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Chapter 17

The Experimental Manipulation of Visual Cortex Efferents

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http://dx.doi.org/10.5772/48632

1. Introduction

The visual cortex is part of the occipital cortex that makes up the primary and secondary visual areas [1,2]. In the primary visual areas of rodents, as in other isocortical areas, two main neuronal types are present: inhibitory interneurons and projecting neurons [3,4]. The inhibitory interneurons belong to several GABAergic subpopulations, while the projection neurons are excitatory pyramidal neurons that are classically distributed in 5 layers, each of which is associated with a preferential projection area [3,4]. Accordingly, the pyramidal neurons of layers II, III and IV give rise to corticocortical connections, while those of layers V and VI project to subcortical structures. In the primary visual cortex pyramidal neurons of layer V project to superficial collicular layers and they give rise to collaterals that project to the pontine nuclei.

The aim of this review is to describe the sprouting capacities of these projecting neurons and to evaluate several strategies to enhance these capabilities in adult animals, principally considering work carried out in rodents. In the first part, we will discuss the sprouting of the corticocollicular ipsilateral connection in young animals. This connection originates in layer V pyramidal neurons and its post-lesional sprouting capacities diminish significantly after the end of the critical period (postnatal day 45). We will also discuss the use of siRNAs to knockdown the expression of molecules that inhibit post-lesional axonal sprouting in adults. Lastly, we will describe alterations in sprouting and synaptic size in the corticocortical visual connections.

2. Differential lesion responses of neonatal and adult visual cortex efferents

The visual system is widely used as a model to study plasticity, given the compartmentalized arrangement of its main stations. In rodents, most of the retinal ganglion

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axons cross the optic chiasm to the contralateral side [5,6]. Thus, retinal deafferentation is a convenient experimental means of investigating the plastic response mechanisms to central nervous system (CNS) lesions.

The superior colliculus (SC) is a layered mesencephalic structure that can be divided into two main compartments: the superficial strata that are mainly devoted to visual function; and the intermediate and deep strata that process multisensorial information [7-9]. The superficial layers are composed of the stratum zonale (SZ), stratum griseum superficiale (SGS), and stratum opticum (SO), and they receive their main afferent input from the retina and primary visual cortex.

In rats, virtually all retinal ganglion cells project to the contralateral SC [6,10] and the majority of optic axons reach the SC prenatally, with the remainder reaching their target in the early postnatal days [11]. Layer V pyramidal neurons of the primary visual cortex (VC1) project to the ipsilateral SC [12-14], with the first visual cortical axons that reach the SC arriving on postnatal day (P) 4/5. At this stage, the axons only appear in the SO. From P7 to P13, these projections spread out to the ventral region of the SGS and the intermediate layers, and between P13 and P19, connections are restricted to the superficial strata of the SC, ultimately forming the organizational pattern seen in adults [15]. Although both retinocollicular and corticocollicular terminals densely innervate superficial strata of the SC, the former ramify more densely in the SZ and upper SGS, while the latter project to the lower SGS and upper SO [12-14]. During development, retinal and primary visual cortex fibers undergo multiple plastic changes, which include axonal growth, target path finding, axonal pruning and projection refinement [15-17]. This results in the formation of a precisely organized topographic map that represents the visual field in the SC in a point-to-point fashion.

CNS lesions or pathologies, and the deprivation of visual stimuli, can alter the final visual corticocollicular organization, predisposing this system to phenomena of neuroplasticity [18]. The capacity to respond to CNS lesions through plastic changes varies depending on the age at which the injury takes place. Thus, during early postnatal development, while connections are being established, neuronal projections exhibit significant capacity for regeneration and reorganization in response to neuronal damage. However, this post-lesional response becomes considerably diminished in adulthood. A remarkable number of publications have described changes in the organization of neuronal connections following neonatal CNS injury. In the visual system, retinal deafferentation at birth results in severe alterations of the afferent systems that project to SC superficial layers [19]. For example, removal of SC input in neonatal rodents results in an aberrant ipsilateral retinotectal projection [20-24], whereas retinal deafferentation in adults has no such effect [25-27]. Gradual, continuous plastic changes have been described in the ipsilateral retinal axons of adult rats subjected to contralateral retinal lesions at P21, in contrast to the fast plastic response observed in neonatal rats evident within 48 hours of lesion [28].

Neonatal lesions of the visual cortex give rise to an aberrant projection to the contralateral SC [29] and expansion of the ipsilateral corticocollicular projection from the remaining unlesioned visual cortex [30]. These plastic responses during early postnatal development

may occur due to axonal sprouting and/or the blockade of developmentally regulated axonal retraction. It has been suggested that axons continuously compete for postsynaptic sites in the CNS. Indeed, it is likely that during development this competition is essential for the formation and refinement of projections, although an equilibrium is reached in the mature nervous system that results in the stabilization of neuronal connections [31,32].

