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Protein kinase A modulates retinal ganglion cell growth during development

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ABSTRACT

During development, retinal ganglion cells (RGCs) extend their axons toward their thalamic and mesencephalic targets. Their navigation is largely directed by guidance cues present in their environment. Since cAMP is an important second messenger that mediates the neural response to guidance molecules and its intracellular levels seem to decrease significantly following birth, we tested whether modulation of the cAMP/protein kinase A (PKA) pathway would affect the normal development of RGC axons. At postnatal day 1, hamsters received a unilateral intraocular injection of either 0.9% saline solution, 12 mM of the membrane-permeable cAMP analogue (dibutyryl cAMP; db-cAMP), or 10 μM of the PKA inhibitor KT5720. Intraocular elevation of cAMP significantly accelerated RGC axonal growth while inhibition of PKA activity decreased it. Moreover, when highly purified RGC cultures were treated with forskolin (an activator of adenylate cyclase) or cAMP analogues (db-cAMP and Sp-cAMP), neurite length, growth cone (GC) surface area and GC filopodia number were significantly increased. This indicates that intraocular elevation of cAMP acts directly on RGCs. Since these effects were prevented by PKA inhibitors, it demonstrates that cAMP also exerts its action via the PKA pathway. Taken together, these results suggest that the cAMP/PKA cascade is essential for the normal development of retinothalamic projections.

Introduction

Following differentiation, RGCs extend their axons to form functional connections with their target cells in the thalamus and the superior colliculus (SC). These axons, tipped at their distal end by the GC, navigate through relatively long distances in a highly directed manner in order to establish functional synapses with thalamic and SC neurons. This is achieved with the help of extracellular guidance molecules which steer RGC axon growth by regulating GC morphology by means of attractive and/or repulsive mechanisms. Consequently, upon binding of a guidance molecule to its receptor, it relays its signal to intracellular second messengers which modulate the GC's response accordingly (Huber et al., 2003). The cyclic nucleotide cAMP is a key intracellular second messenger that plays an essential role in numerous neuronal functions such as cell survival, axon regeneration, and GC behaviour to guidance cues. For example, elevated intracellular cAMP levels enhance the survival rate of CNS neurons (Meyer-Franke et al., 1995; Hanson et al., 1998; Hu et al., 2007). Various mechanisms have been proposed to explain this phenomenon. In RGCs, an increase in survival rate is attributed in part by cAMP's ability to facilitate the cells' responsiveness to trophic factors (Meyer-Franke et al., 1998). cAMP's role in axon regeneration is well documented (Spencer and Filbin, 2004; Teng and Tang, 2006). Furthermore, treatment of injured neurons with a cocktail of trophic factors and cell permeable analogues of cAMP induces elevated numbers of regenerating axons, even promoting their re-growth through inhibitory environment (Cui et al., 2003; Lu et al., 2004; Rodger et al., 2005; Hu et al., 2007). In addition, *in vitro* development of dissociated and highly purified rat dorsal root ganglion cells (DRGs) and RGCs depend upon endogenous cAMP levels (Cai et al., 2001). cAMP also regulates neurite growth by influencing GC behaviour to guidance cues. This is supported by the fact that manipulating the cAMP signalling pathway can alter the response of the GC to a given guidance molecule (Song and Poo, 1999; Bouchard et al., 2004; Nicol et al., 2007).

Despite a large body of evidence describing the role played by cAMP and its downstream effector, PKA, in cell survival and axon regeneration, their role during development *in vivo* remains unknown. The aims of the present study are to determine how the cAMP/PKA pathway modulates neonatal development of RGCs, and to test the hypothesis that modification of retinal cAMP levels will alter retinal axon growth. Our results demonstrate that *in vivo* retinal cAMP levels decrease abruptly following birth. We also show for the first time that an increase in endogenous cAMP levels during development accelerates physiological projection growth by directly acting on RGCs and modulating the cAMP/PKA pathway.

Materials and methods

All animal experiments were approved by the *Comité de déontologie de l'expérimentation sur les animaux* from the University of

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Table 1
Experimental design

Experimental conditions		Number of hamsters per age group					
		P0	P3	P5	P7	P9	P12
ELISA[a]		3	3	3	3	3	
Normal growth (saline)[b]			3	3	3	3	3
Intraocular injections[b]	Control — saline			5			
	db-cAMP			5			
	KT 5720			5			

[a] $n=6$ retinae per age group.
[b] 4–8 consecutive coronal slices per animal.

Montreal and were handled in accordance with the recommendations provided by the Canadian Council on Animal Care. The distribution of the animals in the different experimental groups appears in Table 1.

Reagents

Bovine serum albumin (BSA), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), dibutyryl cAMP (db-cAMP), DNase, forskolin (FSK), Hoechst 33258, insulin, KT5720, laminin, poly-D-lysine, progesterone, putrescine, pyruvate, Rp-cAMP, selenium, Sp-cAMP, apotransferrin, triiodo-thyronine, and trypsin were purchased from Sigma (Oakville, ON). B27, Dulbecco's phosphate-buffered saline (DPBS), foetal bovine serum (FBS), glutamine, and Neurobasal media were purchased from Invitrogen (Burlington, ON). Papain solution was from Worthington Biochemicals (Lakewood, NJ), normal donkey serum (NDS) from Jackson Immuno, (West Grove, PA) and ovomucoid from Roche Diagnostics (Indianapolis, IN). Primary antibodies rabbit-anti-mouse-macrophage and mouse-anti-Thy-1.2 monoclonal IgM (μ chain specific) were obtained from Accurate Chemical (Wesbury, NY), and rabbit-anti-GAP-43 was from Chemicon International (Temecula, CA). Secondary antibodies goat anti-rabbit IgG (H+L) and goat anti-mouse IgM (μ chain specific) were from Jackson Immuno (West Grove, PA), and Alexa Fluor conjugated secondary antibodies (Alexa 488 and Alexa 546) were from Invitrogen. Avidin-biotin-peroxidase complex ABC Kit and donkey anti-goat biotinylated secondary antibody were from Vector Labs (Burlingame, CA). The B fragment of the cholera toxin (CTb) and goat anti-CTb antibody were from List Biological Laboratories (Campbell, CA). cAMP Parameter Assay Kit (KGE002) was purchased from R&D Systems (Minneapolis, MN).

cAMP ELISA

Total retinal cAMP levels were quantified using a cAMP Parameter Assay Kit. The right and left retina were dissected on ice, weighed, frozen and kept at −80 °C for further processing. Tissues were homogenised in a lysis buffer included in the kit and cAMP levels were measured in triplicate according to the manufacturer's instructions. Results are expressed as pmol/μg of wet tissue.

