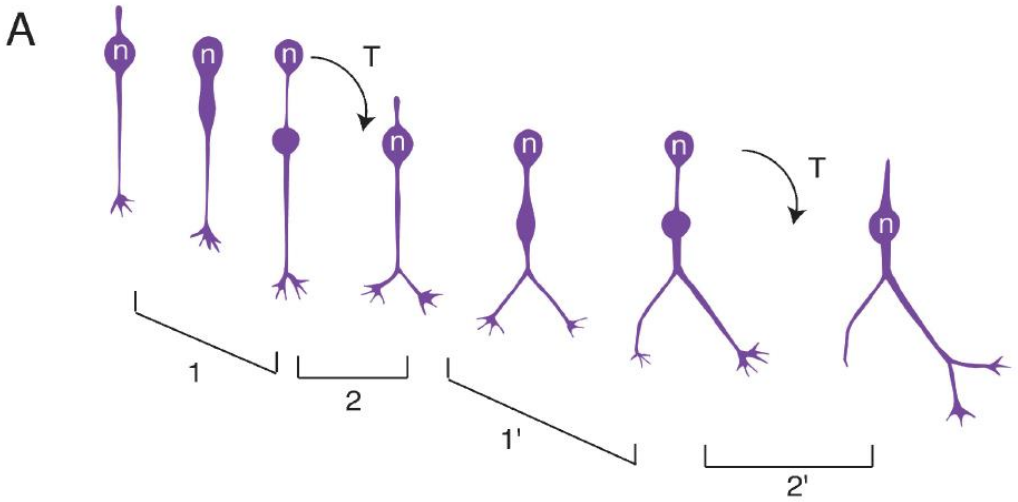
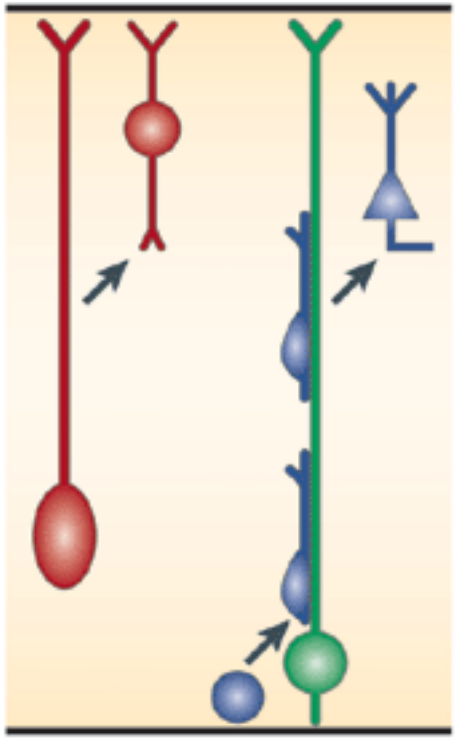
Come fanno gli assoni ed i neuroblasti migranti a “decidere” in quale direzione andare?

- distinzione fra chemotassi e chemochinesi

- approcci sperimentali per studiare la migrazione direzionale cellulare, *in vitro* ed *in vivo*
- esempi da articoli

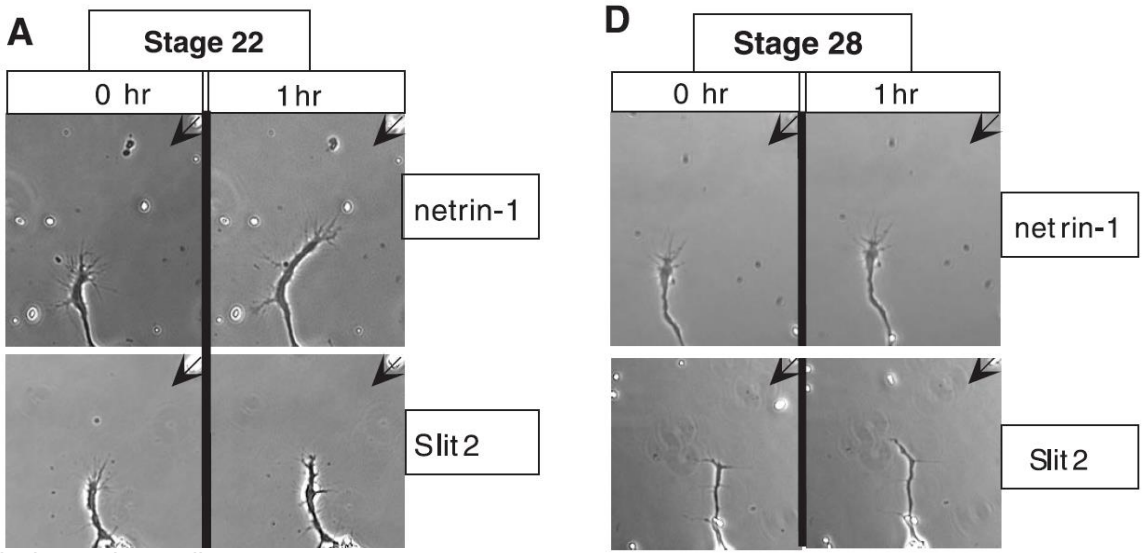




Comunicazione cellula-cellula: attrazione o repulsione?

- studio di un approccio sperimentale per studiare il fenomeno dell'attrazione, della repulsione, del silenziamento dell'attrazione, mediante analisi di articoli originali

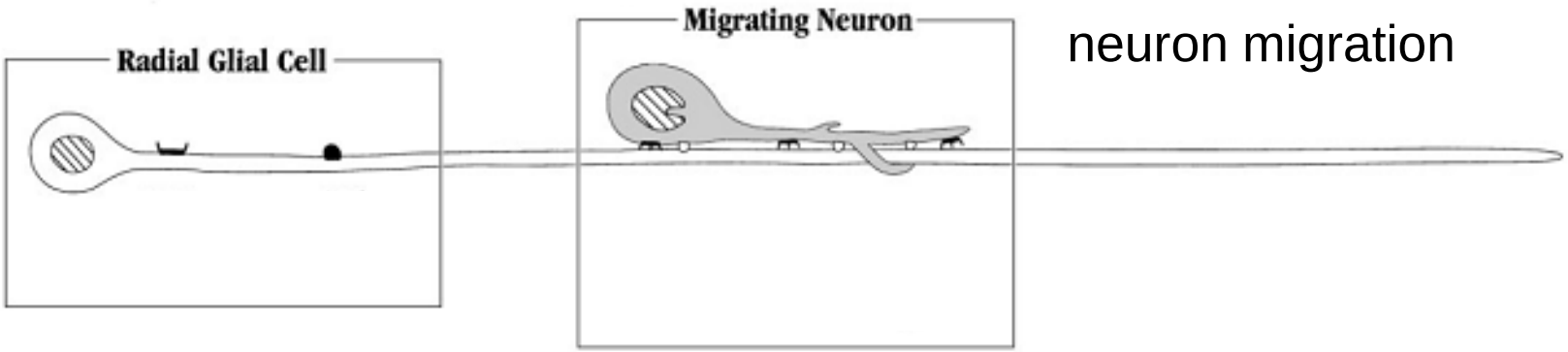
- studio delle strategie utilizzate dalle cellule per trasformare un segnale attrattivo in repulsivo, mediante analisi di articoli originali



Neuronal migration, axon pathfinding & cell-cell interactions

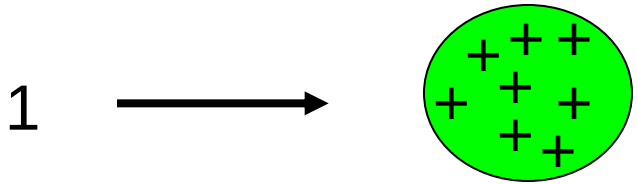
Neuronal migration and axon pathfinding

- migrating neurons are guided by molecular cues that also guide the **projection of axons**



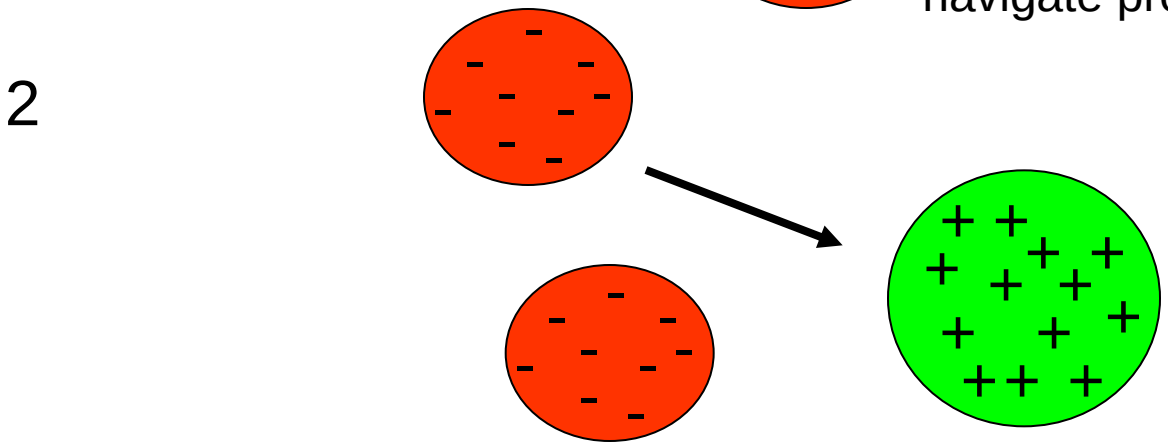
neuron migration

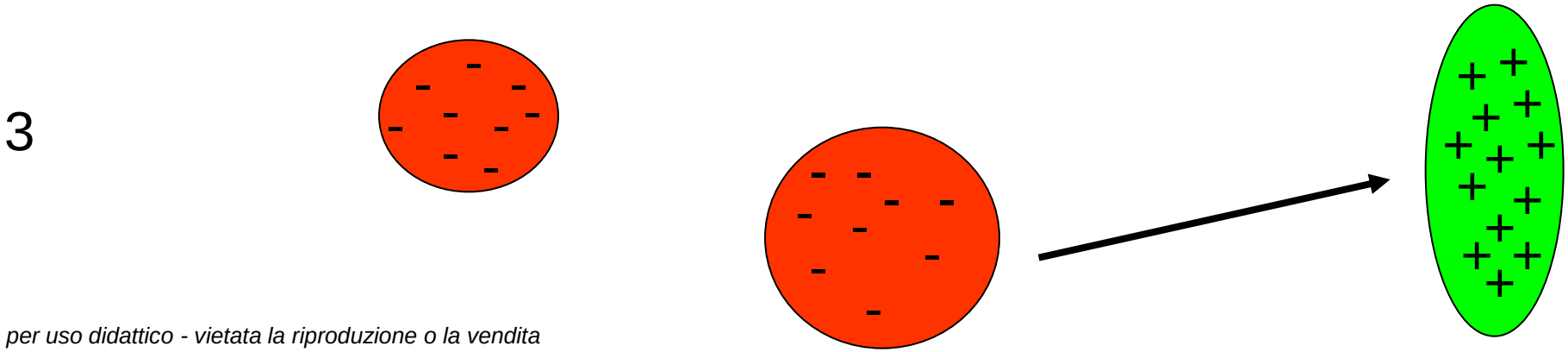
axon pathfinding

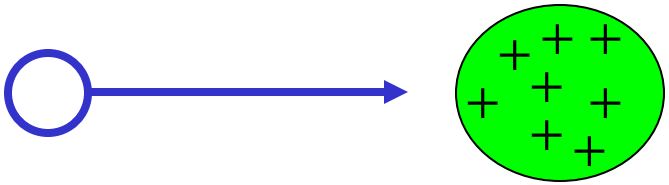


• as neurons or axons move long distances in the developing embryo, they make use of intermediate targets to simplify their navigation into short, manageable segments

• these intermediate targets produce both attractants and repellents, which neurons and axons recognize in sequential order to navigate properly

