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Transient Pioneer Neurons Are Essential for Formation of an Embryonic Peripheral Nerve

MONIKA KLOSE* AND DAVID BENTLEY

In developing nervous systems, many peripheral and central pathways are established by early arising populations of pioneer neurons. The growth cones of these pioneer neurons can migrate while embryonic distances are short and while intervening tissue is relatively uncomplicated. Are these pioneers necessary? In grasshopper embryos, a pair of pioneer neurons arise at the tips of limb buds and extend axons through the limb to the central nervous system. Growth cones of later arising sensory neurons migrate along the pioneer axons. After ingrowth of sensory axons, the pioneer neurons die. If the pioneer neurons are prevented from differentiating by heat shock, then the sensory growth cones that would have migrated along them are blocked and fail to reach the central nervous system. Thus, the pioneer axons are necessary for successful migration of these sensory growth cones. By crossing a segment boundary early in embryogenesis, the pioneers circumvent an incompatibility between differentiated segment boundary cells and growth cone migration. Pioneer neurons may resolve similar problems in many systems.

NEURAL GROWTH CONES MIGRATING over substantial distances through peripheral or central tissue may encounter regions through which it is difficult to travel successfully. These difficulties arise because of the mechanical or chemical features of the tissue, or because of the long distances involved and the complexity of the cellular terrain. Many of these problems could be alleviated by the establishment of neural routes early in embryogenesis and, in several systems, such routes have now been shown to be formed by early arising pioneer neurons (*1–9*). We have now tested whether such pioneers are essential.

In the grasshopper embryo, the first axons in the limb are those of a pair of afferent pioneer neurons found at the limb tip (*1*). The limb position at which the pioneers arise, and where the cell bodies remain, is in the tibia near the tibia-femur limb-segment boundary (Fig. 1A) (*5, 6*). When the pioneer growth cones emerge, they immediately cross this limb segment boundary and then migrate along a specific route to the central nervous system, founding a major limb nerve (5b1) (Fig. 1G). Because of their high affinity for growth cones, differentiated limb-segment boundary cells appear to arrest growth cone migration and to block proximal growth (*10–14*). When first crossed by the pioneer growth cones, the tibia-femur boundary cells have not yet developed their high-affinity characteristics but they do so shortly after the passage of the pioneer growth cones. Much later in embryogenesis, the axons of the distal proprioceptive and exteroceptive sensory neurons in the dorsal tibia cross the tibia-femur boundary by growing centrally along the pioneer axons and forming one of the two major nerves in the tibia. Later differentiating neurons that arise proximal to the tibia-femur boundary and whose growth cones migrate along the pioneer axons are usually able to grow normally when the pioneers are ablated (*6*). We have now examined the behavior of growth cones from neurons that arise distal to the tibia-femur boundary in the absence of the pioneer neurons.

Egg clutches of the grasshopper *Schistocerca americana* were obtained from a colony maintained at Berkeley (clutches hatch in 20 days at 32°C). The pioneer neurons are siblings derived from a precursor cell that begins to round up for division at about the 28% stage of embryogenesis (*15, 16*). Pilot experiments showed the pioneers to be most susceptible to heat shock at about the 27% stage. Therefore, eggs estimated by dissection of embryos from the same clutch to be at the 27% stage of development were heat-shocked by immersion in water at 45.5°C to 47.5°C for 30 min and then incubated at 32°C for 2 to 5 days (Table 1). Embryos were then fixed, labeled with a neuron-selective antibody (*11, 17, 18*), and viewed in epifluorescence (Fig. 1).

Cultured control embryos (Fig. 1) and embryos subjected to a 45.5°C heat shock were not lacking pioneer neurons and had normal nerve pathways. In 25% to 50% of embryos subjected to 46.5°C or 47.5°C, the pioneers were missing in at least one limb bud (Table 1). No cells that were labeled with the neuron-specific antibody were found at the position normally occupied by the pioneers, and no supernumerary cells were labeled elsewhere in the limb. Limbs lacking pioneers were found at all stages of development examined (35% to 55%), and no pioneers that appeared to be prematurely degenerating were observed. We conclude that in most of the pioneer-free limbs, the pioneers failed to differentiate.