Intraocular injections

Syrian golden hamsters (Charles River, St-Constant, QC) were used for intraocular injections. These mammals are born with a premature nervous system (Clancy et al., 2001). Twenty four hours following birth, at postnatal day 1 (P1), hamsters received a unilateral injection of 2 μl solution of CTb, a highly sensitive anterograde tracer, with either 0.9% saline solution, 12 mM of the membrane-permeable cAMP analogue db-cAMP or 10 μM of the PKA inhibitor KT5720. Briefly, under an operating microscope, a small incision was made in the eyelids to access to the right eye. The injections were administered using a glass micropipette attached to a 10 μl Hamilton syringe. The micropipette was carefully inserted into the vitreous at an angle to avoid damage to the lens. Following the injection, the eyelids were closed with surgical glue (Vetbond; 3 M, St-Paul, MN).

Perfusion

At P5, four days postinjection, anesthetised hamsters were perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed overnight at 4 °C and cryoprotected by infiltration of buffered sucrose. The brains were then frozen and kept at −80 °C until further processing.

Immunohistochemistry

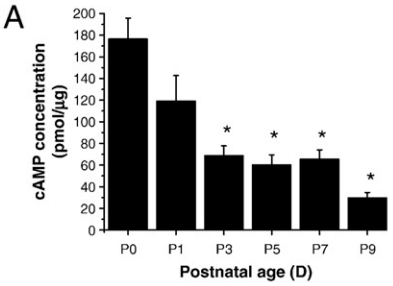
Transported CTb was visualized by immunohistochemistry according to a protocol previously described (Angelucci et al., 1996; Matteau et al., 2003). Briefly, 40 μm thick coronal sections of tissue were incubated in 90% methanol and 0.3% H_2O_2 in 0.1 M PBS (pH 7.4) for 20 min. They were then rinsed and incubated in 0.1 M glycine in PBS for 30 min, followed by an overnight incubation (4 °C) in PBS containing 4% NDS, 2.5% BSA, and 1% Triton X-100. Sections were subsequently rinsed and immersed for 48 h at room temperature in a solution containing goat anti-CTb diluted 1:4000 in PBS with 2% NDS, 2.5% BSA, and 2% Triton X-100. Afterwards, sections were rinsed and incubated in 2% NDS and 2.5% BSA/PBS for 10 min. This was followed by a 1-h incubation in donkey anti-goat biotinylated secondary antibody diluted 1:200 in PBS with 2% NDS, 2.5% BSA, and 1% Triton X-100. Tissue was rinsed, incubated in 2% NDS and 2.5% BSA in PBS for 10 min, and subsequently processed by an avidin-biotin-peroxidase complex ABC Kit (diluted 1:100 in PBS) for 1 h, in the dark and at room temperature. Sections were then rinsed and preincubated in 3, 39-diaminobenzidine tetrahydrochloride (DAB) in PBS for 5 min. The peroxidase was visualized by adding 0.004% H_2O_2 to the DAB solution for 2–4 min. Sections were finally washed five times (1 min each) with PBS, mounted on gelatine-chrome alum-subbed slides, air-dried, dehydrated in ethanol, cleared in xylenes, and coverslipped with Depex (EMS, Hatfield, PA).

