Maturation of the mammalian dorsal root entry zone – from entry to no entry

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Interfaces between glial cell precursors of the PNS and CNS are established early in development and form the sites where sensory axons enter and motor axons exit the developing CNS. The molecular and cellular interactions that lead to the formation of these glial interfaces are only now becoming apparent. New in-vitro techniques are providing clues as to how the maturation of PNS–CNS glial interfaces generates barriers to regenerating axons.

molecules that repel growing axons. What is the basis for the change in the properties of PNS–CNS glial interfaces, from a conduit for growing axons in development, to a ‘barrier’ to their reconnection to the mature spinal cord after injury? Here, we first describe what is known of the origins of glial interfaces in early development, and their influence on the formation of axon exit and entry points along the neuraxis. We then review recent progress in identifying the cellular and molecular components of the DREZ that might confer its barrier properties.

**The formation of PNS–CNS glial interfaces**

Astrocytes and Schwann cells have different embryological origins in vertebrate development: from neural tube and neural crest, respectively. PNS–CNS glial interfaces arise at the site of exit–entry points of the neural tube, potentially by a tripartite interaction between cells derived from the neural crest, the processes of neuroepithelial cells, and the intervening basal lamina of the neural tube. Of these, the contribution of neural-crest cells has been the best characterized. In the chick, Le Douarin et al.13 have shown that a subset of neural-crest cells, migrating in the ventrolateral pathway alongside the neural tube, take part in the formation of exit–entry points (Fig. 1A, B). This subset is a late-migrating population that selectively expresses c-cad7, a member of the cadherin family of cell-adhesion molecules, initially in the dorsal midbrain at stage 10, and a prerequisite for exit–entry points. Niederländer and Lumsden13 excised neural crest-derived neural-crest cells into r4 of a stage-10–11 chick host, and transplanted age-matched quail neural crest to the prospective ventral exit points of the basal lamina of the neural tube define the sites of exit–entry points, it is possible that restricted domains of the basal lamina of the neural tube define the sites where the migrating c-cad7-expressing neural-crest cells arrest. Thus, the function of c-cad7 might be to aggregate neural-crest cells, ensuring coherent migration to the same loci, but the signals to stop migration at specific points on the neural tube are possibly mediated by distinct adhesive mechanisms. A further possibility is that specific groups of neuroepithelial cells differentiate at the prospective exit–entry points and degrade the basal lamina by secreting proteases themselves or by inducing the attachment of neural-crest cells, or both. Thus, a population of chick neuroepithelial cells at the prospective ventral exit points penetrates the basal lamina of the neural tube at stage 17, when the first motor axons emerge from the ventral exit points. In particular, matrix metalloproteinases (MMPs) specific for glycoprotein substrates of the extracellular matrix (ECM) might be involved, such as the MMP Stromelysin-1, which is expressed by neuroepithelial cells in the chick.

A recent study further implicates neuroepithelium in determining positional specification of exit–entry points. Niederländer and Lumsden13 excised neural crest from rhombomere (r)15, in which an exit–entry point does not arise, from a stage-10–11 chick host, and transplanted age-matched quail neural crest from r4, in which the exit–entry point of the facial nerve normally develops. However, this failed to generate an inappropriate exit–entry point in r1 of the host, implying that the initial signals for exit–entry point formation are derived from neuroepithelium and not neural crest.

Glia at PNS–CNS interfaces, generated as a result of such cellular interactions, can be grouped into three categories. Most Schwann cell–CNS glial interfaces are segregated into distinct dorsal (sensory-axon) entry points and ventral (motor-axon) exit points (Fig. 1D). However, in some regions of the hindbrain, motor and sensory axons exit or enter the CNS at common sites (Fig. 1E), whilst in others, distinct exit points for ventral motor axons are also produced (for example, cranial nerves VI and XII) (Fig. 1F). This pattern is also evident in the cervical spinal cord, where common dorsal exit–entry points are produced transiently during development, while distinct ventral motor-axon exit points also arise (Fig. 1F).

