Neural circuit formation in the cerebellum is controlled by cell adhesion molecules of the Contactin family

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Cell adhesion molecules of the immunoglobulin superfamily (IgSF CAMs) have been implicated in neural circuit formation in both the peripheral and the central nervous system. Several recent studies highlight a role of the Contactin group of IgSF CAMs in cerebellar development, in particular in the development of granule cells. Granule cells are the most numerous type of neurons in the nervous system and by forming a secondary proliferative zone in the cerebellum they provide an exception to the rule that neuronal precursors proliferate in the ventricular zone. Granule cells express Contactin-2, Contactin-1 and Contactin-6 in a sequential manner. Contactins are required for axon guidance, fasciculation and synaptogenesis, and thus affect multiple steps in neural circuit formation in the developing cerebellum.

Introduction

The cerebellum has long been known for its role in motor learning and coordination of smooth movements. Only during the last decade, the importance of the cerebellum for cognitive processes, emotions and language processing has been appreciated. Children with cerebellar hypoplasia were often found not only to suffer from congenital ataxia but also to have mild to moderate speech and cognitive problems. Prenatal alcohol exposure resulting in fetal alcohol syndrome (FAS) was shown to reduce the volume of the anterior vermis and to be linked to problems in verbal learning and cognitive performance. Malformations of the cerebellar lobules and changes in the volume of the vermis have been found in patients diagnosed with fragile X syndrome, Down syndrome and Williams syndrome. Recent studies linked aberrant cerebellar development to dyslexia and to attention deficit and hyperactivity disorder (ADHD). Furthermore, changes in cerebellar morphology have been discussed in the context of autism and schizophrenia.

The cerebellum originates from the anterior-most rhombomere of the hindbrain. In mice, cerebellar development spans from embryonic day 9.5/10 (E9.5/10) to postnatal day 15 (P15). The ventricular zone gives rise sequentially to neurons of the deep cerebellar nuclei, Purkinje cells and finally, GABAergic interneurons, such as basket, stellate and Golgi cells. The most numerous type of neurons, the granule neurons, arise from the rhombic lip. From there granule cell precursors migrate rostrally over the surface of the developing cerebellar anlage to generate the second proliferative zone, the external granular layer (EGL). In the outer EGL, granule cell precursors proliferate and start to differentiate (Fig. 1). In the inner EGL, also called premigratory zone, granule cells extend bipolar processes parallel to the pial surface of the cerebellum, hence the name parallel fibers. Finally, granule cells extend a third process perpendicular to the parallel fibers. Along the fibers of Bergmann glia cells granule neurons migrate radially inwards through the developing Purkinje cell layer to form the internal granule cell layer (IGL). The parallel fibers left behind form glutamatergic synapses with Purkinje cell dendrites in the developing molecular layer.
Granule cell dendrites in the IGL form synapses with mossy fibers that have a very characteristic shape, called rosette. Climbing fibers represent a second type of cerebellar input. They contact Purkinje cells. Purkinje cells produce the output of the cerebellum by projecting to the neurons of the deep cerebellar nuclei.

Granule Cell Development

Granule cells start to migrate from the rhombic lip tangentially over the roof of the fourth ventricle around E13 in the mouse. Recently, the transparency of zebrafish larvae has been exploited to visualize long-distance migration of granule cells from the rhombic lip with time-lapse imaging. These studies demonstrated a role of the fish ortholog of N-Cadherin, Cadherin-2, in this type of granule cell migration. A variety of additional molecules known to affect axon guidance and cell migration in the developing nervous system have been implicated in granule cell migration from the rhombic lip (reviewed in ref. 7). After reaching their final position, granule cell precursors proliferate in the external granule cell layer, close to the pial surface.

Figure 1. Contactin family members are expressed sequentially during cerebellar development. Granule cells proliferate close to the pial surface in the outer part of the external granular layer. Postmitotic granule cells in the deeper EGL (inner EGL) start to extend processes in a bipolar manner.Cntn-2 is the first Contactin family member that is expressed on parallel fibers (red). Older granule cells start to express Cntn-1 (green), as well as NrCAM and NgCAM. NrCAM is also expressed in Purkinje cells (yellow). After migration granule cells downregulate NgCAM but start to express Cntn-6 (purple). Both Cntn-1 and Cntn-6 are required for synaptogenesis. Development proceeds from left to right (arrow). Bars on the right indicate expression domains of Contactins, as well as NrCAM and NgCAM. EGL, external granular layer; MCL, molecular layer; PCL, Purkinje cell layer; IGL, inner granular layer.