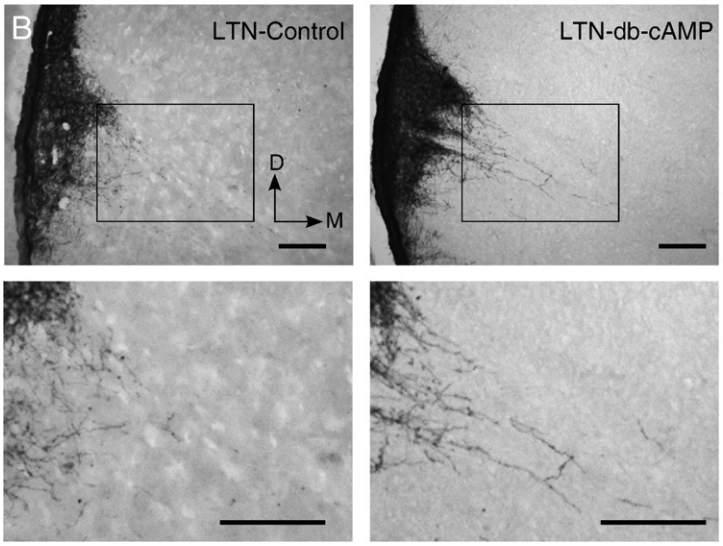
Purified retinal ganglion cell culture

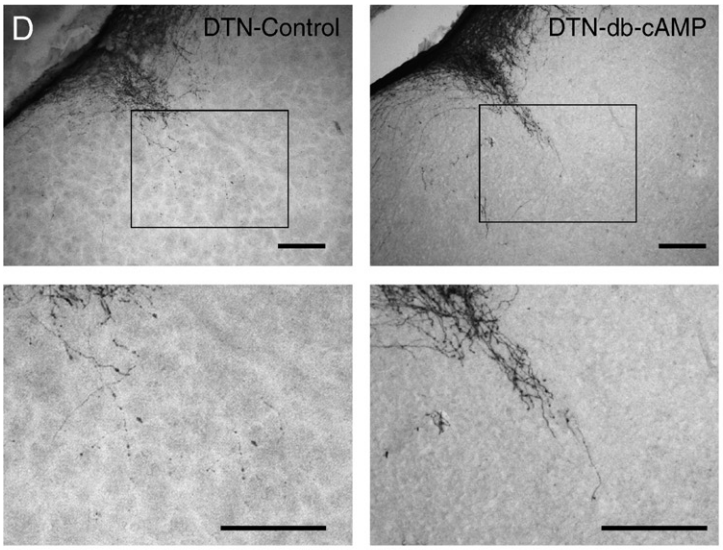
Retinal ganglion cells (RGC) from P7-P8 mice (Charles River, St-Constant, QC) were purified and cultured according to a protocol previously described by Barres et al. (1988). In brief, following enucleation, retinae were dissected and enzymatically dissociated, at 37 °C for 30 min, in a papain solution (15 U/ml in DPBS) containing 1 mM L-cysteine. The retinae were then triturated sequentially, with a 1 ml pipette, in a solution containing ovomucoid (1.5 mg/ml), DNase (0.004%), BSA (1.5 mg/ml) and rabbit antibodies directed against mouse-macrophage (1:75) to yield a suspension of single cells. The suspension was centrifuged and washed in a high concentration ovomucoid-BSA solution (10 mg/ml for each in DPBS). The dissociated cells were resuspended in DPBS containing BSA (0.2 mg/ml) and insulin (5 μg/ml).

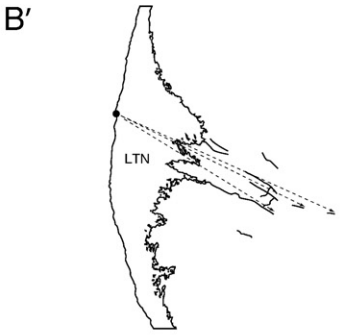
RGCs were purified using the two-step panning procedure (Barres et al., 1988; Meyer-Franke et al., 1995). In short, to remove macrophages, the retinal suspension was incubated at room temperature in petri dishes coated with affinity-purified goat anti-rabbit IgG (H+L). The nonadherent cells were then transferred to a petri dish that had been coated with affinity-purified goat anti-mouse IgM (μ chain specific) followed by anti-Thy-1.2 monoclonal IgM. The adherent RGCs were first released enzymatically by incubating them in a 0.125% trypsin solution at 37 °C and 5% CO_2 followed by manually pipetting an enzyme inhibitor solution (30% FBS in Neurobasal) along the surface of the dish.

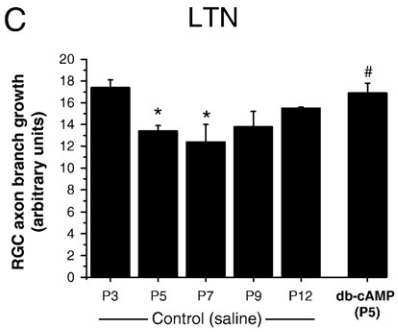
Purified RGCs were plated on poly-D-lysine (10 μg/ml) and laminin (5 μg/ml) coated glass coverslips (number 0 Deckgläser; Carolina Biological, Burlington, NC) in 24-well plates. RGCs were cultured in 600 μl of serum-free medium modified from Bottenstein and Sato (1979). Neurobasal media were supplemented with B27, selenium, putrescine, triiodo-thyronine, transferrin, progesterone, pyruvate

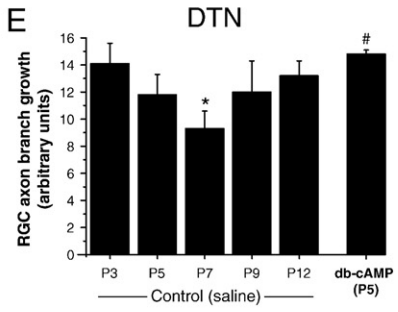

A
cAMP concentration (pmol/µg)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
*
P0
P1
P3
P5
P7
P9
Postnatal age (D)

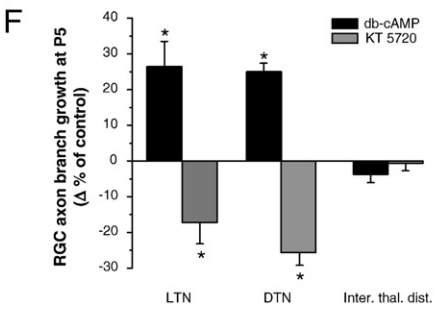

B
LTN-Control
LTN-db-cAMP
D
M


D
DTN-Control
DTN-db-cAMP


B′
LTN


C
LTN
RGC axon branch growth (arbitrary units)
20
18
16
14
12
10
8
6
4
2
0
*
*
#
P3
P5
P7
P9
P12
db-cAMP (P5)
Control (saline)


E
DTN
RGC axon branch growth (arbitrary units)
16
14
12
10
8
6
4
2
0
*
#
P3
P5
P7
P9
P12
db-cAMP (P5)
Control (saline)


F
RGC axon branch growth at P5 (Δ % of control)
40
30
20
10
0
-10
-20
-30
db-cAMP
KT 5720
*
*
*
*
LTN
DTN
Inter. thal. dist.

