Molecular and Cellular Neuroscience 41 (2009) 429–439

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Differential roles of Netrin-1 and its receptor DCC in inferior olivary neuron migration

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ARTICLE INFO

Article history:
Received 12 November 2008
Revised 10 April 2009
Accepted 23 April 2009
Available online 3 May 2009

Keywords:
Guidance
Pathfinding
Floor plate
Mice
Precerebellar neurons
Olivary neurons
Retrograde tracing

ABSTRACT

Netrin-1 was previously shown to be required for the tangential migration and survival of neurons that will form the inferior olivary nucleus (ION). Surprisingly, the compared analysis of mutant mice lacking either *Netrin-1* or its major receptor *DCC* reveals striking phenotypic differences besides common features. Although ectopic stops of ION cell bodies occur in the same positions along the migratory stream in both mutants, the ION neurons' number is not affected by the lack of DCC whereas it is reduced in *Netrin-1* mutant mice. Thus, cell death results from the absence of Netrin-1 and not from neuron mis-routing, arguing for a role of Netrin-1 as a survival factor *in vivo*. The secretion of Netrin-1 by the floor plate (FP) is strictly required – whereas DCC is not – to avoid ION axons' repulsion by the FP and allows them to cross it. Leading processes of neurons of other caudal precerebellar nuclei (PCN) cannot cross the FP in either mutant mouse, suggesting differential sensitivity or mechanism of action of Netrin-1 for leading processes of ION and other PCN neurons.

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Introduction

During the development of the caudal hindbrain, neurons that will form PCN migrate from their birthplace in the germinative neuroepithelium (rhombic lip) to the position they occupy in the mature brain (Rakic, 1990). PCN neurons provide an interesting comparative model of neurophilic tangential migration, including the neurons that will form the external cuneatus nucleus (ECN), the lateral reticular nucleus (LRN) and the inferior olivary nucleus (ION) (Altman and Bayer, 1987a,b). They differ by distinct migratory routes. ION neurons migrate through the submarginal stream (Altman and Bayer, 1987a,b; Bourrat and Sotelo, 1988, 1991), whereas neurons that will form the LRN and the ECN migrate through the marginal stream. All PCN neurons first extend a leading process that initiates the circumferential migratory route and reaches the floor plate (FP) (Bourrat and Sotelo, 1988), then their nuclei translocate inside the leading process (Gilthorpe et al., 2002; Causeret et al., 2002). Although the leading processes of all caudal PCN neurons cross the FP, the behaviour of their soma differs. The cell bodies of ECN neurons and of LRN neurons cross the FP and continue their translocation until reaching their proper destination in the contralateral rhombencephalon. In contrast, cell bodies of ION neurons stop before crossing the FP and aggregate to form club-shaped masses (Altman and Bayer, 1987a,b; Bourrat and Sotelo, 1990). Thus, PCN cell bodies do not respond identically to signals from the FP. Consequently, IONs develop a contralateral projection to the cerebellum whereas LRN/ECN develop an ipsilateral projection.

The FP acts as an intermediate target and is a source of both contact and diffusible molecules that guide axons and cell body migration. In previous studies, we showed that Netrin-1, the first chemotropic factor that has been discovered to be secreted by the FP, was involved *in vivo* and *in vitro* in the migration of ION neurons (Bloch-Gallego et al., 1999; Causeret et al., 2002). Netrin-1 also attracts other PCN neurons *in vitro*, such as LRN or ECN neurons (Alcantara et al., 2000) and directs the tangential migration of pontine neurons (Yee et al., 1999). In the absence of Netrin-1, ION cell bodies are located ectopically along their migratory pathway and ION axons mainly project ipsilaterally to their cerebellar target instead of contralaterally as in wild type (WT) mice, which could be due to an abnormal crossing of ION cell bodies or to the inability for axons to cross the FP (Bloch-Gallego et al., 1999). Additional experimental evidence was required to support one of these two hypotheses. Although the receptor Deleted in Colorectal Cancer (DCC) was implicated in pontine neurons responses to Netrin-1 gradient (Yee et al., 1999), the involvement of DCC in Netrin-1 responses for ION and LRN/ECN neurons had not been investigated so far.

