Original Article

Brain Derived Neurotrophic Factor and Superior Collicular Extract Regulate the Expression of the 1.6 Subfamily of Voltage-gated Potassium Channels in the Developing Rat Retina *in vitro*

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Purpose: To evaluate the role of brain derived neurotrophic factor (BDNF) and superior collicular extract (SCE) on the expression of the 1.6 subfamily of voltage-gated potassium channels (VG Kv 1.6 channels) in retinal ganglion cells (RGCs) of rats in an *in vitro* model.

Methods: Neonatal retinal cultures were supplemented with trophic factors of interest, namely BDNF and SCE, at 0 DIV (days *in vitro)*, 6 DIV and both 0 and 6 DIV. The expression of VG Kv 1.6 channels was evaluated by immunostaining with anti Kv 1.6 and immunofluorescence was measured by confocal scanning laser microscopy on 4, 6, 8, 10 and 12 DIV. The immunofluorescence indirectly measured the quantity of ion channels being expressed.

Results: RGCs were identified by their soma size. BDNF and SCE enhanced RGC survival by enhancing extensive neurite outgrowth, and increased the expression of VG Kv 1.6 channels; the effect of SCE was more significant than BDNF. Trophic factors also enhanced the survival of RGCs by increasing the expression of ion channels thereby contributing to spontaneous bursts of action potentials in the early stages of RGC development.

Conclusion: The expression of delayed rectifier VG Kv 1.6 channels in RGCs may determine membrane excitability and responsiveness to trophic factors, this plays a key role in the refinement of developing retinal circuits.

Keywords: Brain Derived Neurotrophic Factor; Superior Collicular Extract; Retinal Ganglion Cells; Potassium Channels

J Ophthalmic Vis Res 2012; 7 (2): 139-147.

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Received: November 22, 2011 **Accepted:** February 13, 2012

INTRODUCTION

The development of the nervous system takes place in two main steps; first an extensive preliminary network is formed and later it is pruned and trimmed to establish the final form. This refinement is achieved by mechanisms that include cell death, selective growth and loss of neurites, and the stabilization and elimination of synapses. A competition for the limited amount of target derived survival signals is thought to underlie the death of many central and peripheral neurons during nervous system development. Neurons continue to depend on these survival signals even in a mature brain.[1-3] These neurons compete vigorously to migrate,

innervate target neurons and access necessary trophic factors to fuel this process.

Apparently, there is survival of the fittest, since up to 50% of many types of neurons normally die within this period of brain maturation. Those neurons that fail to obtain adequate amounts of neurotrophic factors (due to improper connections) are eliminated by a wave of cell death. Being an extension of the central nervous system, retinal development occurs before any light-induced neural activity is initiated and this is thought to be part of the genetic profile of the cells. Retinal ganglion cells (RGCs) require continuous stimulation by extracellular signals in order to survive.[4] Blockade of electrical activity leads to cell death and disrupts the normal divergence pattern of RGC axons to their primary target, the lateral geniculate nucleus (LGN) of the thalamus, indicating that spontaneous activity in the retina plays a critical role in normal development of the adult visual system.[5] The survival signaling mechanisms not only control the number of neurons, but also specify their location, size and connectivity. Understanding how survival and growth is controlled by extracellular signals is fundamental to understand how the central nervous system develops and repairs itself. Excitability and discharge behavior of neurons depends on the highly variable expression pattern of voltage-gated potassium (VG Kv) channels throughout the nervous system. Accordingly, the present study aims to evaluate the effect of two target derived trophic factors, the superior collicular extract (SCE) and brain derived neurotrophic factor (BDNF), on delayed rectifier voltage gated potassium channels (VG Kv 1.6) in the developing rat retina *in vitro*.

METHODS

The current study was approved by NIMHANS animal care and ethics committee. Neonatal Wistar rat pups (P0) were used for this study. For *in vivo* experiments, three pups were used for each postnatal day and five mid-peripheral retinal sections from a total of six eyeballs each (5x6=30 sections) were utilized to compile the presented data. For *in vitro* experiments, three repetitions, six fields were scanned for each set of four wells on 24 multi-well plates/DIV.

