30 genes with a greater than ±2 fold change in expression between the various groups are displayed in Figure 2B. Comparison between

growing and target-dependent RGC groups revealed a statistically significant relative increase in expression of IGF-1R and CREB1, and a significant relative decrease in expression of CAMK2g and PLCg mRNAs in growing RGCs. Comparatively large fold increases in mRNA levels in growing RGCs were also noted for CNTFRa, BDNF, GSK3B, Akt3 and PTEN. Fewer genes differed when comparing growing versus transitional, and transitional versus target-dependent, RGC groups. CAMK1 expression was significantly lower in transitional compared to target-dependent RGCs (Fig. 2B), but overall the extent of any fold change was generally less in these comparisons. Exceptions were large fold increases in Akt3, GSK3B and CNTFRa gene expression and a more than 10 fold decrease in neurexin 1 gene expression in growing versus transitional RGCs, and increased expression of IGF-1R, Stk24 and IL6R in transitional versus target-dependent RGCs.

Discriminant analysis and significant differences between growing and target-dependent RGCs.

To further assess changes in gene expression between groups, we used discriminant analysis, a multivariate statistical technique used for differentiating groups using multiple quantitative variables. Discriminant scores confirmed that all replicates could be allocated with 100% confidence to their appropriate group/developmental stage based on the pattern of gene expression. An ANOVA comparing the canonical scores revealed significant differences between conditions and Tukey post hoc tests confirmed that each condition was significantly different from the other two (growing vs target-dependent $p<0.0001$; transitional vs target-dependent: $p<0.0001$; growing vs transitional: $p=0.023$; Fig. 2C). Genes that contributed most to these differences were BDNF, CAMK1, Akt1 AND Akt3.

Pathway analysis and intracellular signalling.

To further examine the potential involvement of signalling pathways in RGCs during the transition to target dependence, discriminant analysis was performed on genes known to be

associated with particular growth or survival associated pathways, focussing on systems downstream of BDNF and TrkB (Fig. 3), and the CNTF receptor complex (Fig. 4).

BDNF signalling via TrkB activates three distinct pathways, all partially covered by our gene arrays (Fig. 3B, C). Genes that are components of the CAMK downstream pathway (green, Fig. 3A, B) were significantly different between growing, transitional and target-dependent RGC groups. Genes in the MAPK downstream pathway (blue, Fig. 3A, B) were also significantly different, with expression of MAPK isoforms increased in growing compared to non-growing RGCs. A significant difference in the expression profile of the Akt signalling pathway (pink, Fig. 3A, B) was seen, primarily attributed to an increase in expression of Akt isoforms in growing RGCs (Fig. 3C). Additionally, the growing and target-dependent RGC groups showed a significantly different gene expression profile for at least some factors involved in CNTF signalling, with a peak of CNTFRa and downstream Stat1 and Stat3 expression in actively growing, late-born RGCs (Fig. 4). Transcripts for IL6st (also known as gp130) also appear to be more prevalent, particularly in transitional RGCs.

Tissue culture – RGC survival in the presence or absence of BDNF.

Data were obtained from 4 separate culture preparations of purified P1 RGCs previously labelled with BrdU on either E15 or E18. Each of the 72 wells was initially plated with about 15,000 cells. Counting areas were randomly sampled (see Methods). Independent of BrdU label, in total about 35,000 βIII-tubulin$^+$ RGCs were counted. Based on these counts we estimated that, in the presence of BDNF, on average 18.4% and 14.3% of initially plated RGCs remained viable at 24 hours and 48 hours respectively, compared to 11.4% and 6.2% in the absence of BDNF. Expression of clearly defined, multiple βIII-tubulin$^+$ processes was also affected by neurotrophic support (Fig. 5); irrespective of birthdate, with BDNF 65.5% and 52.9% of surviving RGCs

expressed processes at 24 hours and 48 hours, compared to 41.5% and 33.4% in the absence of this neurotrophin.

On the critical issue of viability of RGCs identified by birthdate, we calculated the average number (± SEM) of E15 or E18 $BrdU^+$ RGCs (BrdU label in greater than 50% of the nucleus) in the presence or absence of BDNF at 24 and 48 hours. There was no change in E18 RGC counts in the two conditions, indicating that these late-born cells did not require exogenous BDNF for survival; however there was a significant (*Mann-Whitney U test , $p<0.05$) loss of E15 RGCs between 24 and 48 hours (Fig. 6). Consistent with this, in addition to the overall loss of viable β-III $tubulin^+$ RGCs at 48 hours, the proportion of these surviving β-III $tubulin^+$ RGCs that were born on E15 was decreased, and the proportion of E18 $BrdU^+$ RGCs increased, in 48 hour cultures that lacked BDNF (Table 2). Many surviving E18 RGCs continued to express processes (Fig. 5B, C), thus the distinction between early- and late-born RGCs was even greater when only analysing process-bearing RGCs. Without BDNF, between 24 and 48 hours the proportion of surviving βIII-$tubulin^+$ E15 RGCs in the cultures was reduced by almost half, whereas the proportion of E18 RGCs increased from 1.5% to 3.6% (Table 2).

In the second series of *in vitro* studies, E18 BrdU labelled RGCs were isolated and cultured for either 24 or 48 hours in the absence of BDNF. After fixation, RGCs were immunostained with antibodies to BDNF (Fig. 5D-G). Immunostaining was substantially reduced when antibodies were pre-absorbed with recombinant BDNF protein (data not shown). Although BDNF positivity was not entirely restricted to the $BrdU^+$ population (many surviving neurons would be from outside the labelling window), $BrdU^+$ RGCs were immunopositive for BDNF, with qualitatively greater staining at 48 compared to 24 hours.