**Development of PNS–CNS glial interfaces and interactions with axons**

Although the changes in antigenic phenotype associated with the maturation of CNS glial cells and Schwann cell precursors have been well documented, few studies have focused specifically on developing glia at PNS–CNS interfaces. Boundary-cap cells in the trunk region of the mouse are the first neural-crest cells at this axial level to express the transcription factor Sox2 at embryonic day (E)10.5 and subsequently to become positive for S-100, a marker of differentiated Schwann cells, by E12.5 (Ref. 17). Early phenotypic changes in Sox2 expression at such sites have not been reported. However, in the rat spinal cord, the only definitive astrocytic marker, the cytoskeletal protein GFAP, is first detected in the distal processes of radial glia at the margins of the ventral, then dorsal spinal cord, at E16 and E19 respectively, coincident with the growth of the final cohort of axons through the glial interfaces in these regions. Could these studies imply that the Schwann cells and CNS glia that populate the interfaces mature before glia elsewhere in the nervous system? Precocious development might be required for appropriate interactions to take place between the earliest arriving axons and the glial cells at PNS–CNS interfaces. Thus, it is known that in the case of primary afferents, there occurs a protracted ‘waiting period’ at the surface of the dorsal grey matter, in the vicinity of the DREZ. Recent work suggests that this stalling of axons is regulated by the local expression of Semaphorin (D), a member of the semaphorin family. In the mouse, neurotrophin 3 (NT3)-dependent muscle sensory afferents are the first to grow into the grey matter of the spinal cord at E14.5, to reach their ventral motorneurone targets, coinciding with the age at which the growth of their axons becomes insensitive to the repulsive effects of SemD in tissue culture. In contrast, NGF-dependent small-diameter afferents, whose growth continues to be inhibited by SemD in vitro, grow into their target fields within the superficial laminae of the dorsal horn at E17.5, only after expression of SemD mRNA in the spinal cord has receded ventrally. In addition, another semaphorin, Semc, is expressed in the nerve roots of the mouse trunk between E12.5 and E14.5, and thus by analogy, might also be involved in confining the growth of sensory afferents.
Postnatal changes at the DREZ generate a barrier to axon growth

Shortly after birth, changes occur in the organization of the mammalian DREZ that might be correlated with the inability of regenerating primary sensory axons to re-enter the spinal cord after injury. Astrocytes extend processes up to 100 μm into the dorsal roots between basal lamina tubes of Schwann cells, and gaps are present in this intervening basal lamina at the DREZ (Ref. 23). This organization is not only thought to confer mechanical strength on the DREZ (Ref. 24), but also increases the surface area of direct contact between Schwann cells and astrocytes, generating a unique environment at the interface25. Moreover, this cellular organization ensures that astrocytic processes are the first CNS elements that are encountered by regenerating primary sensory axons.

In key experiments carried out by Carlstedt3,4 on the influence of age on the ability of injured rat sensory afferents to reconnect with the spinal cord, a ‘critical period’ was identified, between birth and one week, when significant numbers of injured axons were able to regenerate into the cord. In older animals, regenerating labelled axons were observed stopping at, or turning back from, the DREZ. Electron microscopic studies in adult rats have shown that regenerating axons stop growing precisely at the astrocytic processes within the DREZ (Ref. 26). Growth cones stop growing at the astrocytic processes, which is proposed to be analogous to the physiological stop pathway within the neurones, which is proposed to be analogous to the mechanisms whereby growth cones stop at their appropriate targets27,28.

Further evidence for the role of mature astrocytes in preventing regenerating axons (and possibly Schwann cells) from crossing the DREZ is also provided by experiments in which the dorsal spinal cord of the rat is depleted of glia by X-irradiation soon after birth. This treatment generates gaps in the glial limitans through which Schwann cells migrate ectopically into the spinal cord along astrocyte- and oligodendrocyte-free sensory afferents. Invasion by Schwann cells is limited to the glia-depleted areas of the CNS and the few remaining astrocytes appear to block the further migration of Schwann cells. Examination of dorsal root lesions that had been generated two weeks after X-irradiation revealed that several regenerating sensory axons entered the spinal cord through the astrocytic-free DREZ (Ref. 30).

What is known of the identity of the molecules that might contribute to the DREZ barrier? Tenascin and sulphated proteoglycans have been implicated as inhibitors of axon growth that are expressed by DREZ astrocytes31 and in other regions of the developing CNS (Refs 22,33). Thus, Silver et al. reported that both tenascin and sulphated proteoglycans become concentrated at the CNS side of the rat DREZ towards the end of the critical period25. Although a later study indicated that tenascin mRNA and protein are strongly expressed by rat DREZ astrocytes from birth26, following injury to the dorsal root in rats older than the critical age, these molecules become highly concentrated at the DREZ and within proximal regions of the dorsal horn27, in association with extensively branched ‘reactive’ astrocytes25. Reactive astrocytes are a major component of the glial scars that are formed around CNS lesions in adult rats, and through which axons fail to regenerate28.

In vitro, myelin-free plasma membranes isolated from glial scars in lesioned brains of adult rats have been shown to inhibit the growth of neurites from dorsal root ganglia, and septal and hippocampal
neurones of embryonic rat, whilst membranes isolated from uninjured brains supported neurite outgrowth\(^4\). This growth-inhibitory effect could be removed by pretreatment with proteoglycan-degrading enzymes\(^7\). Furthermore, purified chondroitin-sulphate proteoglycans\(^8\) and tenascin\(^9\) have been shown to act as barriers to growth of CNS neurites in vitro when presented as sharp substrate boundaries. However, the increased expression of inhibitory molecules is only one possible mechanism that might account for the failure of axon growth at the mature DREZ. Another possibility is that maturation of astrocytes, both in vivo and in vitro, might downregulate the production of cell-adhesion molecules that promote axon growth\(^4\), in tandem with the upregulation of barrier molecules. This raises the question of whether maturation changes that are intrinsic to normal astrocytes are themselves responsible for the acquisition of barrier properties at the DREZ.