In previous studies, we observed an enlargement of the visual corticocollicular terminal field in rabbits after neonatal removal of contralateral retinal inputs [33], and an alteration in the plastic response to injury when the same lesion was performed in adults [34]. The anterograde axonal tracer biotin dextran amine (BDA) was used to label the corticocollicular connection emerging from layer V pyramidal neurons of the primary visual cortex in 3 different experimental groups: (i) adult rats (P60) subjected to neonatal (P1) optic nerve transection; (ii) adults rats subjected to optic nerve transection in adulthood; and (iii) control adults rats. The animals were sacrificed 10 days after BDA injection and the superior colliculi extracted for histochemical analysis. As BDA was injected into the region of the primary visual cortex that represents the lower temporal visual field [35-36], corticocollicular terminal fields were localized within the posterolateral quadrant of the SC [37] in all experimental animals. In agreement with previous studies [14,33,38], we observed a tight topographical organization of the visual corticocollicular terminal field. In control animals, the corticocollicular terminal field was column-shaped, extending from the SO up to the pial surface, and it was restricted to a small portion of the collicular surface. Fibers ascending from the SO gave rise to dense axonal networks in the lower half of SGS, and they branched to reach the upper half of this stratum and the most superficial SZ, where the fibers were oriented parallel to the collicular surface [34].

Visual deprivation in neonatal animals results in significant expansion of the corticocollicular visual terminal fields, which invaded the entire lateromedial extension of the visual collicular strata. However, the axons tended to concentrate in the posterolateral quadrant of the collicular surface, indicating that the gross topography of the connection was maintained, despite deafferentation [34]. Molecules involved in target path finding, such as ephrins and their receptors, may play a crucial role in determining retinotectal topography [39-41]. Neonatal deafferentation also significantly alters the direction of fiber projection, resulting in horizontal and oblique orientation in the majority of fibers within the SGS in deafferented animals. We previously reported a similar effect in rabbits [33]. However, the expansion of this terminal field may reflect the maintenance of collaterals during postnatal development [38,42] or active sprouting processes. Previous studies reported that in neonatal animals, corticocollicular fibers only appear in the SO [38,43]. As we observed a large density of fibers occupying almost the entire extension of the most superficial strata, SZ and SO, we can assert that an active process of axonal pruning occurred after neonatal deafferentation.

The labeled visual corticocolicular terminal fields in rats subjected to retinal deafferentation in adulthood were columnar, with no changes in extension, although staining was most intense in the upper half of the SGS and in the SZ [34]. Anterograde labeling with BDA allowed clear morphological identification of presynaptic boutons, and quantification of

boutons in the terminal fields revealed a maximal density in the SGS and the SO [34]. Similar results were obtained by counting autoradiographic particles following [^{3}H]-leucine injection into the primary visual cortex [38]. Despite occurring in neonatal deafferented animals, the increase in bouton density in the absence of notable axonal arborization suggests that new synaptic terminals are formed and thus, we conclude that adult visual corticocollicular afferents maintain a certain degree of plasticity. Comparable synaptogenic responses in the adult corticorubral axons have been described following red nucleus deafferentation [44]. Cytoskeletal proteins like GAP-43 have been implicated in axonal growth [45], and GAP-43 expression in the visual cortex is abundant during postnatal development but it decreases in adulthood [46]. These observations may explain the differences in axonal branching between deafferented neonates and adults. Indeed, we also found that immature vimentin-expressing astrocytes are abundant in the neonatal SC [47], where they may induce local sprouting after retinal deafferentation.

In conclusion, our findings demonstrate that the capacity for post-lesional remodeling is partially retained by the adult central nervous system.

3. Molecular determinants involved in the dampening of the plastic response during adulthood

There is evidence accumulating that glial scar-associated molecules and myelin-derived molecules are molecular determinants that contribute to the diminished ability of adult neurons to regenerate their axons and reorganize their connections following CNS lesions. The glial scar is a meshwork composed of reactive astrocytes, oligodendrocyte precursors, meningeal fibroblasts and microglia that migrate to the lesion site to mediate tightly linked processes. Not only is it an impenetrable physical barrier to regenerating axons but it is also an important source of molecules that directly inhibit regeneration. After neuronal injury, reactive astrocytes and meningeal fibroblasts in the glial scar rapidly enhance the production and release of extracellular matrix molecules, such as the chondroitin sulfate proteoglycans (CSPGs), which are important inhibitors of axonal growth [48-49]. In addition, molecules involved in axonal path finding, such as ephrins and ephrin A4 receptor [50,51], semaphorin 3A [52-54] and Slit proteins [55], have been implicated in the mechanisms by which the gliar scar prevents axonal growth [56].

Myelin also mediates the inhibition of axonal growth in the CNS and for 30 years, post-lesional products of CNS myelin have been known to specifically inhibit axonal extension [57]. Subsequent studies confirmed that CNS myelin and mature oligodendrocytes contain molecular components that restrict axonal regeneration [58-61]. Several proteins expressed by oligodendrocytes have been identified as myelin-associated inhibitors on the basis of their ability to inhibit neurite outgrowth and induce growth cone collapse. Of these, Nogo [62-64], myelin associated glycoprotein (MAG) [65,66], and oligodendrocyte myelin glycoprotein (OMgp) [67] are considered the main contributors to the inhibitory effects of CNS myelin.

