not mimic the variable local environments in which these fish live. Nevertheless, our laboratory represents a novel environment, and thus tests the abilities of the three stocks to survive, grow, and reproduce in a new location. The near constant conditions of our flow-through incubator most closely match the thermally stable environment that the Monkey Spring stock has historically encountered. Yet with this apparent advantage, the homozygous Monkey Spring stock still performed most poorly overall. The most heterozygous stock (Sharp Spring) exhibited the highest survival, growth, fecundity, and developmental stability. Tule Spring topminnows, which were intermediate in heterozygosity, also exhibited intermediate growth and fecundity, but their survival equaled that of the Sharp Spring stock.

It is not our intention to imply that the enzyme polymorphisms detected in the electrophoretic study are the primary determinants of fitness in these fish. These genetic markers represent only a small fraction of the entire genome. Nonetheless, historical processes such as severe population bottlenecks, founder events, inbreeding, and migration are expected to affect allelic diversity at all loci to a similar extent, with the possible exception of those under strong selection. Thus, we suggest that population-level estimates of mean heterozygosity reflect the recency and severity of these historical processes (*14*).

Despite uncertainty regarding the predictive power of electrophoretic estimates of genomic heterozygosity, our results are consistent and unequivocal: the Sharp Spring stock currently offers the best choice for stocking in the Gila River system. These topminnows are less likely to suffer the negative effects associated with historical processes that lead to low heterozygosity. Additionally, the establishment of self-sustaining populations of these fish in reclaimed habitats should be rapid because of their higher fecundity and survival, thereby preventing further erosion of genetic variation. Continuous monitoring of the "genetic health" of stocked populations in reclaimed habitats is essential, and periodic restocking might be necessary to supplement genetic variation at isolated localities that are likely to experience wide fluctuations in population size.

The species recovery program that now includes Sharp Spring topminnows for stocking reclaimed habitats in the Gila River system (*15*) should spread the abundance of remnant genetic variation, thereby enhancing the likelihood that this diversity will be preserved. Although hybridization might provide a means of maximizing genetic diversity and fitness of a hatchery stock, indiscriminate mixing could lead to outbreeding depression, a decrease in fitness attributable to negative interactions between differentially adapted genotypes (*16*). Remnant natural populations, such as the Monkey Spring topminnows, should be maintained as isolated reservoirs of potential genetic variation.

Similar studies of fitness components are not feasible with many endangered species. For some species the time to perform such studies would forestall necessary immediate action. However, the small viviparous desert fish examined in this study are ideal for testing many hypotheses regarding the genetic and evolutionary consequences of various species recovery programs.

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Subplate Neurons Pioneer the First Axon Pathway from the Cerebral Cortex

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During the development of the nervous system, growing axons must traverse considerable distances to find their targets. In insects, this problem is solved in part by pioneer neurons, which lay down the first axonal pathways when distances are at a minimum. Here the existence of a similar kind of neuron in the developing mammalian telencephalon is described. These are the subplate cells, the first postmitotic neurons of the cerebral cortex. Axons from subplate neurons traverse the internal capsule and invade the thalamus early in fetal life, even before the neurons of cortical layers 5 and 6, which will form the adult subcortical projections, are generated. During postnatal life, after the adult pattern of axonal projections is firmly established, most subplate neurons disappear. These observations raise the possibility that the early axonal scaffold formed by subplate cells may prove essential for the establishment of permanent subcortical projections.