DISCUSSION

In the developing rat, a significant proportion of RGCs die at the time their axons are growing into and innervating central target sites such as the SC (Dallimore et al., 2002, 2010). Using custom qPCR arrays to quantify gene expression in birth-dated neonatal RGCs isolated by LCM, we have for the first time obtained snapshots of gene expression in maturing CNS neurons *in situ*. This novel methodology allowed an unprecedented investigation of the expression status of identified RGCs, at different stages of maturation *in vivo*, without confounding issues associated with analysis of whole retinal tissue or analysis of gene expression *in vitro* on cultured or cell sorted RGCs. We have identified subtle differences in gene expression in RGCs that (i) are growing independently towards their target, (ii) have axons in the process of target ingrowth, and (iii) have completed ingrowth and now require central targets for trophic support. These differences, discussed in detail below, include the down-regulation of endogenous BDNF expression as RGCs innervate central targets, along with changes in expression of genes involved in signalling pathways downstream of TrkB. There were also maturational changes in responsiveness to cytokines and other growth factors. Although further work is needed to validate and clarify these results, the approach utilized in this study should eventually assist in unravelling the complex factors involved in circuit formation during visual system development.

The switch to target dependency in developing RGCs – BDNF.

We obtained two lines of evidence suggesting that RGCs switch to a particular dependency on target-derived BDNF as their axons enter retino-recipient sites in the brain. The first comes from our qPCR array laser capture experiments, both of which demonstrated that BDNF mRNA expression in growing late-born RGCs was considerably greater than in isolated RGCs with

axons already in central targets. While previous work has shown that BDNF is expressed by RGCs (Cohen Cory et al., 1996; Frost, 2001; Rohrer et al., 2001; Vecino et al., 2002), and that RGCs express the cognate TrkB receptor (Cohen Cory et al., 1996; Suzuki et al., 1998; Rohrer et al., 2001; Vecino et al., 2002; Marshak et al., 2007), the clearly defined developmentally regulated change in BDNF transcript levels in different birth-dated RGC cohorts reported here is a new finding. Importantly, growing CNS-derived RGCs also exhibited increased expression of transcripts associated with survival- and growth-related pathways downstream of TrkB. These included mRNA for signalling proteins downstream of TrkB-Ras which promote survival and neurite outgrowth through MAPK phosphorylation of CREB (Mayr & Montminy, 2001), and lead to up-regulation of a wide range of anti-apoptotic genes, including BDNF itself (Korsmeyer, 1999; Mayr & Montminy, 2001; Mayr et al., 2001). In actively growing RGCs, pathways downstream of TrkB-Akt were also activated, consistent with a role for Akt1 in inhibiting pro-apoptotic factors and promoting survival in response to neurotrophic stimulation (Korsmeyer, 1999; Brunet et al., 2001).

TrkB expression has also been reported to be developmentally regulated (Rickman and Brecha, 1995; Ugolini et al., 1995), but we were unable to detect any change. Previous studies in rat have shown that exogenous application of BDNF or the related neurotrophin NT-4/5 temporarily protects P4/P5 RGCs after central target ablation (Cui & Harvey 1995; Spalding et al., 1998), and BDNF supports the survival of cultured neonatal RGCs (this study – cf. Johnson et al., 1986; Cohen-Cory et al., 1996; Meyer-Franke et al., 1995), which it should be noted have also been axotomized during the process of isolation and cell culture. Furthermore, BDNF is expressed in the SC and is retrogradely transported by RGCs (von Bartheld, 1998; Ma et al., 1998; Frost, 2001; Rohrer et al., 2001; Marotte et al., 2004). Our new data are thus consistent with the proposal that BDNF has an important role in maintaining cell autonomous RGC survival before axonal ingrowth into central targets. Such an interpretation echoes the autocrine

(and/or paracrine) supply of neurotrophins that occurs in peripheral sensory neuron populations prior to target innervation (Wright et al., 1992; Davies, 1996).

Our second line of evidence comes from the *in vitro* data showing that the dependency of purified P1 RGCs on exogenous BDNF for survival is related to their date of birth and thus their axon growth status at the time of isolation. In the absence of exogenous BDNF the number of cultured E15 $BrdU^+$ RGCs declined by over 50% between 24 and 48 hours while late-born E18 $BrdU^+$ RGCs survived for at least 48 hours. Additionally, E18 RGCs maintained expression of endogenous BDNF throughout their time in culture, consistent with these cells still being target-independent. Thus temporal differences in the acquisition of target dependency and onset of PCD in early-versus late-born RGCs are maintained in culture, and in the absence of *in vivo* environmental signals, suggesting that such differences may be intrinsically determined (cf. Davies et al. 1987; Vogel & Davies 1991; Davies, 1996).

Despite these various observations suggesting BDNF via TrkB acts as a survival factor for developing RGCs, the number of RGCs is not reduced in BDNF knockout mice (Cellerino et al., 1997), and RGCs survive the postnatal period in mice lacking TrkB (Rohrer et al., 2001). Depletion of TrkB using fusion proteins does increase the peak rate of RGC death in neonates but does not alter final cell number (Pollock et al., 2003). After early gene deletion there may be compensation (via NT-4/5 actions and/or cytokine expression, or signalling through other receptors) that maintains RGC viability, however conditional Cre-mediated deletion of BDNF as neurons mature also did not affect RGC viability or myelination (Rauskolb et al., 2010). Note however that in these mice some BDNF (5% of control) was still measurable by ELISA in cerebral cortex, but BDNF levels were not reported for retinal tissue.