Limbs without pioneer neurons had normal morphology. The circumferential constrictions that mark the developing tibia-femur and other limb-segment boundaries were present and appeared normal (Fig. 1). Several identified nerve cells, pairs of cells, or groups of cells arise in the limb soon after the pioneers (*5, 6*). These neurons were found at their normal locations in most limbs (Fig. 1). We conclude that the lesion resulting from the heat shock was quite

Table 1. Effect of heat shock of 27% stage embryos on genesis of the pioneer neurons (30-min heat shocks; temperatures ± 0.5°C). Embryos at this stage had 0, 1, or 2 differentiated abdominal segments.

Conditions	Embryos treated	Embryo mortality	Number of limb buds lacking pioneers					Embryos lacking pioneers (%)†
			2 days*	3 days	4 days	5 days	Total	
Control	12	0	0/15‡	0/39		0/12	0/66	0
45.5°C	9	0	0/24	0/24		0/6	0/54	0
46.5°C	24	0	1/18	5/69	2/21	5/30	13/138	25–42
47.5°C	20	4	1/12	0/48		8/55	9/115	25–50

*Duration of post-shock incubation. †Pioneers missing in at least one limb bud (uncertainty in percent of embryos arises because embryos were processed histologically as unmatched halves). ‡For example, of 15 limb buds examined, none were lacking pioneers.

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

*Present address: Max-Planck-Institut für Verhaltensphysiologie, 8130 Seewiesen b. Starnberg, Federal Republic of Germany.

specific and did not, per se, disrupt the normal differentiation of the limb.

The first growth cones to cross the tibia-femur boundary by growing along the pioneer axons extend from the sensory neurons of the subgenual organ (SGO), an internal proprioceptive sensory organ (which also subserves audition in some orthopterans). These sensory neurons arise at about the 38% stage and their growth cones reach the pioneer neuron somata at about the 40% stage (*6*). In pioneer-free limb buds at the 40% stage or later, proximal migration of the SGO growth cones was arrested at the tibia-femur segment boundary [Fig. 1 and cover (*19*)]. In limbs at the 45% stage, some circumferential spreading of SGO processes along the tibia-femur boundary was observed (Fig. 1F). By the 55% stage, an unusually thick fascicle of fibers, containing axons of both internal and cuticular sensory neurons, terminated in a compact tuft at the boundary (Fig. 1H). Therefore, in the absence of the pioneers, no growth cones from more distal cells that normally project along the pioneers successfully crossed the tibia-femur boundary. The tibia contains a second major nerve (5b2) that lies on the posterior, ventral side of the limb and forms from a different, later arising, set of neurons (*6*). This nerve formed normally in all limbs, again suggesting that there was no abnormal development of the boundary itself (Fig. 1, E through H). We conclude that the failure of the dorsal nerve (5b1) to cross the boundary was due to the absence of the pioneer neurons, and that these neurons are essential for formation of the nerve.

Differentiated limb-segment boundaries appear to present a major obstacle to the proximal migration of afferent growth cones (*10–14*). Pioneer growth cones, for example, appear unable to cross normally the trochanter-coxa segment boundary in the absence of a pair of pre-axonogenesis (guidepost) neurons located just across that boundary (*20*). Since the proximal portion of the limb differentiates before the distal portion, this creates a timing problem in limb embryogenesis: the tibia-femur boundary differentiates well before the more distal sensory neurons that must cross it have been generated. Our results show that this problem appears to be solved by the presence of a temporary nerve, produced by the transient pioneer neurons, that bridges the boundary when the growth cones of the sensory neurons arrive. Although the pioneers themselves are also sensitive to the boundary once it has differentiated, they cross it before it becomes an obstacle (*11–14*).