IN HIGHER MAMMALS, THE GENESIS OF neurons destined for the adult cerebral cortex is preceded by a period dedicated to the production of two transient populations of neurons, the subplate cells and the marginal zone cells. In the cat, [^{3}H]thymidine birth-dating studies have shown that these cells are generated between embryonic day (E) 24 and E30 (gestation, 65 days) (*1*), and the neurons of the six cortical layers are generated only thereafter (*2*). The later-born cortical cells then insert themselves between the early-generated neurons, splitting the

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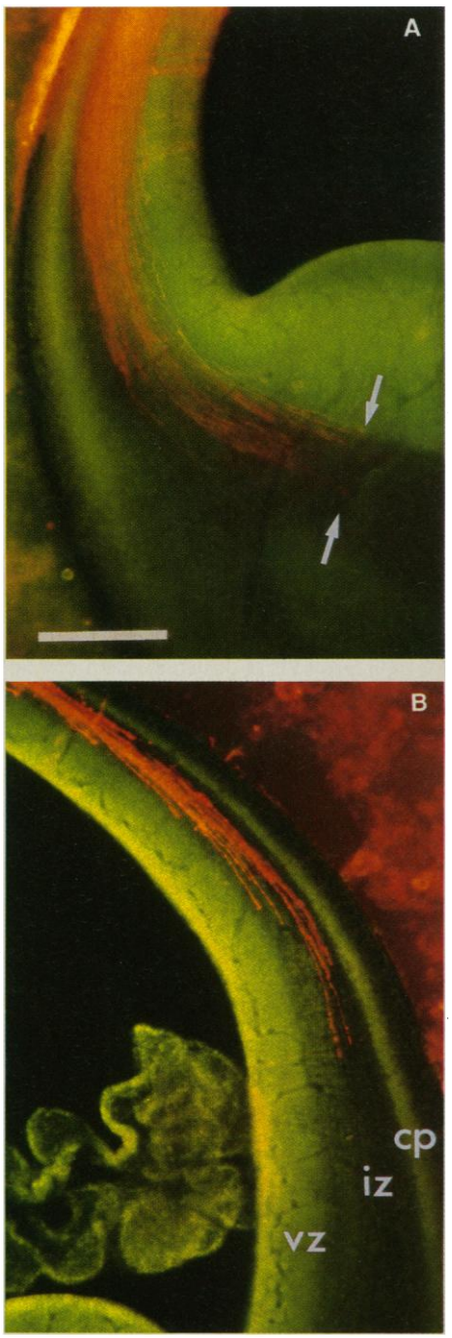

Fig. 1. DiI-labeled axons in E30 cat fetuses after cortical injections of DiI, shown in coronal sections counterstained with a fluorescent Nissl stain (*12*). (**A**) DiI-labeled axons traveling through the intermediate zone and entering the internal capsule (arrows). Axons were labeled after placement of a crystal of DiI into the temporal cortex (the lateral wall of the telencephalon), at and above the top left of the figure. (Also visible close to the injection are cells and radial processes labeled directly by DiI.) (**B**) DiI-labeled axons from a more dorsal and posterior injection site, in the presumptive visual cortex, travel anteriorly and ventrally toward the internal capsule but do not enter it. Many axons terminate in prominent growth cones. Abbreviations: cp, cortical plate; iz, intermediate zone; and vz, ventricular zone. Scale bar, 350 μm.

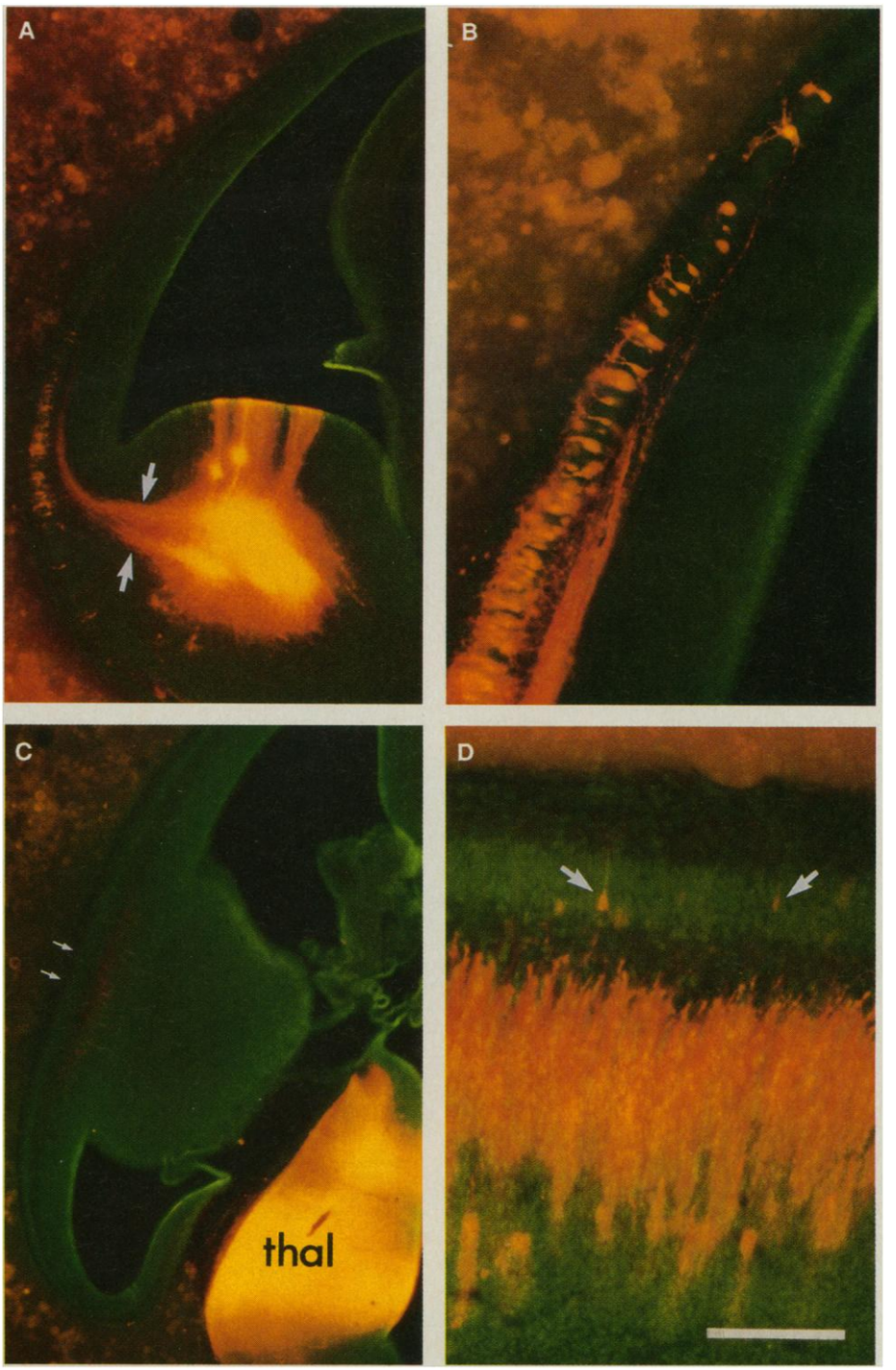

Fig. 2. Subplate neurons are retrogradely labeled after DiI injections into either the internal capsule (A and B) or the thalamus (C and D) at E30 in the cat, showing that subplate cells are responsible for the descending axons seen in Fig. 1. (**A**) Coronal view of a DiI injection site in the internal capsule, which retrogradely labels cells situated in the lateral wall of the telencephalon. The internal capsule is demarcated by arrows. (**B**) Increased magnification of retrogradely labeled subplate neurons in the dorso-lateral telencephalon after DiI injection in the internal capsule. The cell bodies are found in the cortical plate, which at this age is composed only of the subplate neurons, whereas the axons run in the intermediate zone. (**C**) A DiI injection site restricted to the thalamus (thal) of an E30 fetus, shown in the horizontal plane of section. Increased magnification of the area marked by arrows is shown in (D). (**D**) Injection of DiI into the thalamus results in the anterograde labeling of many ingrowing thalamocortical axons (dense labeling in the internal capsule and intermediate zone, below the cortical plate) and in the retrograde labeling of a small number of subplate neurons in the temporal cortex (arrows), implying that the axons of thalamic and subplate neurons must pass each other in the internal capsule. Scale bar: (A) 600 μm; (B) 200 μm; (C) 850 μm; and (D) 150 μm.

