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Specification of Neuronal Connectivity: ETS Marks the Spot

Minireview

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One of the most remarkable aspects of nervous system development is the specification and formation of functionally appropriate synaptic connections. Wiring up roughly 10 billion neurons into meaningful circuits may seem like a hopelessly complicated problem to address, but there are few other problems that are as central to the study of nervous system development. Work from the last three decades has brought increasing insight into the cellular and molecular mechanisms that underlie the patterning of appropriate connections, and progress in this field has come in part from the realization that, from a developmental perspective, the problem of neuronal connectivity can be broken down into three separate developmental events. The first is the specification of neuronal subtypes, a process that leads to the generation of groups of neurons that share certain functional properties. The second is pathfinding, a process that allows axons from functionally related neurons to grow to their appropriate target regions. The third is the formation of appropriate synaptic connections, a process that leads to the formation of functionally appropriate neural circuits. Advances in our understanding of the first two processes have been recently reviewed elsewhere (Lumsden and Krumlauf, 1996; Tessier-Lavigne and Goodman, 1996); the focus of this review is a discussion of some recent progress in the field with regard to the problem of specification of synaptic circuits during development.

Much of our insight into the mechanisms that underlie the formation of appropriate synaptic connections comes from the studies on the development of the circuit that mediates the stretch reflex. In this simple circuit, the peripheral branch of the sensory axons innervate a limb muscle and fire action potentials upon detecting a rapid mechanical stretch in the muscle. The sensory neurons convey this information to the spinal motor neurons via monosynaptic synapses that the central branch of the sensory axons make onto the dendrites of the motor neurons. The axons of these motor neurons in turn project to the muscle and induce a contractile response. Studies on the development of this circuit indicate that the underlying connections are specified with exquisite precision and require coordinated interactions among the motor nerve, the muscle target, and the sensory nerve. While much is known about the cell biology of the process, the molecular mechanisms that mediate the formation of these precise connections are not well understood.

In a current study, the Jessell, Lance-Jones, Anderson, and Saito laboratories (Lin et al., 1998 [this issue of *Cell*]) provide compelling evidence that the establishment of this functional neural circuitry depends on the coordinate expression of individual ETS-family transcription factors by both motor neurons and corresponding presynaptic sensory neurons. Further, this study establishes an essential role for the peripheral target in directing the expression of similar ETS transcription factors in both classes of neurons. Although the requirement for specific ETS proteins in circuit formation is not yet firmly established, this work provides a logical framework for beginning to understand how specific connections between the motor neurons, the sensory neurons, and the muscle may be established during development.

Motor Neuron Pools Are Specified with Regard to Muscle Targets before Motor Nerve Formation

A discussion of the mechanisms that specify reflex circuits requires a brief review of the organization of motor neurons within the spinal cord (see Figure 1). In the chick, the motor neurons that innervate the limb musculature are located at the brachial and lumbosacral levels in the lateral motor columns (LMC) of the spinal cord. Within the LMC, motor neurons that innervate ventral muscles are located medially (LMCm), and those that innervate dorsal muscles are located laterally (LMCl) (Landmesser, 1978) (Figure 1C). LMC motor neurons are generated starting at stage 15 in the chick, with the LMCm neurons becoming postmitotic before the LMCl neurons (Hollyday and Hamburger, 1977). The LMCm and LMCl motor neurons can be further subdivided into motor pools that correspond to groups of motor neurons that innervate a specific muscle. Motor neurons of the LMCs can be distinguished from other motor neurons by patterns of expression of LIM homeodomain (LIM-HD) proteins, and this molecular signature provides an important tool for defining the factors that affect motor column determination (Tsuchida et al., 1994; Tanabe and Jessell, 1996; Ensini et al., 1998).