**Culture model systems enable the interaction of axons with the DREZ to be studied in novel ways**

To test whether maturing astrocytes acquire the ability to inhibit axon growth, it is necessary to confront growing primary sensory axons with the uninjured DREZ, a scenario that is impossible in vivo. However, this has been made possible by adapting an *in-vitro* cryoculture approach\(^42\). In this technique, sensory neurones are cultured on thin cryostat sections of nerve tissue, an environment that closely resembles the one that would be encountered in vivo. The ECM and cell-surface molecules are preserved, whilst cells within the frozen tissue are non-viable and are no longer able to secrete soluble factors. This allows the differential effects of substrate and soluble factors to be studied. By preparing longitudinal cryostat sections of the dorso-lateral spinal cord of the rat, it has been possible to incorporate the DREZ and attached dorsal roots and use these as substrates for cultures of dissociated neurones\(^44\) of the dorsal root ganglia (Fig. 2). Neurones that adhere to the dorsal roots extend neurites along the lamina tubes of Schwann cells towards the DREZ, as they would in vivo, where they might grow across to the spinal cord, stop, or turn back along the dorsal root. A major advantage of this approach is that DREZ from various developmental stages, both before and after the hypothesized critical period, can be employed as substrates, enabling us to study more closely how the development of this region influences neurite growth. Thus, we have found that neurites growing from neurones of neonatal dorsal root ganglia cross the newborn (postnatal day 0, P0) DREZ more readily than P6 or adult DREZ (Fig. 3A, B). This supports the idea that inhibitors of axon growth appear at the DREZ during a critical period within the first postnatal week\(^4\) that is independent of injury-induced responses by glial cells. This might indicate that the sulphated proteoglycans that accumulate at the DREZ by P6 are sufficient to halt growing primary sensory axons, but the possibility remains that other, as yet uncharacterized, molecules might also be involved. Significantly, neurite outgrowth on the spinal cord or dorsal root, immediately central or peripheral to the DREZ, was similar at different ages that encompassed the critical period, suggesting that changes intrinsic to the DREZ are initially responsible for developing a barrier to regenerating axons. By adulthood, both the DREZ and the CNS adjacent to it serve as poor substrates for outgrowth of neurites of the neonatal dorsal root ganglia, suggesting that after the critical period additional inhibitory molecules become expressed generally within the CNS.

**Influence of age of neurones on their interactions with the DREZ**

By testing a range of ages of neurones in cryoculture, we were also able to separate the influence of development of neurones of the dorsal root ganglia on the ability of neurones to cross the DREZ. We found that neurones from early embryonic neurones were less sensitive to the P6 DREZ inhibitors\(^44\) (Fig. 3C) than neurones from more mature neurones, suggesting that, in tandem with changes at the DREZ.
during the critical period, there are corresponding maturational changes in the expression of axonal receptors for ligands that influence growth. A parallel approach in vivo involves transplanting allografts of embryonic dorsal root ganglia into adult rats. In these animals some immature axons were found to have entered the spinal cord of the adult host, although it is unclear whether they actually grew through the DREZ. Both studies are consistent with an emerging general principle that immature neurones are better able to extend axons within the mature CNS environment, possibly as a result of their lack of receptors that recognize inhibitory ligands. The complementary findings that have been obtained from both in-vivo and in-vitro studies on maturation of the DREZ are summarized in Fig. 4.
Future prospects

Current knowledge on the provenance of exit and entry points of the neural tube is limited, but recent work suggests that cells of neuroepithelial origin, rather than of the neural crest, determine the sites where these points form in development. Further information on the molecular and structural properties of these immature glial environments should enlighten us as to the optimal conditions for axon growth across such interfaces.

The mechanisms that underlie the failure of injured axons to regenerate across mature interfaces remain equally elusive. Complementary in-vivo and in-vitro studies that focus on the DREZ offer the prospect of circumventing some of the complexity encountered elsewhere in the CNS, and provide new leads as to the identity of the molecules responsible. Until recently, the most likely mechanism involved changes in the composition of the ECM in the vicinity of the DREZ, which were proposed to be instrumental in blocking the reconnection of lesioned primary afferent axons with the spinal cord. However, the spatiotemporal pattern of expression of SemD in the developing spinal cord, and its selective repulsive effects on ingrowth of sensory afferents, have been invoked more recently to explain the patterning of primary afferent inner- sory afferents, have been invoked more recently to explain the patterning of primary afferent inner-

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