Preparation of superior collicular extract

Superior colliculi are a pair of subcortical structures in the midbrain. They are the primary target nuclei for rat RGCs. Extracts from the superior colliculi, which are a rich source of trophic factors was prepared according to the procedure described in earlier studies.[6,7]

Superior collicular tissue was collected from postnatal rat pups (P0-P3) and stored at -70°C until used; 4 g of this tissue was homogenized in 12ml of 200mM Tris-HCl, pH 7.4, at 40°C. The homogenate was centrifuged at 100,000G for 1 hour at 40°C, the supernatant was collected, and the concentration of the buffer was adjusted to 20mM Tris-HCl, pH 7.4, using ice-cold water. This extract was stored at -20°C until used.

Preparation of retinal cultures

Retinal cultures were prepared according to the method described previously.[8,9] Neonatal rats (P0) were sacrificed by decapitation and the eyes were removed in Hanks' balanced salt solution (HBSS; GIBCO-BRL, Carlsbad, CA, USA) under sterile conditions in a laminar flow hood (Class II, Klenzaids, Mumbai, India). Eyeballs were punctured at the posterior pole adjacent to the optic nerve stump. The eyeballs were split open using a pair of fine forceps to dissect the retina free of meninges and lens. The retina was then mechanically dissociated using an 18G needle in Dulbecco's modified essential medium (DMEM; Invitrogen, Carlsbad, CA, USA).

Cell plating

The retinal cells were seeded onto 13mm glass coverslips (Blue Star, Chennai, India) kept in 24 multi-well plates (Nunc, Langenselbold, GmbH, Germany). The coverslips were precoated with poly-L-Lysine (25µg/ml, Sigma-Aldrich, St. Louis, MO, USA) for better adherence. Subsequently, cells were grown in DMEM supplemented with 10% fetal calf serum (GIBCO-BRL, Carlsbad, CA, USA) at a density of $1.5x10^5$ cells in an incubator with 5% CO2 at 37°C (Thermo Fischer Scientific,

Hera Cell 150, Dreieich, Germany). The medium was changed on alternate days. The cultures were treated with 0.1µl of SCE and 0.01µg/1µl of BDNF (Sigma-Aldrich, St. Louis, MO, USA) on 0 DIV, 6 DIV and both 0 and 6 DIV. Control cultures were untreated while vehicle control cultures were treated with 1µl of phosphate buffered saline (PBS). The cultures were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 20 minutes according to the protocol.

Identification of RGCs *in vitro*

RGCs were identified on the basis of their soma size.[10] The soma size of an RGC is reported to be larger than 12µM while those of other retinal neurons are smaller than 10µM; however, we also identified RGCs by indirect immunohistochemistry using RGC specific Thy.1.1 antibody[11] (Sigma-Aldrich, St. Louis, MO, USA).

Immunostaining of VG Kv 1.6 channels

Culture coverslips were permeabilized with Triton X-100 (0.25%, Sigma, St. Louis, MO, USA) in PBS for 5 minutes at room temperature. To avoid non-specific staining, the coverslips were incubated for half an hour in 10% normal goat serum (NGS; GIBCO-BRL, Carlsbad, CA, USA) diluted in PBS. Subsequently, the coverslips were incubated with polyclonal primary antibodies (anti VG Kv 1.6; Alomone Labs, Israel) at 1:75 dilution in PBS and 0.025% anti fungal agent sodium azide (Sigma, St. Louis, MO, USA) for 16 hours in a humidified chamber at room temperature. After three washes (15 minutes each) in PBS containing 1% NGS and 0.1% Triton X-100, the coverslips were incubated with goat anti-rabbit fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:100 dilution; Sigma-Aldrich, St. Louis, MO, USA) for l hour at room temperature. The coverslips were washed and coverslipped with anti-fading mounting medium (0.1% diazabicyclo octane, DABCO, in 65% Glycerol in PBS, Sigma-Aldrich, St. Louis, MO, USA) and sealed with nail varnish (Lakme, Mumbai, India).

Confocal imaging and fluorescence image analysis of VG Kv 1.6 channels

Bio-Rad MRC-1024 confocal scanning laser microscope, working with an argon laser at wavelengths of 488 and 568nm, was used for qualitative and quantitative evaluation of immunofluorescence of VG Kv 1.6 channels.[12] A digital image of the specimen consists of up to 1024×1024 pixels with 256 gray levels. One such image corresponds to an optical slice or section through the specimen in the focal plane of the microscope. The gain was adjusted for each sample set until the brightest fluorescence was observed so that the peak white and pixel intensity covered the full range from 0-256 on the 256 point gray scale. ×20 objective lenses were used and the qualitative data were collected on zoom 4 setting of the computer. The microscope illumination was kept constant and the optical density over a blank area on each slide was set at zero so that background density could be subtracted. The detector black level was set at about its midpoint and the ramp parameters were left on their default 0-256. Mean optical density was obtained by averaging several frames for each age group studied and represented as fluorescence/unit area (Scion image analysis, NIH program).