LMC motor neurons extend an axon toward their limb muscle targets via the motor nerve, and by stage 23/24 the growth cones of these axons have reached the base of the developing limb. Upon entering the limb mesenchyme, the nerve splits into dorsal and ventral branches, which contain axons from the lateral and medial LMCs, respectively. By stage 35 the motor axons have innervated their appropriate muscle targets. Several perturbation experiments suggest that by the time lateral column motor neurons become postmitotic they are determined with regard to their specificity for appropriate muscle targets. For example, if the limb is rotated before innervation by the motor nerve, the nerve innervates the appropriate muscles although the muscles are now in ectopic locations (Lance-Jones and Landmesser, 1980; Ferns and Hollyday, 1993). Similarly, if a segment of the neural tube is rotated at stage 15 to cause an anterior–posterior reversal, the motor axons are able to grow to their appropriate muscles as long as the rotation does not involve more than 2–3 spinal segments (Lance-Jones and Landmesser, 1981). Such early specification of motor neurons is not unique to chicks, and elegant cell transplantation experiments in the zebrafish embryo have indicated that individual motor neurons are determined with regard to peripheral muscle innervation even before they extend an axon (Eisen, 1991). The ability

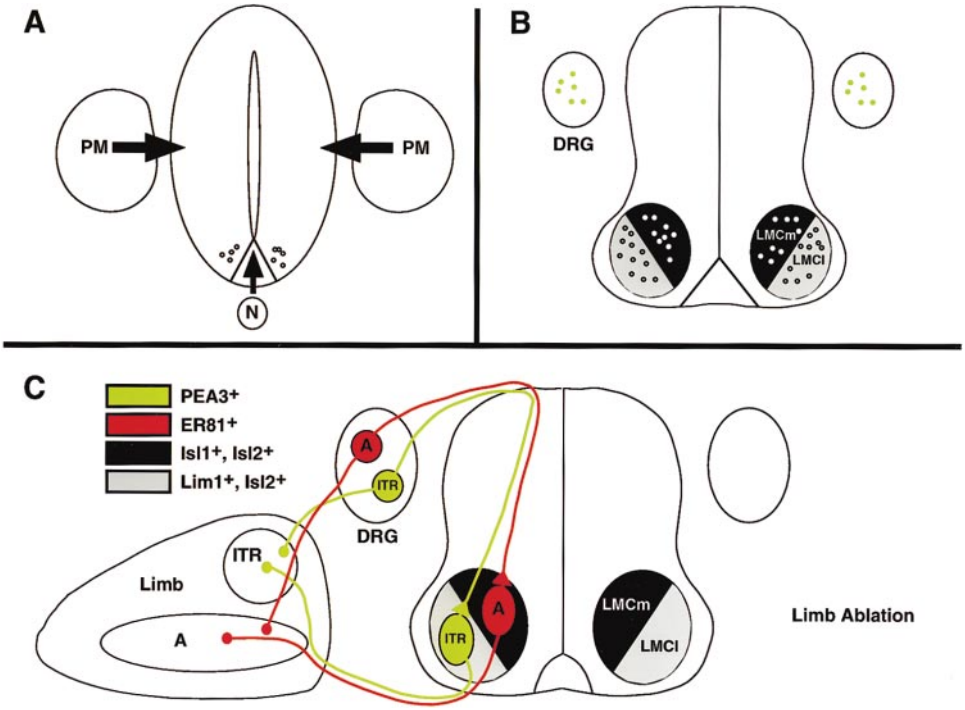

Figure 1. Sequential Specification of Motor Neurons during Development

(A) At early neural tube stages mesodermal signals from the notochord (N) and paraxial mesoderm (PM) are required for motorneuron differentiation and axial specification of lateral motor column (LMC) subtypes, respectively (small circles represent LMC neurons).

(B) Following early mesodermal and LMC inductive events, the medial LMC (LMCm) and lateral LMC (LMCl) have formed and can be identified by their patterns of LIM-HD expression (black = Isl1$^+$, Isl2$^+$; gray = Lim1$^+$, Isl2$^+$). At this time a small number of developing dorsal root ganglia (DRG) sensory afferents are PEA3$^+$ (green); however, none are ER81$^+$ (red). Around this time ETS expression is beginning in subsets of LMC neurons that are starting to segregate into individual motor pools (not shown).