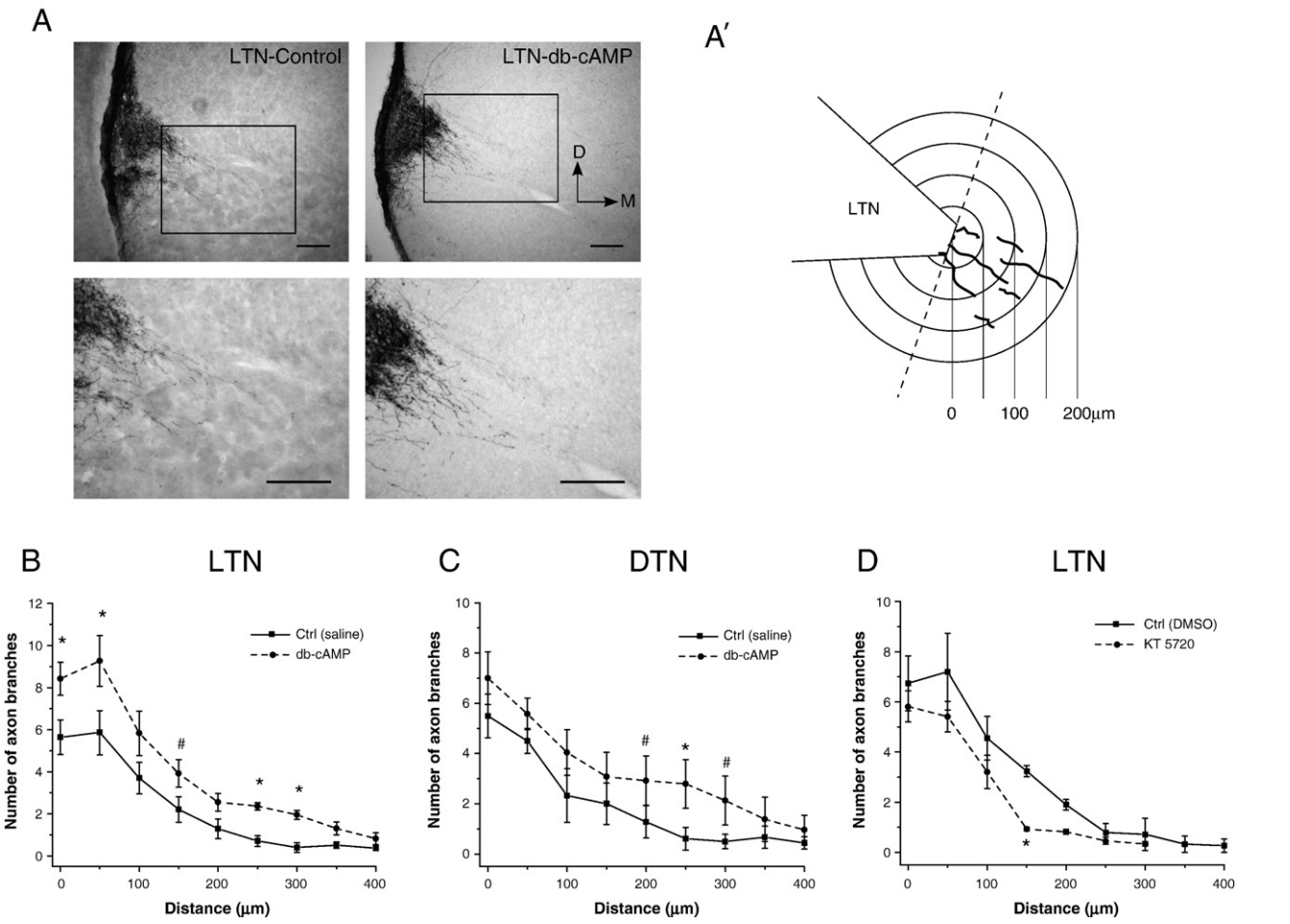


Fig. 2. Retinal projection density. A–D. Evaluation of retinal branch density using Sholl's technique in LTN and DTN. A. Photomicrographs showing retinal-LTN projections in control and db-cAMP treated groups. Retinal projections are shown at higher magnification (2×) in the lower panel. Note that the retinal projections are more numerous in the treated group. Orientation pointers indicate the dorsal and medial parts of the thalamus. Scale bar = 100 μm. A′. An illustration of the method used to quantify the number of retinal axon branches. B and C. The number of axon branches diminishes along the medio-lateral axis in LTN and DTN. D. KT5720 decreased axonal branches in LTN. * and # indicate significant changes compared with the P5 control group ($p<0.01$ and $p<0.05$, respectively).

(1 mM), glutamine (2 mM), ciliary neurotrophic factor (CNTF; 10 ng/ml), brain-derived neurotrophic factor (BDNF; 50 ng/ml), insulin (5 μg/ml), and FSK (10 μM). RGCs were cultured at 37 °C and 5% CO_2. All experiments on purified RGCs were performed 36–40 h following platting.

Immunocytochemistry

RGC cultures were washed with PBS (pH 7.4), fixed in 4% paraformaldehyde (pH 7.4) and blocked with 2% normal goat serum (NGS) and 2% BSA in PBS containing 0.1% Tween 20 (pH 7.4) for 2 h at room temperature. Cells were then incubated overnight at 4 °C in blocking solution containing anti-GAP-43 (1:1000). The following day, cells were washed and labelled with Alexa 488 secondary antibody and Hoechst 33258, and the coverslips were mounted with Geltol (Thermo Scientific, Pittsburgh, PA).