NgR, a GPI-linked protein with multiple leucine-rich repeats, is the receptor for Nogo-66, and it mediates the signaling cascade that inhibits axonal growth [68]. More recent studies have shown that MAG and OMgp can also bind to NgR to exert their inhibitory actions [69-71]. The neurotrophin receptor p75 ($p75^{NTR}$) forms a complex with NgR that mediates axonal growth inhibition and that initiates the signaling cascade triggered by myelin derived inhibitors [67,71,72]. $p75^{NTR}$ is not ubiquitously expressed in the adult brain, whereas almost all mature CNS neurons respond to inhibition by myelin. Thus, it is likely that other proteins assume the function of $p75^{NTR}$. TROY is an orphan member of the tumor necrosis factor receptor (TNFR) superfamily that is widely expressed by both embryonic and adult neurons [73,74], and it has been identified as a functional homolog of $p75^{NTR}$ that may contribute to the inhibitory effects of myelin [75,76]. Nonetheless, the role of TROY as a signal transducing receptor in the inhibition of axonal growth remains unclear, as its expression has not been consistently demonstrated in the adult CNS [77]. Lingo-1, the third component of this receptor complex [78], belongs to a large family of proteins that contain leucine-rich repeats and immunoglobulins [79]. Physical association of Lingo-1, NgR and $p75^{NTR}$ results in the formation of a tripartite receptor complex that mediates the inhibitory signaling triggered by myelin inhibitors [78], and the intracellular signaling cascade this complex activates alters the Rac1/RhoA balance in growth cones. RhoA, Rac1 and Cdc42 are widely expressed members of the small GTPase family that regulate actin dynamics and microtubule assembly. Rac1 and RhoA exert antagonistic effects on growth cone dynamics via their effector-kinases, PAK1 and ROCK, stimulating growth cone motility and inducing collapse, respectively. In the damaged nervous system, myelin-derived inhibitors alter the Rac1 and RhoA signaling equilibrium, augmenting RhoA activity at the expense of Rac1 activity [80, 81]. RhoA activation activates the sequential ROCK/LIM kinase/cofilin signaling cascade, resulting in the depolymerization of actin filaments and subsequent growth cone collapse [82]. This intracellular mechanism can be influenced by several molecules, including MAG, Nogo, OMgp, Netrin-1, ephrins and CSPGs, and it has been proposed as the convergence point of several inhibitors of axonal growth that exert similar functions [78,80,83-85].

4. Strategies to promote corticocollicular sprouting after visual deafferentation in adulthood

Several strategies have been described to promote the regeneration and reorganization of neuronal connections following CNS injury. Regeneration of mature damaged axons has been demonstrated using antibodies against myelin-derived inhibitors. For example, treatment of adult rats with anti-Nogo-A IN-1 after spinal cord lesions promotes significant axonal sprouting and regeneration over long distances caudal to the lesion site, accompanied by motor improvements and restoration of sensorial function [86-88]. Similarly, in animal models of spinal cord injury and stroke, the intrathecal administration of antibodies that effectively neutralize Nogo-A activity enhances regeneration of the corticospinal tract fibers, restoring damaged neuronal circuits and promoting functional recovery [89,90]. In support of these findings, the intrathecal administration of anti-Nogo-A

antibodies in monkeys subjected to cervical spinal cord hemisection promotes extensive functional recovery, increased sprouting and regenerative axonal elongation [91].

Other strategies to promote axonal regeneration and reorganization following adult CNS lesions have been described in transgenic animal models. Nogo-A single knockout and Nogo-A/B double knockout mice exhibit dramatic increases in axonal sprouting and extension after spinal cord injury, accompanied by substantial locomotor recovery [92,93]. While no increase in axon regeneration was observed in another study in either Nogo-A/B double knockout or Nogo-A/B/C triple knockout mice [94], a more recent study using the optic nerve crush model in Nogo-A/B/C triple knockout mice reported significant axon regeneration [95], suggesting Nogo influences in axon regeneration *in vivo*.

Blockade of RhoA and ROCK activation with C3 transferase and Y-27632 antagonists, respectively, enhances axonal growth in myelin substrates *in vitro* [83,84,96] and *in vivo* [83,84]. However, the effectiveness of these antagonists appears to depend on their mode of administration, as C3 transferase was not effective in all *in vivo* studies [96]. Since the discovery of RNA interference [97], numerous studies have focused on inhibiting target molecules using siRNAs that specifically silence the expression of target mRNAs [98]. Several studies have reported the promotion of neurite outgrowth *in vitro* following siRNA administration. For example, siRNAs against $p75^{NTR}$ disinhibit dorsal root ganglia neurite outgrowth in the presence of MAG [99]. Likewise, siRNA-mediated silencing of components of the inhibitory signaling cascade, including $p75^{NTR}$, NgR and RhoA mRNA, enhances dorsal root ganglia neurite outgrowth in the presence of CNS myelin, with RhoA knockdown exerting the strongest effect [100]. A recent study using a murine model of multiple sclerosis demonstrated that systematic administration of siRNAs against Nogo-A promotes functional recovery accompanied by a significant increase of GAP43 expression, a protein expressed in growing axons. Based on these findings, the authors suggested that axonal repair may underlie the improved clinical outcome in mice treated with siRNAs against Nogo-A [101].