(C) After motor neurons and sensory neurons contact their respective peripheral and central targets, the matched pattern of ETS protein distribution is apparent. Sensory and motor neurons that contact the same muscle and form a single motor unit express the same profile of ETS proteins (shown here for the **A** and **ITR** motor units). Limb ablation results in a loss of ETS protein expression in both sensory and motor neurons; however, motor column specification remains unchanged.

of emerging motor axons to make appropriate target choices soon after the motor neurons become postmitotic suggests that motor neurons in different motor pools must be molecularly distinct by the time they extend an axon.

ETS Proteins Are Specific Markers for Individual Motor Pools

The demonstration that LIM-HD proteins define subclasses of motor columns prompted a search for a distinct class of transcription factors that might serve to distinguish motor pools from one another. In the current study Lin et al. (1998) report that members of the ETS family of transcription factors (which have previously been characterized as targets of the Ras-MAPK signaling pathway and influence the differentiation of a variety of invertebrate and vertebrate cell types [Wasylyk et al., 1998]) are expressed in discrete populations of motor neurons in the embryonic chick ventral spinal cord. Importantly, retrograde labeling experiments with horseradish peroxidase (HRP) indicate that individual motor pools express a specific complement of ETS and LIM-HD proteins. For example, HRP injections into the adductor (**A**) muscle (a ventral muscle) retrogradely label a population of motor neurons that expresses the ER81 ETS protein and also the LIM-HD proteins Isl1 and Isl2 (Figure 1C). Therefore, this expression profile defines this ER81$^+$ **A** motor pool as an LMCm motor pool. Similarly, HRP injections into the iliotrochanterici (**ITR**) muscle (a dorsal muscle) retrogradely label a motor pool that expresses the PEA3 ETS protein and also the LIM-HD proteins Isl2 and Lim1, defining this PEA3$^+$ **ITR** motor pool as an LMCl motor pool. A detailed analysis of several lumbosacral motor pools shows that the combinatorial expression of ETS and LIM-HD proteins serves to define uniquely many motor pools.

Since previous neural tube reversal experiments have shown that signals from the paraxial mesoderm (PM) help establish the rostrocaudal identity of specific motor pools during a critical period up until stage 15 (Figure 1A), patterns of ETS protein expression should also reflect this influence if they are markers for distinct motor pools. Indeed, Lin et al. report that following neural tube reversal at stage 13 when paraxial mesodermal signals are capable of respecifying motor pools, the distribution of ETS and LIM-HD proteins shows that specific motor pools ultimately are found to reside in their normal rostrocaudal position. Neural tube reversal at stage 15, when this respecification is no longer possible, results

in ETS and LIM-HD expression profiles that show a rostrocaudal reversal of motor pool identity. These observations indicate that subsets of motor pools can be uniquely identified molecularly during spinal cord development based on the pattern of ETS and LIM-HD protein expression.

ETS Proteins Are Expressed by Subsets of Sensory Neurons

Sensory neurons, which reside in the dorsal root ganglia (DRG), can be broadly divided into cutaneous and muscle afferents, which characteristically express receptors for various neural growth factors. The cutaneous afferents convey sensory information from the skin, and predominantly express receptors for NGF (TrkA) or BDNF (TrkB). The muscle afferents, which innervate muscle spindles, express the neurotrophin-3 (NT-3) receptor TrkC. As previously described, the muscle afferents make highly specific connections with motor neurons in individual motor pools during development, suggesting that molecular recognition mechanisms might be involved in the patterning of these connections. Lin et al. provide evidence that ETS proteins are also expressed in the sensory neurons, and that subpopulations of sensory neurons and their motor neuron targets share ETS protein expression patterns.