In addition, in *Netrin-1* mutant mice, the number of ION neurons was observed to be reduced at birth (Bloch-Gallego et al., 1999) and

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doi:10.1016/j.mcn.2009.04.008

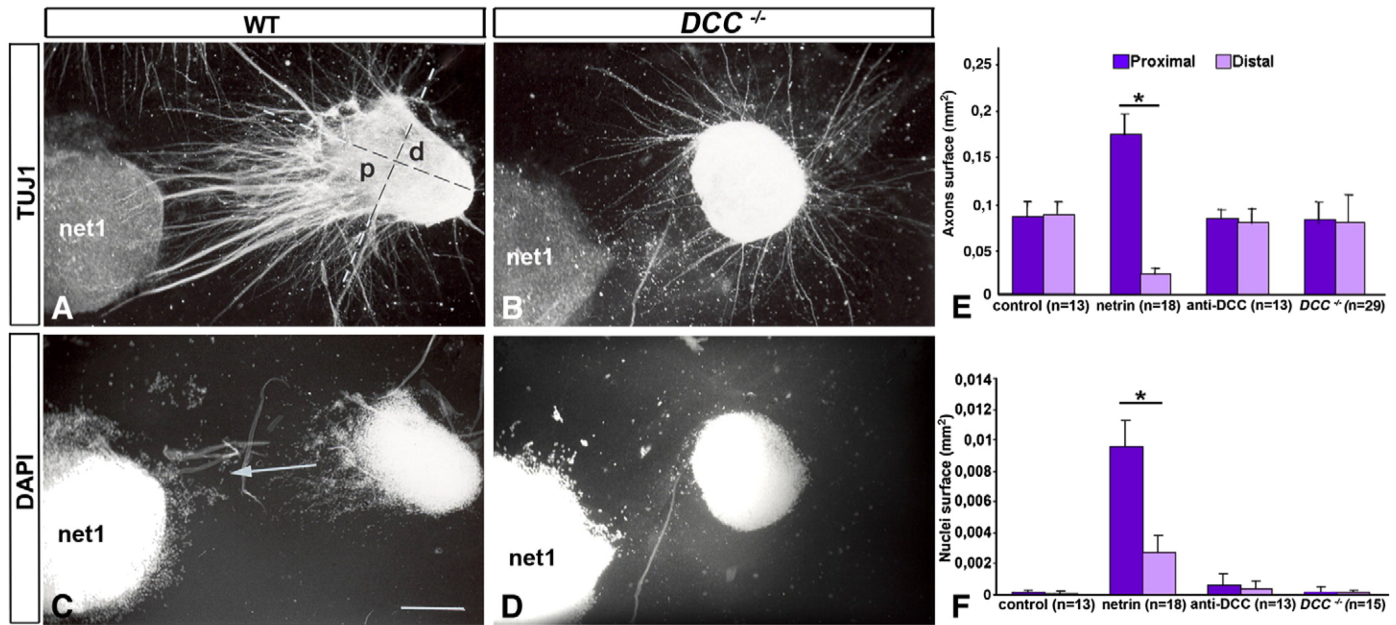

Fig. 1. DCC is required for ION neurons' attractive response to Netrin-1 in vitro. E11.5 rhombic lip explants containing ION neurons are faced with Netrin-1-secreting cells (net1), cultured for 3 days and then analyzed for axon outgrowth and nucleokinesis through Tuj1 and DAPI staining respectively. (A, B) Axon outgrowth from WT and $DCC^{-/-}$ rhombic lip explants. In WT explants ($n=18$) (A), Netrin-1 induces oriented axon outgrowth in the proximal quadrant (p), but not in the distal one (d), whereas when explants from *DCC* mutant embryos ($n=29$) (B) are used, axon outgrowth is no more promoted by Netrin-1-secreting cells. (C, D) Nucleokinesis in WT and $DCC^{-/-}$ rhombic lip explants. Netrin-1-secreting cells induce nuclei migration from WT explants ($n=18$) (C), but not from $DCC^{-/-}$ explants ($n=15$). (E) Bar graphs showing total axon outgrowth toward (proximal) and away (distal) from control cells or Netrin-1-secreting cells were obtained upon threshold quantifications of Tuj1 staining on WT, $DCC^{-/-}$ or anti-DCC blocking antibodies treated rhombic lip explants. (F) Bar graphs showing nucleokinesis toward (proximal) and away (distal) from control cells or Netrin-1-secreting cells were obtained upon threshold quantifications of DAPI staining on WT, $DCC^{-/-}$ or anti-DCC blocking antibody treated rhombic lip explants. Quantification were performed using the Student's *t*-test, and * is for $p<0.01$.

we had proposed that it might play the role of a survival factor via its dependence receptors, members of the DCC and Unc5 families (Llambi et al., 2001).