Quantification method

Photomicrographs were taken with an inverted Olympus IX71 microscope (Olympus Canada, Markham, ON) and an Evolution VF camera (MediaCybernetics, Bethesda, MD). Images were analyzed with Image Pro Plus 5.1 image analysis software (MediaCybernetics). Axon branch growth was quantified on consecutive photomicrographs of coronal slices of brain tissue comprising the lateral terminal nucleus (LTN) and the dorsal terminal nucleus (DTN). On each photomicrograph, the distance between the lateral border of the nucleus of interest and the tips of the longest axon branches were measured (Figs. 1B and B′). To take into account for differences in brain sizes, axon branch lengths were normalized with the interthalamic distance (distance between the right and left lateral borders of the thalamus). Axon collateral density was quantified on consecutive photomicrographs comprising the LTN and DTN using an adaptation of the Sholl's technique (Sholl, 1953)

Fig. 1. cAMP and retinal projection growth *in vivo* during development. A. In hamsters, during the first week of development, cAMP concentration in the retina decreases significantly with age (six retinae per time-point). ($^{*}p<0.001$). B and D. During normal development, RGCs send their projections towards the lateral terminal nucleus (LTN), and the dorsal terminal nucleus (DTN). Upper panel: photomicrographs show P5 retinal projections in LTN (B) and DTN (D) in control and db-cAMP treated groups. Retinal projections are shown at higher magnification (2×) in the lower panel. B′. An illustration of the LTN and retinal projection branches pointing towards the medial part of the thalamus. Arrowed dotted lines indicate the distance between the lateral border of the thalamus and the tip of the farthest projections. C and E. Retinal axon branches in LTN (C) and DTN (E) of control (saline) groups (P3 to P12) slow their development between P5 and P7 before resuming normal growth ($^{*}p<0.05$). Note that db-cAMP treated animals are comparable to P9 and P12 but significantly different from P5 controls ($^{\#}p<0.05$). RGC axon branch growth was quantified by measuring the distance between the lateral border of the nucleus of interest and the tip of the longest axon branches. The latter was normalized to the interthalamic distance (distance between the right and left lateral borders of the thalamus). F. At P5, compared with the control group, the db-cAMP treated group increased retinal axon branch length in LTN and DTN. A decrease in branch length was induced by KT5720, PKA inhibitor. ($^{*}p<0.005$). Orientation pointers indicate the dorsal and medial parts of the thalamus. Scale bar = 100 μm.

(Figs. 2A and A′). Values are expressed as the mean ± SEM. Statistical significance of differences between means was evaluated by analysis of variance (ANOVA) with Sheffe's *post hoc* test (Systat).

Results

The objective of the present study was to investigate the implication of the cAMP/PKA pathway in axon guidance during RGC development. First, we examined the effects of modulating retinal cAMP levels *in vivo* on RGC projections development during the first neonatal week. Then, using highly purified RGC culture, we investigated the direct action of cAMP on RGCs by stimulating the cAMP-dependent-PKA.

Retinal cAMP levels decrease during postnatal development

Compared with rats and mice, hamsters have shorter gestation period. Consequently, they are born with a relatively premature nervous system (Clancy et al., 2001). In hamsters, cAMP concentration in the retina decreases significantly during the first week of neonatal development. In comparison with P0 retinae, cAMP levels at P3 decrease by more than half. Retinal cAMP concentration reaches its lowest level at P9 which corresponds to an 85% reduction compared with P0 retinae (Fig. 1A). These results support previously reported observations in isolated rat DRGs and RGCs (Cai et al., 2001).

Development of retinal projections

During development, RGC axons navigate through distinct anatomical structures before reaching their targets. Axons enter the optic disc, penetrate the optic nerve head, cross the optic chiasm and finally connect with their thalamic (lateral geniculate nucleus — LGN and lateral posterior — LP) and midbrain (SC) targets in the appropriate hemisphere. In hamsters, by P3, virtually all RGCs have reached their targets (Bhide and Frost, 1991; Clancy et al., 2001). Consequently, the intraocular injection of an anterograde tracer would intensely label the LGN, LP and SC thus making difficult the evaluation of RGC axon collateral growth at P5. To circumvent this problem, we chose to investigate RGC branch growth in the accessory visual pathway composed of LTN and DTN (Figs. 1B and D). Visual projections to these nuclei are sparse, well defined and easy to visualize. They are involved in mediating visuomotor reflexes underlying the generation of optokinetic nystagmus (Ling et al., 1998; Bai et al., 2001). During the first postnatal week, retinal axon branches grow in distinct phases. Between P3 and P7, RGCs projecting collaterals in the LTN and the DTN slow their development (Figs. 1C and E). The decrease in their length between P3 and P7 is statistically significant. By the second postnatal week, RGC collaterals resume their growth, and at P12, the branch length is comparable with the P3 group (Figs. 1C and E). Retinal axon collaterals in the LTN and DTN decrease in number as they grow medially further away from the optic tract.

The cAMP/PKA pathway plays an essential role during retinal projection development

A single monocular injection of db-cAMP at P1 results in a significant increase in retinal collateral length. Compared with the untreated P5 aged group, the db-cAMP group (P5) shows a significant increase in RGC axon collateral length in the LTN and the DTN (Figs. 1B and D respectively, and F). Furthermore, an increase in retinal cAMP levels accelerates retinofugal axon collateral development in the P5 (cAMP-treated) group with branch length similar to untreated P9 and P12 groups (Figs. 1C and E, respectively). Collateral branches in the LTN and the DTN project medially from the optic tract located on the lateral edges of the thalamus. db-cAMP treated groups show a higher axon branch density at various distance points along the medio-lateral axis (Figs. 2A, B and C). This demonstrates that increasing cAMP levels accelerates the normal development of RGC projections. Conversely, blocking basal PKA activity via intraocular injection of KT5720 slows down RGC development by diminishing branch growth and density in the LTN and DTN (Figs. 1F and 2D, respectively). Moreover, increasing intraocular db-cAMP does not enhance the transitory aberrant projections observed in the thalamic somatosensory nuclei (ventrobasal nucleus — VBN; Fig. 3A) (Frost, 1984; Langdon and Frost, 1991) neither does it induce aberrant projections in the auditory thalamic nuclei (medial geniculate nucleus — MGN; Fig. 3B) reported in rewired hamsters (Bhide and Frost, 1999; Frost et al., 2000; Ptito et al., 2001).

cAMP acts directly on RGCs modulating the shape of their growth cones and controlling the extension of their axon

The retina is a complex neural tissue composed of several cell types assembled in precise functional architecture (Rapaport et al., 2004). Most of these cells, intrinsically or in response to a stimulus, express different factors which influence their development (Isenmann et al., 2003; Amato et al., 2004). An important question in understanding