Prior to the onset of the formation of monosynaptic connections between muscle sensory afferents and motor neurons, ~70% of DRG sensory afferents that express these ETS proteins express both PEA3 and ER81. In addition, at this time some ETS-expressing sensory afferents do not show coexpression of TrkC, which is expressed on more mature muscle sensory afferents. As these sensory neurons begin to form monosynaptic connections with motor neurons, however, these expression patterns change dramatically. Most of these sensory afferents (~90%) now express PEA3 or ER81 but not both ETS proteins, and all of these sensory neurons express TrkC and not TrkA.

To address the relationship between the sensory afferents and motor neurons that express the same ETS proteins, Lin et al. made HRP injections into muscles to label retrogradely sensory and motor neurons that share the same peripheral muscle target. These experiments reveal that many groups of sensory afferents and motor neurons that contact the same muscle show a similar pattern of ETS expression. For example, ~95% of the sensory afferents that innervate the **A** muscle express ER81, which is also expressed by the motor neurons that innervate the **A** muscle (Figure 1C). Similarly, ~90% of the sensory afferents that innervate the **ITR** muscle express PEA3, which is also expressed by the motor pools that innervate the **ITR** muscle. This correlation is not exact, however, since a low but significant number of ETS^+ sensory afferents contact ETS^- motor neurons, and at least one $ETS^-/PEA3^-$ motor pool receives ~50% of its sensory afferent input from $PEA3^+$ sensory afferents. While these exceptions indicate that additional factors must contribute to precise matching of sensory and motor neurons that define an individual motor unit, the general finding that there is a striking match between ETS expression profiles for certain motor pools and their sensory afferents strongly suggests that the specification of these connections is regulated by the expression of common ETS proteins between these neuronal populations. It will be important to determine whether or not other neurons that innervate specific motor pools, such as spinal inhibitory interneurons, also express ETS proteins.

Target-Derived Signals Induce both Sensory and Motor Neuron ETS Expression

The expression of common ETS proteins in subsets of motor and sensory neurons suggests that the expression of these proteins may be coordinately regulated by a peripheral signal. Given that these neurons innervate common muscle targets, is it possible that both motor pool and muscle sensory afferent specification depends on target-derived signals? To address this issue Lin et al. performed limb ablation experiments between stages 16 and 20, prior to the onset of ETS protein expression and also the onset of axon outgrowth into the limb, but after the time when paraxial mesodermal signals have already defined motor column identity. The early (stage 16/17) hindlimb ablations result in a dramatic loss of PEA3 and ER81 expression in both sensory and motor neurons on the operated side of the spinal cord (not accounted for by cell death) indicating that a peripheral signal is required for ETS protein expression in these neuronal populations. Later (stage 19) limb ablations do not result in a loss of ER81 expression in the **A** motor pool indicating the motor axons and sensory afferents need to detect the peripheral signal only briefly for persistent ETS expression. It should be noted, however, that these experiments do not prove that a peripheral signal confers motor pool identity, since it is possible that the peripheral signal induces ETS protein expression in previously specified motor neuron pools. Examining ETS protein expression in animals where motor axons are forced to innervate ectopic muscles should help resolve this issue.

While the limb ablation experiments suggest that a signal from the periphery is necessary for ETS protein expression in motor neuron pools and in sensory neurons, they do not reveal the specific location and identity of the peripheral signal. It is reasonable to speculate that the peripheral signal that specifies motor pool ETS expression should be produced by the target muscle, but cell biological experiments suggest that although the motor nerve innervates the peripheral muscle mass with great precision, the signal for nerve patterning may not be muscle derived. This possibility is suggested by somite removal and somite reversal experiments in which the motor nerve invades the limb mesenchyme and separates into dorsal and ventral branches although the muscle-derived signals are missing or aberrant (Lance-Jones, 1988; Phelan and Hollyday, 1990). Therefore, the signal responsible for the dorsal–ventral patterning of the nerve and muscle-specific innervation may be produced by the connective tissue in the developing limb or the overlying epidermis. It will be instructive to determine if ETS protein expression in individual motor pools is altered in such somite perturbation experiments since this would provide insight into the role of the muscle target in inducing ETS expression in the spinal cord.