Through comparative analysis of *Netrin-1* and *DCC* mutant mice, we report observations that provide cues for understanding the distinct involvement of Netrin-1 and DCC in ION survival and for various caudal PCN leading processes in crossing the FP during their tangential migration.

Results

We have further investigated the role of Netrin-1 in neuronal PCN migration, i.e. the positioning of cell bodies, axon outgrowth and guidance, and the development of the cerebellar projection. To this end, we have compared the phenotypes of *DCC* and *Netrin-1* mutant mice. Here we detail the differences and similarities that we observed during PCN migratory process in ligand and receptor mutant mice.

DCC is required for ION axon attraction, promotion of axon outgrowth and permissivity of nuclear migration in response to Netrin-1 in vitro

To assess the role of DCC in axon outgrowth and neuronal migration (nucleokinesis) for ION neurons in response to Netrin-1, *in vitro* experiments were carried out. Rhombic lip explants from E11.5 embryos were used since they are enriched in ION neurons due to the peak of ION neurons' birthdate at this specific stage compared to other PCN populations (Causeret et al., 2002, 2004). They were taken from WT or *DCC* mutant embryos and were faced with either mock or Netrin-1 secreting 293-EBNA cells in a collagen matrix. ION axon outgrowth and nucleokinesis were analyzed in these coculture assays after 3 days in culture. The explants were immunostained with class III β-tubulin (Tuj1) antibodies and the cell nuclei were visualized with DAPI. In this type of assay, we have previously shown that a Netrin-1 source promoted extensive and oriented axon outgrowth, as well as nucleokinesis from WT explants ($p<0.01$) (Causeret et al., 2002; Figs. 1A, C and quantification in E and F) whereas only a slight degree of axon outgrowth and no cell body migration occurred when WT explants were presented with control cells (not illustrated, quantification in Figs. 1E and F). The coculture assays were next performed with E11.5 rhombic lip explants taken from *DCC* mutant embryos ($n=29$). In this case, neuronal processes were much shorter, axon outgrowth was greatly decreased and followed a random pattern of growth (proximal/distal ratio of 1.2 ± 0.5; Fig. 1B and quantification in Fig. 1E). In addition, very few cell bodies left the explants (Fig. 1D and quantification in Fig. 1F). Similar results were observed when we added blocking anti-DCC antibodies to WT explants ($n=13$): axon outgrowth lost its directionality (proximal/distal of 1.1 ± 0.3 — which is comparable with the neuronal processes in WT explants faced with control cells (quantification in Fig. 1E)) and nucleokinesis was impaired (quantification in Fig. 1F). As a control, we used an antibody directed against the intracellular domain of DCC: the addition of this antibody had no effect on axonal oriented outgrowth and nucleokinesis (not shown). These results indicate that DCC is required for the chemoattractive effect of Netrin-1 on ION axons and cell bodies *in vitro*, and that the presence of both Netrin-1 and DCC promotes axon outgrowth.

Netrin-1 masks a repulsive activity of the floor plate

We next examined responses to FP explants, which provide a source of Netrin-1 but also of other potential chemotropic cues. To determine the role of Netrin-1 in responses to FP explants, we co-cultured rhombic lip explants taken from E11.5 WT embryos with FP explants from either WT or *Netrin-1* mutant embryos. When rhombic lip explants were confronted with FP explants dissected from E11.5 WT embryos, both oriented outgrowth (Fig. 2A) and nucleokinesis occurred (not shown) toward the FP explants ($n=22$; $p<0.01$, P/D ratio $=3.60\pm0.80$ for axon outgrowth) (quantification in Fig. 2E). However, both events were affected when explants were faced with FP from *Netrin-1* mutant embryos. Surprisingly, although no nuclear migration occurred in the absence of Netrin-1 (not shown),

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