subplate and marginal zones into their two distinct locations (*1, 3*). At later fetal ages, subplate neurons achieve a remarkable degree of morphological maturity. They express neuron-specific markers, are immunoreactive for certain neurotransmitters, receive synaptic contacts, and form local circuit and interhemispheric connections (*4–8*). Finally, in postnatal life, about 90% of subplate neurons undergo programmed cell death (*1, 9, 10*). Several observations suggest that subplate neurons may function during later fetal life as the temporary target of waiting axonal systems (*11*). However, the very early presence of subplate cells in the cerebral cortex suggests another possible function: subplate neurons might lay down some of the first cortical axonal pathways.

To investigate this possibility we used the lipophilic fluorescent tracer 1,1-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI; Molecular Probes) to trace the connections of the cerebral cortex in aldehyde-fixed fetal brains (*12*). We first examined fetuses at E30 to visualize selectively the axons of the earliest generated neurons in the absence of any contribution from the later generated neurons of the cortical layers. Because of known gradients of development in the telencephalon, DiI was placed in the cerebral wall at a variety of locations. In every case, the DiI revealed an axon pathway headed directly toward the internal capsule, the gateway between the cortex and subcortical structures. The axons of this pathway (Fig. 1) are restricted to the intermediate zone of the cerebral wall, the cell-sparse region separating the ventricular zone from the cell-dense cortical plate just below the pial surface (*13*). Many of these simple axons ended in growth cones.

There was a marked spatial gradient of development in this early projection. When DiI was placed in the lateral cerebral mantle, labeled axons streamed ventrally within the intermediate zone of the telencephalon and then turned medially to enter the internal capsule (Fig. 1A). More dorsal placements of DiI into cortex further away from the internal capsule (Fig. 1B) labeled axons that ran toward the capsule without quite reaching it. Thus, at E30, all of the injections labeled cortical axons directed toward the internal capsule. However, only those axons from the sites closest to the capsule actually traversed it to form the first long-distance subcortical projections. Such projections were not seen at E26, the earliest age examined: in these experiments, the growth cones of labeled cells extended within the intermediate zone only short distances from the injection site.

To identify the cells responsible for forming the early subcortical projection, two sets of experiments were done. In the first, DiI was placed in the internal capsule at E30 (*14*) to label all of the cells sending axons subcortically. Such an injection retrogradely labeled many projecting neurons restricted to the lateral wall of the telencephalon (Fig. 2, A and B). These neurons were found in the cell-dense cortical plate. The axons of most originated directly from the cell body and, once within the intermediate zone, turned toward the internal capsule. The dendrites of many of these cells were directed toward the pial surface and branched within the marginal zone; occasionally, cells had dendrites directed downward toward the intermediate zone.

Several lines of evidence indicate that these early projecting cells are the subplate neurons. Previous [^{3}H]thymidine birth-dating studies have shown that virtually none of the neurons that make up the adult cortical layers have been born at E30 (*2*), and that the cortical plate at E30 is almost exclusively composed of the early generated subplate neurons (*1*). As mentioned above, marginal zone neurons are also present at E30; however, these neurons do not seem to contribute to the thalamic projection since they were not retrogradely labeled with DiI placed in subcortical structures.

