in vivo; hence, the observations in vitro presumably reflect the same mechanism. In assessing the mode of action of erythropoietin, therefore, it is necessary to understand this proliferative response and to ask whether differentiation into enucleate, hemoglobin-rich erythroid cells is the end of a single program, the expression of which is due to one hormone-cell interaction, or whether the proliferative and maturational responses to erythropoietin are due to discrete signals that may be characterized and, ultimately, experimentally controlled. The availability of the clonal lines described here now permits an examination of this question.

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8. For these studies we used human urinary erythropoietin prepared from an anemic donor. The specific activity in vitro was measured at 20 U/mg by a mouse bone marrow technique, with NIH lot M-7-TaLSL used as a standard. Rauscher erythroleukemia cells also respond to human erythropoietin of higher specific activity and to sheep plasma erythropoietin (Connaught).
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Opioid Receptors Undergo Axonal Flow

Abstract. *Previous studies have indicated the presence of opiate receptors on axons of the rat vagus nerve and on other small diameter fibers. In examinations of the effect of ligation on the distribution of receptors in the vagus nerve by in vitro labeling light microscopic autoradiography, a large buildup of receptors was found proximal to the ligature. This result indicates an axonal flow of receptors.*

Axoplasmic flow and axonal and dendritic transport are responsible for delivery of various macromolecules to distant parts of the neuron (*1*). Although the mechanisms of these transports are unknown, their properties have been studied (*1*). We report evidence that receptors undergo such movement.

Several biochemical investigations have indicated the presence of opiate presynaptic receptors on axons and nerve terminals (*2*). Light microscopic autoradiographic studies have demonstrated opiate receptors in association with the rat vagus nerve fibers and with other small diameter nerve fibers (*3*, *4*). It was suggested that some of these vagal receptors may be undergoing axonal transport or axoplasmic flow (*4*). In vitro labeling autoradiography, a highly sensitive and quantitative measure of receptors (*5*), has proved adequate for measuring opiate receptors in the vagus. By using this technique, we detected opiate receptors in the nodose ganglion and receptor buildup on the proximal side of a ligature of the vagus nerve.

Surgical exposure of the nodose ganglion and vagus nerve in the neck region of the rat was performed as described in

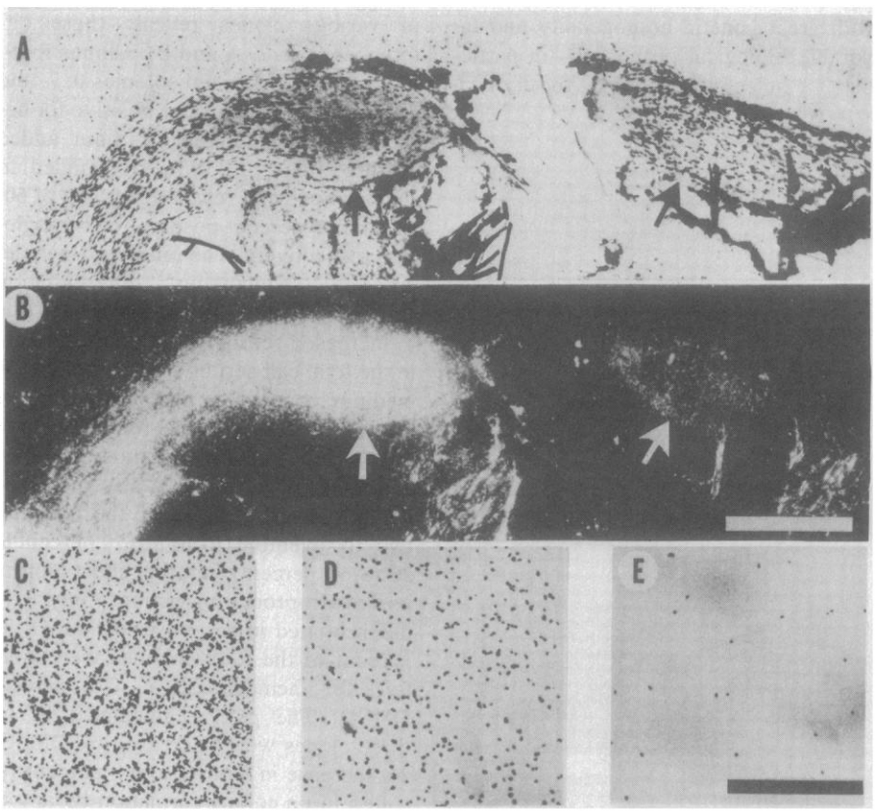

Fig. 1. (A) Light micrograph showing a section along a ligated nerve. The nerve was removed from the neck and embedded in a brain paste to maintain a good consistency for sectioning. The ligation was placed slightly to the right of center where there is an actual separation of proximal (left) and distal (right) sides. Arrows point to the nerve sheath or edge of nerve on either side of where the tie was made. Dark streaks to right of both arrows are fragments of the silk ligature. The autoradiographic grains marking receptors cannot be seen at this magnification with brightfield microscopy. (B) A darkfield of the same section as in (A). The grains appear as white dots and the tissue cannot be seen. The grain density is so high on the proximal side that the individual grains cannot be distinguished. Scale bar, 500 μm. (C) A high-power brightfield taken directly above the left arrow in (B) showing the grain density on the proximal side. (D) Grain density on the distal side directly above the right arrow in (B). (E) Grain density from an adjacent section that was coincubated with naloxone to produce a blank. The photograph shows the same part of the nerve as that in (D). Scale bar, 50 μm. Autoradiograms were exposed for 3 months. The receptors were labeled with [^{3}H]DAMA.

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(*4*). Ligation of the nerve approximately 1.5 cm below the nodose ganglion was made with size 4-0 silk, nonabsorbable, surgical sutures. At various times after ligation, a section of nerve with at least 3 to 4 mm on each side of the ligation was removed, mounted in a brain paste, and frozen on a microtome chuck. Nodose ganglia were treated similarly.

Sections (8 or 10 μm) of the ganglia and nerve were processed for the autoradiographic localization of opiate receptors as described in (*5*). After preincubation with 100 m*M* sodium and 50 μM guanosine triphosphate (GTP) for 15 minutes at room temperature to dissociate endogenous opioids more rapidly (*5*), slide-mounted tissue sections were incubated in either 4 n*M* ^{3}H-labeled [D-Ala$_2$,MetNH$_2^5$]enkephalin (DAMA, Amersham Corp., 25 Ci/mmole), or ^{125}I-labeled [D-Ala^2,MePhe^4,Met(*O*)-ol]-enkephalin (FK 33-824, Sandoz; labeled by R. Goodman), or 6 n*M* [^{3}H]naloxone (New England Nuclear, 50 Ci/mmole) for 40 minutes at room temperature and given two 5-minute washes. In some experiments adjacent slide-mounted sections were coincubated with 1 μM naloxone to produce blanks and others with 10 μM GTP. The sections were dried and apposed to emulsion-coated covers to generate autoradiograms (*5*).

Examination of opiate receptor distribution in sections of ganglia revealed receptors in association with the fiber bundles between the neuronal cell bodies, as expected. There was also a marked association of receptors with the majority of, but not all, neuronal cell bodies. Examination of the DAMA-labeled opiate receptor distribution in sections of nerve ligated for 20 hours revealed a striking buildup of binding sites on the proximal side of the ligation (Figs. 1 and 2). Grain counts revealed a ratio of proximal to distal grain densities of about 5 or 6 to 1 (818 ± 228 to 153 ± 85 grains per 1000 μm^2 with [^{3}H]DAMA; N = 27). The ratio was less in nerves ligated for 7 hours and not significantly different from unity in nerves ligated for only 1 to 2 minutes before processing. Similar results were obtained in sections labeled with [^{3}H]naloxone.

These observations indicate an axonal movement of receptors. It is possible that the movement is produced by the mechanisms of axoplasmic flow or axonal transport (*1*). Other investigators have observed axonal transport of other substances in the vagus (*6*). Particulate-bound proteins, which would include receptors, may be carried by fast rather than slow transport (*7*). Our estimated rate suggests that opiate receptors are moving slowly, that is, in the range of 3 to 10 mm per day, but more accurate measurements may reveal a faster flow (*8*).

Our pharmacological studies (*3–5, 9*) on the vagal binding sites suggest that these are opiate receptor sites rather than some nonspecific binding site for the radiolabeled peptide used for Fig. 1. (i) The sites bind agonist and antagonist radiolabeled drugs such as naloxone, diprenorphine, etorphine, and dihydromorphine, as well as enkephalin-like peptides such as [D-Ala^2-D-Leu^5-enkephalin, FK 33-824, and DAMA; (ii) they show displacement by unlabeled naloxone; (iii) they show stereospecificity in that levallorphan but not dextrallorphan displaces the binding; and (iv) they show decreased binding of FK 33-824 in the presence of GTP. A number of studies (*10*) show that these ligands, under appropriate conditions, bind to opiate receptors.