Similar situations and solutions may be widespread in embryogenesis. Laser lesion of pioneer neurons in cricket cercal sensory appendages results in the formation of many small sensory axon bundles rather than large nerve trunks (*21*). In the central nervous system of grasshoppers, commissural and longitudinal pathways are established by a group of central pioneer neurons, some of which appear to die or to undergo a later transformation of phenotype (*2–4*). In fish and amphibians, some spinal cord pathways are established by a population of pioneer neurons, Rohon-Beard cells, which also are transient (*7, 8*). Recently it has been shown that the mammalian telencephalon contains a massive population of subplate neurons which pioneer the first cortico-thalamic pathways and then disappear (*9*). These observations suggest that pioneer neurons are important in the successful establishment of neural pathways in many systems.

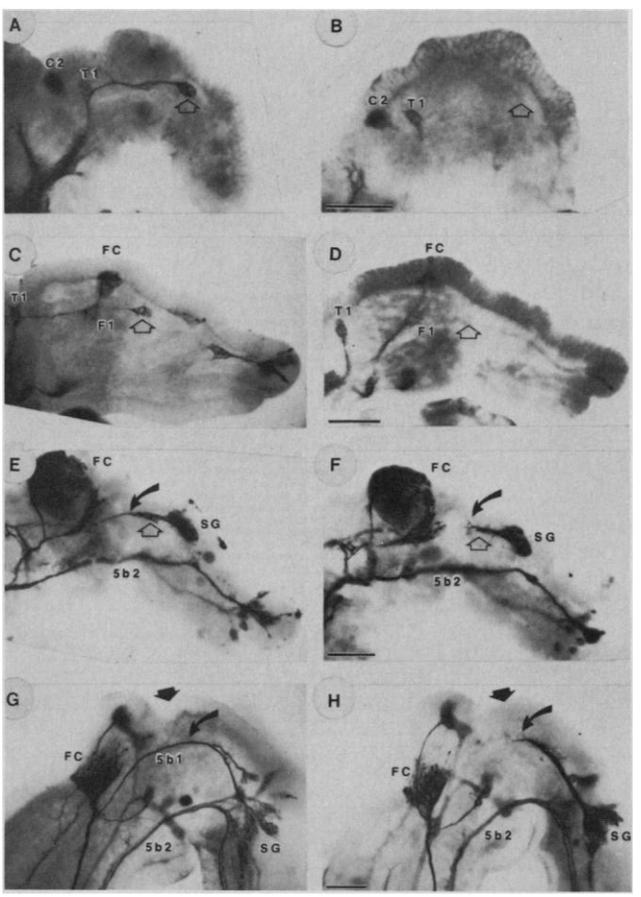

Fig. 1. Afferent nerves and neurons in control limbs (left) and in experimental limbs missing the pioneer neurons (right). Images are photomicrographs of whole-mounted limb buds labeled with the neuron-specific antibody to horseradish peroxidase. (**A**) Control limb, 35% stage. The pioneer neurons (arrowhead) have extended growth cones that have just completed their migration through the limb. Identified neurons Tr1 (T1) and Cx2 (C2) are labeled in their normal locations. (**B**) Experimental limb, 35% stage. No cells are labeled at the pioneer location (arrowhead). Identified neurons Tr1 (T1) and Cx2 (C2) are labeled in their normal locations. (**C**) Control limb, 38% stage. The pioneer neurons (arrowhead) have extended axons from their normal location (FC, femoral chordotonal organ neuron Fe1, F1). (**D**) Experimental embryo, 38% stage. No cells have labeled at the pioneer location (arrowhead). The axon of F1 has extended in an abnormal direction [compare to (C)]. (**E**) Control limb, 45% stage. The growth cones of sensory neurons from the SGO (SG) have reached the pioneer neurons (arrowhead). Just proximal to the pioneer neuron cell bodies, the fascicle (curved arrow) of pioneer and sensory axons crosses the tibia-femur segment boundary. Nerve 5b2 can be seen on the ventral and posterior side of the limb. (**F**) Experimental limb, 45% stage. No cell bodies have labeled at the pioneer location (arrowhead). Sensory neurons of the SGO (SG) extended growth cones (curved arrow) that have encountered, but not crossed, the tibia-femur segment boundary. Nerve 5b2, on the ventral and posterior side of the limb, has crossed the boundary normally. (**G**) Control limb, 55% stage. The pioneer neurons normally die at about this stage (*22*). A robust nerve 5b1 (curved arrow), containing axons of SGO and cuticular sensory neurons, crosses the tibia-femur segment boundary (arrowhead). (**H**) Experimental limb, 55% stage. An abnormally thick fascicle of SGO and cuticular sensory axons terminates abruptly (curved arrow) where it encounters the tibia-femur segment boundary (arrowhead). Nerve 5b2 crosses the border normally on the opposite side of the limb. Orientation: dorsal, up; proximal, to left. Bars, 100 μm.