A second set of experiments was performed to identify some of the possible subcortical targets of these earliest axonal projections. Injections of DiI into the cortex at E36 indeed labeled anterogradely many axons and growth cones that have reached the thalamus and superior colliculus (*15*). However, we cannot be certain that all of these axons belonged to subplate neurons, because at this and later ages the neurons of the deeper cortical layers might also contribute to the projection. Thus, to examine whether subplate cells send the first axons to these different targets, DiI was placed in the thalamus or tectum at various ages to retrogradely label any neurons contributing to the projections (*16*). Placement of DiI in the thalamus at E30 resulted in the retrograde labeling of exclusively subplate neurons, which were restricted to the lateral wall of the cerebral mantle (Fig. 2, C and D). However, very few cells were labeled after a thalamic injection; this is not surprising since few axons have yet grown into the thalamus, and because axons may not follow identical routes after passing through the internal capsule. At E36, when cortical DiI injections revealed the arrival of many more axons within the thalamus, subplate neurons distributed over a broader tangential extent could be retrogradely labeled after thalamic DiI injections (*15*).

Subplate neurons that project to the thalamus can also be readily identified in older fetuses (Fig. 3). In this experiment, DiI was placed in the dorsal thalamus of an E51 fetus. A variety of cellular elements were labeled after the diffusion of dye in both anterograde and retrograde directions, including the projection neurons of the deep cortical layers and a subpopulation of subplate neurons. One such subplate cell located deep within the intermediate zone is shown in Fig. 3C; it has the distinctive inverted pyramidal morphology and beaded processes typical of many subplate neurons (*4–7, 17, 18*). Similar experiments performed at E43, E47, E55, E60, and postnatal day (P) 7 all resulted in the retrograde labeling of subplate neurons and cortical neurons of the deep layers (*15*). These observations indicate that subplate neurons not only extend the first axons to the thalamus, but that many retain a thalamic connection at later fetal and neonatal ages (*19*).

Subplate neurons also project to at least one other subcortical target, the superior colliculus (SC). A representative subplate cell that was retrogradely labeled from the SC of a ferret kit at postconception day 56 (*16*) is shown in Fig. 3D. Thus, the axons of subplate neurons extend to two known subcortical targets, the thalamus and the SC. At present, we do not know whether all descending pathways from the cortex are pioneered by the axons of subplate neurons.

In the development of axonal projections from the cerebral cortex, it has commonly been assumed that initial projections originate from the neurons of the cortical plate that will ultimately form the adult connection. However, we have demonstrated here that the initial projections to subcortical structures originate from a transient population of subplate neurons that will essentially not contribute to these projections in the adult (*19*). It is also known that subplate neurons can extend corticocortical (*6, 7, 17*) and interhemispheric (*4*) axons. Thus, these cells may be responsible for laying down the initial pathways between the cortex and other cortical and subcortical targets.

Since subplate neurons extend the first axons to leave the telencephalon en route to the thalamus, they may serve a role as pioneer neurons. Initially described in the developing insect nervous system, pioneer neurons are the first cells to send axons to distant targets, forging routes that are followed by later growing axons (*20*). A striking parallel can be drawn between subplate cells and a pioneer neuron of the vertebrate nervous system, the Rohon-Beard cell of the spinal cord of simple vertebrates (*21*). Rohon-Beard neurons are among the first generated neurons of the spinal cord (*22*); they receive functional synaptic inputs from skin mechanoreceptors (*23*), pioneer central axo-

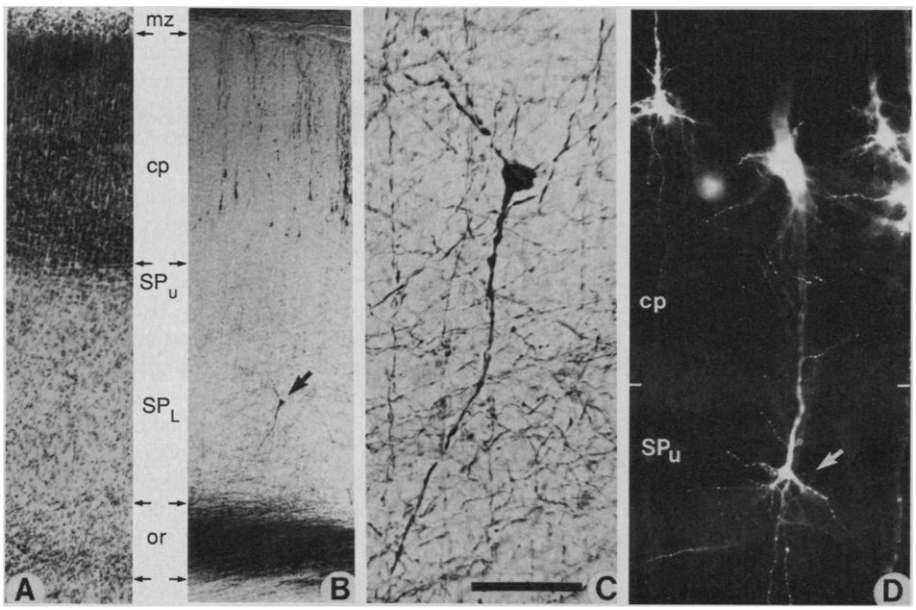

Fig. 3. Subplate neurons retrogradely labeled from the dorsal thalamus (A to C) or superior colliculus (D), two subcortical targets of the pioneering subplate axons. (**A**) Nissl-stained section showing the organization of the cerebral wall of the cat at E51. (**B**) Subplate neuron (arrow) and neurons of the deep cortical layers retrogradely labeled from an injection of DiI into dorsal thalamus on E51. The optic radiations containing both thalamocortical and corticothalamic axons are densely labeled as well. This DiI-labeled section was photoconverted (*16*) to reveal detailed features of cellular elements. (**C**) Morphological detail of the retrogradely labeled subplate neuron shown in (B). The distinctive inverted pyramidal morphology and beaded processes are characteristic of many subplate neurons. (**D**) Fluorescent subplate neuron (arrow) retrogradely labeled with DiI from the superior colliculus of a P14 ferret. Also labeled is an upper tier of layer 5 neurons, the cells that form the permanent corticotectal projection. Abbreviations: mz, marginal zone; cp, cortical plate; SP_U, upper subplate; SP_L, lower subplate; and or, optic radiations. Scale bar: (A and B) 500 μm; (C) 50 μm; and (D) 150 μm.

nal projections (*21*), and then disappear late in development (*24*). Subplate neurons are also known to receive synaptic inputs (*4, 7, 8*) and to generate postsynaptic potentials in slices of fetal cortex (*25*). These similarities suggest that subplate neurons may not only act early on as pioneers, but may also subserve an essential physiological function during fetal life.

The question arises to what extent the axons of subplate neurons interact with other axons in navigating their pathways. One possibility is that the descending axons of subplate neurons could be guided to their targets by the ascending axons from the thalamus. However, though the two sets of axons do indeed cross each other in the internal capsule (Fig. 2), the intracortical pathways traversed by subplate axons are formed before the arrival of thalamic axons and therefore are uniquely pioneered. Furthermore, subplate neurons ultimately project to other subcortical structures, such as the SC, that have no reciprocal ascending axons and therefore cannot be involved in axon guidance. Another possibility is that a small subset of subplate axons have a special role in navigation. The observed spatial gradient in axon outgrowth (Fig. 1) suggests that the most proximal subplate neurons pioneer the pathway through the internal capsule, and then axons from more distal subplate cells piggyback onto it. A similar phenomenon is observed as Rohon-Beard neurons in the spinal cord form the longitudinal pathway; however, it is known that each Rohon-Beard neuron has the ability to pioneer the pathway in the absence of neighboring Rohon-Beard cells (*21*).

Ablation studies on both vertebrate Rohon-Beard neurons and insect pioneer neurons have demonstrated that later growing axons may require the presence of the pioneer pathway in order to find their targets (*21, 26*). If the subplate neurons are truly analogous to these other pioneers, we would predict that the presence of the axon pathway formed by subplate neurons will prove necessary for the normal development of adult subcortical projections, a prediction that could be tested by ablating the subplate neurons during fetal life.

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

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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.

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