The portions of the vagus nerve that we ligated contain sensory afferents, whose cells are in the nodose ganglia, and motor fibers, whose cells are in the medullary nuclei, including nucleus ambiguus and dorsal motor nucleus of the vagus. Which fibers are the receptors on? Evidence supports a localization to the sensory fibers. Previous studies (*4*) indicate a loss of binding in the nucleus tractus solitarii (a recipient of sensory fibers) after vagotomy. Also, the present study found a notable association of binding with cells in the sensory nodose ganglia. However, we can not rule out that motor fibers also have receptors since nucleus ambiguus in the medulla contains a high density of receptors (*3*), and these could extend along the axons of these neurons. It is not clear whether all the receptors are undergoing transport and what their functions might be. We have speculated that they are destined for use in axo-axonic synapses or that they are presynaptic receptors possibly for circulating opioid hormones that could affect the sensory fibers (*3, 4*).

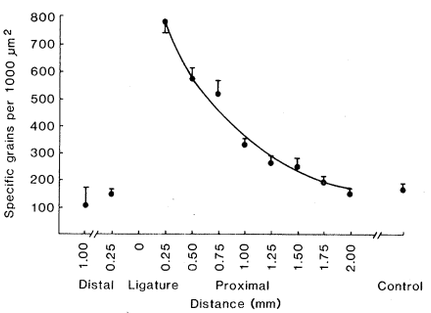

Fig. 2. Autoradiographic grain densities along a ligated vagus nerve. The data were obtained from tissue labeled with [^{3}H]DAMA from four animals prepared and processed as described in Fig. 1. Each animal yielded three or four sections, all of which were counted. Counting was done randomly through a ×40 oil objective and an eyepiece with an attached, calibrated grid. The data are means ± standard errors of the mean; N = 20 to 27 measurements at each point. Counts were made at several distances on either side of the ligature. Counts from controls were obtained from unligated nerves from three animals. Nonspecific binding, measured in adjacent sections, was subtracted at each point. A large buildup at the ligature on the side proximal to the cell body in the ganglion is evident. When reasonable assumptions are made about the binding affinities of [^{3}H]DAMA and [^{3}H]-naloxone to the axonal receptors (*10*), the large increase in binding on the proximal side of the ligature cannot be explained by a change in affinity for the ligands (K_d) but must at least include a significant change in the number of binding sites (B_{max}) of axonal receptors.

Perhaps it is not surprising that receptors undergo axonal or dendritic flow, or both. After synthesis in the perikaryon, the receptor protein must be moved to distant areas. Thus, axonal flow may be a common property of most or all receptors.

Note added in proof: We have recently found that muscarinic cholinergic receptors undergo axonal flow in the vagus and sciatic nerve. While this report was in press, Laduron (*11*) reported axonal flow of muscarinic receptors in the dog splenic nerve.

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8. If we estimate the rate of flow of receptors by dividing the receptor accumulation per day by the receptor content of the vagus per millimeter of nerve, we obtain a rate of 3.5 mm per day from the data in Fig. 2. This estimation assumes (i) that all the receptors are flowing, (ii) that there is no swelling of the nerve, (iii) that the flow rate is constant over the entire period of ligations, and (iv) that the ligation does not affect the flow rate. Previous experiments suggest that these assumptions may not be totally valid. For example, in studies of axonal transport of acetylcholinesterase, it was found that only 10 percent of the axonal enzyme was moving (*1*). Our microscopic studies suggest that some swelling occurs on the proximal side of the ligature. There is a technical limitation in that the grain density on the proximal side (Fig. 1 and 2) is somewhat beyond the limits of linearity compared to that on the distal side. For example, in an experiment with [^{3}H]naloxone with shorter exposures, the proximal to distal ratios were 10 or 12 to 1 (*9*), a result that could double our estimated rate. Thus, the actual concentration of radioactivity on the proximal side compared to that on the distal side is higher than the grain density would suggest in Fig. 2, and a more accurate estimate of the receptor flow rate could well be larger.
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Superior Colliculus: Control of Eye Movements in Neonatal Kittens

Abstract. *Activation of the neonatal cat superior colliculus can produce organized eye movements before visual stimuli are capable of activating visual neurons in the colliculus. These findings are consistent with the hypothesis that eye movement development precedes, and is necessary for, visuomotor integration.*

The ability to respond appropriately to visual cues (visuomotor integration) develops gradually with experience. Hein *et al.* (*1*) demonstrated that eye movements that produce image displacements across the retina are necessary for visuomotor development. Since central neurons responsive to visual stimuli (*2–4*) and the optical apparatus itself (*5*) require months to mature, the gradual association of visual stimuli and motor responses may be due, in large part, to the long maturational course of the sensory apparatus related to "seeing." The motor apparatus may already be functional in very young animals.