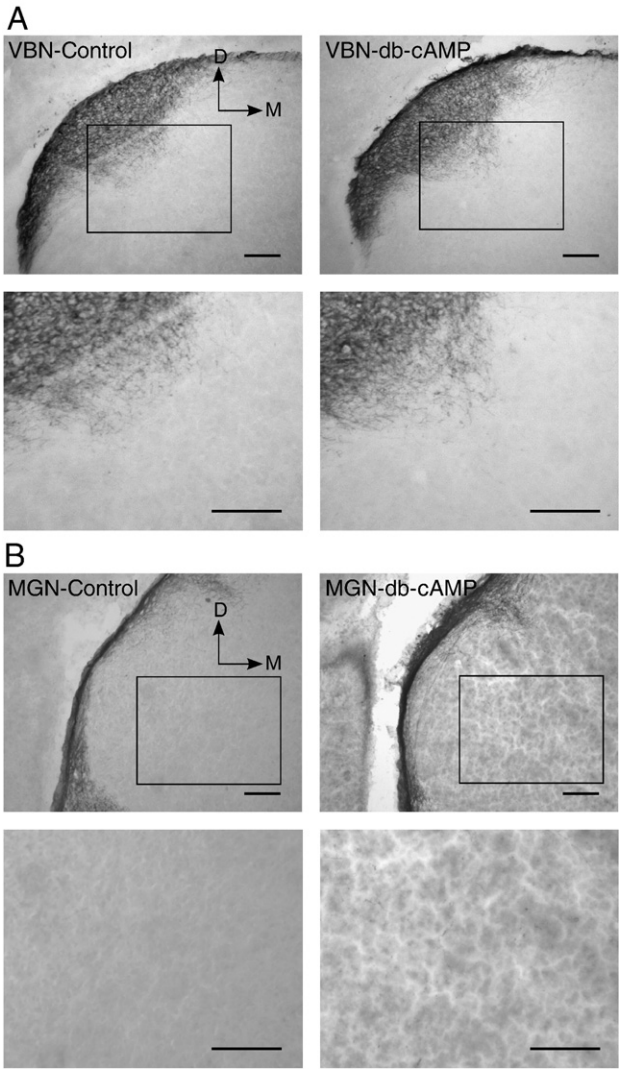


Fig. 3. Retinal projections in the VBN and the MGN. Increase in intraocular cAMP levels does not induce aberrant axon growth. A. During normal development, RGCs send transient projections towards the VBN. Upper panel: photomicrographs show P5 retinal projections in VBN in control and db-cAMP treated groups. Transient projections are shown at higher magnification (2×) in the lower panel. B. Photomicrographs showing P5 retinal projections in MGN in control and db-cAMP treated groups. The MGN is shown at higher magnification (2×) in the lower panel. No differences were observed in both structures for the two groups. Orientation pointers indicate the dorsal and medial parts of the thalamus. Scale bar = 100 μm.

the mechanisms that underlie the increase in thalamic projection growth following an intraocular injection is whether cAMP acts directly on RGCs. To address this question, we treated cultures of highly purified RGCs with various pharmacological agents known to modulate the cAMP/PKA pathway. Treating these cultures with activators of this pathway (FSK and Sp-cAMP) significantly increases