514 It is difficult to reconcile these various observations to our current, and previously published,
515 findings. TrkB expressed in the absence of BDNF does not trigger neuronal death and is thus
516 not a 'dependence receptor' (Nikoletopoulo et al., 2010). However, lack of ligand-induced
517 kinase activity in TrkB receptors may alter numerous down-stream pathways that indirectly
518 mediate RGC death. RGCs do express p75 and/or the related Troy, both members of the tumour
519 necrosis factor receptor family that possess an intracellular death domain (Mandemakers and
520 Barres, 2005; Ahmed et al., 2006). Perhaps the well-described survival effects of either BDNF
521 or NT-4/5 on neonatal RGCs *in vitro*, or after injury *in vivo*, are related to altered expression of
522 p75/Troy and TrkB. Yet numerous studies have documented pro-survival effects of BDNF and
523 NT-4/5 in the *normal uninjured* rodent visual system; application of BDNF to the SC increased
524 RGC survival (Ma et al. 1998), and RGC loss was reduced after intravitreal NT-4/5 injection
525 (Cui and Harvey, 1995). Excess 'target-derived' BDNF also prevented the retraction of the
526 normally transient ipsilateral retinotectal pathway (Isenmann et al., 1999). Co-injection of
527 blocking antibodies to BDNF and NT-4/5 in the SC in neonatal rats significantly increased RGC
528 death, as did injection of these same antibodies into the retina, although it is important to note
529 that this increase was considerably less after intraretinal antibody application, clearly to be
530 expected if only late-born not yet target-dependent RGCs were affected (Spalding et al. 2004).
531 Finally, Carpenter et al. (1986) and Spalding et al. (2004) described the extensive loss of
532 neonatal RGCs after kainic acid lesions in the SC, a technique thought to cause loss of target
533 neurons in the SC without concomitant axonal injury.
534
535 <u>The switch to target dependency in developing RGCs – other growth factors.</u>
536 Expression of cytokine signalling related transcripts was also significantly different between
537 RGCs at different stages of target ingrowth. Actively growing RGCs expressed relatively higher
538 levels of CNTFRa mRNA, a sub-component of the cytokine receptor (Stahl & Yancopoulos,
539 1994), and its downstream effectors STAT1 and STAT3, known to promote survival and growth

(Peterson et al., 2000; Park et al., 2004; Ng et al., 2006). There was also increased expression in transitional and growing RGCs of IL6st, another sub-component of the cytokine receptor, and IL6R. It has been reported that exogenous CNTF does not support the survival of RGCs *in vitro* (Meyer-Franke et al., 1995) or P4/P5 RGCs *in vivo* (Spalding et al., 2005). However, intraocular application of leukemia inhibitory factor did reduce RGC death to some extent after early SC ablation (Cui and Harvey, 1995). Given the very small population of late-born RGCs, without information about birth date any positive effects of cytokines would be less obvious within the overall RGC population. It is worth noting here that in the adult rat CNTF is a powerful driver of RGC survival *and* long-distance axonal regeneration (Fischer, 2012; Harvey et al., 2012).

There was significantly greater expression of IGF-1R in growing versus target-dependent RGCs, and relatively greater expression of this receptor in transitional RGCs. IGF-1 is known to have protective effects on neonatal (Gutiérrez-Ospina et al., 2002) and adult (Kermer et al., 2000) RGCs, and IGF-1R expression is necessary for the expression of neurites from adult RGCs *in vitro* (Dupraz et al., 2013). Note that signalling through this receptor also involves PI3-K and Akt pathways, the latter consistently upregulated in growing RGCs (Fig. 3) (Kermer et al., 2000). Transitional RGCs also appeared to express greater levels of the kinase Stk24, also known as MST3b, implicated in the regrowth of adult RGC axons (Lorber et al., 2009).

Not all transcripts for pro-survival proteins were more abundant in actively growing RGCs. Expression of genes encoding proteins downstream of TrkB via PLCg, a pathway implicated in moderating survival in response to synaptic interactions and neuronal activity (Tao et al., 1998; Ming et al., 1999; Reichardt, 2006) was reduced, presumably because these actively growing RGCs had not yet formed synaptic connections. In this regard, there was a large fold difference between growing and transitional RGCs in expression of transcripts for the presynaptic protein neurexin 1 (Fig. 2B), to be expected given that this protein is needed during the process of