Postmitotic granule cells come to lie in the deeper zones of the EGL, thus separating the EGL into an outer and an inner layer. The inner EGL, also called premigratory zone, contains postmitotic granule cell precursors that start to extend their first processes in a bipolar manner. As suggested by their name, these processes are formed in a parallel manner to processes from neighboring granule cell precursors and parallel to the pial surface.

Cell Adhesion Molecules of the Contactin Family are Expressed Sequentially in Developing Granule Cells

The Contactin group of immunoglobulin superfamily cell adhesion molecules (IgSF CAMs) contains six members: Contactin-1/F3/F11, Contactin-2/TAG-1/Axonin-1, Contactin-3/Big-1, Contactin-4/Big-2, Contactin-5/FAR-2/NB-2 and Contactin-6/NB-3. They contain six immunoglobulin domains followed by four fibronectin type III repeats and a glycosylphosphatidylinositol (GPI) anchor that links them to the membrane.

The first Contactin family member expressed by granule cells extending bipolar processes is Contactin-2/TAG-1/Axonin-1 (Fig. 1). Cntn-2/TAG-1/Axonin-1 is well known for its role in axon guidance in different parts of the nervous system. It was shown to affect guidance, but not growth, of commissural axons in the spinal cord. In contrast, Cntn-2 affected both growth and guidance of sensory axons.

Similarly, Cntn-1/F3/F11 was implicated in axon growth and guidance. Cntn-1 is expressed by older granule cell neurons in the molecular layer and is barely overlapping with Cntn-2 expressed in the inner EGL and upper molecular layer. Cntn-1 is downregulated but still detectable in postmigratory granule cells in the IGL.

Finally, Cntn-6/NB-3, the third family member that has been functionally characterized in cerebellar development is expressed largely postnatally in mice. It overlaps with Cntn-1 but not with Cntn-2 and is found in granule cells during synaptogenesis.

Cntn-5/FAR-2/NB-2 is expressed by subpopulations of Purkinje cells and neurons of the deep cerebellar nuclei. So far, no functional characterization of Cntn-5 is available. Similarly, neither functional
data nor detailed expression patterns are available for Cntn-3/BIG-1 and Cntn-4/BIG-2.

**Contactin Family Members Regulate Guidance and Fasciculation of Parallel Fibers**

As mentioned above, granule cells express Cntn-2/TAG-1/Axonin-1 as soon as they extend processes in the inner EGL. Knockout mice lacking Cntn-2 were not found to have any obvious motor deficits and the cerebellum was not different from controls. However, knocking down Cntn-2 in the developing cerebellum of the chicken embryo using ex ovo RNAi, resulted in aberrant formation of parallel fibers. Rather than forming the typical T-shape, granule cell axons extended toward the pial surface, and thus failed to extend parallel to each other and to form an appropriate molecular layer. Granule cell axon extension was not affected in the absence of Cntn-2/TAG-1/Ax-1 both in vitro and in vivo. It is not clear, how axonal navigation is mediated by Cntn-2. Homophilic interactions of Contactin-2 have been demonstrated in addition to a multitude of heterophilic interactions in cis and trans.

Because expression of Cntn-2/TAG-1/Ax-1 precedes expression of Cntn-1/F3/F11 and Cntn-6/NB-3, as well as NrCAM and NgCAM, the guidance effect of Cntn-2 may be mediated by a homophilic interaction or a yet unidentified receptor on parallel fibers. Downregulation of Cntn-2/TAG-1/Ax-1 did not induce changes in expression patterns of other Contactin family members or affect expression of other IgSF CAMs, like L1/NgCAM or NrCAM.

A close relative of Cntn-2/TAG-1/Axonin-1, Cntn-1/F3/F11 is expressed on parallel fibers only slightly later. Contactin-1 expression was highest on older parallel fibers in the molecular layer of the cerebellum. Mice lacking Contactin were ataxic by P10 and died by P18. Overall, the morphology of the cerebellum was normal despite a reduction in size by 17.5%.

Based on both in vitro and in vivo analyses Contactin-1 was concluded to be required for fasciculation and compaction of parallel fibers. Detailed analysis of parallel fiber orientation revealed aberrant projections of a late-developing population in the outer portion of the ML. While parallel fiber orientation was normal in the inner ML, axons of late-born granule cells extended in parallel rather than perpendicular to Purkinje cell dendrites. It is unclear whether absence of Cntn-1 affects the navigation of a specific sub-population of granule cells or whether the observed defects are due to defasciculation of parallel fibers.