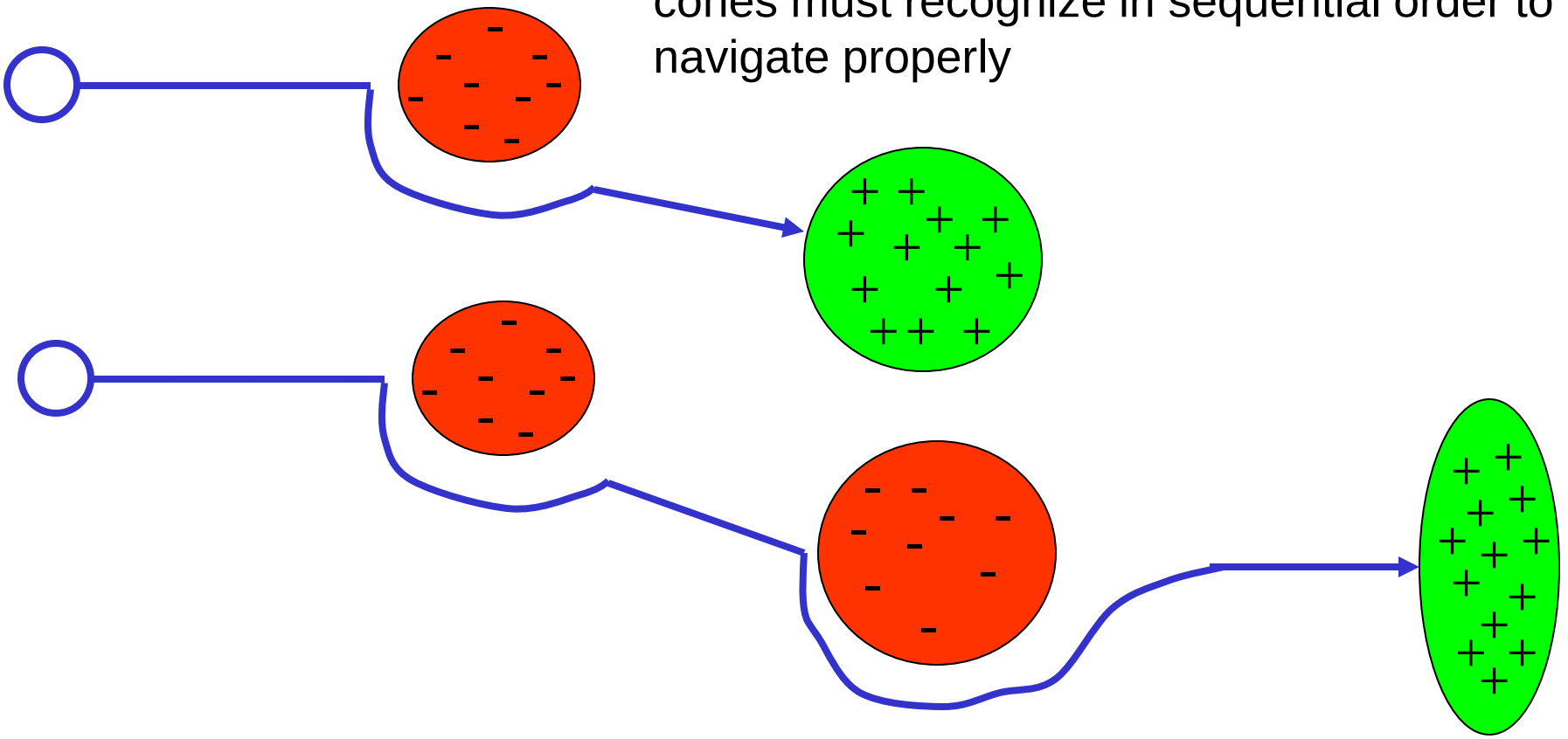




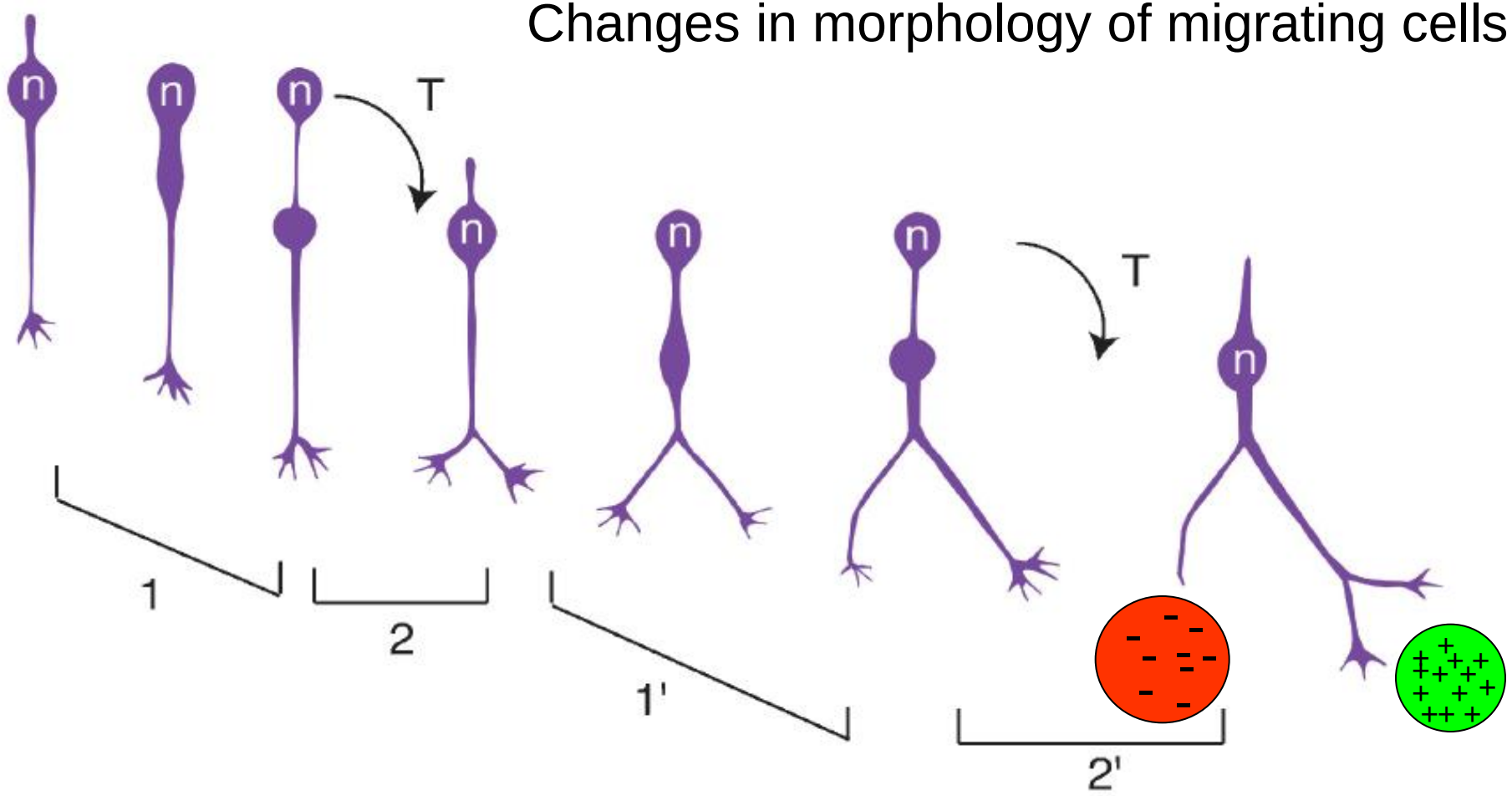


• as axons grow long distances in the developing embryo, they make use of intermediate targets to simplify their navigation into short, manageable segments

• these intermediate targets produce both attractants and repellents, which axonal growth cones must recognize in sequential order to navigate properly

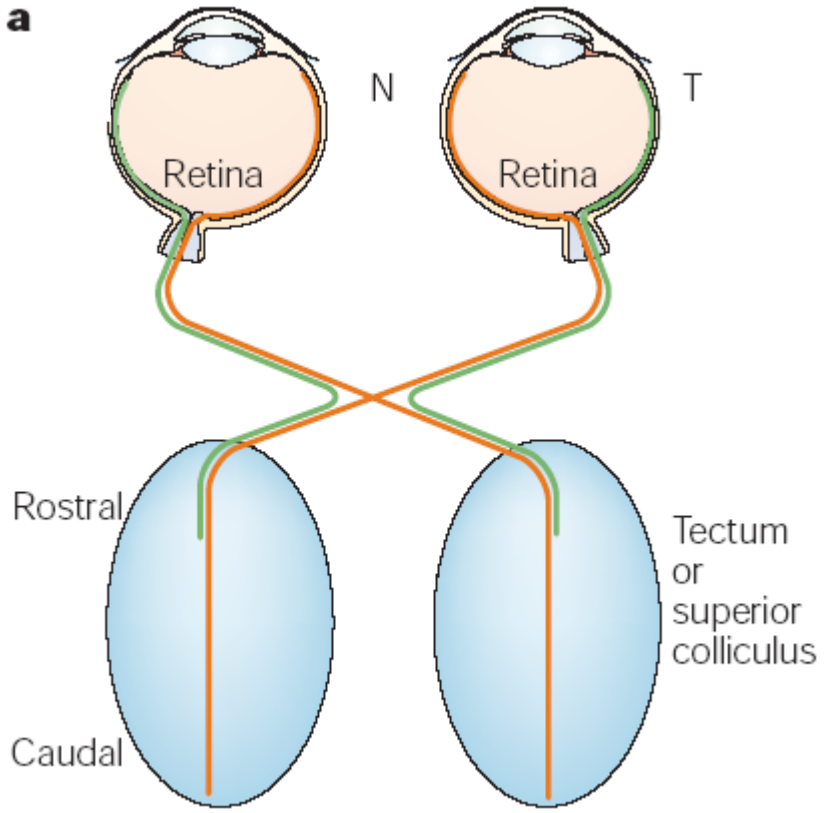


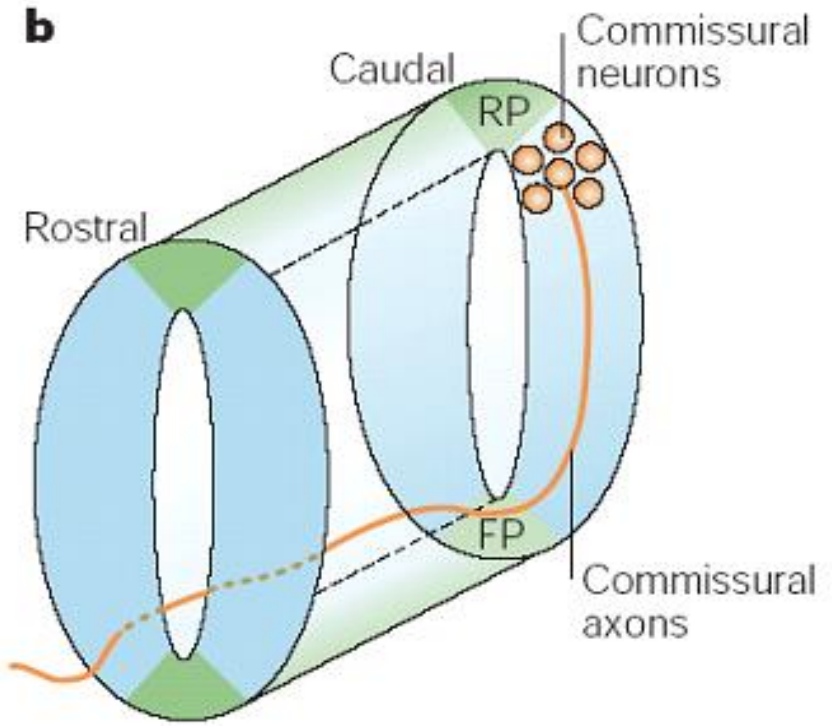
Changes in morphology of migrating cells



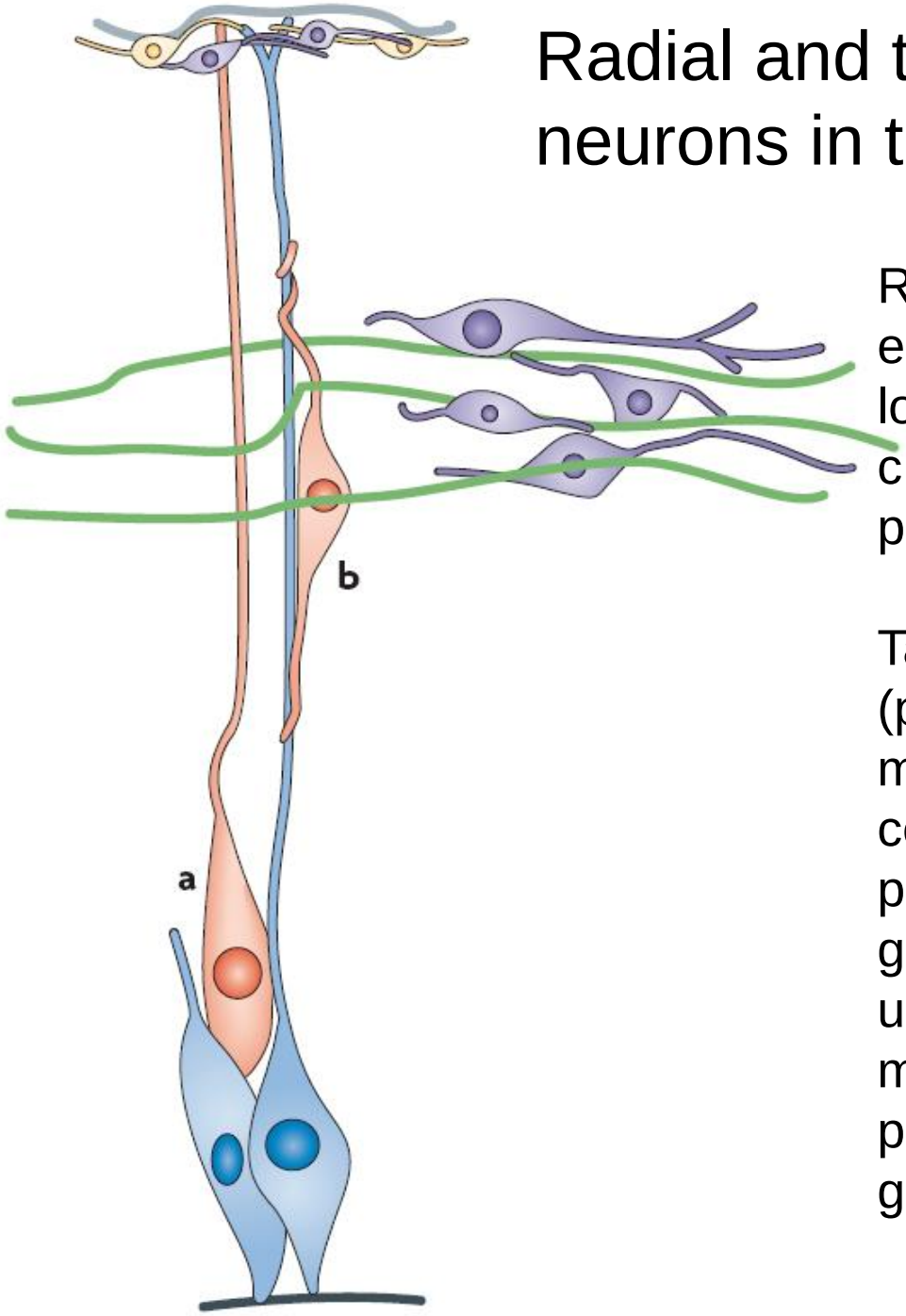
During migration, cells regularly split their leading growth cone and produce new branches at their front. Neurite outgrowth is highly dynamic. Following leading growth cone splitting, cells quickly extend diverging paired branches.
Then, one branch is retracted; the other one further divides and receives the nucleus. Growth cones stop their migration and often split during nuclear translocations, whereas leading neurite(s) elongate(s) during resting phases.

Molecular control of axon pathfinding





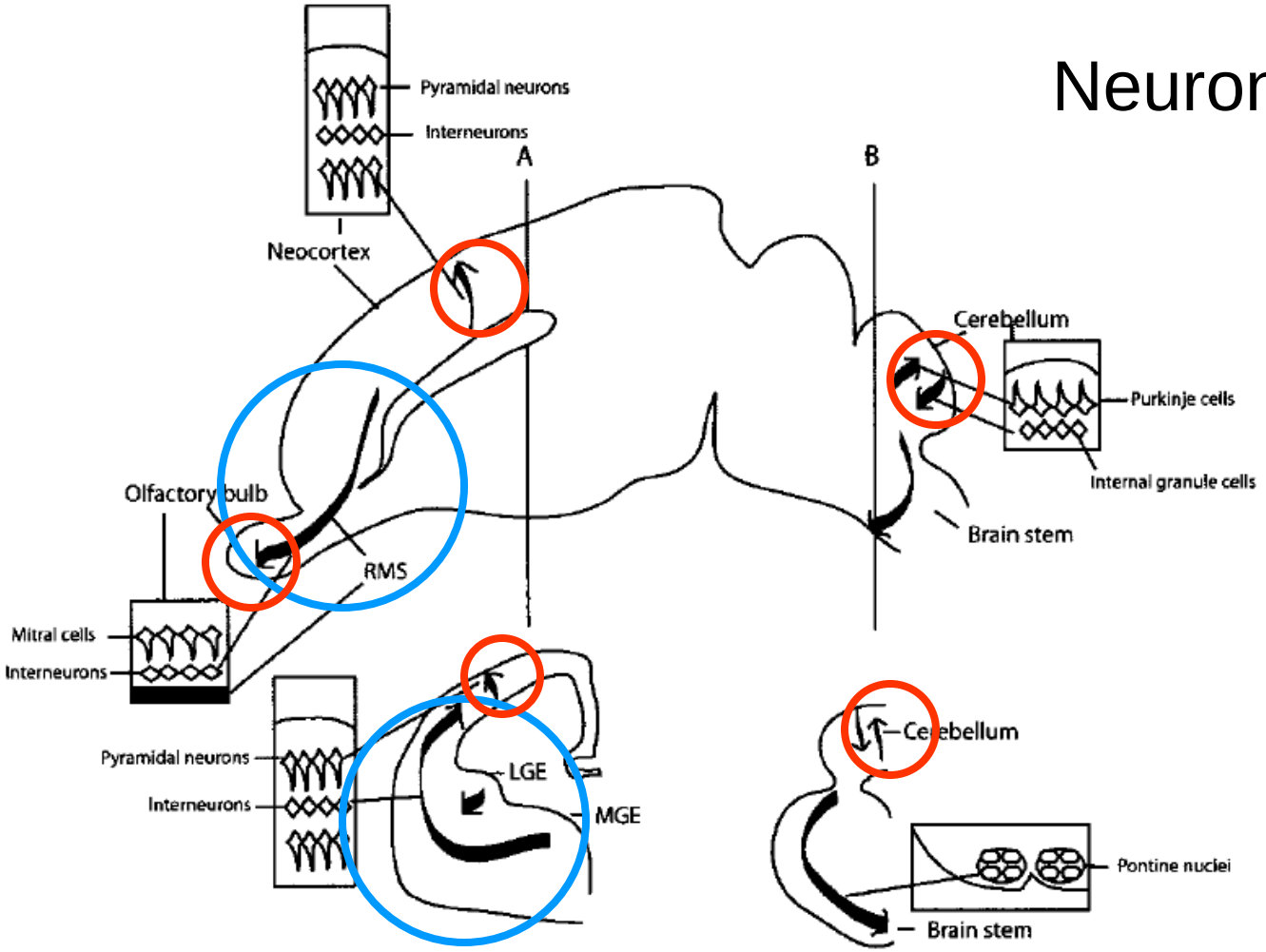
Radial and tangential migration of neurons in the developing cortex.



Radially migrating neurons either use somal translocation with a long leading process (**a**) or migrate in close apposition (**b**) to a radial glial process (blue).

Tangentially migrating neurons (purple) invade the cortex along the marginal zone or through the cerebral wall in a manner that is presumed to be independent of radial glia. These neurons are thought to use corticofugal fibres (green), marginal zone neurons (yellow) or the pial membrane (grey) as migratory guides.

Neuronal migration



• **radial migration** is involved in the development of pyramidal cells in the cortex and cerebellar granule cells

• **tangential migration** is important for the development of interneurons in the cortex and olfactory bulb, and pontine nuclei of the brain stem

A, B: Coronal sections at the levels of A and B. Abbreviations: RMS, rostral migratory stream; LGE and MGE, lateral and medial ganglionic eminences

Table 1. Directional Guidance cues involved in CNS neuronal migration in vivo and in vitro

Ligands	Receptors	Defects in CNS neuronal migration in vivo	Neuronal migration in vitro
Slits	Robo	—	1. Slit repels postnatal SVZa cells[37] 2. Slit repels prenatal SVZ cells of GE[43]
Netrins	DCC	1. Abnormal pontine nuclei in DCC and netrin-1 mutants[46]	1. Netrin-1 attracts pontine nuclei[11]
	Unc-5h	2. Abnormal cerebellar development in unc-5h3[64]*	2. Netrin-1 repels postnatal cerebellar granule cells and prenatal SVZ cells[48,49] 3. Anti-DCC antibody blocks directed migration of postnatal SVZa cells[47]
Semaphorins	Neuropilin Plexin	1. Abnormal GABAergic interneurons in the striatum in neuropilin-2 mutants[50]	—
Ephrins	Eph	—	1. Disruption of Eph-B/Ephrin-B system affects the migration of postnatal SVZa cells[51]

*Unc-5h3/RCM mutant mice showed abnormal development of cerebellum. However, it is still unclear that the defect is primarily caused by migration abnormality or other reasons.

Slit-Robo-Netrin-DCC

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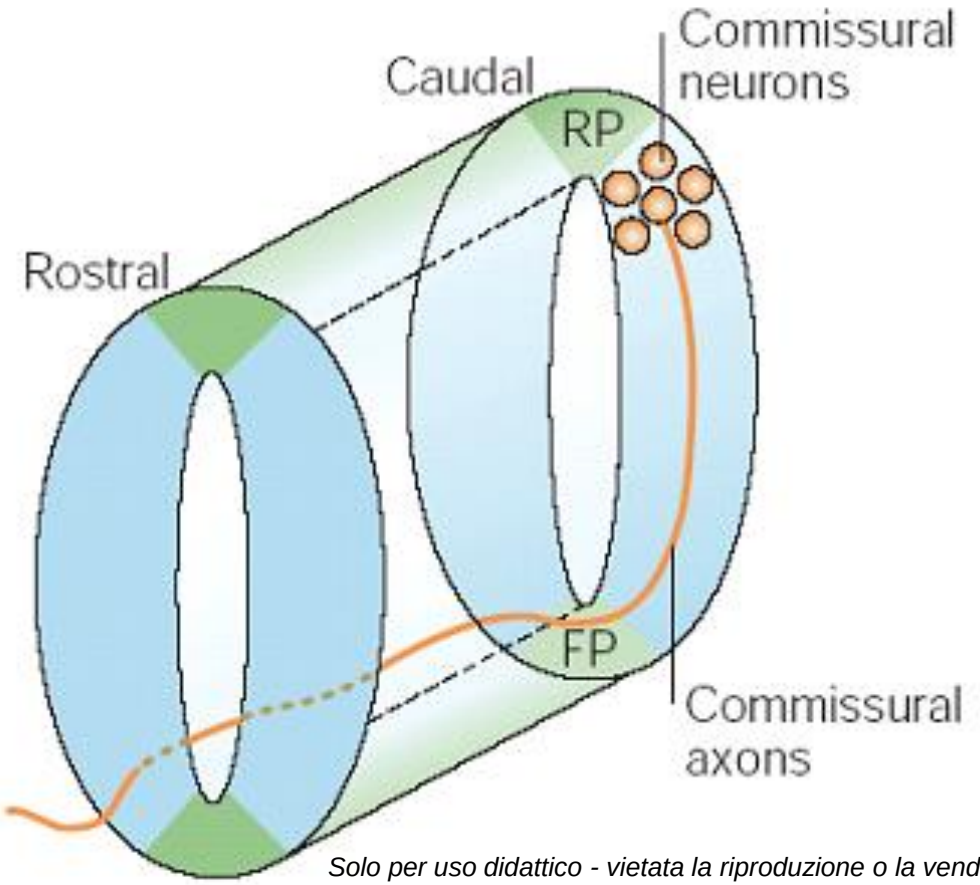
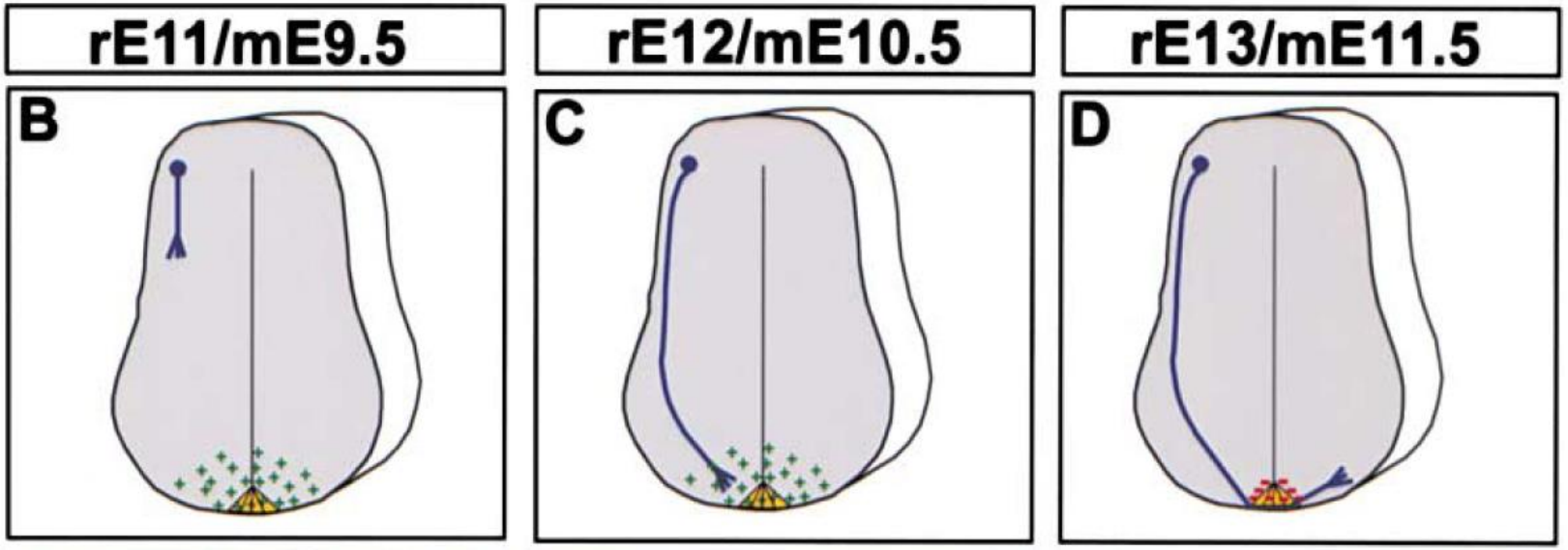
• after being initially attracted to their intermediate targets, growth cones must undergo a change in responsiveness to continue on their migratory route, losing responsiveness to the attractants that led them to their intermediate target and gaining responsiveness to repellents produced by that same target

• this change must be tightly regulated, so that growth cones can move on to the next stage in their trajectory only once they have passed through their intermediate target

• the ventral midline of the nervous system of both vertebrates and invertebrates has served as a model system for understanding the mechanisms by which axons interact with intermediate targets

• Commissural neurons, a subset of interneurons, use the ventral midline as a key intermediate target on their way to their final targets in the contralateral half of the body





• in vertebrates and insects, commissural axons are initially drawn to the midline by attractant proteins (which include members of the netrin family)

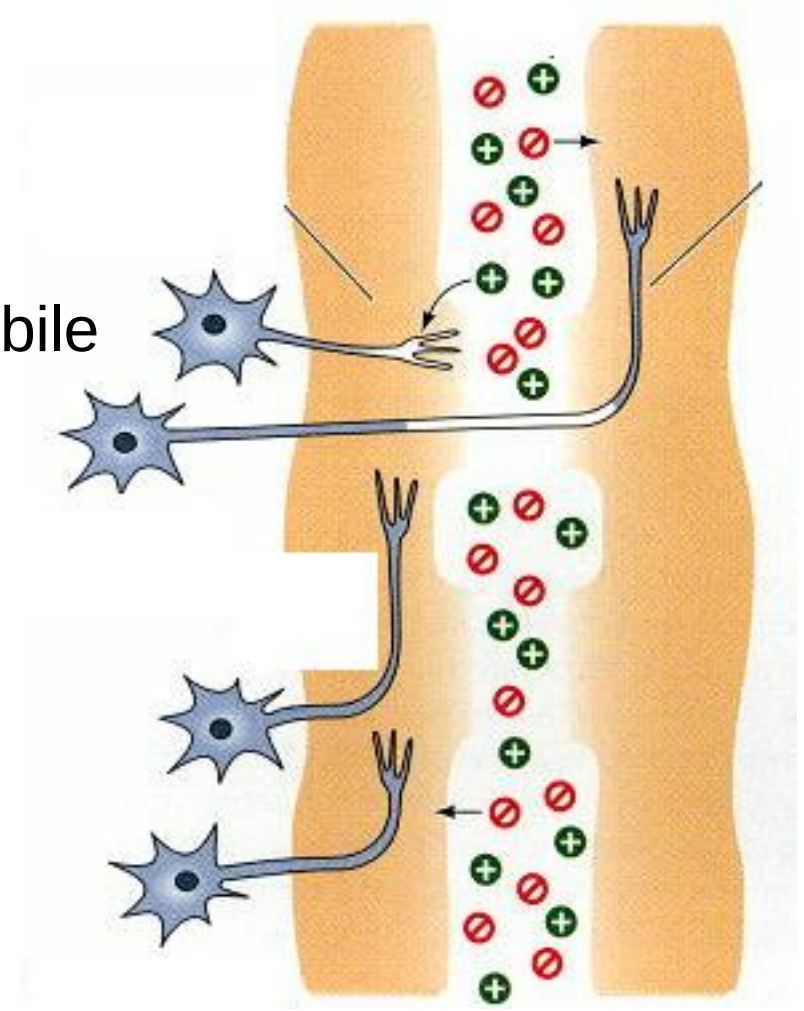
• upon crossing the midline and reaching the contralateral side, however, these growth cones turn longitudinally, lose responsiveness to netrins and become sensitive to repellents made by midline cells (which include Slit proteins)

• this switch prevents commissural axons from re-crossing the midline and allows them to move on toward their final targets

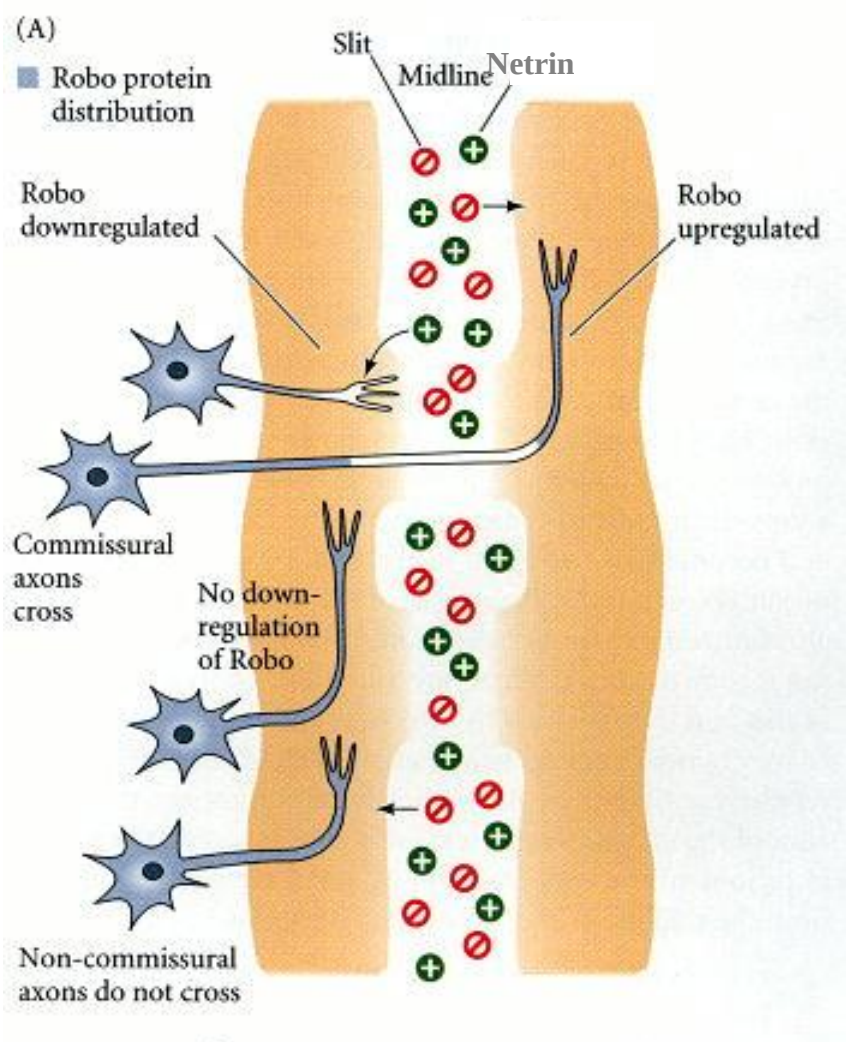
Come fa la midline a passare da regione attrattiva a regione repulsiva?

The midline secretes **netrin** protein, which is stimulatory to commissural axons, and **Slit** protein, which is inhibitory to non-commissural axons.

Come fa un assone ad essere sensibile all'**attrazione** o alla **repulsione?**



Simplified model for chemotactic factors directing commissural axons to cross the midline while keeping other axons on one side of the midline.



• the midline secretes netrin protein, which is stimulatory to commissural axons, and Slit protein, which is inhibitory to non-commissural axons

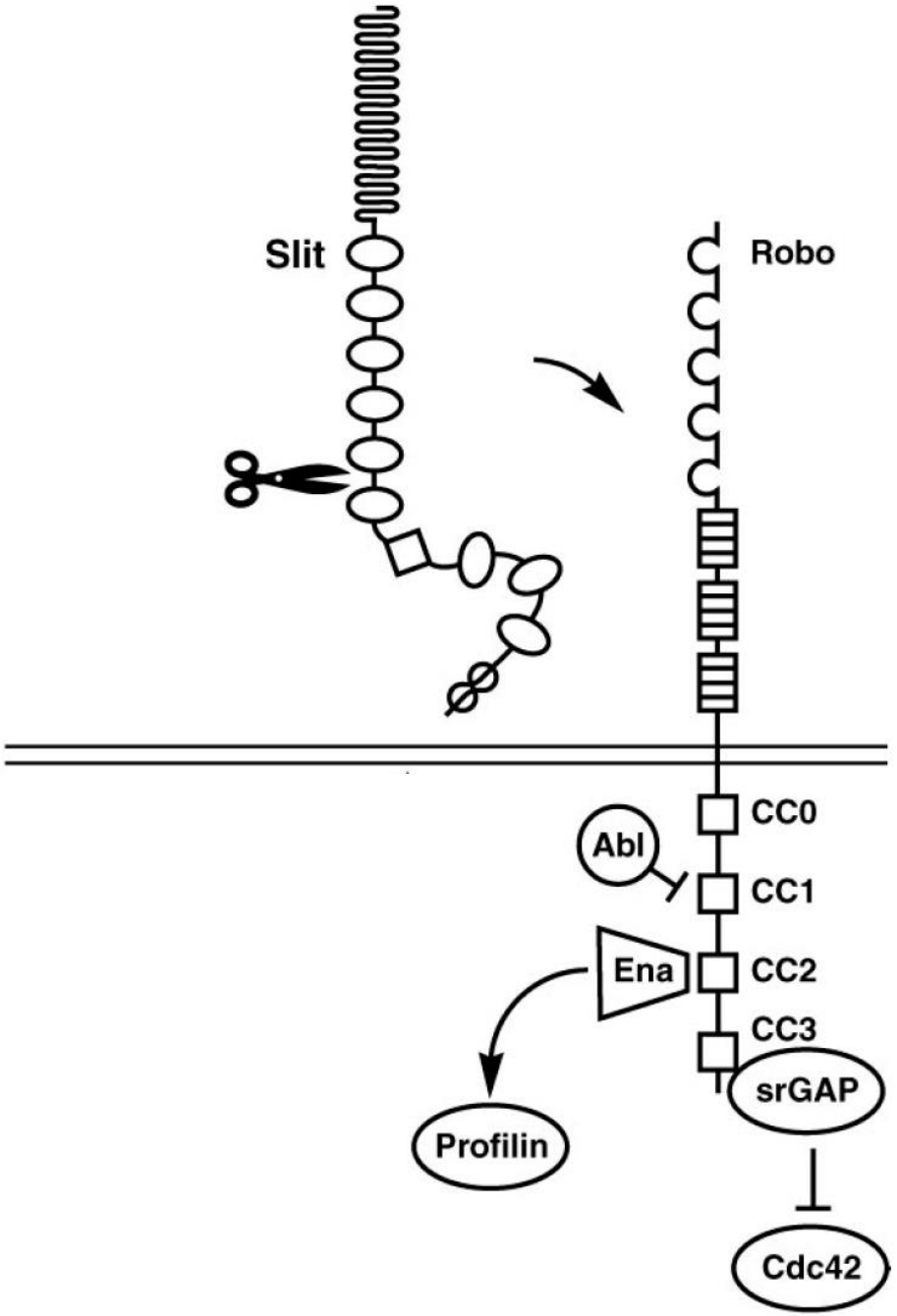
• when they reach the midline, commissural axons have little or no Robo protein, the receptor of Slit

• stimulated by netrin, these axons cross the midline. Once across the midline, they re-express Robo, and therefore cannot return

• non-commissural neurons express Robo and therefore are inhibited from crossing the midline

SLIT (ligando)-Robo (recettore)

- axon pathfinding
- axonal and dendritic branching
- neuronal cell migration



Drosophila

Robo -/-: quale fenotipo vi aspettate di trovare?

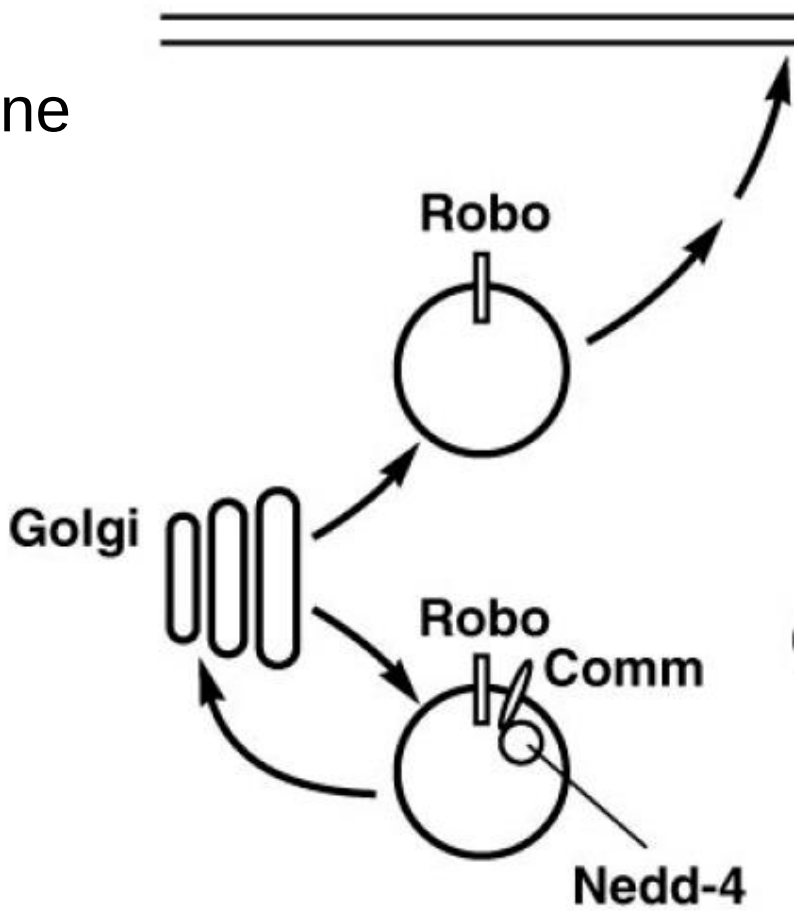
Robo: *roundabout* (*Drosophila*)

-/- attraversamenti multipli

Comm: *commissureless*: regola (down-regolandolo) il livello di espressione di Robo (interagisce con Robo sottraendolo alla superficie)

-/- no attraversamento della midline

+++ tutti gli assoni attraversano



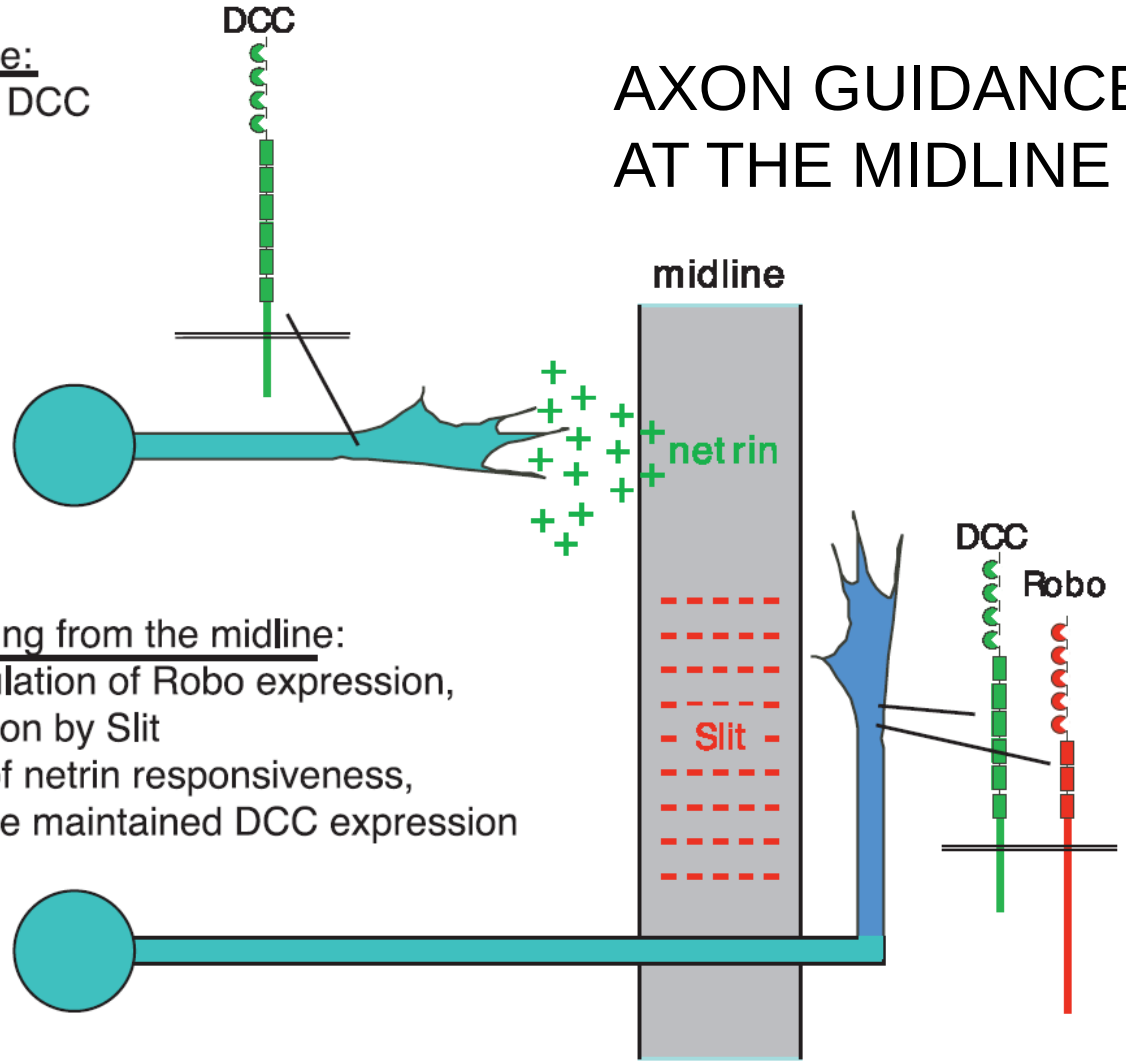
Hierarchical Organization of Guidance Receptors: Silencing of Netrin Attraction by Slit Through a Robo/DCC Receptor Complex

Elke Stein and Marc Tessier-Lavigne*

Department of Anatomy and Department of Biochemistry and Biophysics, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143, USA.