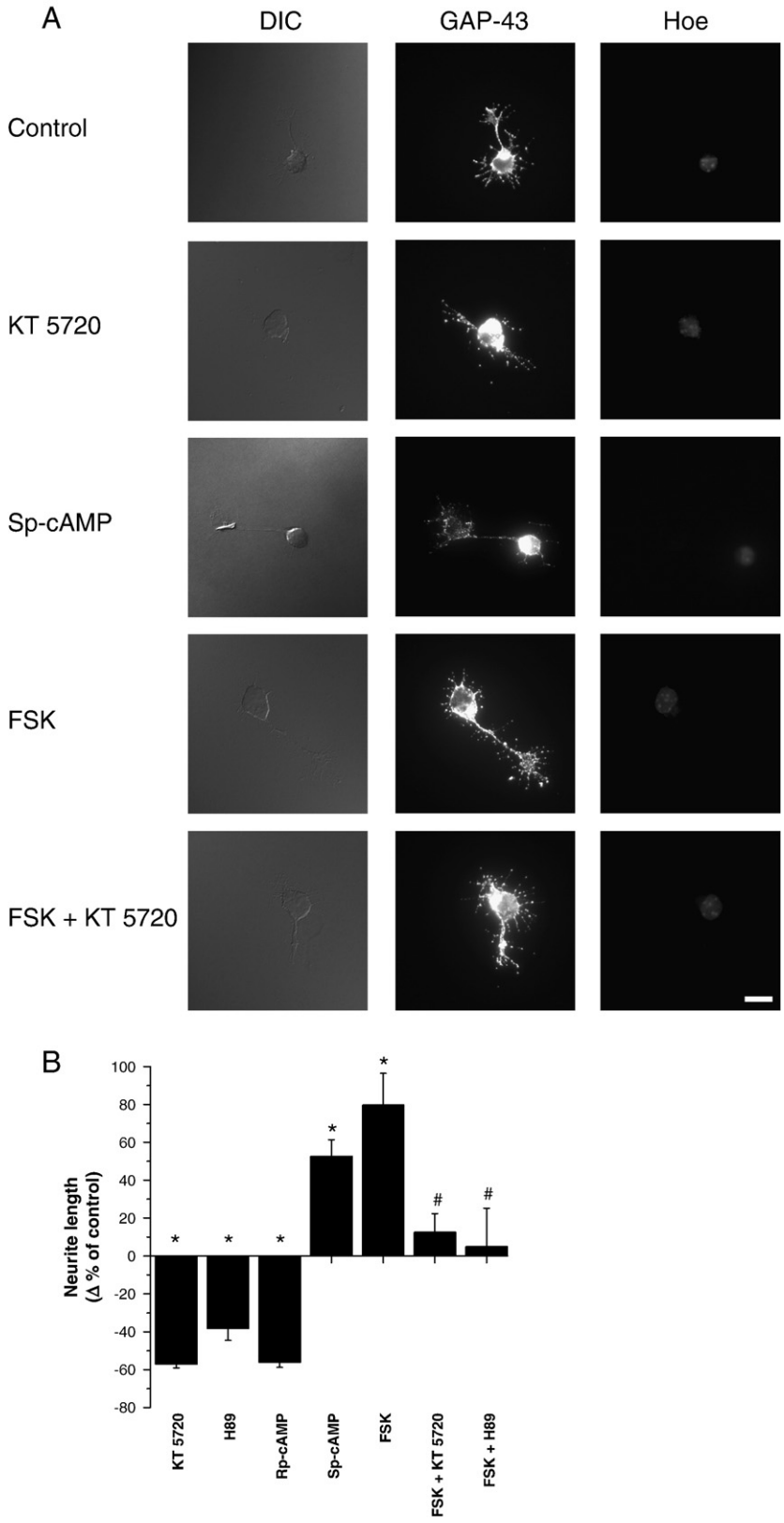


Fig. 4. cAMP and PKA modulate RGC projection growth. A. Cultures of highly purified RGCs were treated with pharmacological modulators of the cAMP/PKA pathway 36–40 h after platting. B. When RGCs were treated with cAMP/PKA pathway activators (FSK and Sp-cAMP), neurite length significantly increased. This increase was blocked with PKA inhibitors (KT 5720 and H89). Blocking basal PKA activity induced a significant decrease in neurite growth. * indicates a significant change compared with the control group and # with the FSK group ($p<0.001$). Scale bar = 20 μm.

neurite growth (Figs. 4A and B). Furthermore, exposing RGC cultures to pharmacological agents that increase intracellular cAMP levels (FSK) and to PKA activators (db-cAMP and Sp-cAMP), augments significantly GC surface area and filopodia number at the GC (Figs. 5A, B, and C). The cAMP induced increases in length, GC surface area and filopodia number are blocked when RGCs are treated with a combination of cAMP increasing agents and PKA-selective pharmacological inhibitors (KT5720, H89, Rp-cAMP; Figs. 4B and 5B, and C).