566 synaptogenesis (Barker et al., 2008). Finally, we detected increased levels of pro-apoptotic Bax
567 and GSK-3B mRNA, although the concomitantly increased levels of Akt might inhibit the
568 function of these two proteins (Tokuoka et al., 2002; Zhou & Snider, 2006; Alabed et al., 2010).
569 Down-regulation of Akt when RGC axons reach their target could provide a rapid and sensitive
570 mechanism for regulating RGC death.
571
572 Interpretation of gene expression – RGCs at a transitional stage of target dependency.
573 In comparing growing (E18/P1) to transitional (E18/P5) RGCs, many of the differences in gene
574 expression were similar to those found for growing versus target-dependent RGCs. Significant
575 differences were also detected between transitional and target-dependent RGCs using
576 multivariate analysis, although such differences were fewer, with generally lower fold changes.
577 Expression of transcripts for proteins in the PLCg pathway was reduced in target-dependent
578 compared to transitional RGCs, including significantly lower levels of CAMK1 mRNA
579 expression. Perhaps the effects of this activity-dependent survival pathway become more
580 important as RGCs begin the process of target innervation.
581
582 The development of retina-to-brain connectivity.
583 During visual system development, BDNF also acts as a terminal arborization factor and
584 promotes the formation and stabilization of synapses (Hu et al., 2005; Marshak et al., 2007). The
585 high level of BDNF we observed in actively growing RGCs seems to conflict with findings that
586 delivering BDNF to the axons of developing RGCs causes long-distance axon extension to
587 cease, and triggers collateral arborization and synapse formation (Cohen-Cory & Fraser, 1995).
588 However, in development at least, BDNF can be delivered to the maturing retina without
589 impeding long-distance RGC axon extension (Lom et al., 2002; Cohen-Cory et al., 2010).
590 Perhaps the effect of BDNF on RGC axon outgrowth during development depends on whether it
591 activates TrkB receptors in the axon terminal or the cell body (Lom et al., 2002; Cohen-Cory et

592 al., 2010; Park and Poo, 2012). Alternatively, our new data also pointed to developmental
593 changes in RGC sensitivity to cytokines and other factors, such as insulin-like growth factors.
594 Such factors acting as intermediaries en route to targets could facilitate/promote axonal growth
595 in concert with the role of BDNF in sustaining RGC viability.
596
597 Although our experiments did not determine the cause of BDNF down-regulation in RGCs as
598 they transition to dependence on target-derived trophic support, we can speculate on some of the
599 relevant candidates identified in our expression screen. One possible candidate is the
600 transcription factor CREB, a known driver of BDNF transcription, which is transiently switched
601 off in RGCs in transition and may thus signal a change in transcriptional targets. In this context
602 our initial qPCR suggested that there may be altered expression of members of the NFAT
603 transcription factor family, important in mediating neurotrophin signalling during initial axonal
604 growth (Graef et al., 2003). In addition numerous transcription factors and epigenetic processes
605 including DNA methylation, histone modifications and microRNA binding could be responsible
606 for developmental changes in BDNF expression (Chen et al., 2003; Numakawa et al., 2010;
607 Zheng et al., 2012; Karpova, 2014; Varendi et al., 2014). Many of these regulatory mechanisms
608 are activity-dependent and can direct the mRNA to different locations within the neuron, thus
609 influencing protein function.
610
611 Maturational changes in post-translational processing of BDNF may also be important.
612 ProBDNF is cleaved to mature BDNF and these different forms influence RGC axonal growth
613 state via direct and indirect mechanisms (Cohen-Cory et al., 2010). Mature BDNF preferentially
614 activates TrkB, directly promoting survival and axonal growth, whereas proBDNF binds to the
615 p75NTR-sortilin receptor complex to promote apoptosis, or to the p75NTR-NgR-LINGO
616 complex to inhibit neurite outgrowth (Mandemakers and Barres, 2005). Furthermore, the form
617 of BDNF may indirectly change the growth state of RGCs by influencing the outcome of Eph

receptor signalling as RGC axons innervate the target. ProBDNF association with p75/TROY (Marler et al., 2010) suppresses EphA-mediated arborization (Marler et al., 2010; Poopalasundaram et al., 2011), but mature BDNF signalling has the reverse effect, promoting branching via the association of the EphA receptor with TrkB (Marler et al., 2008; Marler et al., 2010; see also Cohen-Cory et al., 2010). The complex interaction of BDNF and Eph receptor signalling raises the intriguing possibility that down regulation of BDNF expression in developing RGCs may be necessary to enable retinotopic mapping in response to precisely regulated levels of target-derived BDNF (Goodhill & Richards, 1999; Feldheim et al., 2000; Marotte et al., 2004).

Technical considerations.

The experiments reported here are, to our knowledge, the first to compare mRNA expression in identified, birth-dated CNS neurons using LCM. The use of BrdU *in vivo* to help define the spatial-temporal maturation of birth-dated olfactory sensory neurons has recently been reported (Rodriguez-Gil et al., 2015), although this group did not use LCM to isolate individual neurons for gene expression profiling. As detailed in the Results, we encountered several technical issues associated with undertaking qPCR on RGCs captured from fixed retinal sections that had been processed for EdU and Brn3a. Despite the collection of over 25,000 individual RGCs to provide RNA for qPCR, RNA degradation from necessary fixation and IHC protocols likely resulted in the failed amplification and variability seen for some genes in the panel. In addition, the variance we often found between samples may reflect a number of real biological variables. Although our estimates here apply to the time required to reach the SC, RGC axons also grow into other targets including the lateral geniculate nucleus and other visual brain regions en route. This, together with delivery of EdU over several hours, may contribute to the variability observed, especially in the transitional E18/P5 cohort, even within identified RGCs of similar but not identical birthdate. Finally, as discussed above, protein function can be altered by post-

transcriptional and post-translational processing, as well as by subtle changes to intracellular localization, which are not captured by our qPCR data. This may explain why some genes, such as some Bcl-2 family members, showed no clear-cut changes in expression during the process of target innervation. In future studies it will clearly be of interest to investigate protein expression and epigenetic changes in microdissected CNS neurons identified by day of neurogenesis.

Conclusion.