AXON GUIDANCE EVENTS AT THE MIDLINE

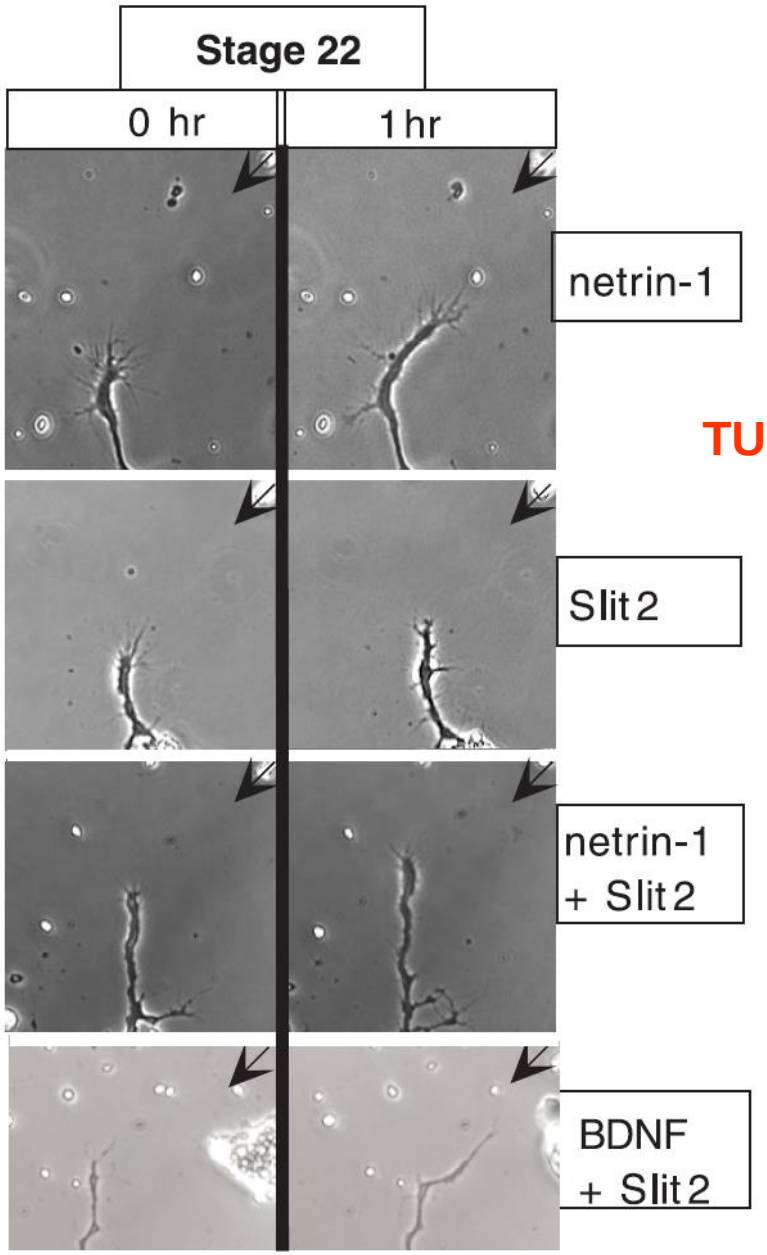
1. Attraction to midline:
netrin activation of DCC

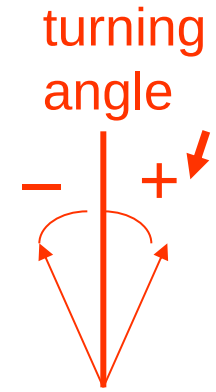


2. Crossing and moving from the midline:
Event 1: Upregulation of Robo expression, repulsion by Slit
Event 2: Loss of netrin responsiveness, despite maintained DCC expression

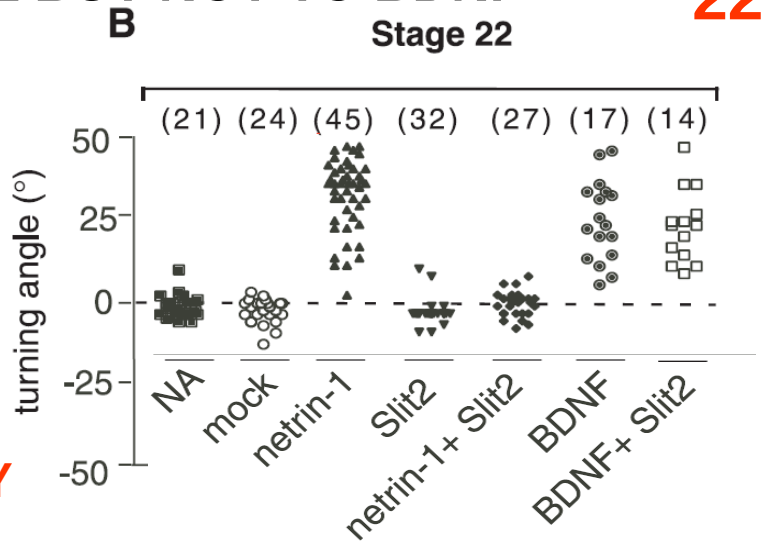
In vertebrates, insects, and nematodes, commissural axons are attracted by netrin protein(s) secreted by midline cells, which attract by activating a receptor of the DCC family on growth cones. After crossing the midline, axons change their responsiveness, such that they are repelled by the midline. In *Drosophila* (and likely in vertebrates as well) this involves up-regulation of the Robo receptor on the postcrossing portions of the axons, so they become responsive to the midline repellent Slit. Axons that cross the midline also lose responsiveness to the netrin attractant, despite maintained expression of the DCC receptor (at least in vertebrates).

SLIT2 SILENCES ATTRACTION TO NETRIN-1 BUT NOT TO BDNF



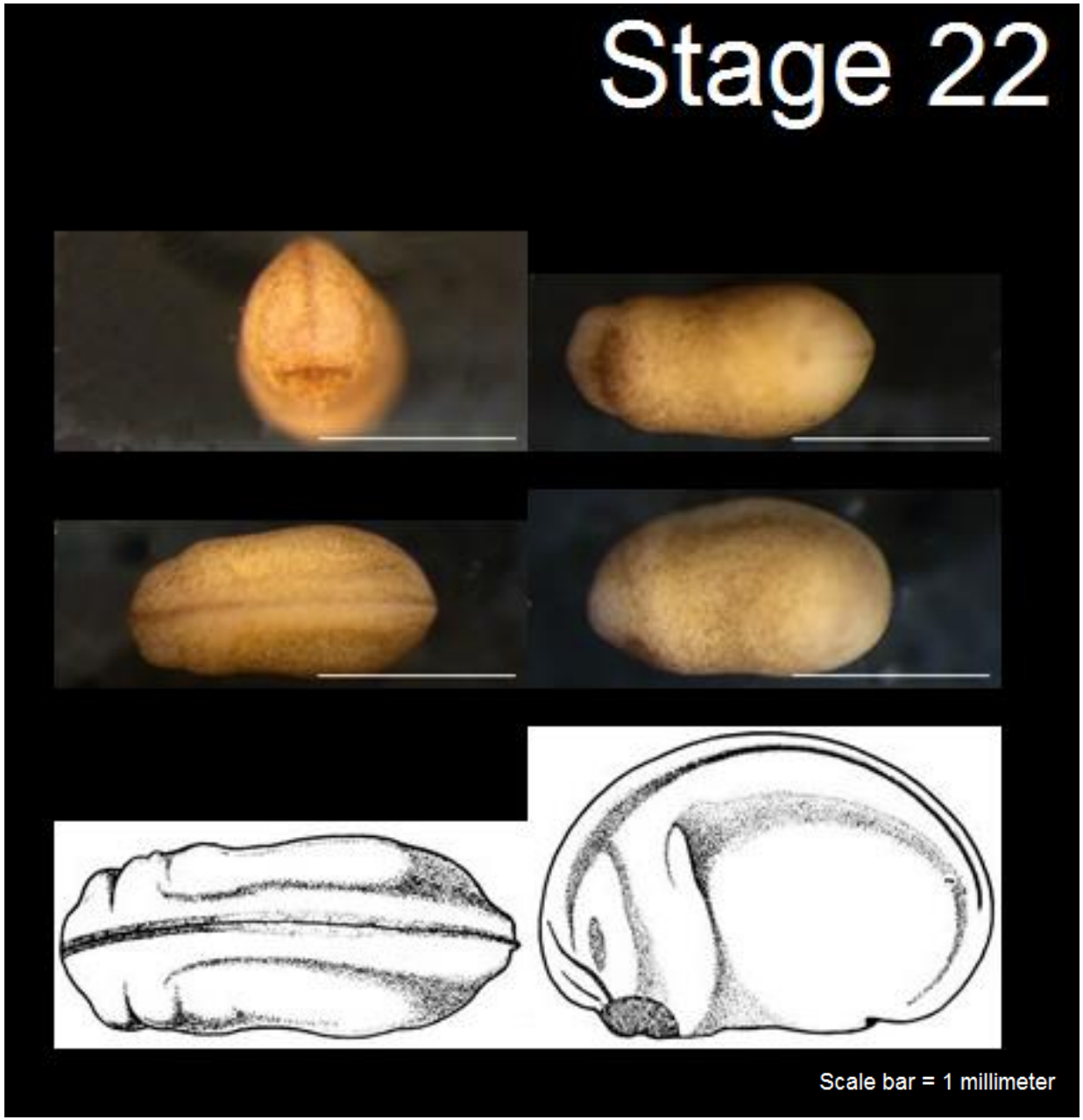


TURNING ASSAY

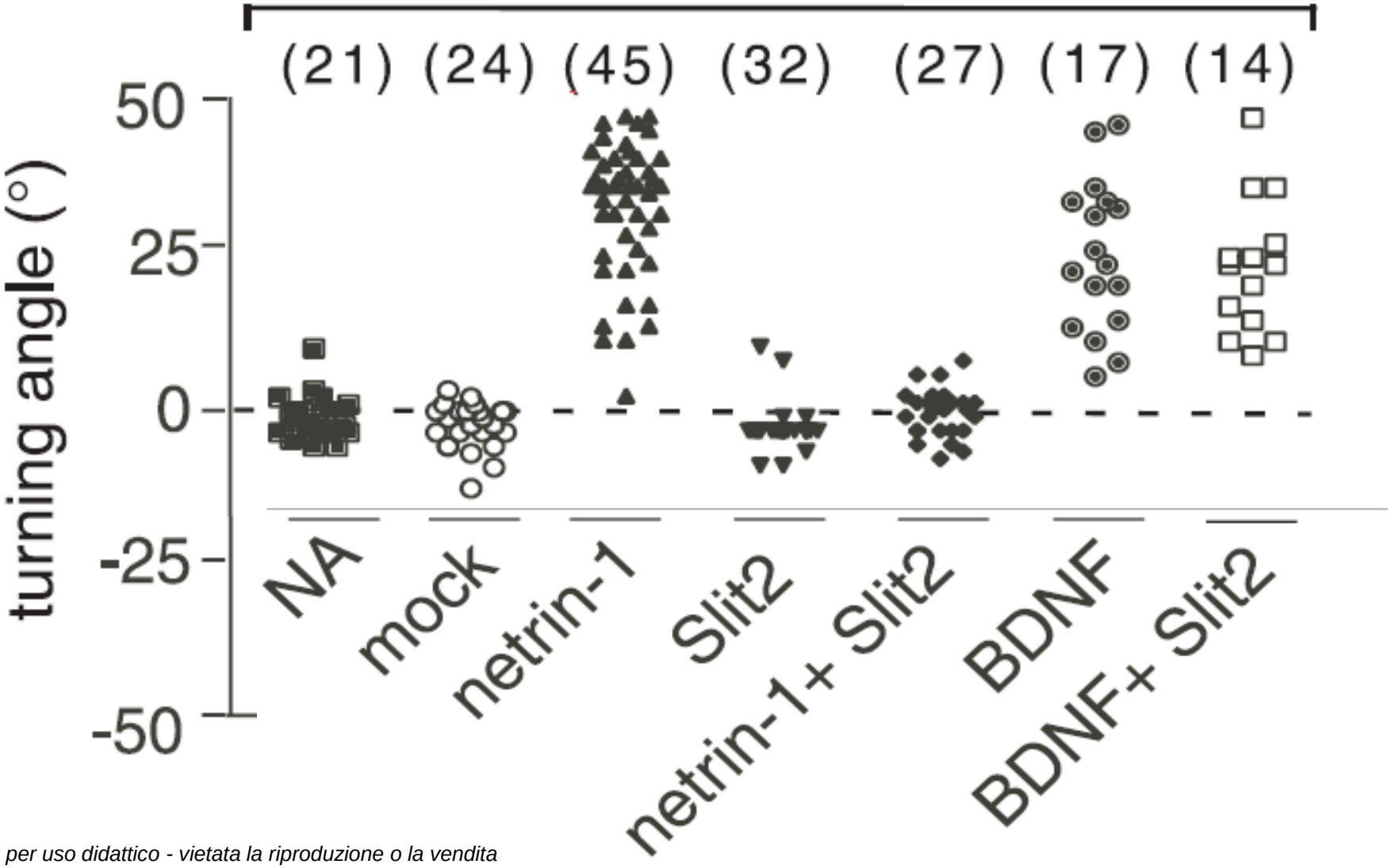


When growth cones of neurons from stage 22 Xenopus embryos are exposed to a gradient of netrin-1 for 1 hour, they turn toward the source. This attractive response requires the function of the netrin receptor DCC. In contrast, the same axons exposed to a gradient of Slit2 protein did not show a directional response. Nevertheless, when growth cones were exposed to a gradient of netrin-1 and simultaneously exposed to Slit2 (in the pipette or in the bath), the attractive effect of netrin-1 was completely abolished (silenced) in all cases. This silencing effect of Slit2 appeared specific for attraction by netrin-1, because Slit2 did not block the attractive effect of brain-derived neurotrophic factor (BDNF), which attracts these axons by activating the trkB receptor in these cells.