We previously demonstrated that visual cells in an area of the brain that plays a role in visuomotor integration (the superior colliculus) are not functional before 7 days of age and show a protracted developmental course thereafter (*2, 3*). On the basis of these observations we speculated that a structure such as the superior colliculus, whose role is normally to integrate sensory information and motor responses, might set the stage for visuomotor integration by developing the capacity to move the eyes even before visual sensation becomes a significant source of information (*6*). We now report that kittens are capable of some spontaneous eye movement even at 2 days of age and that electrical stimulation of the superior colliculus in the animals elicits eye movements with many of the same features seen in the adult (*7*).

Twenty-six kittens 2 to 77 days of age and six adult cats were studied. Prior to the experimental session, a stainless steel chamber was implanted on the skull over a cranial opening (*8*). Mounting bolts were fixed to the skull to provide an atraumatic means of securing the head during testing, and the entire assembly was held in place with anchoring screws and dental acrylic. On the experimental day, ketamine hydrochloride, a short-acting anesthetic, was administered, the animal was placed in a retaining bag, and the head was fixed to a stereotaxic headholder by the implanted bolts. The eyelids were opened when necessary, the eyes anesthetized with 0.5 percent proparacaine hydrochloride, and a small mirror was glued to each cornea. Light beams were reflected from the mirrors (Fig. 1) onto a translucent hemisphere so that two spots of light were clearly visible on this surface and moved as the eyes moved. The displacement of the light spots was exactly twice that of the eye movements in degrees of arc.

Testing began as soon as the anesthetic began to wear off and the animal moved either spontaneously or in response to tactile stimuli (*9*). A monopolar electrode approached the superior colliculus through the visual cortex in 0.5-mm steps. At each step, a 70-msec, 700-μA "search" stimulus consisting of 0.1-msec rectangular pulses at 200 pulses per second was delivered. When an eye movement was produced at a given locus, its threshold (*10*) was determined. The electrode was subsequently lowered in 0.25-mm increments, and movement thresholds were determined at these sites. Several stimulation series were conducted with various stimulus in-

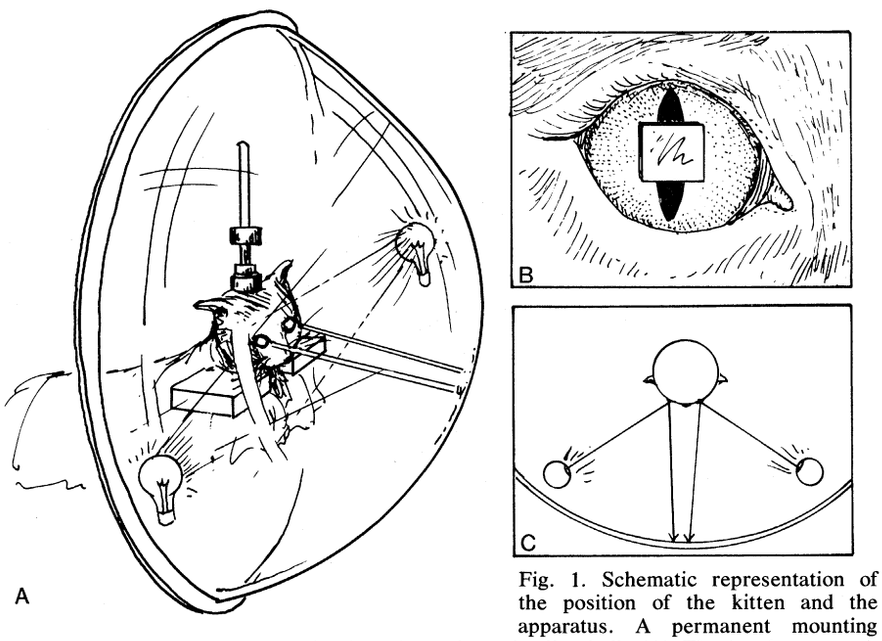

Fig. 1. Schematic representation of the position of the kitten and the apparatus. A permanent mounting bolt and hollow cylinder were implanted on the animal's skull. (A) The head was positioned at the geometric center of a translucent (not transparent, as drawn) hemisphere, and electrodes reached the superior colliculus through the hollow cylinder. (B) Small mirrors were fixed to the anesthetized eyes, and (C) beams of light, reflected from these mirrors, appeared as spots of light on the hemisphere. A pattern of 10° concentric circles was scribed on the hemisphere; eye movements caused movement of the light spots whose excursions were traced directly on the hemisphere's surface.

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