5. Disinhibition of axonal growth by small interfering RNAs against the Nogo Receptor and RhoA

Given the essential role of myelin-derived molecules in the inhibition of neurite outgrowth, we studied the effect of NgR and RhoA knockdown, key mediators of the signaling cascade that promotes actin depolymerization and subsequent growth cone collapse, and that triggers inhibition of axon growth [82]. We investigated whether these interventions result in the expansion of the corticocollicular connection in rats subjected to unilateral retinal deafferentation in adulthood, a response that normally only occurs when this lesion is induced neonatally. To this end, we administered a single injection of siRNAs against NgR or RhoA into the left primary visual cortex immediately after the enucleation of the right eye in two-month-old Sprague Dawley rats. After four days, the animals received a microinjection of the anterograde tracer BDA 10,000 at the site of siRNA administration. Seven days later the animals were perfused and the nervous tissue processed for

histochemical analysis. Control rats received the same siRNA injections into the primary visual cortex. The effect of the siRNAs on NgR and RhoA mRNA levels were measured by qRT-PCR in the cortex beneath the injection site.

Microinjection of siRNAs against NgR and RhoA into the primary visual cortex of adult enucleated rats promoted a mild expansion of the ipsilateral visual corticocollicular terminal field, although in both cases the centre of the field presented a characteristic column-like shape extending from the SO up to the pial surface, a similar pattern to that seen in non-siRNA treated animals. Likewise, following siRNA injection, many fibers were observed running parallel to the pial surface, mainly located within the ventral half of the SGS and running away from the terminal field center towards the middle line. Moreover, several growth cone-bearing axons were observed in these cases, suggesting active axonal growth (Fig. 1, 2).

To confirm the inhibitory effect of siRNAs on NgR and RhoA mRNA expression in the primary visual cortex, and hence the involvement of these molecules in the reorganization of the visual corticocollicular field in adult rats subjected to retinal deafferentation, relative mRNA levels were quantified by qRT-PCR 24 hours after siRNA injection. This revealed significant decreases in NgR and RhoA mRNA levels (44.8 ± 7.3% and 21.67 ± 10.53%, respectively, relative to controls: Fig. 3).

These results demonstrate that siRNA-mediated abolition of the expression of key mediators of axonal growth inhibition, such as NgR and more notably RhoA, promotes axonal outgrowth after adult CNS injury. Indeed, recent studies using different approaches to reduce the expression of molecules involved in axonal growth inhibition have reported similar beneficial effects on axonal growth. For example, the administration of monoclonal antibodies or peptide antagonists improves axonal and functional regeneration in rats subjected to spinal cord lesions [102-104]. An increase in the number of regenerated retinal ganglion cells axons passing through and growing beyond the injured optic nerve has also been described in an NgR double negative mutant model [105]. Recent studies also demonstrated that siRNA knockdown of $p75^{NTR}$ increases dorsal root ganglia neurite outgrowth in the presence of MAG [99], while the reduction of NgR expression levels using small hairpin RNAs augments axonal growth in neuronal cultures [106].

Several authors have reported increased neurite outgrowth following RhoA inactivation, both *in vitro* [80,83,84,96,100,107] and *in vivo* [83,84]. In our study RhoA knockdown resulted in a greater expansion of the visual corticocollicular terminal field. Similarly, siRNA knockdown of $p75^{NTR}$, NgR and most significantly, RhoA, was shown to disinhibit dorsal root ganglia neurite outgrowth in the presence of myelin [100]. It was suggested that in addition to myelin-derived inhibitory ligands, which act by binding to NgR, other neurite growth inhibitors including ephrins, semaphorins and CSPGs, may converge on the RhoA signaling pathway leading to growth cone collapse [108,109]. Thus, NgR knockdown may block the inhibitory action of myelin derived ligands alone, with no influence on other inhibitory ligands. Nonetheless, RhoA knockdown could block the convergent signaling from all inhibitory ligands.

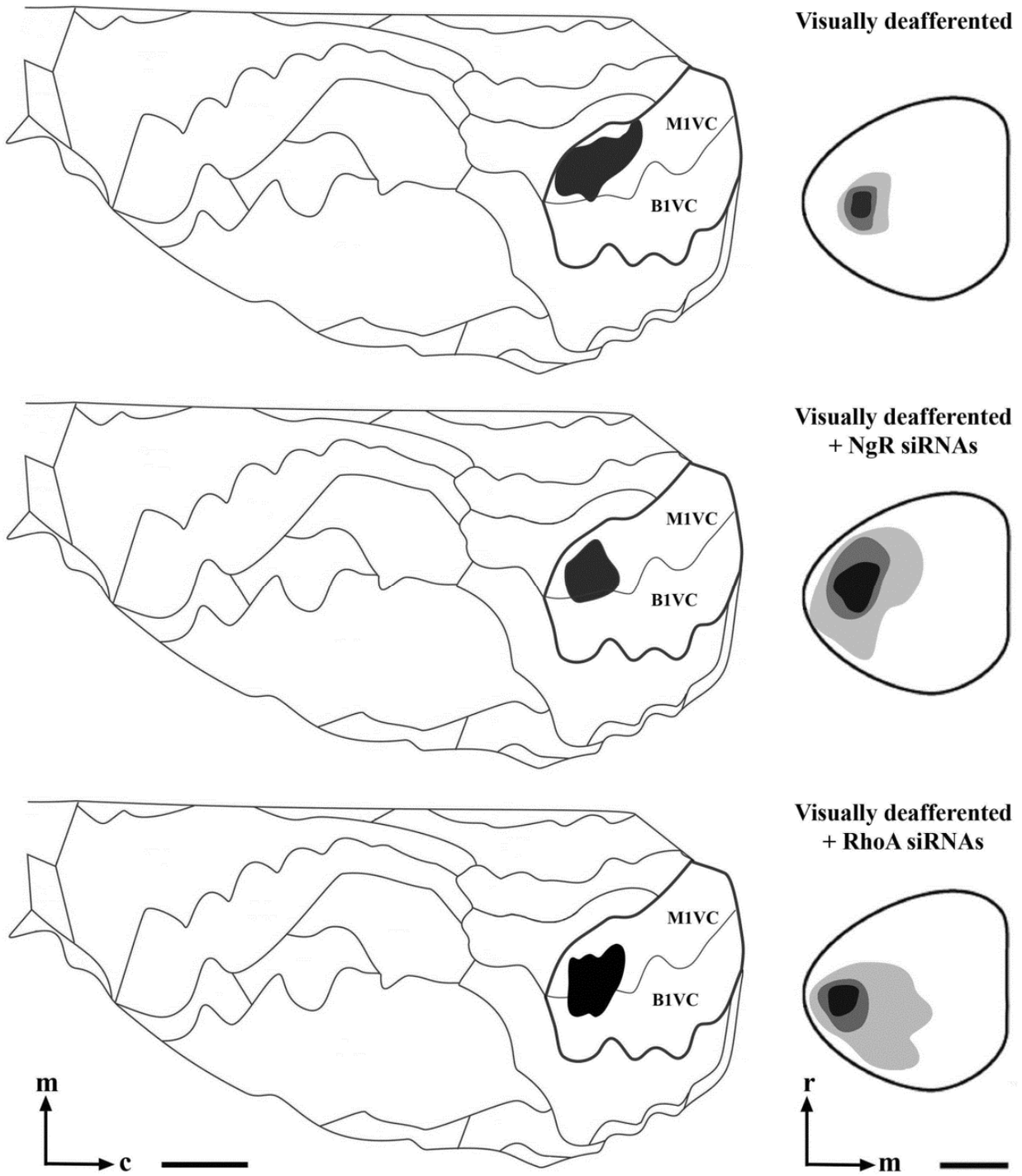


Figure 1. Scheme showing a dorsal view of the site of BDA injection into the primary visual cortex (left) and the projection site in the superior colliculus (right) in different experimental conditions. The administration of siRNAs against NgR and RhoA led to the expansion of the visual corticocollicular terminal field in animals subjected to retinal deafferentation in adulthood. The black areas in the SC denote regions with the greatest density of fibers, while the grey shaded areas denote regions of decreasing axonal density. M1VC, monocular primary visual cortex; B1VC, binocular primary visual cortex; c, caudal; m, medial; r, rostral. Scale bars = 2 mm (left) and 1 mm (right).