Statistical Analysis

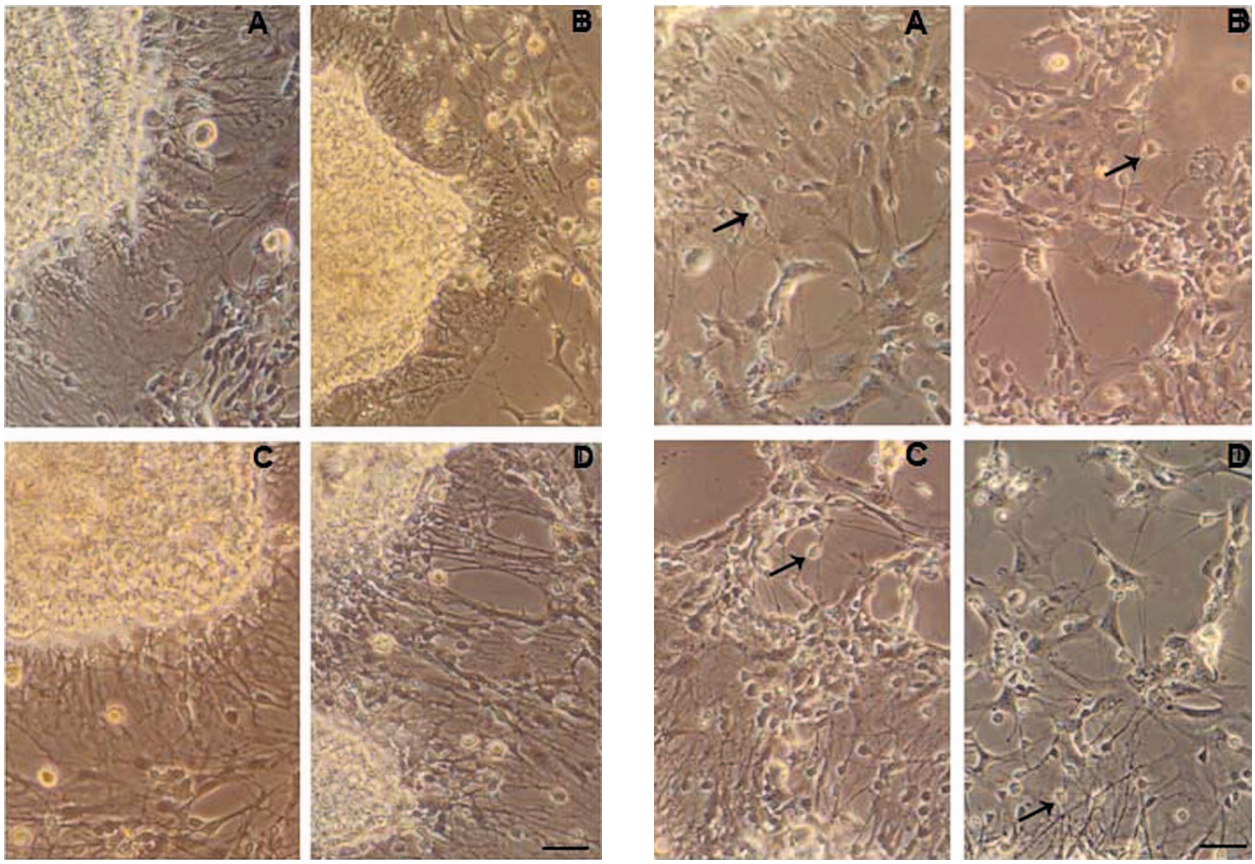
Data analysis was performed using appropriate statistical tests (SPSS 7.5). P-values less than 0.05 were considered as significant. Data on the effect of BDNF and SCE on immunoreactivity of VG Kv 1.6 *in vitro* were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post-hoc test.