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19. The metathoracic limb at the 55% stage of development of an embryo that was heat-shocked at the 27% stage of development and then incubated at 32°C. Neurons in the limb are labeled with a neuron-specific antibody, and the whole-mounted limb is imaged in pseudo-color in a laser confocal scanning microscope. The general morphological characteristics of the limb, including the shapes, relative sizes, and positioning of limb segments, are normal. Neurons in the limb are normal except that the tibial portion of nerve 5b1 (the detail in Fig. 1H), containing sensory axons from the subgenual organ and from cuticular sensillae, terminates abruptly and fails to cross the tibia-femur limb-segment boundary. This appears to be due to the heat shock block of differentiation of a pair of pioneer neurons, and the subsequent failure of the pioneer axons to provide a neural bridge across the segment boundary.
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Mapping Neuronal Inputs to REM Sleep Induction Sites with Carbachol-Fluorescent Microspheres

JAMES J. QUATTROCHI,* ADAM N. MAMELAK, ROGER D. MADISON, JEFFREY D. MACKLIS, J. ALLAN HOBSON

The cholinergic agonist carbachol was conjugated to latex microspheres that were fluorescently labeled with rhodamine and used as neuroanatomical probes that show little diffusion from their injection site and retrogradely label neurons projecting to the injection site. Microinjection of this pharmacologically active probe into the gigantocellular field of the cat pontine brain stem caused the awake cats to fall into rapid eye movement (REM) sleep indistinguishable from that produced by free carbachol. Three-dimensional computer reconstruction of the retrogradely labeled neurons revealed a widely distributed neuronal network in the pontine tegmentum. These pharmacologically active microspheres permit a new precision in the characterization and mapping of neurons associated with the control of behavioral state and of other cholinergic networks.

THE EVOLUTION OF TECHNIQUES for the central administration of pharmacological agents to mimic, enhance, or block the various neurotransmitters involved in the regulation of behavioral and physiologic functions has been dramatic in recent years. Within the field of sleep physiology, for example, the microinjection of cholinergic agonists into the brain stem pontine tegmentum of adult cats elicits immediate and prolonged rapid eye movement (REM), or desynchronized (D), sleep signs and "D sleep"–like behavior (*1*).

However, these cholinergic agonists show unacceptably wide diffusion within tissue. They can diffuse a mean radial distance of 1 mm from the injection site 1 hour after injection, even when volumes as small as 100 nl are used (*2*). Such widespread diffusion precludes discrete localization of injection sites and confounds the identification of neuronal populations that, once activated, generate the observed changes in behavioral state.