Single-cell laser microdissection is a powerful tool for assessing gene expression in individual cells *in situ*. To our knowledge this method has not previously been used to obtain snapshots of gene expression in developing mammalian neurons specifically identified by their day of neurogenesis, at a defined state of *in vivo* maturation. Such an approach presents technical challenges; nonetheless we have been able to show that gene expression in rat RGCs born on a particular day is correlated with their developmental stage of axonal outgrowth and target innervation. Thus studies that define developmental changes in gene expression based solely on the post-conception age of the animal may not give an accurate representation of gene expression changes across development and beyond. While further work is needed to clarify and extend the present work, our data suggest that the transition to target dependency in neonatal rat RGCs is linked to the down-regulation of endogenous BDNF expression and altered signalling in downstream pathways, as well as changes in responsiveness to cytokines and perhaps other growth factors. We hypothesize that the combined effects of decreased responsiveness to axogenic factors and decreased intra-retinal BDNF expression allows TrkB expressing RGCs to become more sensitive to target-derived BDNF, important for the onset of local arbor formation, and for the establishment and stabilization of ordered retinotectal connectivity.

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TABLES

Table 1. Gene primers in the Qiagen Custom RT^2 Profiler PCR Array CAPR11291R.

Brief function, relevant citations, and RefSeq number are listed for each gene. Arrows in the right hand column indicate a greater than ±2 relative fold change (FC) in gene expression when comparing expression levels between E18/P1-E15/P0, E18/P1-E18/P5, and E18/P5-E15/P0 groups. ↕, relative change in expression, either up or down, depending on pair-wise comparison; see Fig. 2B for details. <2, genes with a change in expression less than ±2. -, genes that failed to amplify in one or more samples, or amplification was evident but only after cycle 45 and therefore discounted.

Gene	Function	RefSeq no.	FC
Adcyap1	Stimulates cAMP production; up-regulated over development (Boeshore et al., 2004; Wang et al., 2007)	NM_016989	-
Akt1	Downstream of PI3K; critical in neurotrophin-mediated growth and survival (Kaplan & Miller, 2000; Perkinton et al., 2002; Campos et al., 2003)	NM_033230	↑
Akt3	Downstream of PI3K; critical in neurotrophin-mediated growth and survival (as above)	NM_031575	↕
ApoE	Involved in CNTF signalling, regulated over development (Fischer, 2012)	NM_138828	↓
Arg1	Downstream from cAMP in survival and regeneration; critical in overcoming myelin inhibition; regulated over development (Cai et al., 2002; Moore & Goldberg, 2011)	NM_017134	-
ATF3	Transcription factor; interacts with CREB and c-JUN; up-regulated after injury, promotes regeneration (Seijffers et al., 2006; Moore & Goldberg, 2011)	NM_012912	-
Bad	Pro-apoptotic protein (Isenmann et al., 2003; Wilson & Di Polo, 2012)	NM_022698	-
Bax	Pro-apoptotic protein (as above)	NM_017059	↑
Bcl2	Anti-apoptotic protein; necessary for neuronal survival; over-expression reduces PCD (Brunet et al., 2001; Campos et al., 2003)	NM_016993	<2
Bcl2l1	Anti- or pro-apoptotic protein depending on splice variant (as above)	NM_031535	-
Bcl2l11	Pro-apoptotic protein; downstream of FOXO transcription factors (as above)	NM_022612	-
BDNF	Promotes RGC survival; axon branching; synaptogenesis (Numakawa et al., 2010)	NM_012513	↑
CAMK1	Phosphorylates CREB; prolongs ERK activation; regulates axon growth (Lonze & Ginty, 2002; Perkinton et al., 2002; Jiao et al., 2005)	NM_134468	↓
CAMK2d	Phosphorylates CREB; prolongs ERK activation; regulates axon growth and dendritic architecture; involved in depolarization-induced survival (as above)	NM_012519	↕

Gene	Function	RefSeq no.	FC
CAMK2g	Phosphorylates CREB; prolongs ERK activation; regulates axon growth, dendritic architecture, neurite length; involved in depolarization-induced survival (as above)	NM_133605	↓
Casp8	Apoptotic factor; expressed after injury; mediates apoptosis through FOXO1 (Brunet et al., 2001; O'Driscoll et al., 2006; Johnson et al., 2009)	NM_022277	-
Casp9	Apoptotic factor; activates effector caspases; involved in PCD (as above)	NM_031632	-
Cdc42	Rho family GTPase; positive regulator of actin dynamics, microtubule formation, growth cone formation and axon elongation; acts in opposition to RhoA (Zhou & Snider, 2006; Major & Brady-Kalnay, 2007; Hausott et al., 2009)	NM_171994	↑
Cdh1	Adhesion factor; regulates axon growth and patterning (Konishi et al., 2004)	NM_031334	-
CNTFR	Receptor for CNTF; promotes growth and survival (Yip & So, 2000; Lingor et al., 2008)	NM_001003929	↑
CREB1	Transcription factor; critical for survival and growth signalling (Lonze & Ginty, 2002; Moore & Goldberg, 2011)	NM_031017	↑
DCC	Guidance factor; receptor for netrin-1; involved in guidance towards optic disc (Koeberle & Bähr, 2004; Erskine & Herrera, 2007)	NM_012841	-
DCX	Microtubule-associated protein; growth promoting (Blackmore et al., 2010)	NM_053379	-
DSCAM	Guidance factor; involved in self-avoidance (Fuerst et al., 2008; Fuerst et al., 2009)	NM_133587	<2
EFNA2	Ephrin A2 ligand; involved in inhibition; expressed in development and after axotomy in adult (O'Leary & Wilkinson, 1999; Cang et al., 2008)	NM_001168670	-
EGFR	Receptor for EGF; involved in myelin inhibition; required for development of some CNS regions (Hannila & Filbin, 2008; Hausott et al., 2009)	NM_031507	-
ELK1	Transcription factor; regulates cell cycling; pro-survival effects (Hausott et al., 2009)	XM_001055949	-
EphA4	Receptor for Ephrin A and B; involved in inhibition (O'Leary & Wilkinson, 1999; Reber et al., 2004; Lindqvist et al., 2010)	NM_001162411	<2
Fos	Involved in regulation of TrkB expression (Chang et al., 2004; Park et al., 2004)	NM_022197	<2
FOXO3	Pro-apoptotic transcription factor; inhibited by neurotrophin signalling via Akt and SGK (Brunet et al., 2001)	NM_001106395	-
Gap43	Protein required for axon growth (Schaden et al., 1994)	NM_017195	-
GHR	Growth hormone receptor; pro-survival effects on developing RGCs via CREB (Sanders et al., 2008)	NM_017094	↓
GSK3B	Influences cytoskeleton assembly; involved in pro-apoptotic signalling; apoptotic function inhibited by Akt (Brunet et al., 2001; Tokuoka et al., 2002; Alabed et al., 2010)	NM_032080	↑
HAND1	Transcription factor, required for sympathetic neuronal survival (Doxakis et al., 2008)	NM_021592	<2
HAND2	Transcription factor, required for sympathetic neuronal survival (as above)	NM_022696	-