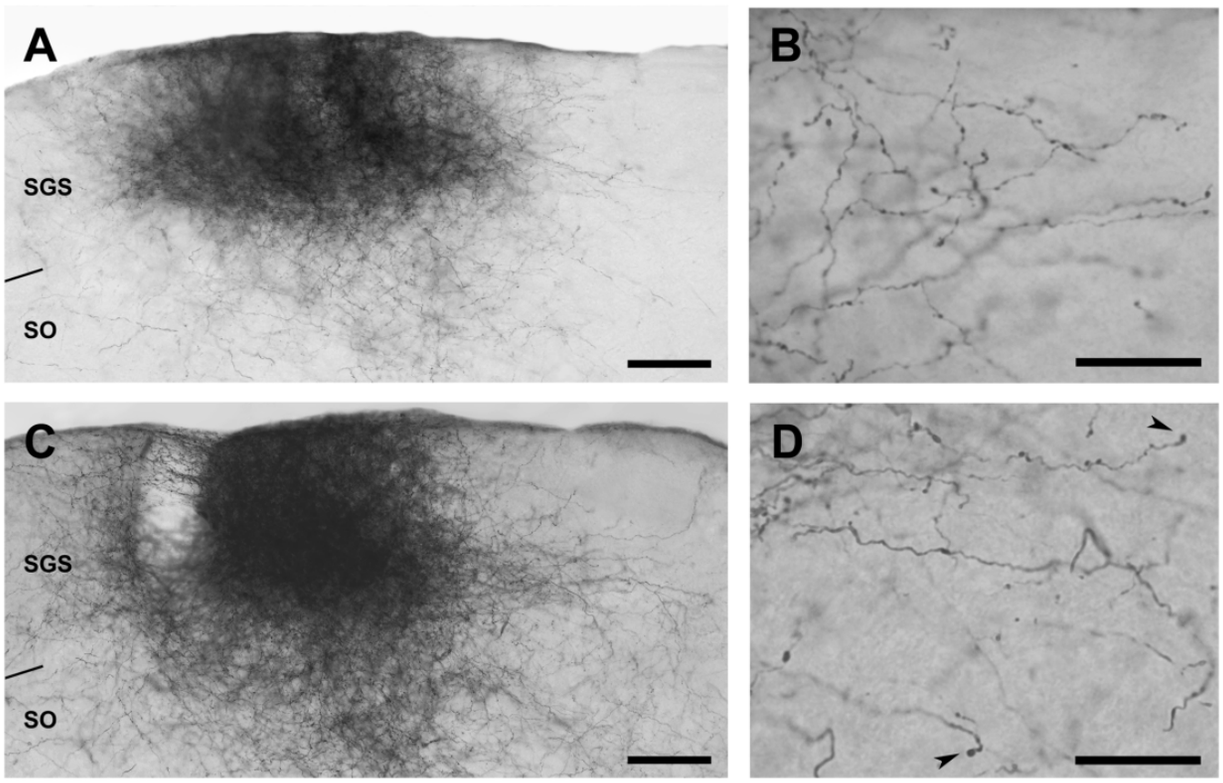


Figure 2. Photomicrographs of BDA-labeled visual corticocollicular terminal fields following administration of siRNAs against NgR (A,B) and RhoA (C,D) in rats visually deafferented in adulthood. (A,C) The microinjection of siRNAs in the primary visual cortex, the projection origin, evoked an increase in terminal field extension, with fibers running towards the lateral SC and the medial edge. (B,D) Detail of axons in the SGS running away from the terminal field, some exhibiting terminal thickening (arrowheads), which may indicate the presence of vestigial growth cone. SGS, stratum griseum superficiale; SO, stratum opticum. Scale bars = 100 μm (A,C) and 30 μm (B,D).

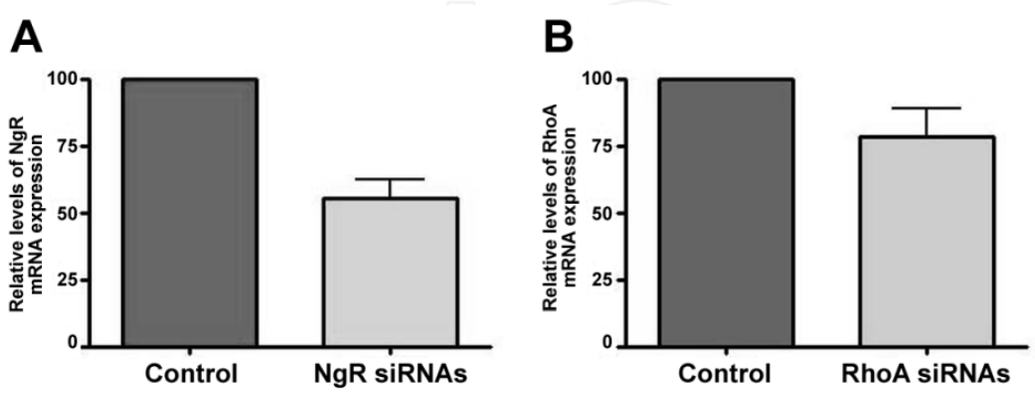


Figure 3. Relative expression of Nogo Receptor (A) and RhoA (B) mRNA in the primary visual cortex following administration of their corresponding siRNAs. Weaker NgR and RhoA mRNA expression in the siRNA-treated groups than in the untreated controls was detected. The relative mRNA levels are normalized to YWHAZ and TATA box binding protein reference genes.

In summary, our *in vivo* results strongly support the use of siRNAs to silence inhibitors of axonal growth, promoting reorganization and axonal outgrowth of the visual corticocollicular connection in adult enucleated rats following a single siRNA injection.