It will also be important to determine if the same peripheral signal regulates ETS protein expression by both

the motor and sensory neurons. While the notion of a single target-derived signal specifying ETS expression in both populations is attractive, certain cell biological observations suggest that motor neurons and sensory neurons may respond to distinct signals. In general the development of sensory projections to the periphery appears to depend upon signals from the motor nerve. During development, the sensory nerve follows the motor nerve to appropriate muscle targets. If the ventral neural tube is removed from a chick embryo at stage 16, most of the motor neurons fail to form and the motor nerve is absent or severely reduced (Landmesser and Honig, 1986). In these animals the sensory projections to the muscles are grossly altered suggesting that a signal from the motor neurons may be required for the proper development of peripheral sensory projections. Examining the pattern of ETS protein expression in the sensory ganglia in animals where the motor pool is ablated or the motor nerve transected would be useful in determining the contribution of the motor nerve to the molecular specification of sensory neurons.

While the motor nerve may be involved specifying the peripheral projection of sensory neurons, it appears that a signal from the peripheral musculature can specify the central projection pattern of the sensory neurons. In experiments in which the ventral muscles are replaced with dorsal muscles during limb development to create a double-dorsal limb, the ventral motor and sensory nerves innervate the ectopic dorsal muscle. In such manipulated animals, the central branch of the ectopically projecting sensory neurons makes synapses onto the LMC neurons that normally innervate the dorsal muscle. This suggests that the central projection of sensory neurons is specified in response to signals from the periphery (Wenner and Frank, 1995). According to the model proposed by Lin et al., one would expect the pattern of ETS proteins to be respecified in sensory neurons innervating the ectopic dorsal muscle. Such a respecification would lend support to the idea that ETS protein expression may play an instructive role in specifying connections between the central axons of sensory neurons and their target motor pools. Also, examining the pattern of ETS protein expression in the motor neuron pools would allow one to determine if motor neuron pools can be respecified with regard to ETS expression. If such experiments demonstrate alterations in ETS expression that are diagnostic of neuronal respecification, they would support the notion that peripheral signals specify motor pool and sensory neuron identity and do not simply induce a prespecified differentiation program.

While the findings of Lin et al. establish that a peripheral signal is capable of directing patterns of ETS protein expression in motor and sensory neurons, they leave open the identity of such a signal. Identification of these signals and understanding how such signals might regulate coordinated ETS protein expression in motor pools and subsets of sensory neurons should provide important mechanistic insight into the process by which neurons acquire their ETS protein profiles.

Conclusion

The experiments described in the study by Lin et al., together with classic cell biological experiments, suggest a model in which a signal from the peripheral target induces the expression of particular ETS proteins in individual motor pools and subsets of sensory neurons. Once ETS protein expression has been induced, the sensory neurons extend their central axons ventrally and make specific connections with motor neurons in appropriate motor pools. Lin et al. suggest that homophilic cell surface interactions might provide for a selective matching of sensory axons and motor neurons that are part of the same circuit. Further, they note that there is emerging evidence implicating ETS proteins in controlling expression of *cadherin* genes and that at least one member of this family of homophilic cell adhesion molecules (CAMs) is expressed in a motor pool–specific pattern. It will be of interest to explore the possibility that ETS-regulated expression of a CAM by both sensory and motor axons might result in matched central connections.

While such a model is attractive in its simplicity, several important issues are not yet resolved. Most importantly, there is as yet no evidence that ETS proteins are required for the formation of specific connections between central sensory axons and target motor neurons. It is also not known whether peripheral manipulations that alter the connectivity of sensory neurons lead to a respecification of ETS protein expression in those neurons. Still, the evidence from the study of Lin et al. strongly suggests that ETS proteins play a deterministic role in the specification of spinal circuits, and if central aspects of the model receive experimental verification, we will have gained important insight into the processes by which circuits in the developing nervous system may be specified.

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