Similarly, neuroanatomical tracers also diffuse widely and do not identify a discrete population of neurons projecting to the injection site, limiting the understanding of how activation might be triggered under physiological conditions. This is particularly important when mapping the brain stem network associated with D sleep. The locus within the anterodorsal pontine tegmentum that produces D sleep when activated by cholinergic agonists has no cholinergic neurons (*3*). Inputs to this activation zone must, therefore, come from elsewhere and may project from several relatively distant neuronal groups. Because D sleep signs are enhanced by blockade of β-adrenergic receptors (*4*), it is also important to determine whether noradrenergic neurons project to this cholinoceptive zone.

We have developed a new retrograde

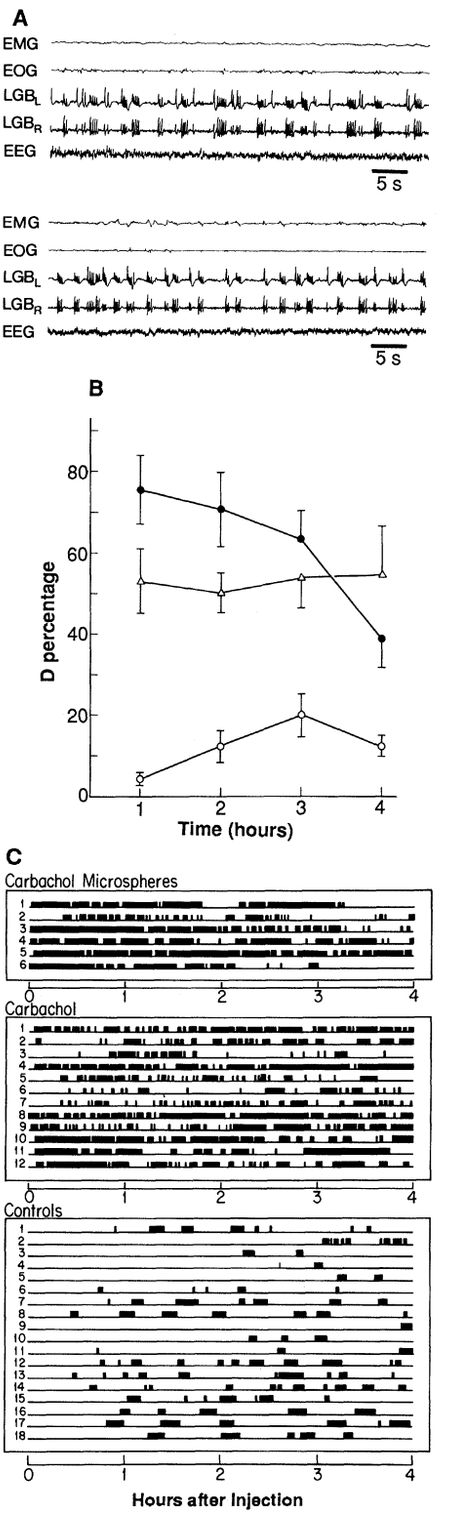

Fig. 1. (**A**) Polygraphic recordings illustrate D sleep induced by free carbachol (top) and that induced by carbachol microspheres (bottom). (**B**) The D% at each hour of recording time is significantly greater for both carbachol microspheres ($n = 6$; $F = 117.87$; $P < 0.0001$) and carbachol ($n = 12$; $F = 113.74$; $P < 0.0001$) than for controls ($n = 18$) (repeated measurement analysis of variance with Scheffe test). Values and error bars represent mean ± SEM. ●, Carbachol microspheres; △, carbachol; ○, controls. (**C**) Temporal distribution of D sleep episodes is shown for each trial by horizontal black bars.

J. J. Quattrochi, A. N. Mamelak, J. A. Hobson, Laboratory of Neurophysiology and Program in Neuroscience, Harvard Medical School, 74 Fenwood Road, Boston, MA 02115.
R. D. Madison, Division of Neurosurgery, Duke University Medical Center and Research Service, Veterans Administration Hospital, Durham, NC 27710.
J. D. Macklis, Department of Neurology and Program in Neuroscience, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed.