

Figure 61 IMMATURE NEURONS from sympathetic ganglia of a newborn rat. In standard medium the non-neuronal supporting cells of the ganglion survive and divide, forming a continuous layer around and beneath the neurons (*top*). Cultures that contain only neurons (*bottom*) can be prepared by growing ganglionic cells in a medium in which neurons survive but non-neuronal cells do not. One such medium contains cytosine arabinoside, which is toxic when incorporated into the nucleic acid molecules of the dividing non-neuronal cells but does not affect the neurons, which merely grow larger.

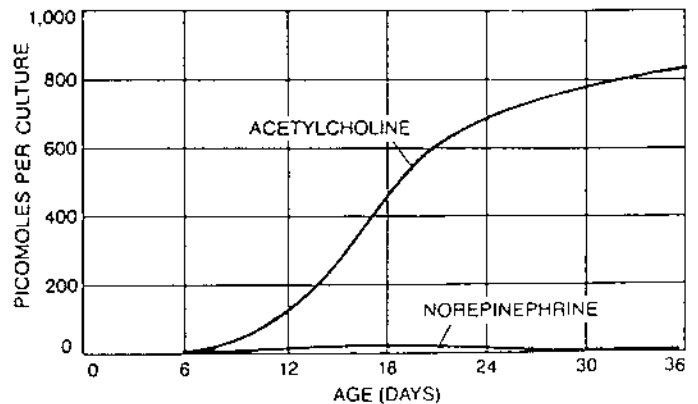
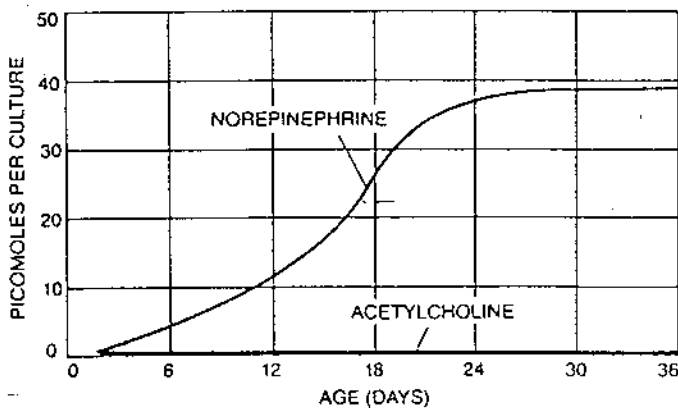
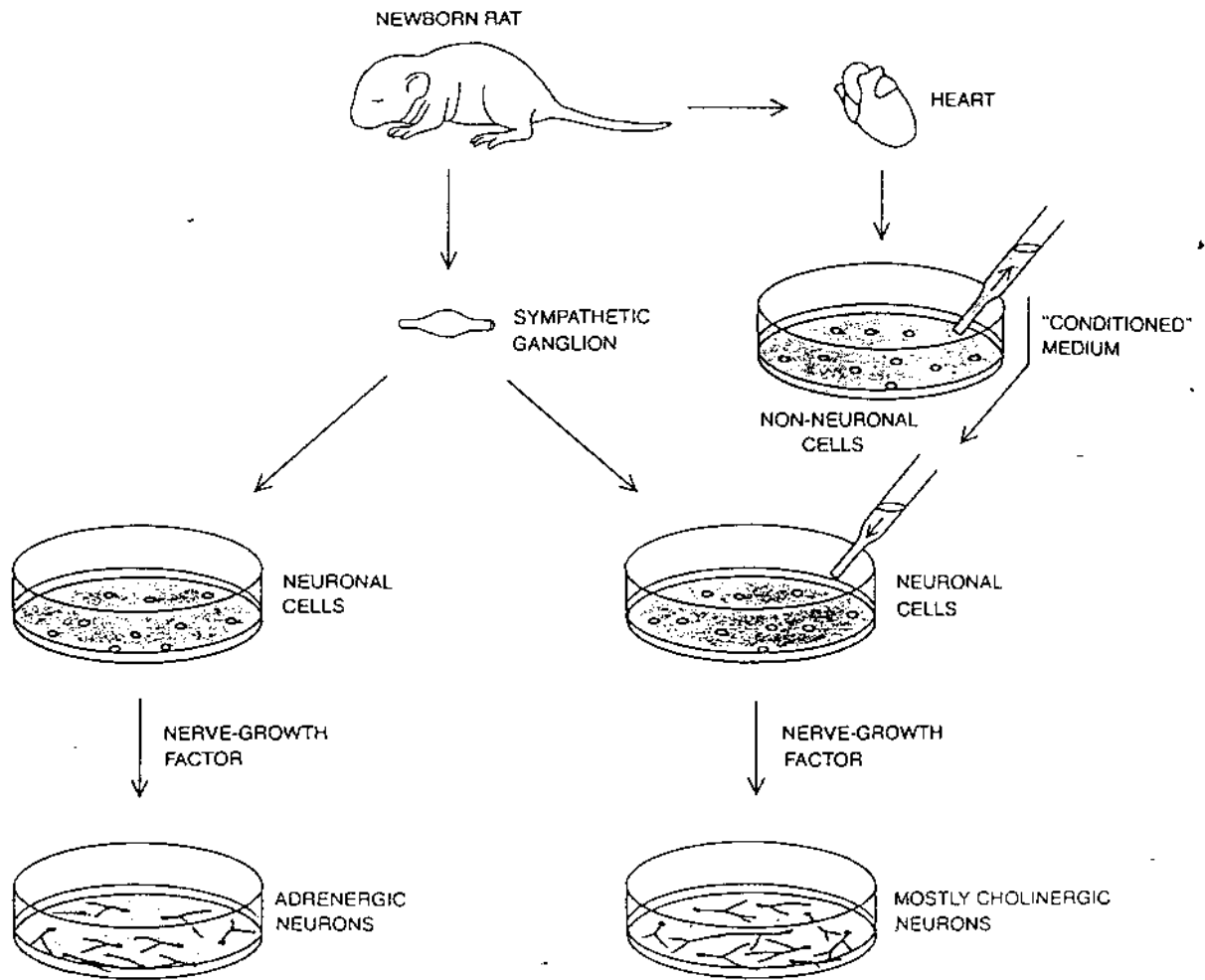


Figure 62 EFFECT OF CULTURE ENVIRONMENT. If immature neurons from the sympathetic ganglia of newborn rats are grown in pure cultures, nearly all manufacture and secrete norepinephrine. If the immature neurons are cultured together with non-neuronal cells, or if they are treated with culture medium that has been incubated with non-neuronal cells, a large majority of the neurons will

manufacture and secrete acetylcholine. In the absence of conditioned medium (*left graph*) the ability to make norepinephrine rises over three weeks and the ability to make acetylcholine remains negligible. In sister cultures grown in 62 percent conditioned medium (*right graph*) the ability to make norepinephrine first rises then declines as ability to make acetylcholine rises.

tions. According to this hypothesis the neurons that express adrenergic properties at the outset are still "plastic" with respect to neurotransmitter choice for a considerable period after birth and can become cholinergic under the influence of conditioned medium. This concept implies that the active ingredient of conditioned medium determines the choice of transmitter and the type of synapses made by a sympathetic neuron without affecting whether the neuron survives or how extensive its axon and dendrites are.

The reciprocity in the expression of adrenergic and cholinergic functions can be ascribed to a "competition" of some kind within each neuron between the prenatal instruction to become adrenergic and the new instruction to become cholinergic. If such a competition exists, one may ask if an individual neuron can express both transmitter systems simultaneously, at least for a short period. We attempted to answer the question by growing single neurons on small beds of heart-muscle cells from a newborn rat for about two weeks. The cells were

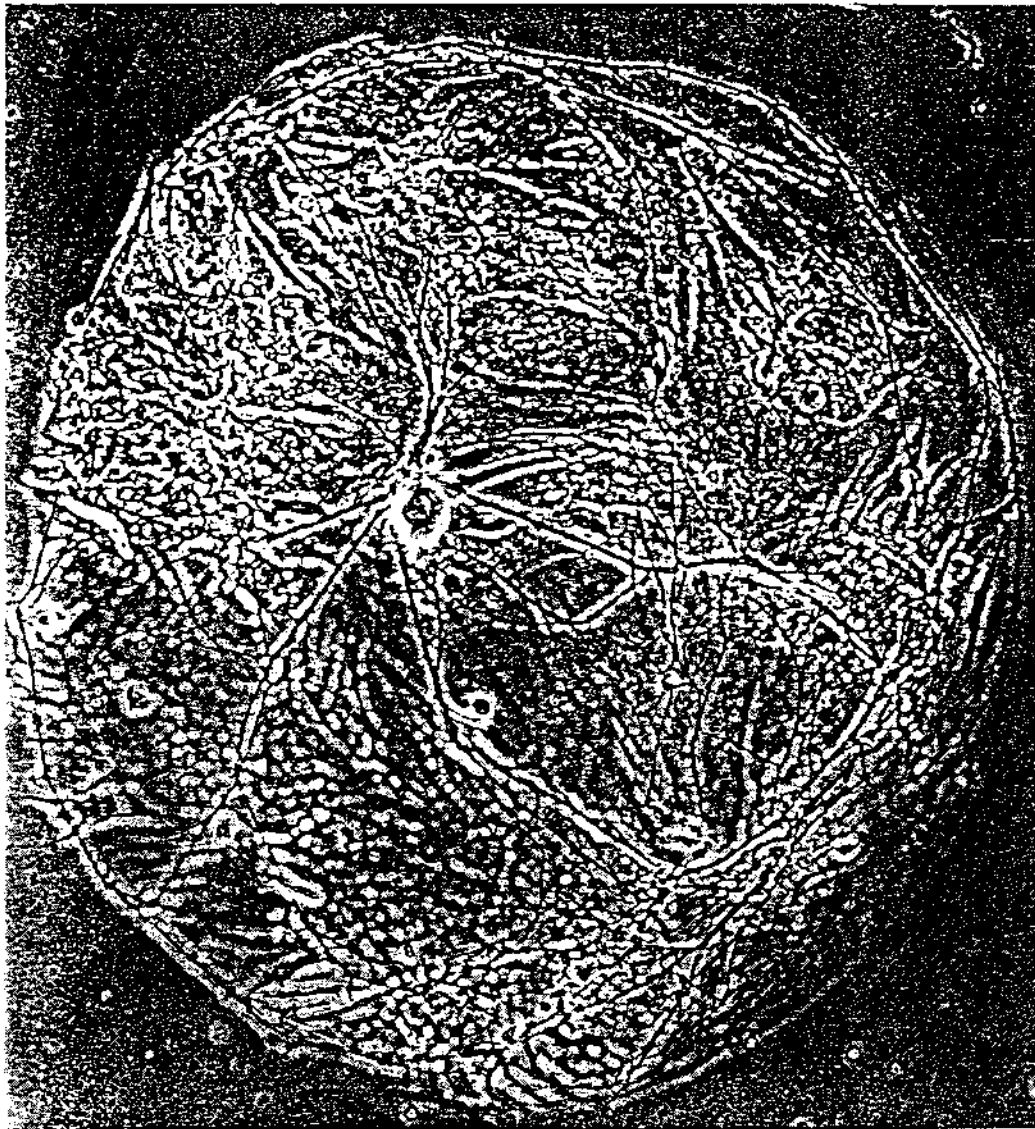


Figure 63 SINGLE NEURON (*center*) extends its branches over a background layer of heart cells in a microculture system. The transmitter secreted by the neuron can be identified by its effect on the cardiac muscle cells, which contract spontaneously and rhythmically in culture. Once the neuron is attached, its threadlike extensions form syn-

aptic connections with some cardiac muscle cells. The neurotransmitter secreted at the synapses is either acetylcholine, which slows the beating of the cells, or norepinephrine, which accelerates it, and at a certain stage in differentiation some neurons secrete both.

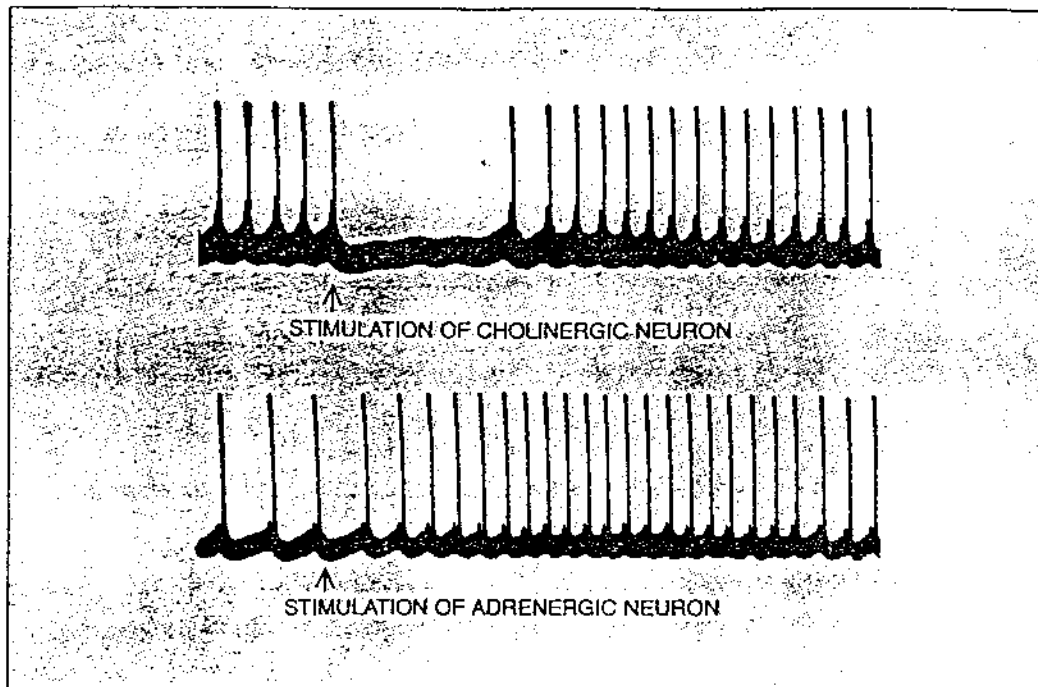


Figure 64 FREQUENCY OF CONTRACTION of the cardiac muscle cells in a single neuron reveals the identity of the neurotransmitter secreted by that neuron. A microelectrode inserted into the neuron stimulates the release of transmitter as a second microelectrode in an innervated muscle cell monitors the cell's contractions. Trace at the

top shows the effect of stimulating a cholinergic neuron: spontaneous contractions of the muscle cell cease temporarily. Bottom trace shows effect of stimulating an adrenergic neuron: contraction frequency of the innervated cell increases.

plated onto tiny disks of collagen about .5 millimeter in diameter to which they attach preferentially. One such disk is shown in Figure 63. The neuron and an adjacent heart cell in such microcultures were impaled with microelectrodes to monitor their electrical activity; in that way the transmitter choice of the neuron could be assayed by triggering the release of the transmitter and observing its effect on the heart cells, which "beat" spontaneously and rhythmically in culture. Unlike the cultured sympathetic neurons, the heart cells possess both cholinergic receptors and electrically active adrenergic receptors; thus a slowing or stopping of the beating indicates the secretion of acetylcholine, whereas an increased frequency of the beating indicates the secretion of norepinephrine. Further evidence for the identity of the transmitter can be obtained by observing the effects of certain drugs that compete specifically with the natural transmitter for binding to the receptors on the heart cells. For example, atropine blocks cardiac acetylcholine receptors, whereas propranolol blocks cardiac norepinephrine receptors.

Working with this technique we have identified

three types of neurons in the two-week-old microcultures. The first type is adrenergic: it excites the heart cells and the effect is blocked by propranolol. The second type is cholinergic: it inhibits the heart cells and the effect is blocked by atropine. The third type of neuron exhibits both cholinergic and adrenergic activity: stimulation of the neuron inhibits the heart with an atropine-sensitive mechanism and then speeds up the heart with a propranolol-sensitive mechanism. Since only one neuron is present in each microculture, it is clear that the same cell mediates both effects. When the microcultures are examined with the electron microscope, numerous dense-core synaptic vesicles are seen in the adrenergic neurons, clear vesicles are seen in the cholinergic neurons and a few dense-core vesicles combined with a large majority of clear vesicles are seen in the dual-function cells. An obvious advantage of studying cultures that contain only one neuron is that it is possible to make an unambiguous correlation between the structure and function of the cell.

These findings establish that a single neuron can express both transmitter systems simultaneously at an immature stage. Dual function may seem to be a

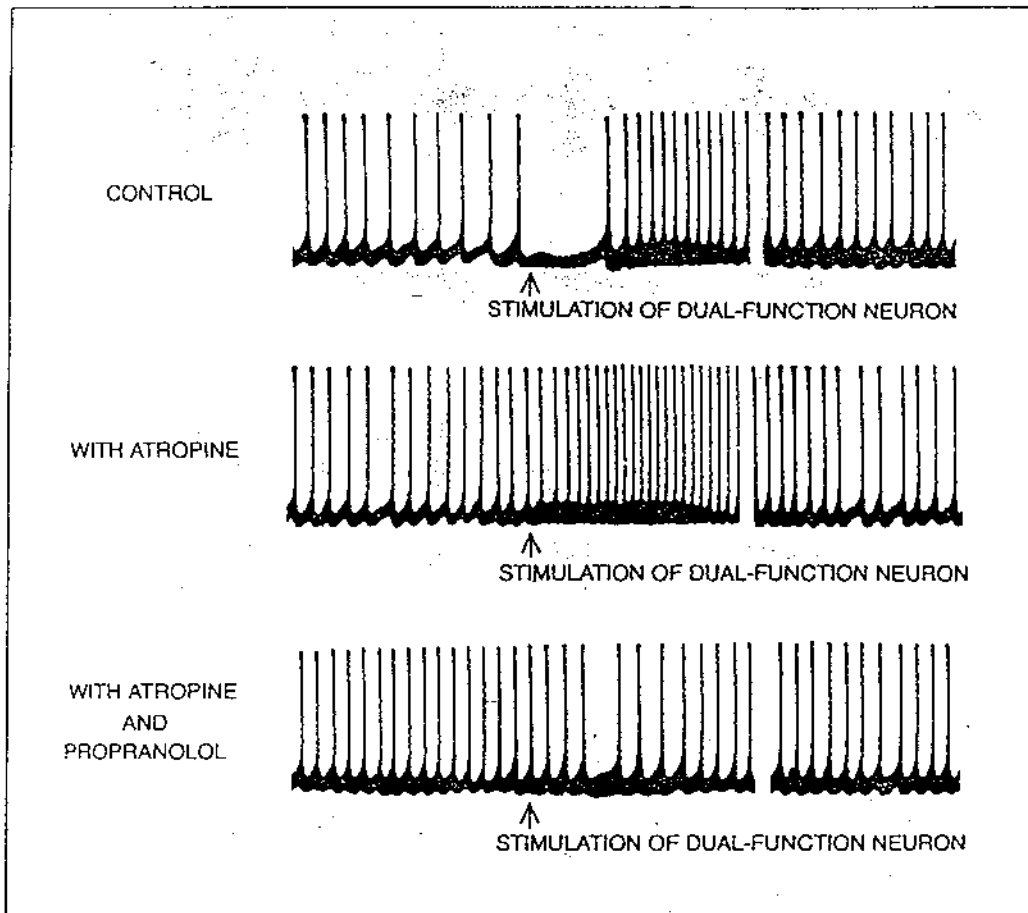


Figure 65 DUAL-FUNCTION NEURON. In the trace at top stimulation of the neuron causes a cessation of the beating of the innervated cardiac muscle cells (mediated by acetylcholine) then a resumption of beating at an enhanced rate (mediated by norepinephrine). In the lower two traces drugs that specifically block either cholinergic or adrener-

gic transmission have been added to culture medium to verify dual function. With atropine the inhibition is removed but the excitation remains intact. By adding propranolol both the excitatory and inhibitory effects of the neuron are blocked.

novel concept, but developing neurons have not previously been investigated in ways that might reveal this behavior. In retrospect dual function is a logical intermediate step in the conversion of an adrenergic neuron to a cholinergic neuron under the influence of conditioned medium. Even if there is no temporal overlap between the synthesis of enzymes and other components involved in manufacture and release of the two neurotransmitters, it is reasonable to assume that the enzymes and synaptic vesicles involved in adrenergic transmission would continue to function for a while after their synthesis had ceased. The precise duration of the dual-function state is not yet known.

After about four weeks the single neurons in microculture have grown so large that biochemical assays can be made on them. The cells are

incubated in a mixture of radioactive tyrosine and choline for eight to 12 hours; then the amount of norepinephrine and acetylcholine that have been synthesized from these precursor molecules is determined. In the absence of conditioned medium virtually all the neurons make detectable quantities only of norepinephrine. In the presence of heart cells, however, a substantial majority of the neurons make only acetylcholine. Under no circumstances is there a significant number of "silent" or dual-function neurons after four weeks in culture.

These findings indicate that most neurons are adrenergic at the time they are put into culture but are susceptible to a "flip-flop" control mechanism that determines their ultimate choice of transmitter. The duration of the transition period during which dual function may be expressed is not known, but by four or five weeks after birth virtually all the