6. Does guanosine enhance corticocortical synaptogenesis?

Considerable effort has been directed towards identifying the specific molecules that guide axonal growth and subsequent synaptogenesis during development, some of which are inductive glial factors [110,111]. Evidence gathered over the last decade has attributed a fundamental role to astrocytes in regulating synaptogenesis and modulating synaptic plasticity during critical periods in different sensory and motor systems [112]. During postnatal development, astrocytes are strongly involved in the formation of synaptic contacts in the CNS, participating in each of the 3 stages of synaptogenesis: (i) the establishment of contacts between neurons; (ii) the formation of the synapse; and (iii) synaptic stabilization or elimination [113]. The role of astrocytes in regulating the synaptic stability of retinal ganglion cells (RGCs) has been studied in detail by culturing purified RGCs in the presence or absence of astrocytes [114]. Astrocytes promote an increase in the number of RGC synapses, although this effect is reversible since when cultured for one week after removing the glia there is a significant reduction in the number of synaptic puncta. The regulatory role of astrocytes in synaptogenesis has also been demonstrated through ultrastructural and physiological studies *in vivo*. Once retinocollicular afferents reach the collicular superficial strata, the synaptic arrangement is closely correlated with the growth and differentiation of astrocytes at the end of the first postnatal week in rats [114], or from P30-P40 in opposums [115].

Matricellular proteins are extracellular regulatory factors secreted by astrocytes that mediate cell-matrix interactions. This is heterogeneous group of proteins includes thrombospondins [116,117], HEVIN [118] and cholesterol [119], which are strongly expressed during development and in response to injury [120,121]. In addition, these matricellular proteins interact with different matrix constituents, growth factors, integrins and other cell surface receptors [122]. Co-culture of purified glutamatergic RGCs with astrocytes results in the secretion of cholesterol by glia, which promotes synaptogenesis [122]. The absence of glial cells from these cultures, or a reduction in the cholesterol content of glia-conditioned medium, diminishes both the number of synapses and GluR2/3 expression by RGCs [123]. While cholesterol production within the CNS is necessary for growth and survival, lipid raft signaling, synaptic vesicle formation and synaptic function [124], increased synaptogenesis and axon pruning requires additional cholesterol production [122]. Recent *in vitro* studies indicate that guanosine increases the efflux of cholesterol from astrocytes [125], the primary source of cholesterol in the nervous system. Moreover, binding of cholesterol to apolipoprotein E (ApoE) promotes synapse formation in RGC cultures [122]. We assessed the synaptogenic effect of guanosine administration *in vivo*, having anterogradely labeled the visual corticocortical connection in young adult male Sprague-Dawley rats by injecting BDA into the primary visual cortex. After BDA administration (24 hr), an osmotic pump was implanted at the site of BDA injection to administer guanosine (300 μM) and 2 weeks after

tracer injection, the animals were sacrificed and the nervous tissue analyzed histochemically. While a clear corticocortical projection from primary to secondary visual areas was observed in all cases, guanosine administration significantly increases the number and size of the presynaptic boutons along the axonal branches that reach the secondary visual areas, while the pattern of visual corticocortical projections was preserved. Laterally running fibers emerged at several different levels, white matter fibers ran close to layer VI, while at the level of layers VI and V, afferent fibers gave off divergent branches to form a dense plexus. A smaller contingent of corticocortical fibers ran horizontally along layers IV and V, and a very superficial group of fibers ran laterally at the level of layer I [126].

We observed 2 plexuses in this efferent connection, a deep plexus in layer IV-VI and another in the superficial layer I, both of which were connected by ascending fibers that gave off scarce divergent branches containing irregularly distributed presynaptic boutons. Treatment with guanosine either increased the number or altered the orientation of the axonal branches of the visual corticocortical connection. Moreover, the number and size of synaptic boutons was significantly higher in these animals, and most were more rounded/oval than those in control animals. Guanosine administration significantly increased bouton density (number/200 μm^2), which was 1.3-fold higher in treated versus control rats ($p<0.02$). Moreover, while the average size of small synaptic boutons did not appear to be affected by guanosine ($0.57 \pm 0.07\ \mu m^2$ vs. $0.47 \pm 0.05\ \mu m^2$ in control animals; $p<0.002$), the larger boutons were significantly larger on average in guanosine-treated rats ($3.76 \pm 0.06\ \mu m^2$ vs. $2.26 \pm 0.1\ \mu m^2$ in control rats; $p<0.002$). These data highlight the synaptogenic specificity of the astrocytic factors elicited by guanosine (Fig. 4) [126].