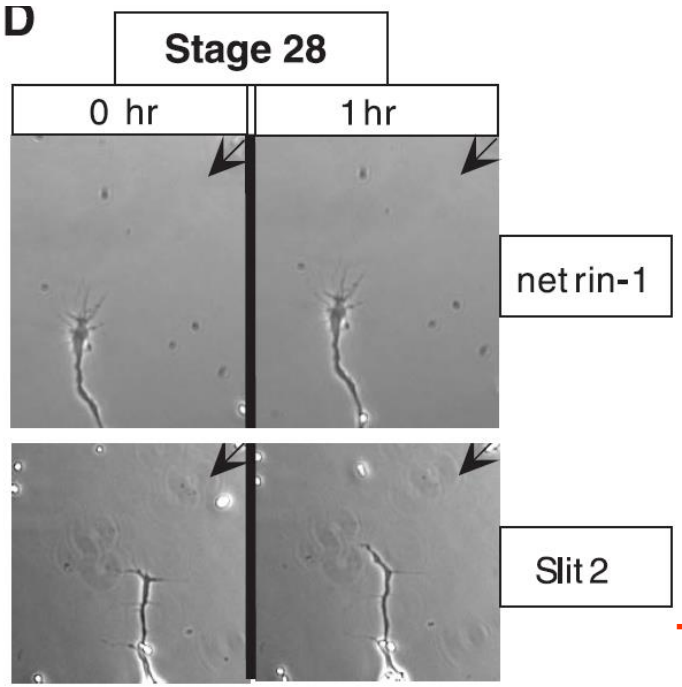
http://www.bio.davidson.edu/people/balom/stagingtable/xenopushome.html

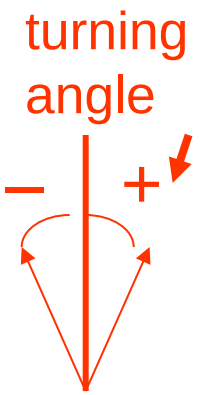


Come si interpreta questo grafico? Quali informazioni mancano rispetto ad un classico grafico con gli istogrammi? Secondo voi, perché gli autori hanno scelto questo tipo di grafico?

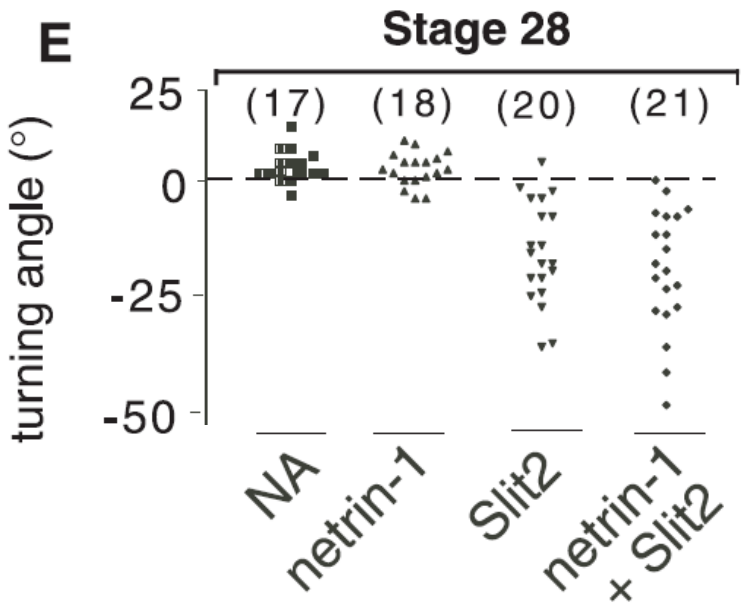


•The finding that Slit2 silences netrin-1 attraction of stage 22 growth cones but does not repel them was unexpected, because Slit2 is expected to function as a repellent.





TURNING ASSAY

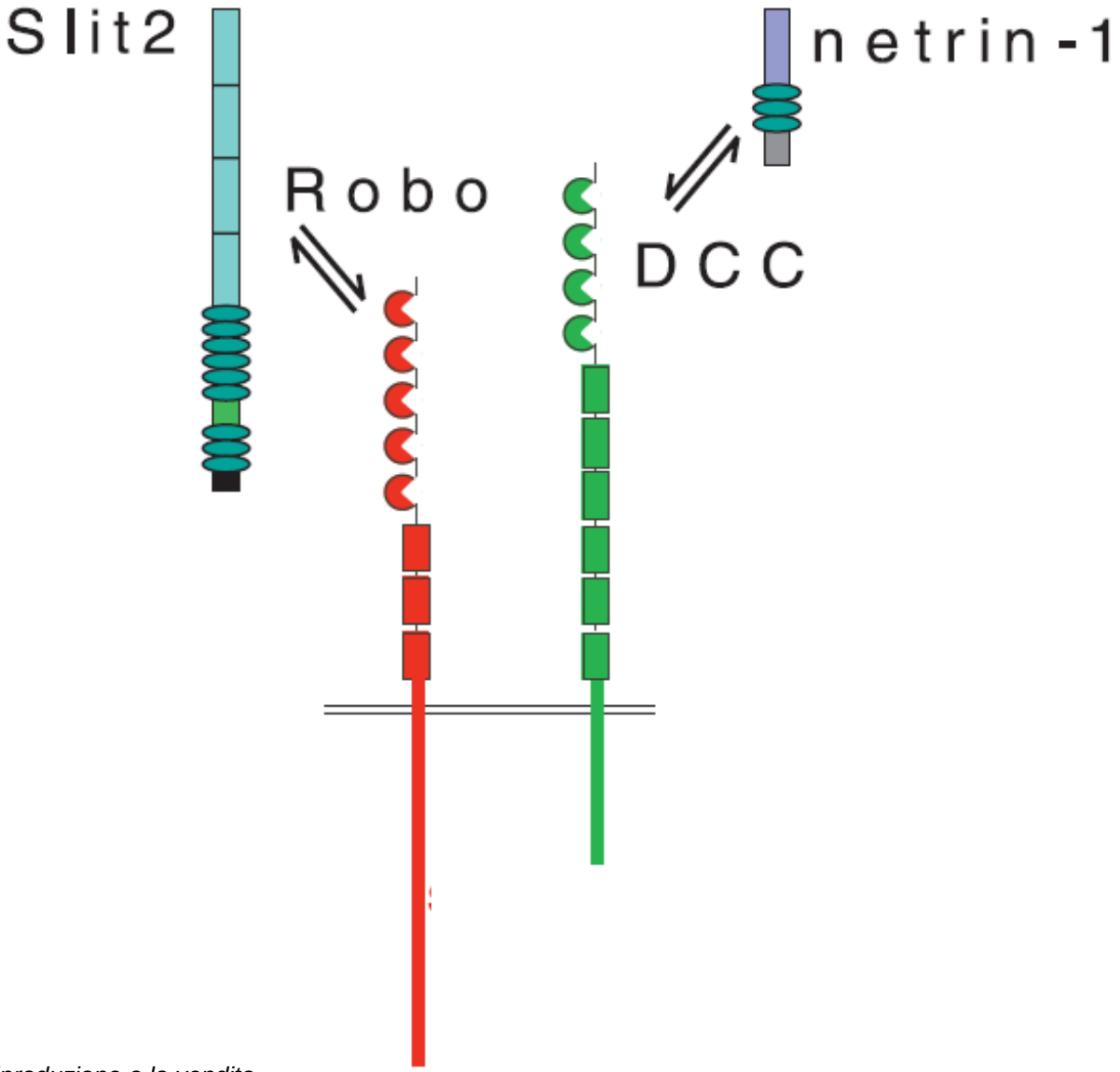


•The axons of older spinal neurons obtained from stage 28 embryos were consistently repelled by Slit2, but did not show any response to netrin-1, likely **because of the absence of DCC expression** in these neurons, as assessed by immunohistochemistry. So it cannot be tested whether Slit2 has a silencing function at that stage as well.

•The differences between stage 22 and stage 28 neurons suggest that the *Xenopus* spinal neurons in these cultures switch their responsiveness to netrins and Slits over time.

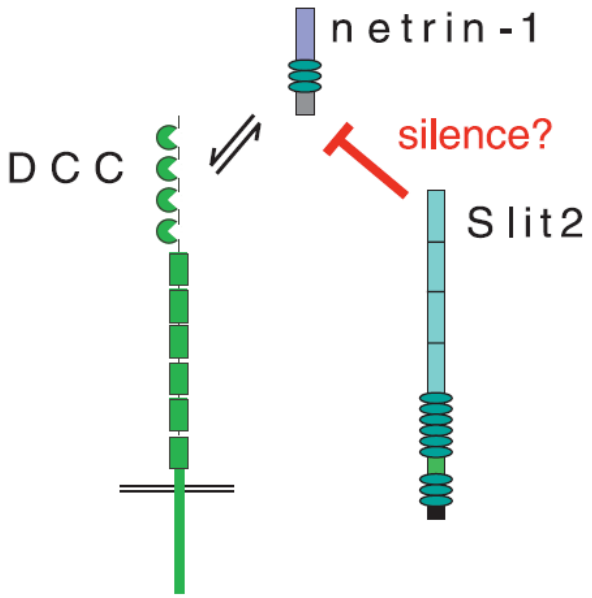
http://www.bio.davidson.edu/people/balom/stagingtable/xenopushome.html

Con quale/i modello/i si può spiegare l'effetto di silenziamento di Slit sull'attrazione mediata dalla netrina?

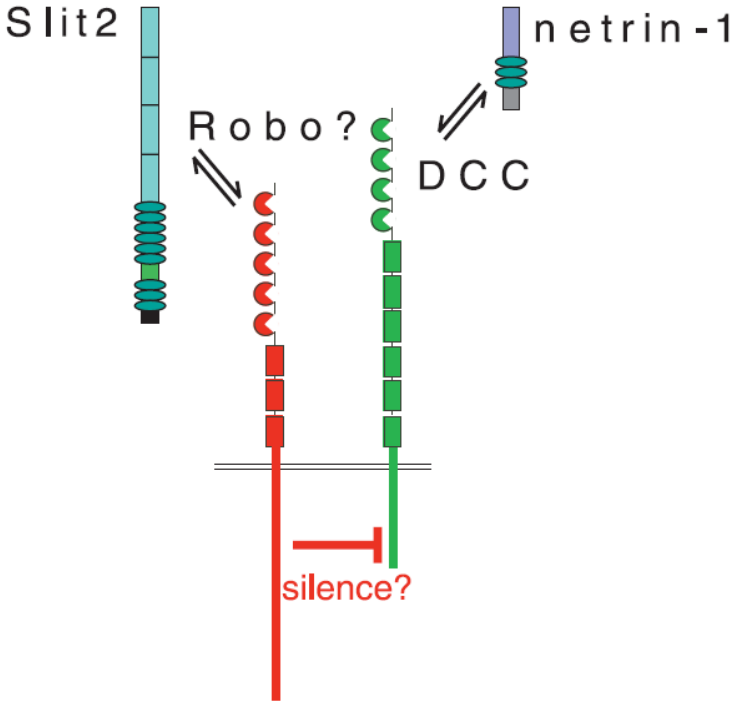


Two models could explain the silencing effect of Slit2 on netrin-mediated attraction.

Model 1:
Ligand-Ligand interaction



Model 2:
Receptor-Mediated Silencing

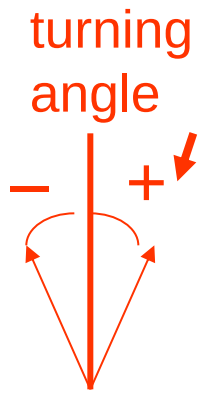


1. because Slit2 can bind netrin-1 directly, silencing might be caused by binding of the two proteins, which could in principle interfere with the netrin-DCC interaction.

2. silencing might be a receptor-mediated event, with Slit2 activating a receptor (presumably a Robo receptor) on growth cones that antagonizes netrin attraction mediated by DCC.