RESULTS

Postnatal retinal cultures were grown and supplemented with trophic factors, BDNF and SCE, according to the study protocol (Fig. 1). Ontogenic expression of VG Kv 1.6 channels was observed in RGCs *in vitro*. VG Kv 1.6 channel immunoreactivity in RGCs was increased from 4 DIV to 12 DIV (Fig. 2). When retinal cultures

1.1. Retinal cultures on 2 DIV

1.3. Retinal cultures on 8 DIV



1.2. Retinal cultures on 4DIV

1.4. Retinal cultures on 10 DIV

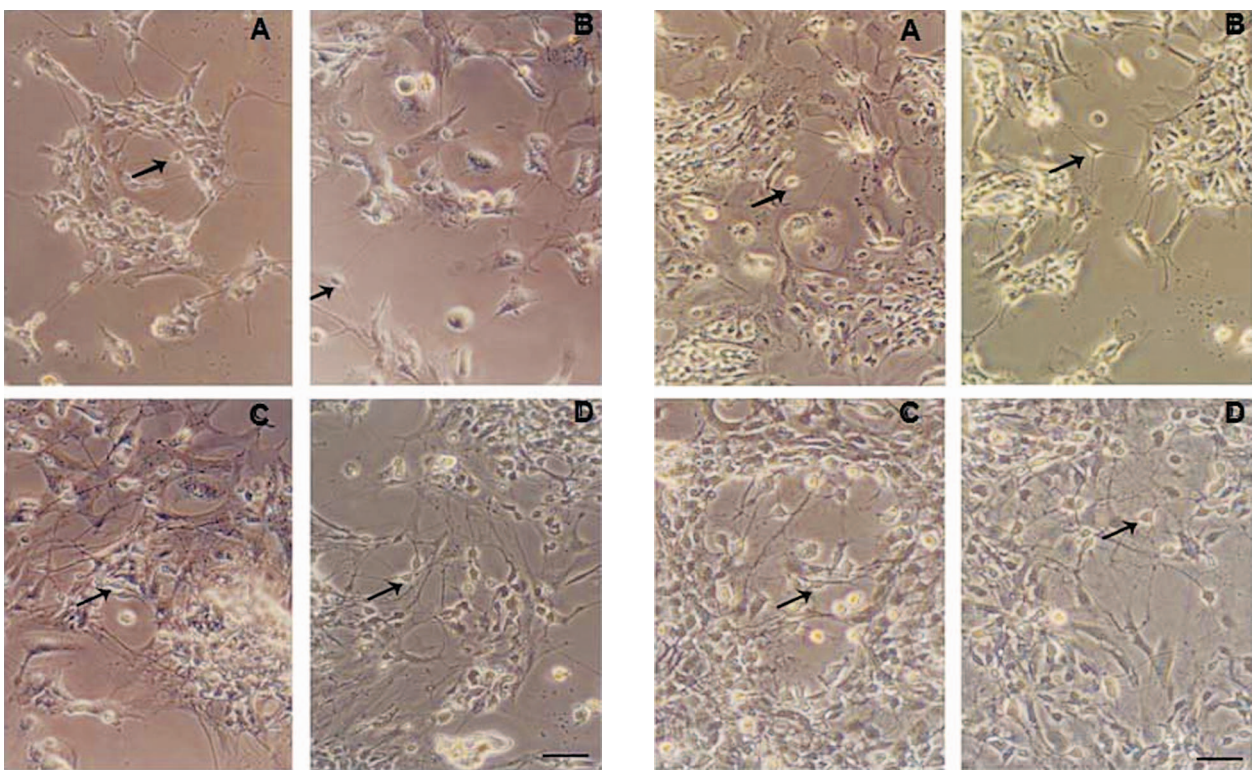


Figure 1. Phase contrast photomicrographs of retinal cultures (supplemented with trophic factors on 0 DIV). (A) Control; (B) Vehicle; (C) BDNF treated; (D) SCE treated. [Scale bar = 50 μm]

were treated with BDNF on 0 DIV, further enhancement of immuno- reactivity of VG Kv 1.6 channels was observed during the early stages of *in vitro* development (4 DIV and 6 DIV) and reached its peak by 8 DIV and remained constant through 10 DIV; by 12 DIV there was again a maximal expression of ion channels. SCE treatment on 0 DIV significantly increased (P<0.01) VG Kv 1.6 channel immunoreactivity on all days as compared to controls and BDNF, with maximal expression of VG Kv1.6 channels on 12 DIV (Table 1A and Fig. 3A).