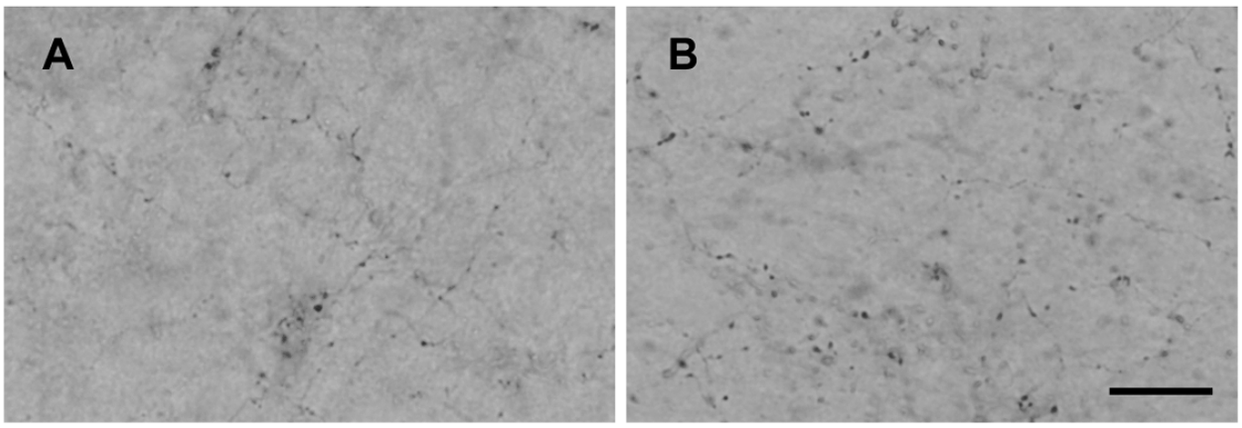


Figure 4. Representative photomicrographs of BDA-labeled visual corticocolicular terminal fields in control (A) and guanosine-treated rats (B). Note the markedly higher density of labeled presynaptic boutons in the guanosine-treated rat than in the control animals. Scale bar = 20 μm.

We propose that synaptogenesis induced by the local application of guanosine *in vivo* may be mediated by factors such as cholesterol, ApoE and pleiotropic factors secreted by astrocytes. Guanosine administration increases both cholesterol and ApoE efflux from astrocytes *in vitro*, supporting a pharmacological role of guanosine in the modulation of cholesterol homeostasis in the brain [125]. Moreover, astrocytes that release guanosine can exert neurotrophic effects and promote neuritogenesis, possibly via MAP-kinase and PI3-

kinase signaling pathways [127]. Previous studies failed to report an increase in synapse number in response to cholesterol administration *in vitro* [116], despite a strong enhancement in synaptic efficacy [119]. However, guanosine increased both the number or size of synaptic boutons in our *in vivo* model. These morphological changes were observed at least one week after 7 days of local guanosine administration and it is likely that this effect progressively diminishes over subsequent days, as is usually observed in nervous structures following lesion. Nonetheless, the synaptogenic effects promoted by reactive astrocytes in denervated terminal fields can last for months [34]. The larger synaptic boutons generated following guanosine administration may reflect the accumulation of presynaptic components such as mitochondria, synaptic vesicles and presynaptic receptors, elements that could eventually exert a modulatory effect upon functional aspects of neurotransmission, such as transmitter release or presynaptic potentiation. It remains unclear whether the proportion of synapses that contact neurons varies after guanosine administration, as astrocytes promote cholesterol-mediated glutamatergic synaptogenesis but they induce GABAergic synaptogenesis via a different mechanism [128], raising the possibility that the majority of new synapses are glutamatergic. Changes in the proportion of inhibitory and excitatory synapses may trigger homeostatic mechanisms [129] that maintain the synaptic activity of the connection within certain functional limits.

Synaptogenesis occurs both during development and adult life. In addition to the aforementioned factors, several other factors promote synaptogenesis in mature nervous systems, including GDNF (glial derived neurotrophic factor) and sex hormones, particularly in areas that display strong synaptic plasticity [130,131]. *In vivo* studies of the neocortex [132,133] revealed the ongoing growth and retraction of dendritic spines, accompanied by the elimination and formation of synapses. While we have only examined labeled projecting axons, synaptogenic effects may also extend to axons emerging from interneurons. Therefore, it may be of interest to analyze GABAergic synaptogenesis in the area surrounding the site of implantation of the osmotic pump that supplies guanosine in this experimental paradigm.

The increase in the number and size of a significant proportion of synapses after guanosine administration indicates a potentiation of axon growth that may promote reinnervation after partial experimental lesion of a neural pathway, or after elimination of a specific afferent connection projecting to a given brain region. We are currently investigating other strategies to inhibit molecules that restrict axonal sprouting and regeneration, including the injection of siRNAs against the p75 receptor and LINGO-1 into the contralateral visual cortex following monocular retinal deafferentation, with encouraging preliminary results.

7. Conclusion

In contrast to the classical dogma of neuronal regeneration, the results presented here indicate that both corticocortical and corticosubcortical connections can be manipulated in adult animals. We focused specifically on two connections, namely corticocollicular and corticocortical projections emerging from the primary visual cortex, and we demonstrate

significant post-lesional sprouting of these neurons following specific siRNA knockdown of molecules that inhibit axon regeneration. This strategy is particularly efficacious on a broad range of potential targets. The combination of this knockdown approach with strategies to promote axonal growth by trophic stimuli may be particularly promising for the therapeutic modulation of specific neuronal connections in the future.

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Acknowledgement

Grant sponsors: Fondo de Investigaciones Sanitarias (Ministerio de Sanidad y Consumo) PI05/2046 and PS09/00476; Universidad del País Vasco/Euskal Herriko Unibertsitatea GIU06/15; Gobierno Vasco SA-2010/00095 and GIC10/113; UPV/EHU Predoctoral Fellowship PIFA/01/2006/042.

Technical and human support from SGIker is gratefully acknowledged.

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