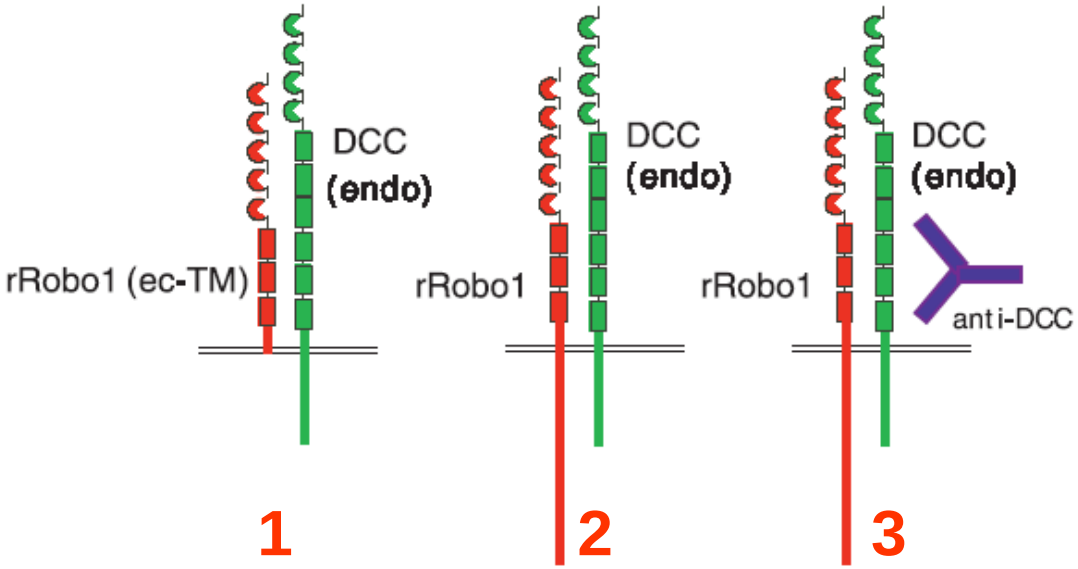
• in all subsequent **TURNING ASSAYS**, exogenous receptors were expressed by injecting *in vitro* transcribed mRNA encoding versions of the receptors of interest [usually tagged with a **Myc** or hemagglutinin (**HA**) epitope tag] into the second blastomere at the four-cell stage of *Xenopus* embryos, together with mRNA encoding **green fluorescent protein (GFP)** as a marker for expression of exogenous proteins

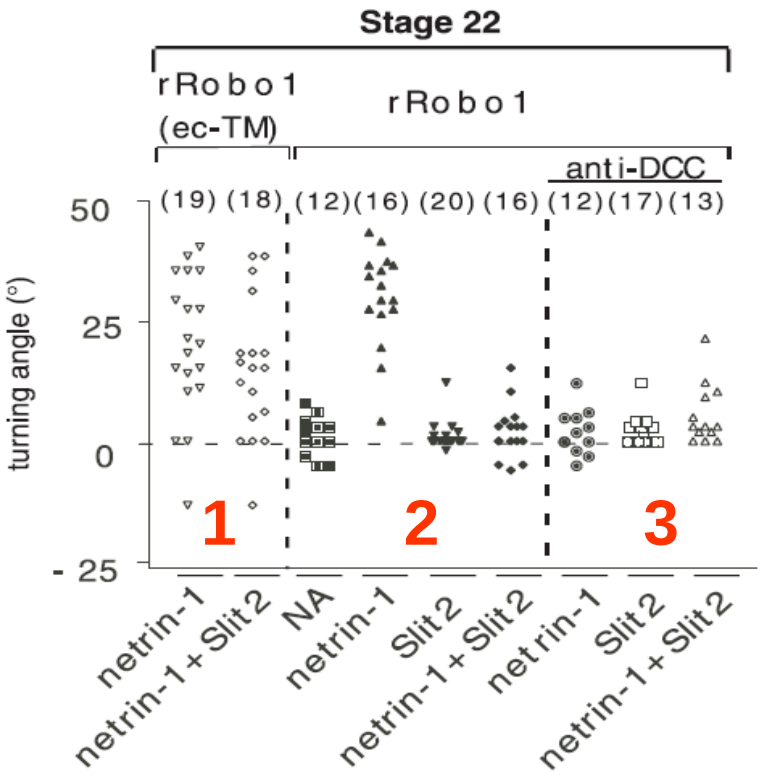
• embryos were allowed to develop to stage 22, and GFP-expressing spinal cord neurons derived from these embryos were assayed for turning responses



TURNING ASSAY

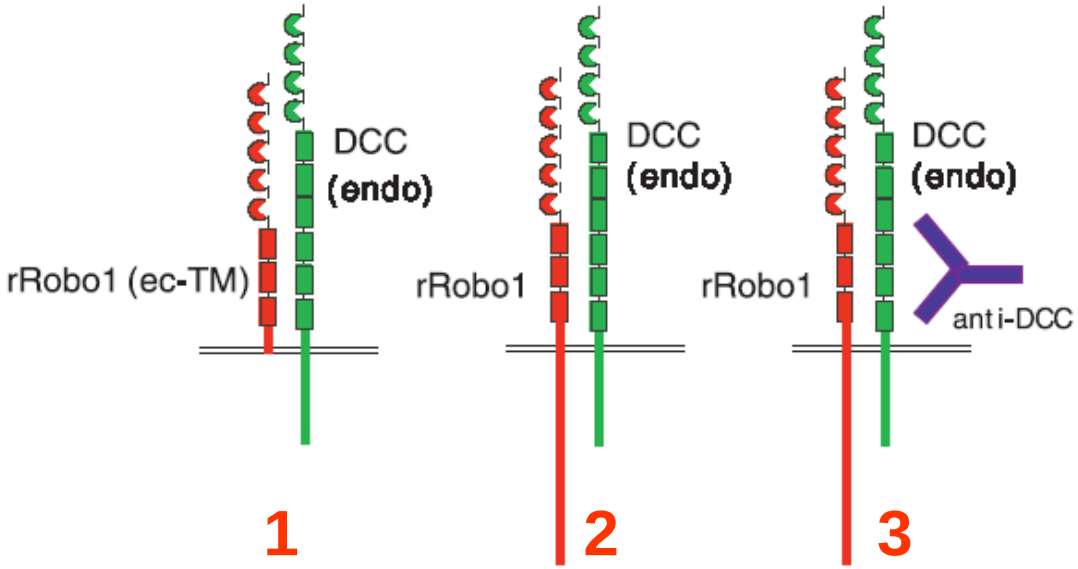
Expression of a truncated Robo receptor (dominant negative) in these neurons to distinguish between model 1 and model 2:

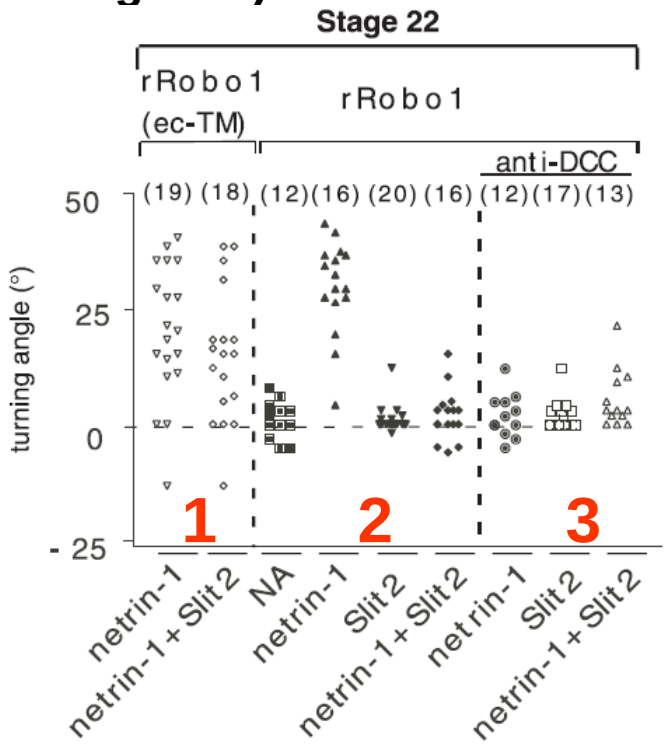




Osservando questi primi esperimenti, quale dei due modelli ipotizzati vi sembra il più plausibile per spiegare il silenziamento dell'attrazione?

Expression of a truncated Robo receptor (dominant negative) in these neurons to distinguish between model 1 and model 2:





A Slit2 no longer silenced the attractive effect of netrin-1; this result is consistent with the involvement of a **receptor-mediated mechanism in silencing**

B expression of full-length rRobo1 in these cells did not interfere with silencing by Slit

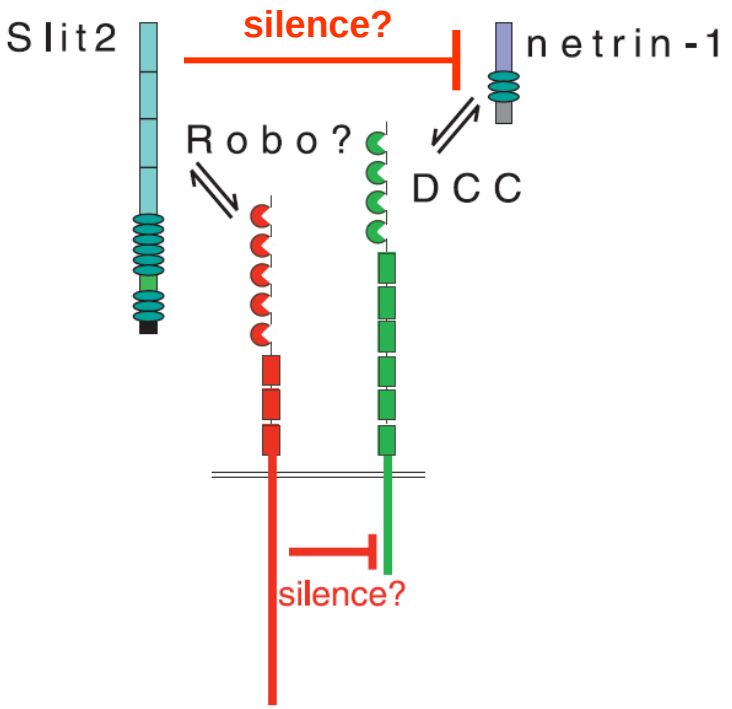
C Slit2 did not repel growth cones expressing full-length rRobo1, indicating that expression of a Robo receptor is not sufficient for repulsion, which presumably requires additional signaling molecules in the growth cone

D the attractive effect of netrin-1 observed in all experimental conditions was blocked by antibodies to DCC, consistent with the requirement of DCC for netrin-mediated attraction

• truncated Robo receptor can block silencing by Slit

→ **receptor - mediated mechanism but....**

Secondo voi il modello 2 (quello dell'interazione fra recettori) è l'unico che può spiegare i dati ottenuti con il recettore privo del dominio citoplasmatico?



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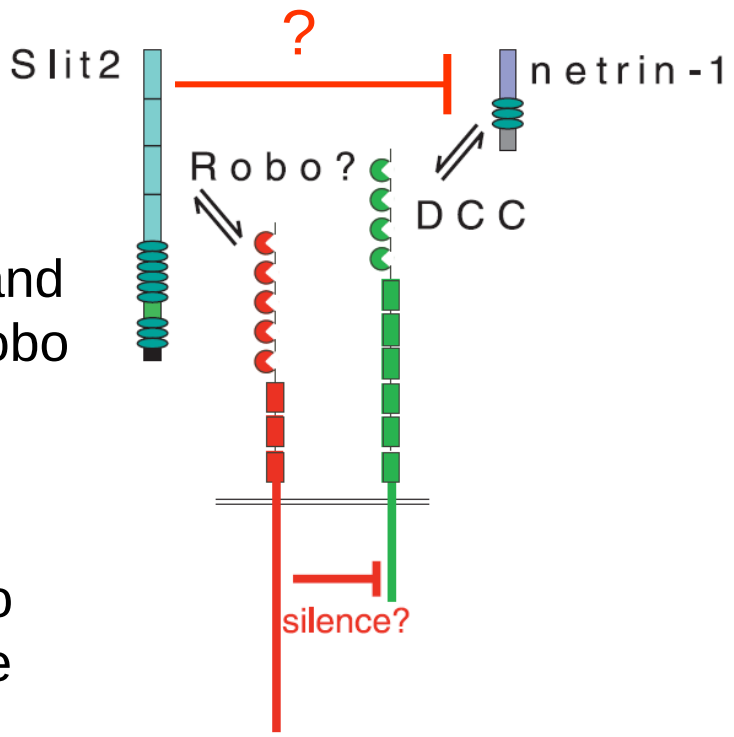
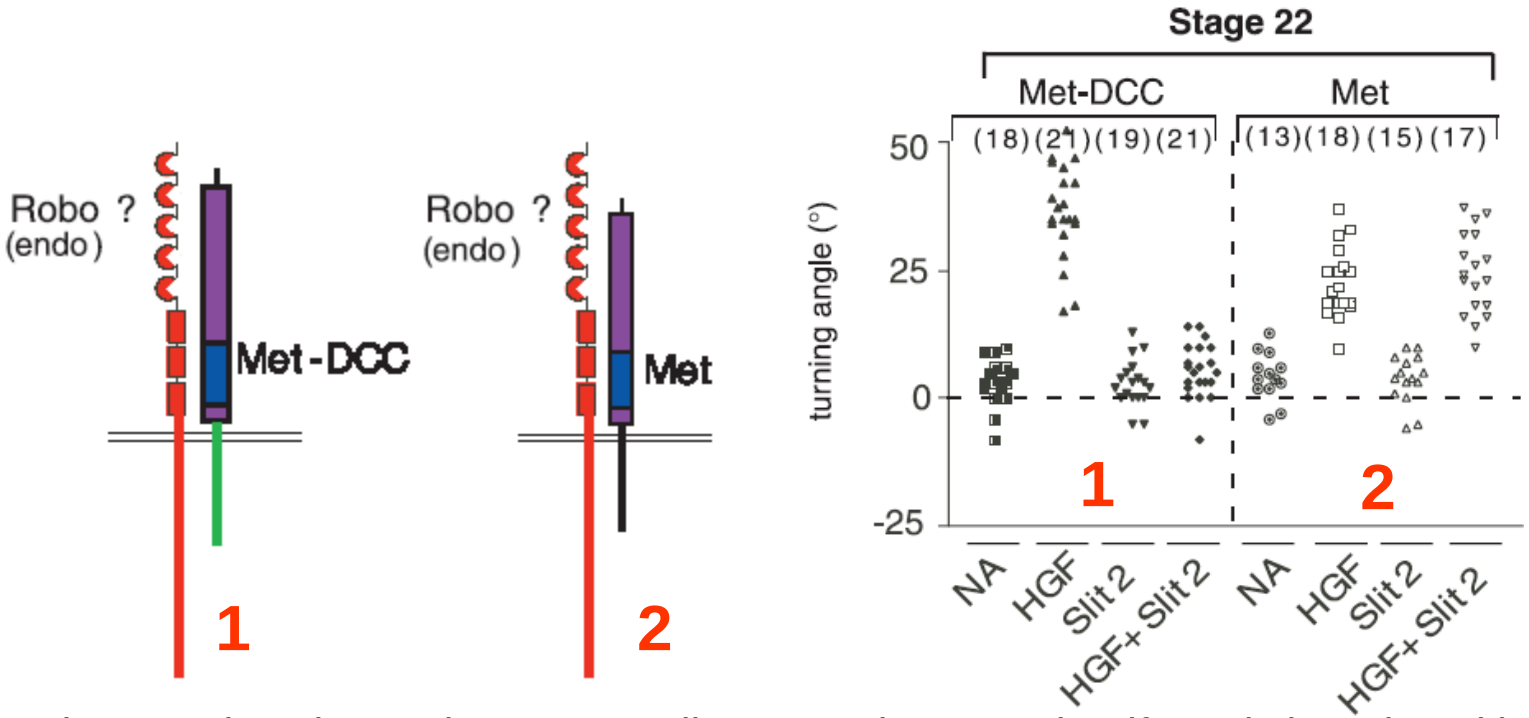


• ....this result is also compatible with a ligand-ligand interaction model of silencing if the exogenous Robo can bind and somehow locally reduce (titrate) the amount of available Slit2 protein

• to more definitively discriminate between the two models, they used chimeric receptors in which the ectodomain of DCC or that of Robo1 is replaced with an exogenous ectodomain: that of the Met receptor tyrosine kinase, a receptor for hepatocyte growth factor (HGF), a soluble chemoattractant

• quali vantaggi dà un costrutto chimerico Met-DCC o Met-Robo?