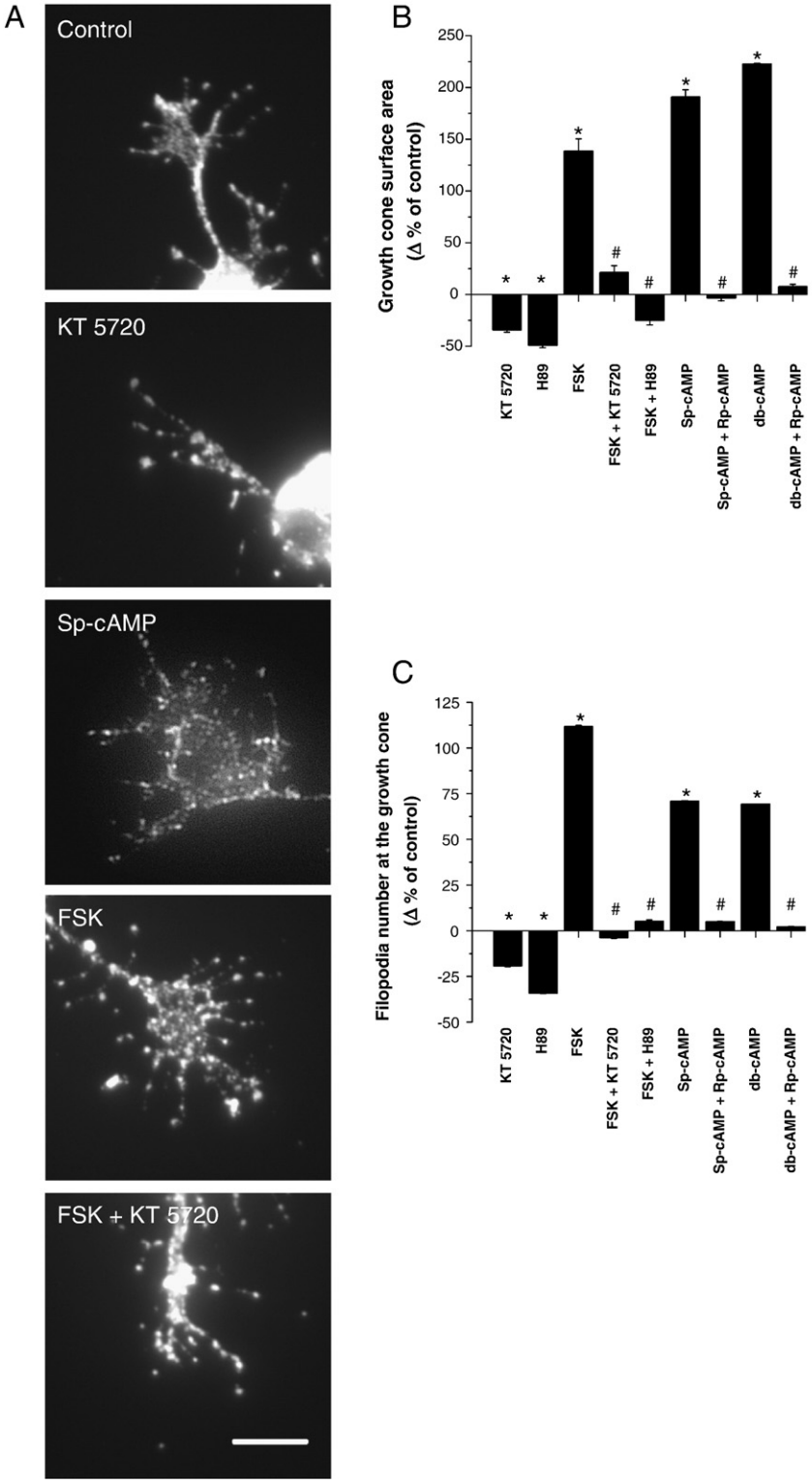


Fig. 5. cAMP/PKA pathway and growth cone morphology. A. Photomicrographs of control, Sp-cAMP, FSK, KT 5720 and FSK+KT 5720 treated RGC growth cones (GCs). B. Pharmacological activation of the cAMP/PKA pathway (FSK, Sp-cAMP and db-cAMP) significantly increases GC surface. This increase was blocked by PKA inhibitors (KT 5720, H89 and Rp-cAMP). Blocking basal PKA activity with KT 5720 or H89 decreased GC surface area. * indicates a significant change compared with the control group and # with FSK, Sp-cAMP, or db-cAMP treated groups ($p<0.001$). C. Activation of the cAMP/PKA pathway (FSK, Sp-cAMP and db-cAMP) increases the number of filopodia at the GC. PKA inhibitors (KT 5720, H89 and Rp-cAMP) prevented this effect. Deactivating basal PKA activity using KT 5720 or H89 induced a significant decrease in filopodia number at the GC. * indicates a significant change compared with the control group and # with FSK, Sp-cAMP, or db-cAMP treated groups ($p<0.001$). Scale bar = 20 μm.

Furthermore, adding exclusively PKA inhibitors to the RGC culture media diminishes significantly neurite growth and modifies GC morphology (Figs. 4A and B and 5A, B, and C). These results confirm our hypothesis suggesting that cAMP acts directly on RGCs by modulating the morphology of the GC and by regulating the axon extension.

Discussion

The present study demonstrates that retinal cAMP levels decrease during early postnatal development. Interestingly, increasing retinal concentration with a single intraocular injection of db-cAMP accelerates RGC growth by activating directly PKA in RGCs. Conversely, inhibition of PKA slows RGC axon branch development in the thalamus (LTN and DTN).

cAMP is an important second messenger for axon growth (Meyer-Franke et al., 1995; Meyer-Franke et al., 1998; Bouchard et al., 2004) and segregation of retinofugal projections in an eye-specific manner (Stellwagen et al., 1999; Ravary et al., 2003; Nicol et al., 2006). Here we report, for the first time, a rapid decline in endogenous retinal cAMP levels *in vivo* in hamsters during the first postnatal week. These results are in agreement with a previous *in vitro* study that reported a significant decrease in endogenous levels of the cyclic nucleotide cAMP in purified rat DRGs and RCGs (Cai et al., 2001). However, our data appear to be in contradiction with a recent study showing a non significant decrease in adenylate cyclase gene (*AC1*) expression in the retina during the first week following birth (Nicol et al., 2006). Since the latter authors did not systematically monitor retinal cAMP levels during postnatal retinal development, it is difficult to evaluate the magnitude of the cAMP production decline resulting from a slight decrease in *AC1* gene expression. It is also possible that the postnatal decrease in cAMP levels is due to a reduction in AC activity.

cAMP also appears to be an important mediator in promoting axon growth during regeneration. Several studies have reported that increasing endogenous cAMP levels with injections of pharmacological agents promote axon regeneration (Qiu et al., 2002; Monsul et al., 2004), and also potentiate growth factor induced axon regeneration (Cui et al., 2003; Lu et al., 2004). Furthermore, these studies indicate that cAMP acts by directly activating PKA. In the present study, we were interested in investigating the effects of a variation in retinal cAMP levels on RGC growth during neonatal development *in vivo*. Our results clearly demonstrate that intraocular cAMP levels modulate normal axon growth during postnatal development and do not induce aberrant axon growth since there was no significant increase in RGC axon branch density in the VBN and the MGN. Although the magnitude of the increase in collateral length is significant, it did not match the 7-fold increase in length observed in isolated embryonic RGC cultures. Our data however are in agreement with the developmental loss in intrinsic axonal growth capability documented in a previous study (Goldberg et al., 2002). Moreover, the fact that the treated P5 group had similar characteristics in terms of axon branch length and density in LTN and DTN to the untreated P9 and P12 groups suggests that cAMP accelerates RGC collateral development. On the other hand, inhibition of basal PKA activity decreased natural RGC axonal growth confirming the physiological importance played by the cAMP/PKA pathway during development.

Elevated intraocular db-cAMP levels could exercise its effects by several mechanisms. It could influence surrounding cells to secrete growth factors which will subsequently affect RGC growth and/or directly act on RGCs. In the present study, we have used the cell culture technique developed by Barres et al. (1988) and Meyer-Franke et al. (1995) that allowed for highly purified RGCs and various pharmacological agents known to modulate PKA activity in order to demonstrate that cAMP acts directly on RGCs. Similar to our results obtained *in vivo*, modulation of the cAMP/PKA pathway in RGC cultures *in vitro* induced RGC neurite growth and raised GC surface area and filopodia number at the GC. These results confirm that increasing intraocular cAMP levels act in part by directly activating RGCs. Inhibition of basal PKA activity induces the reverse effect and supports the role played by the cAMP/PKA pathway during RGC axon extension.

In conclusion, numerous studies have shown that cAMP plays an essential role during GC pathfinding in the developing CNS *in vitro*. The data presented here demonstrate that retinal cAMP concentrations diminish significantly following birth. More importantly, we show, for the first time *in vivo*, that maintaining a high level of intravitreal cAMP levels in mammals born with a premature nervous system promotes RGC axon branch development by directly activating the cAMP/PKA pathway in RGCs. These data confirm the pivotal role played by cAMP during the establishment of the retinothalamic pathway. Consequently, this could potentially be an interesting mechanism by which pathological conditions in premature newborns could be corrected.

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