Cultures supplementation with BDNF on 6 DIV raised the level of immunoreactivity from 8 DIV to 12 DIV as compared to controls, however the difference was not significant on 10 DIV and

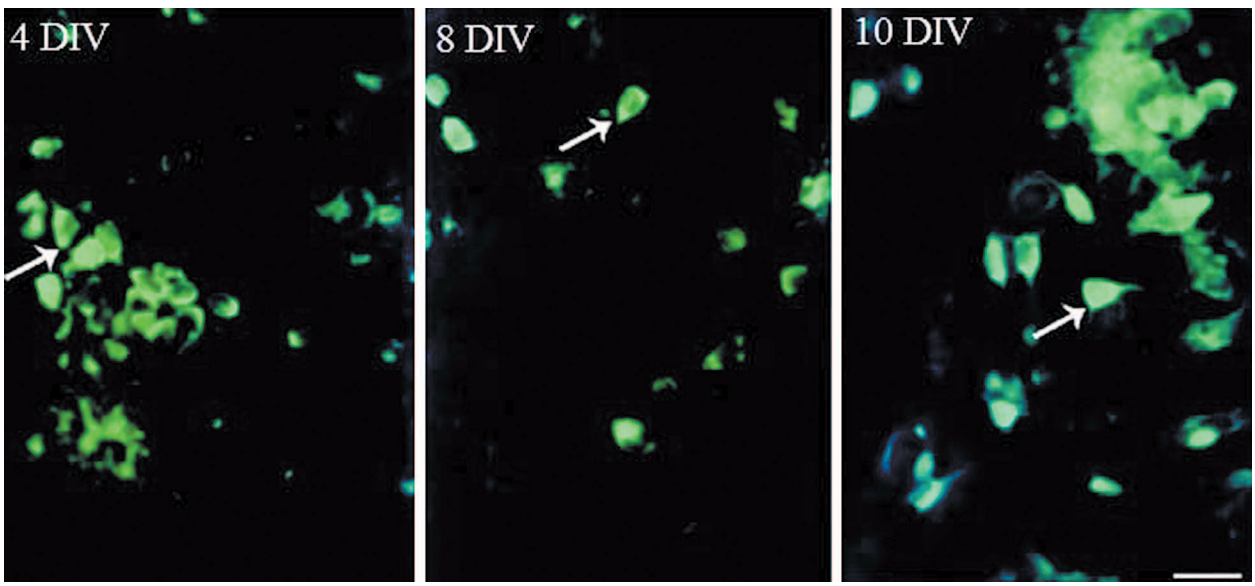


Figure 2. VG Kv 1.6 channels expression in rat RGCs at various days *in vitro*. Immunofluorescence of anti-VG Kv 1.6 at 4 DIV, 8 DIV and 10 DIV. [Scale bar = 20µm]

Table 1. Quantitative expression of VG Kv 1.6 channels immunoreactivity on various days *in vitro* (DIV) following treatment with trophic factors. The values are represented as mean±SD of fluorescence intensity in RGCs as indicated by the gray level (256 is bright fluorescence, 0 is blank) compared to controls. [* P<0.01; ** P<0.001.]

Group/day	4DIV	6DIV	8DIV	10DIV	12DIV
Control	82.9±2.79	86.5±2.25	104.5±2.56	110.8±1.54	111.6±2.10**
Vehicle	81.5±4.31	86.1±2.81	102±2.40	110.7±1.40	112.3±2.13
BDNF	97.6±1.91*	105.9±1.41*	112.8±2.29*	113.1±4.22	124.9±2.26
SCE	110.7±1.40*	123.1±2.44*	130.2±3.42*	134.2±2.68**	151.2±1.42**

A. Trophic factors supplemented on 0 DIV.

Group/day	8DIV	10DIV	12DIV
Control	104.6±2.56	110.2±1.52	111.6±2.16
Vehicle	101.6±2.0	112.4±2.28	113.2±4.32
BDNF	117.2±4.52*	124.8±2.29*	123.6±3.26*
SCE	130.6±3.46**	137.1±3.08**	148.3±3.06**