• *Xenopus* growth cones in cultures do not normally respond to HGF, but if Met is introduced into them, they respond to HGF with attraction

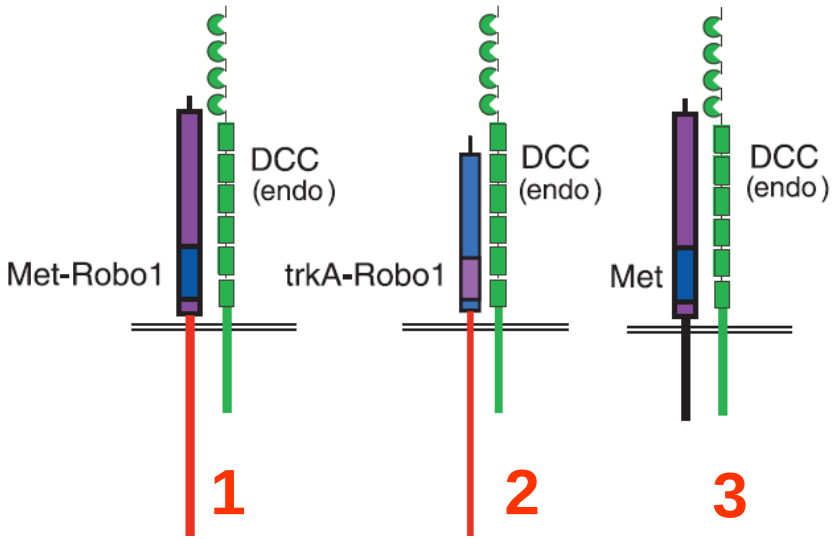
• when a chimeric receptor comprising the Met ectodomain and the DCC transmembrane and cytoplasmic domain is introduced into these cells, HGF induces attractive responses

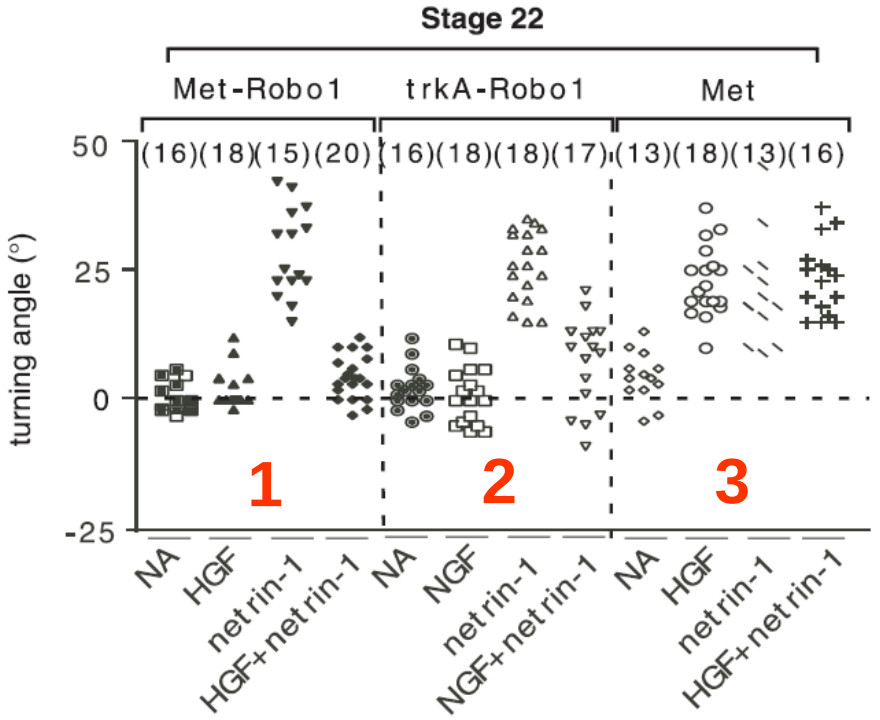
• Slit2 is as effective in silencing attractive responses elicited by HGF binding to the Met-DCC chimeric receptor as it is in silencing netrin-mediated attraction

• Slit2 does not silence attractive responses to HGF that are mediated by the wild-type Met receptor tyrosine kinase

• **silencing is observed only for attraction caused by activation of the DCC cytoplasmic domain**

Could activation of the Robo signaling pathway by a heterologous ligand also lead to silencing of netrin attraction?



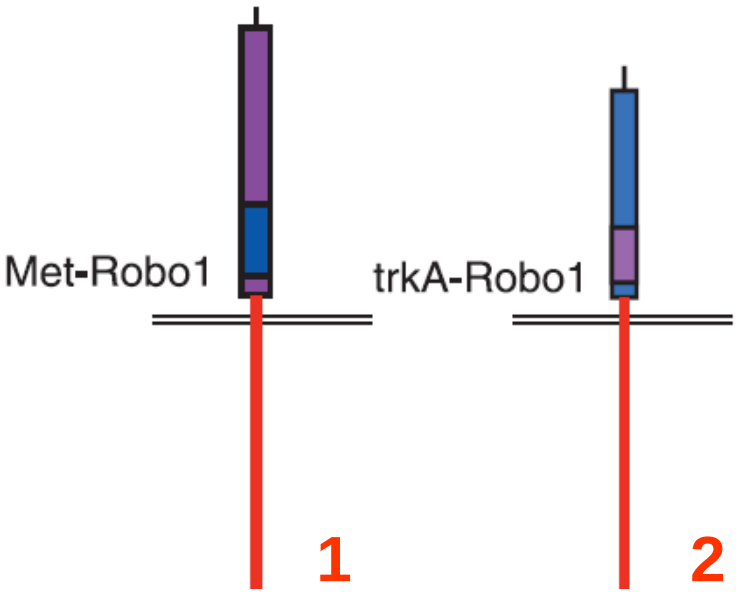


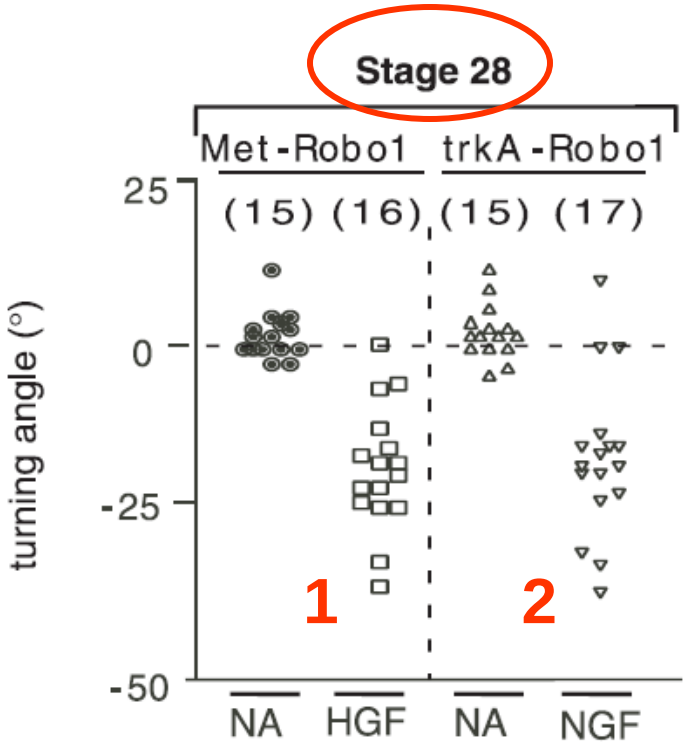
• chimeric receptors comprising the cytoplasmic domain of rRobo1 and the ectodomain of either Met or the trkA (receptor tyrosine kinase for nerve growth factor), were introduced in these cells

• in neurons expressing the Met- Robo1 chimera, as observed with Slit, HGF did not elicit directional responses, but completely silenced the attractive effect of netrin-1

• in neurons expressing the trkA-Robo1 chimera, NGF did not elicit directional responses, but completely silenced the attractive effect of netrin-1

• as a control, introduction of the wild-type Met receptor into these neurons led to attractive responses to HGF, as well as to netrin-1 together with HGF





• **stage 28 neurons** expressing Met-Robo1 or trkA-Robo1 showed clear repulsive responses to HGF or NGF, respectively, responses that were not observed in stage 22 neurons

• this finding supports the idea that **there are differences between stage 22 and stage 28 neurons** that determine whether only silencing or frank repulsion will be elicited by activation of the Robo signaling pathway

• these studies strongly support the **receptor-mediated silencing model** by indicating that attractive responses elicited by activation of a DCC cytoplasmic domain (whether by netrin-1 or by a heterologous ligand acting on a chimeric receptor) can be silenced by activation of a Robo cytoplasmic domain (whether by Slit or by a heterologous ligand acting on a chimeric receptor)

• in the absence of antibodies to *Xenopus* Robo receptors, it cannot formally be proved that Slit is mediating its effects on these axons through an endogenously expressed Robo receptor.

• nonetheless, this assumption is supported by the findings that
-a truncated Robo receptor blocks silencing
-introduction of full-length rRobo1 into these neurons does not alter silencing
-silencing can be elicited by activating Met-Robo1 or trkA-Robo1 chimeras
-Robo mRNA is expressed in stage 22 neurons (RT-PCR data)

→ therefore, **results will be interpreted as if Slit is mediating its effect via an endogenous Robo receptor**

Quale tecnica si può utilizzare per vedere se Robo e DCC formano un complesso?

? Could Robo and DCC form a receptor complex in transfected cells?

RECEPTOR CO-IMMUNOPRECIPITATIONS

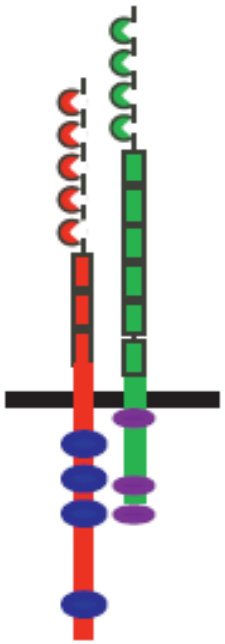
• HA- and Myc-tagged versions of DCC and Robo1 [**DCC(HA)** and **Robo1(Myc)**] were co-transfected into **COS cells**

• 40 hours after transfection, cells were incubated for 20 min at 37°C with ligands (control medium, netrin-1, Slit2, HGF, NGF)

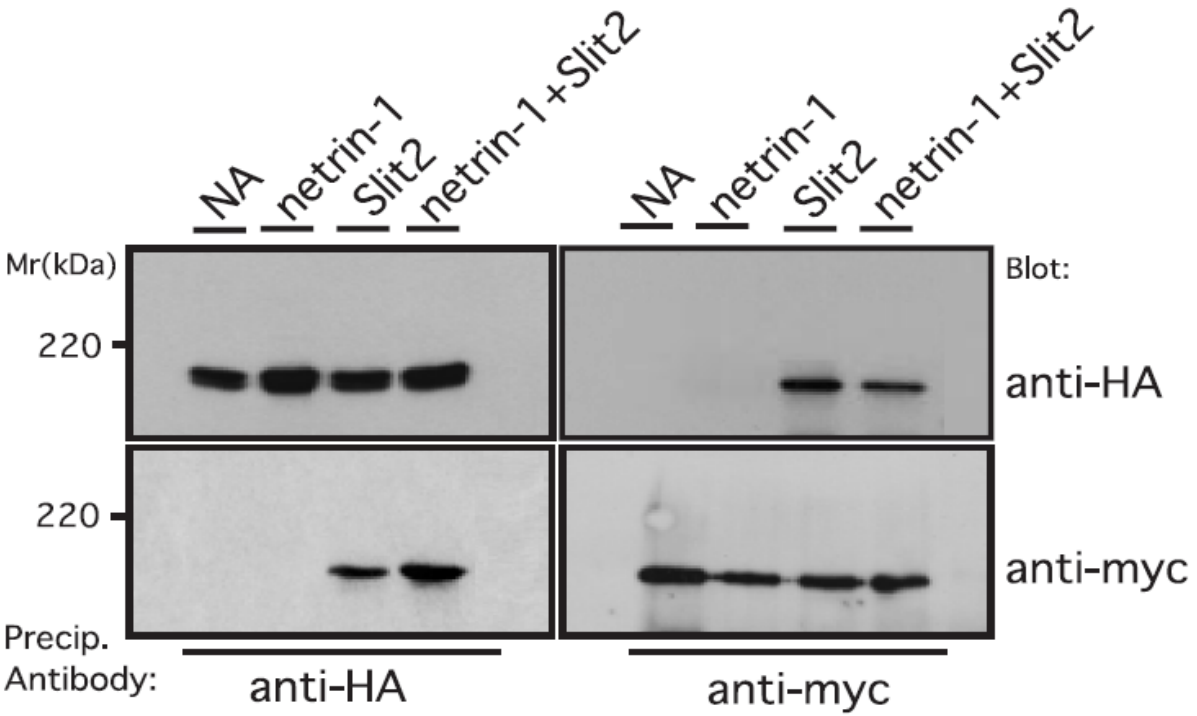
• total proteins were extracted

• proteins were subjected to immunoprecipitation, using the indicated antibodies

• proteins were analyzed by Western blotting



DCC(HA)
+ rRobo1(myc)



- Commentate questo risultato