**Contactin Family Members are Required for Synaptogenesis**

In addition to the aberrant formation of parallel fibers in the ML, mice lacking Cntn-1 were found to have a defect in the formation of synapses between mossy fibers and granule cells, although Cntn-1 was not found in dendrites of granule cells in the IGL. Because Cntn-1 was reported to be expressed in Golgi cells and in mossy fibers, which both contribute to the rosettes formed with granule cell dendrites, Cntn-1 is still located appropriately to contribute to synapse formation in the IGL. In addition, the absence of Contactin-1 was also shown to interfere with the extension of Golgi cell dendrites into the ML.

Interestingly, expression of Contactin-1 from the Cntn-2/TAG-1/Ax-1 promoter interfered transiently with cerebellar development. Size and foliation of the cerebellum were affected between P3 and P30 in transgenic mice. The molecular layer was reduced in width due to a decrease in both neurite outgrowth of granule cells and dendritic arborization of Purkinje cells. Contactin-6/NB-3 knockout mice showed mild but reproducible motor coordination deficits. Consistent with its expression pattern, Cntn-6/NB3 was found to affect late stages of granule cell development. Cntn-6/NB-3 expression levels were very low in the developing cerebellum, increased until P15 and then declined to intermediate levels that were maintained in adulthood. Cntn-6/NB3 was expressed in the developing molecular layer underneath the Cntn-2-positive area. Cntn-2/TAG-1/Axonin-1 and Cntn-6/NB-3-positive zones did not overlap. Both zones did partially overlap with L1/NgCAM expression. Based on the observation that L1/NgCAM staining was altered in Cntn-6 knockout mice, granule cell migration was analyzed in detail. L1/NgCAM was suggested to be expressed on migrating and postmigratory granule cells in the IGL but to be downregulated before synapse formation sets in. The results of the immunohistochemical analyses in the Cntn-6/NB3 knockout mouse suggest that migration of granule cells was not affected, but that cells failed to downregulate L1/NgCAM and failed to start synaptogenesis between parallel fibers and Purkinje cell dendrites.

**Contactins Interact with Other IgSF CAMs in Cerebellar Development**

The IgSF CAM NrCAM was shown to mediate neurite growth from granule cells on a Cntn-1 substrate, but was dispensable for neurite growth on L1/NgCAM. Because Cntn-1 expressed by a feeder layer of CHO cells was shown to reduce neurite outgrowth compared to control cells, the importance of this observation for cerebellar development is unclear. NrCAM is expressed in Purkinje cells and in granule cells in the IGL, but not in the EGL. Thus, NrCAM expression overlaps with Cntn-1 and Cntn-6, but only little with Cntn-2. Mice lacking NrCAM showed a mild reduction in the size of lobes IV and V of the cerebellum but there was no difference in granule cell development. However, the concomitant loss of NrCAM and L1/NgCAM resulted in more severe cerebellar defects. Because NrCAM/L1 double knockout mice died before P8, cerebellar analysis was restricted to non-mature stages and a developmental delay rather than a specific defect in granule cell development could not be ruled out. In mice lacking both NrCAM and L1/NgCAM the EGL was found to be relatively normal but the thickness of the IGL was markedly reduced. Based on their expression patterns, a role of NrCAM and L1/NgCAM in granule cell migration was suggested. This would have been consistent with the observed reduction in IGL thickness in double knockout mice. However, no evidence for a migration defect was found in vivo. In cultures of granule cells
taken from double knockout mice neurites were not maintained and cells failed to survive. Further analyses will be required to understand the molecular mechanisms of NrCAM and L1/NgCAM function in cerebellar development in vivo.

Migration of granule cells, thus, appears to be the only step in development of postmitotic granule cells that is not regulated by IgSF CAMs. In fact, during the last years, a role of Semaphorin6A and PlexinA2 in granule cell migration has been demonstrated.\(^{29,30}\)

**Conclusion**

With the exception of granule cell migration from the EGL to the IGL, all steps in postmitotic granule cell development require IgSF CAMs of the Contactin group. Initial navigation but not extension of parallel fibers requires Contactin-2. Older parallel fibers fasciculate due to the onset of Contactin-1 expression. Contactin-1 expression in Golgi- rather than granule cells, is suggested to contribute to synaptogenesis between mossy fibers and granule cell dendrites in the IGL. Contactin-6, finally, comes up in granule cells during synaptogenesis with Purkinje cells. Additional studies will be required to identify the interaction partners and the signaling pathways by which IgSF CAMs of the Contactin group-pregulate neuronal circuit formation in the cerebellum.

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