B. Trophic factors supplemented on 6 DIV.

Group/day	8DIV	10DIV	12DIV
Control	104.6±2.57	110.3±1.52	111.5±2.10
Vehicle	101.6±2.01	112.4±2.28	113.2±4.39
BDNF	121.5±2.15*	126.8±2.69*	128.7±3.20*
SCE	127.2±4.35*	137.4±3.05**	148.4±3.06.*

C. Trophic factors supplemented both on 0 and 6 DIV.

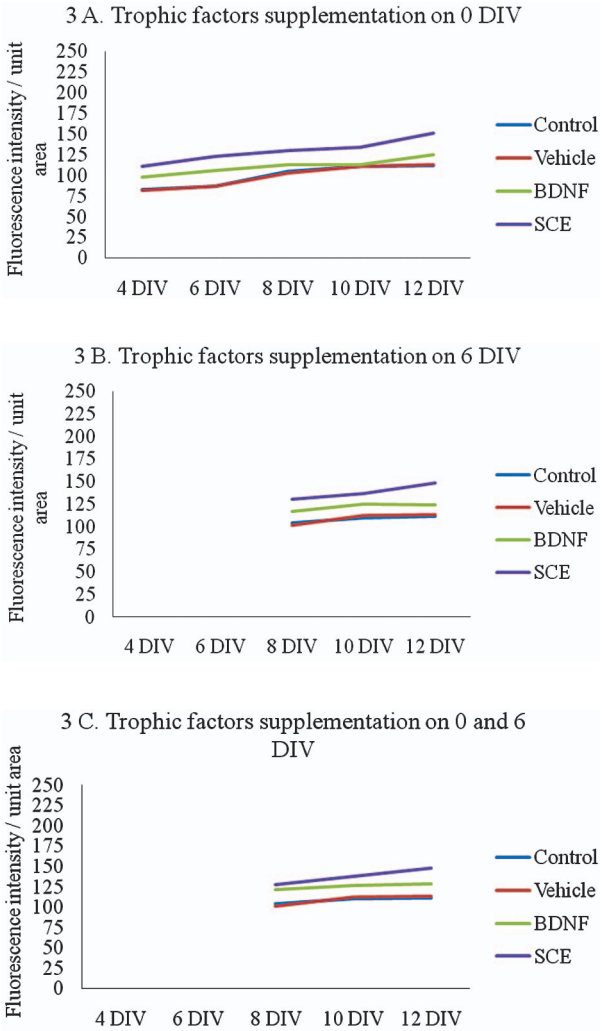


Figure 3. Effect of trophic factors BDNF and SCE on VG Kv 1.6 channel immuno-reactivity in RGCs at various days *in vitro*.

12 DIV. SCE treatment on 6 DIV significantly increased potassium channel immunoreactivity on 10 DIV with no significant change on 12 DIV (Table 1B and Fig. 3B).

When retinal cultures were supplemented with BDNF on 0 as well as on 6 DIV, immunoreactivity was more marked from 8 to 10 DIV as compared to controls; however it was not different from the response obtained from a single dose of BDNF and remained constant through 12 DIV. Meanwhile a significant increase in immunoreactivity was observed between 8 DIV, 10 DIV and 12 DIV in the SCE treated group. The immunoreactivity observed between 10 DIV and 12DIV was not significantly different from the response obtained following a single dose of SCE on 0 DIV and 6 DIV (Table 1C and Fig. 3C).

The expression of VG Kv 1.6 follows a regular developmental pattern. SCE increased the expression of VG Kv 1.6 ion channels as compared to BDNF and controls; nevertheless, peak immunoreactivity remained unchanged following single dose treatment with BDNF or SCE either on 0 DIV or on 6 DIV and double dose on 0 and 6 DIV. SCE also promoted early expression of VG Kv 1.6 ion channels *in vitro*.

DISCUSSION

Voltage gated K^+ channels are key determinants of membrane excitability and contribute to a wide range of neuronal phenotypes observed in the mammalian nervous system.[13] Very little is known about VG Kv 1.6 channels of rat RGCs. At the 15th embryonic day (E15), an outward K^+ current, composed of 4-aminopyridine (4-AP) and tetraethylammonium chloride (TEA) sensitive (I_k, delayed rectifier) components, was observed. Minor change was observed between E15-P5 (5^{th} postnatal day). Lack of developmental changes in I_k supports the view that these channels ensure repolarization after a single action potential but are not specifically responsible for the development of repetitive firing. In the present study, the expression of delayed rectifier VG Kv 1.6 channel immunoreactivity in the early developmental period of RGCs agrees with findings from other regions of the nervous systems.[14-16] The onset of I_k coincides with the beginning of synapse formation in retinal cultures, whereas the development of other voltage activated currents seems to be complete at that stage.[17]

Transient expression of delayed rectifier channels in neuronal soma transmits action potentials at a high frequency.[18,19] Given the high level of expression in most neurons of the brain, the delayed rectifier potassium channels are expected to be a major determinant of neuronal activity.

Survival of RGCs is also increased by neurotrophins and by activation of second messenger cascades associated with neuronal

depolarization.[20] BDNF mRNA is expressed in the superior colliculus, the target tissue receiving the majority of retinal projections.[21,22] In addition, the expression of BDNF in developing visual targets could be activity-dependent. BDNF mRNA levels begin to increase dramatically following innervation by retinal ganglion cell axons in the chick tectum, but decrease after optic nerve transection or injection of tetradotoxin into the eye.[23] Both BDNF and its high affinity receptor trkB, in addition to being expressed in the superior colliculus, are expressed in the retina.[24,25] In our study, besides enhancing cell survival (data not shown) BDNF significantly increased VG Kv 1.6 channel immune reactivity during early stages of *in vitro* development (4 to 6 DIV). BDNF at 40ng/ml concentration promoted the functional development of passive membrane currents and regulated the expression of delayed rectifier K+ channels at the early developmental stage of neural stem cell (NSC).[26]

In the visual system, tectal extracts have been shown to stimulate neurite outgrowth from RGCs in dissociated cell cultures.[27,28] It has been demonstrated that fragments and crude extracts of this center have trophic effects on axotomized ganglion cells both *in vitro* and *in vivo* suggesting that the superior colliculus releases a soluble trophic factor.[29,30] The importance of target tissue for survival of mature ganglion cells was demonstrated in regeneration experiments that involve the transplantation of superior collicular tissue to the site of optic nerve transaction, these experiments reduced loss of ganglion cells and promoted extensive axonal regeneration.[31]

In our studies we used crude superior collicular extract (SCE), which is a cocktail of unidentified trophic factors. The results of the present study with SCE imply that, target derived trophic factors promote the expression of VG Kv 1.6 channels in the developing rat retina more than BDNF. Retinal ganglion cell neurotrophic factor (RGNF), a trophic molecule, was isolated and purified from rat superior colliculi. RGNF carrying the HNK-1 epitope was characterized as a 480kDa superior collicular chondriotin sulfate proteoglycan type C (SCCP). The trophic effect of collicular proteoglycan derived from the superior colliculus on neonatal rats *in situ*, was studied by Huxlin et al.[32] VG Kv 1.6 channel expression also paralleled the expression of synaptic proteins in developing retina reported from this laboratory earlier.[33] Kv-1 channels are responsible for membrane resonance and subthreshold oscillations. Modulation of Kv-1 channel properties will influence the bursting properties of neurons that use resonance and subthreshold oscillations for maintaining excitability.[34] Understanding the process of activity-dependent development could revolutionize our ability to identify, prevent and treat developmental disorders resulting from disruptions of neural activity that interfere with the formation of precise neural circuits.

Although the current study supports the role of trophic factors in regulation of neuronal activity both qualitatively and quantitatively, electrophysiological studies could further unravel the mechanism of action of trophic factors in the modulation of intrinsic neuronal activity. Double labeling of RGCs with Thy 1.1 and Kv 1.6 antibodies may provide precise distribution of Kv 1.6 channels both in ganglion cell layer (GCLs) *in vivo* and RGCs *in vitro*. In this model, we used crude SCE; purification of SCE and protein expression are warranted so that the regulatory role of protein molecules derived from SCE could be better established. Recent research emphasizes the crucial role of voltage dependent potassium channels (Kv) in regulation of apoptosis.[35] Quantification of apoptotic nuclei in RGC systems and expression of Kv 1.6 channels give us an insight to RGC survival and spontaneous electrical activity which in turn play a decisive role in determining functional retinal circuitry.

Acknowledgements

The authors thank the National Institute of Mental Health And Neuro Sciences (NIMHANS) for the research grant and the National Center for Biological Sciences (NCBS) for confocal microscopy facilities.

Conflicts of Interest

None.

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