Gene	Function	RefSeq no.	FC
IGF-1R	Growth factor receptor; promotes RGC survival and growth (Goldberg et al., 2002)	NM_052807	↕
Il6R	Cytokine receptor; promotes RGC survival and growth (Cao et al., 2006; Moore & Goldberg, 2011)	NM_017020	↑
IL6st	Part of the IL6R complex; promotes RGC survival and growth (as above)	NM_001008725	↓
JAK1	Component of JAK/STAT signalling cascade; involved in signalling via IL6R, CNTFR and GHR; regulated in development (Park et al., 2004; Wang et al., 2007)	NM_053466	<2
JAK2	Component of JAK/STAT signalling cascade (Park et al., 2004)	NM_031514	-
Jun	Transcription factor; involved in apoptosis, growth and regeneration (Lu et al., 2003; Tedeschi, 2012)	NM_021835	-
KLF4	Transcription factor; inhibits axon growth, regulated in development (Moore et al., 2009)	NM_053713	<2
KLF6	Transcription factor; promotes axon growth (Veldman et al., 2007; Moore et al., 2009)	NM_031642	-
KLF7	Transcription factor; promotes axon outgrowth (as above)	NM_001108800	↕
KLF9	Transcription factor; inhibits axon outgrowth (as above)	NM_057211	-
L1CAM	Cell adhesion molecule, up-regulated in axon regeneration (Fischer et al., 2004)	NM_017345	-
LIFR	IL6 class cytokine receptor; neurotrophic factor; promotes survival and growth in RGCs (Goldberg et al., 2002; Moore & Goldberg, 2011; Fischer, 2012)	NM_031048	-
LINGO1	Part of Nogo receptor complex (Mi et al., 2004; Fu et al., 2008; Hannila & Filbin, 2008)	NM_001100722	-
MafK	Transcription factor; up-regulated after axotomy (Fischer et al., 2004)	NM_145673	-
MAP2k1	Major neurotrophin signalling pathway (Atwal et al., 2000; Gao et al., 2003; Hausott et al., 2009; Johnson et al., 2009)	NM_031643	-
MAP2k2	Major neurotrophin signalling pathway (as above)	NM_133283	↕
MAPK1	Kinase involved in MAPK signalling (Kaplan & Miller, 2000; Reichardt, 2006)	NM_053842	<2
MAPK14	Kinase involved in both apoptosis and survival signalling; downstream phosphorylation of CREB; possibly involved in neurite outgrowth (as above)	NM_031020	-
MAPK3	Kinase involved in MAPK signalling (as above)	NM_017347	↑
MAPK8	Kinase involved in pro-apoptotic signalling; inhibits Akt, downstream in p75NTR apoptotic signalling; activates c-Jun; regulates microtubule formation (Kaplan & Miller, 2000; Roux & Barker, 2002; Zhou & Snider, 2006)	XM_341399	<2
MAPK9	Kinase involved in pro-apoptotic signalling; involved in developmental apoptosis (Kuan et al., 1999; Le-Niculescu et al., 1999)	NM_017322	<2
mTOR	Kinase; controls protein synthesis required for axon growth; downstream of PI3K (Park et al., 2008)	NM_019906	↓
NFATc3	Transcription factor; involved in axonal growth; neurotrophins alter activity (Zhou & Snider, 2006; Moore & Goldberg, 2011)	NM_001108447	<2
NFATc4	Transcription factor; involved in axonal growth; neurotrophins	NM_001107	<2

Gene	Function	RefSeq no.	FC
	alter activity (as above)	264	
Ngfr	p75NTR; low-affinity neurotrophin receptor; pro-neurotrophin receptor; pro- or anti-apoptotic depending on conditions (Kaplan & Miller, 2000; Roux & Barker, 2002)	NM_012610	-
Nptx2	Role in synaptic plasticity (Fischer et al., 2004)	NM_001034199	-
Nrxn1	Involved in synapse formation (Barker et al., 2008)	NM_021767	↕
Nrxn2	Involved in synapse formation (as above)	NM_053846	-
Ntrk2	TrkB; receptor for BDNF (Atwal et al., 2000; Kaplan & Miller, 2000)	NM_012731	<2
Optn	Involved in axonal transport, regulated in development (Wang et al., 2007)	NM_145081	↑
Pde4b	Enzyme responsible for cAMP hydrolysis; inhibited by neurotrophin signalling (Gao et al., 2003; Hannila & Filbin, 2008)	NM_017031	-
PKCcd	Kinase involved in PI3K and p75NTR/NgR signalling; regulates MAPK activity (Reichardt, 2006; Zhou & Snider, 2006; Major & Brady-Kalnay, 2007)	NM_133307	-
PLCg1	Phospholipase activated through TrkB; extends MAPK signalling; increases intracellular Ca^{2+} (Segal, 2003; Zhou & Snider, 2006; Numakawa et al., 2010)	NM_013187	↓
POU4F1	Transcription factor Brn3a; involved in dendritic branching and architecture (Weishaupt et al., 2005; Badea et al., 2009; Voyatzis et al., 2012)	XM_341372	↓
POU4F2	Transcription factor Brn3b; involved in cell fate determination; critical for RGC differentiation, involved in axonal development (as above)	NM_134355	-
Psip1	Transcription factor; regulates expression of growth-associated genes; involved in dendritic arborization (Zhao et al., 2008)	NM_175765	-
PTEN	Phosphatase; converts PIP_3 to PIP_2; inhibits Akt and downstream signalling (Park et al., 2008)	NM_031606	↑
Ptk7	Transcription factor; downstream in PI3K and MAPK pathways; involved in BDNF-induced survival (Chang et al., 2004)	NM_001106889	-
Rac1	Rho family GTPase; involved in growth cone mechanics; inhibits RhoA; involved in nasal-temporal crossing (Pearse, 2004; Schiller, 2006; Major & Brady-Kalnay, 2007)	NM_134366	↑
RelA	NFkB transcription factor; involved in axonal growth and survival; activated by p75NTR (Moore & Goldberg, 2011)	NM_199267	-
RhoA	GTPase; activates ROCK triggering growth cone collapse; binds to p75NTR (Park et al., 2005; Lingor et al., 2008; Mi, 2008; Hausott et al., 2009)	NM_057132	↑
Rock1	Kinase; downstream of RhoA in mediating growth cone collapse (as above)	NM_031098	-
Rps6	Ribosomal protein; growth and survival promoting (Sun & He, 2010)	NM_017160	-
Rps6ka1	Phosphorylates ribosomal Protein s6; activates STATs, CREB; involved in MAPK survival signalling (Lonze & Ginty, 2002; Chang et al., 2004; Koeberle & Bähr, 2004)	NM_031107	-
Rps6ka2	Phosphorylates ribosomal Protein s6; activates STATs, CREB; involved in MAPK survival signalling (as above)	NM_057128	<2

Gene	Function	RefSeq no.	FC
Rps6kb1	Phosphorylates ribosomal Protein s6; downstream of Akt and mTOR (Manning & Cantley, 2007; Park et al., 2008)	NM_031985	-
Rtn4r	Part of NgR-p75NTR signalling complex; mediates inhibitory signalling dependent on cAMP levels and Arg1 activity (Cai et al., 2002; Schwab, 2004; Fu et al., 2008)	NM_053613	-
SGK1	Kinase; involved in PI3K pathway, similar to Akt; inhibits pro-apoptotic factors e.g. FOXO3 (Brunet et al., 2001)	NM_019232	-
SOCS3	Negative regulator of JAK/STAT signalling; knockout improves regeneration (Sun & He, 2010; Hellström et al., 2011; Fischer, 2012)	NM_053565	-
Sort1	Pro-neurotrophin receptor; complexes with p75NTR; pro-apoptotic (Jansen et al., 2007; Teng et al., 2010)	NM_031767	<2
SOX11	Transcription factor; increases expression of growth-associated molecules (Veldman et al., 2007; Moore & Goldberg, 2011)	NM_053349	-
STAT1	Activated by STAT3 via JAK/STAT cascade (Moore & Goldberg, 2011; Tedeschi, 2012)	NM_032612	↑
STAT3	Component of JAK/STAT signalling cascade; pro-survival signalling (as above)	NM_012747	↑
Stk24	Kinase; critical for axon regeneration in cultured CNS neurons (mst3B, Lorber et al., 2009)	NM_001127494	↑
Tnfrsf19	TROY; takes place of p75NTR with LINGO and NgR (Park et al., 2005; Hisaoka, 2006; Mi, 2008)	Unigene: Rn.202731	-
PPIA	Housekeeping gene (commonly used rat housekeeping gene in laboratory)	NM_017101	-
Rpl10a	Housekeeping gene (Hong Cai et al., 2007)	NM_031065	-
HPRT	Housekeeping gene (Vázquez Chona & Vazquez, 2004)	NM_012583	-

Table 2. Proportion of surviving, birth-dated retinal ganglion cells in the presence or absence of BDNF, 24 and 48 hr after plating.

	E15 BrdU		E18 BrdU	
	24hr	48hr	24hr	48hr
All βIII-tubulin$^+$ RGCs				
With BDNF	28.0%	28.2%	1.5%	2.3%
No BDNF	24.2%	16.9%	1.5%	3.3%
Process bearing RGCs				
With BDNF	37.0%	38.8%	1.4%	2.8%
No BDNF	34.0%	20.0%	1.5%	3.6%

FIGURE LEGENDS

Figure 1

Laser capture microdissection (LCM) isolates individual retinal ganglion cells (RGCs) from tissue sections, the neurons identified by their birthdate. EdU and Brn3a stained retinal section from a pup that received an E18 EdU injection and was perfused at P1. A, B: section before LCM stained for EdU (A) and Brn3a (B); double-labelled (DL) RGCs were tagged (numbers). C, D: section after LCM, stained for EdU (C) and Brn3a (D); tags show spaces left following catapulting of DL cells.

Figure 2

A: Heat map and cluster analysis for all genes with reliable PCR amplification. B: Pair-wise comparisons between actively growing (E15/P0), target-independent (E18/P1) and transitional (E18/P5) RGCs, showing all genes with greater than ±2 fold change. Significant differences are shown in red. C: Plots show canonical scores 1 (X axis) and 2 (Y axis) from a multivariate discriminant analysis of overall gene expression levels ($2^{-\Delta CT}$). The two canonical scores represent 100% of the variance. Axes represent arbitrary units of standard deviation. Circles represent the 95% confidence region to contain the true mean of the treatment groups. Black lines show the coordinate direction (for simplicity, only selected individual gene expression levels are shown here) in canonical space. Note that the length of the lines is not representative of effect size due to the multidimensional nature of the analysis. Comparisons are made on canonical scores using ANOVA with Tukey's post hoc test: E15/P0 vs E18/P1: $p<.0001$ (Canonical 1); E15/P0 vs E18/P5: $p<.0001$ (Canonical 1); E18/P5 vs E18/P1: $p=0.0203$ (Canonical 2).

FIGURE 3:

Analysis of genes in pathways downstream of BDNF signalling. A: Diagram showing the main pathways analysed. Note that some genes in the diagram did not show changes, or were not analysed, but are included for context. B: Plots show canonical scores 1 (X axis) and 2 (Y axis) from a multivariate discriminant analysis of a subset of genes within specific pathways downstream of BDNF. Colors are used to identify distinct pathways but in some cases genes are common to more than one pathway. The two canonical scores represent 100% of the variance. Axes represent arbitrary units of standard deviation. Circles represent the 95% confidence region to contain the true mean of the treatment groups. Black lines show the coordinate direction (for simplicity, only selected individual gene expression levels are shown here) in canonical space. Note that the length of the lines is not representative of effect size due to the multidimensional nature of the analysis. Comparisons are made on canonical scores using ANOVA with Tukey's posthoc test: via PlCg: E15/P0 vs E18/P1: $p < .0001$; E15/P0 vs E18/P5: $p < .0001$; E18/P5 vs E18/P1: $p < 0.0016$. Via Ras: E18/P1 vs E18/P5: $p < .0001$; E15P0 vs E18/P5: $p = 0.0008$; E18/P1 vs E15/P0: $p = 0.0026$. Via GSK3b: E18/P5 vs E15/P0: $p = 0.0483$; E18/P5 vs E18/P1: $p = 0.1186$; E18/P1 vs E15/P0: $p = 0.6579$. C: Histograms showing mean relative expression level of individual genes (±SEM) for each group. Significant differences are indicated with an asterisk.

Figure 4

Analysis of genes in pathways downstream of CNTF signalling. A: Diagram showing the main pathways analysed. Note that some genes in the diagram did not show changes, or were not analysed, but are included for context. B: Plots show canonical scores 1 (X axis) and 2 (Y axis) from a multivariate discriminant analysis of a subset of genes downstream of CNTF. The two canonical scores represent 100% of the variance. Axes represent arbitrary units of standard deviation. Circles represent the 95% confidence region to contain the true mean of the treatment

groups. Black lines show the coordinate direction (for simplicity, only selected individual gene expression levels are shown here) in canonical space. Note that the length of the lines is not representative of effect size due to the multidimensional nature of the analysis. Comparisons are made on canonical scores using ANOVA with Tukey's post hoc test: E18/P1 vs E15/P0: p=0.0177; E18/P5 vs E15/P0: p=0.1261; E18/P1 vs E18/P5: p=0.4446.

Figure 5

Examples of BrdU labelled (green), βIII-tubulin$^+$ (red) retinal ganglion cells (RGCs) purified at P1 and examined after 48hrs in culture in the presence (A) or absence (B, C) of BDNF. A: process-bearing, BrdU$^+$ E15 RGCs are evident at 48hrs in the presence of BDNF. B, C: absence of BDNF resulted in the overall loss of cultured RGCs and reduction in neurite expression, however E18 RGCs continued to survive and many expressed processes. Note the fragmented neurites due to loss of cells between 24 and 48 hours. D-G: E18 RGCs cultured without BDNF immunostained with a BDNF antibody. D: 24hr culture; E-G: 48hr culture. Scale bars A-C, D-G = 50μm.

Figure 6

The mean number (±SEM) per well of E15 or E18 BrdU labelled, βIII-tubulin immunopositive P1 retinal ganglion cells (RGCs) after either 24 or 48hrs *in vitro*, in the absence of BDNF in the culture medium. Note the significant (asterisk, Mann-Whitney U test, $p<.05$) loss of E15 RGCs between 24 and 48hrs, but the numbers of E18 RGCs remained unchanged.