Retrograde Horseradish Peroxidase Transport After Oculomotor Nerve Injury
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We studied the distribution of somatic motor neurons innervating the cat superior rectus 3-6 months after oculomotor nerve injury using intramuscular horseradish peroxidase (HRP). In normal cats, 98% or more of the labelled superior rectus motoneurons were in the contralateral oculomotor subnucleus. Two experimental cats who exhibited little or no evidence of recovery showed few labelled cells (4% of controls) which were distributed in both the ipsilateral and contralateral oculomotor nucleus. The other three experimental cats demonstrated definite signs of recovery, and HRP injections labelled more cells (20% of controls) also distributed in the ipsilateral and contralateral oculomotor subnuclei. This study shows that, after sectioning, the oculomotor nerve regenerates and anomalous connections develop between the somatic motoneurons of the ipsilateral oculomotor nucleus and the superior rectus. These findings support the hypothesis that acquired oculomotor synkinesis developing after third nerve injury results from misdirection of regenerating axons. Invest Ophthalmol Vis Sci 27:975-980, 1986

Following oculomotor nerve injury, some patients develop permanent paradoxical patterns of pupillary, lid, and eye movements referred to as acquired oculomotor synkinesis. The mechanism of acquired oculomotor synkinesis is controversial, and has recently been reviewed in several publications.1-5 The prevailing view, originally proposed by Bielchowsky,6 supported by Bender and Fulton,7,8 and others, posits that regenerating axons of the oculomotor nerve become misdirected and inappropriately innervate the extraocular muscles. The evidence for misdirection is principally based on Cajal’s morphologic studies of nerve regeneration9 and clinicopathologic case reports of patients with acquired oculomotor synkinesis.10,11 However, no one has ever traced a misdirected axon from its central origin to its termination on an extraocular muscle.1 Moreover, clinical evidence, including cases of reversible oculomotor synkinesis,1,3,4 and spontaneously acquired oculomotor synkinesis,1,12,13 appear incompatible with the fixed-wiring implicit in misdirection.1,4 Other explanations have therefore been proposed, including ephaptic transmission1 and central synaptic reorganization.1,4

In order to test the misdirection hypothesis, we used horseradish peroxidase (HRP) to anatomically localize the neurons that innervate the superior rectus muscle after injury to the feline oculomotor nerve.

Materials and Methods

Five cats underwent craniotomy under nembutal anesthesia (IP, 35 mg/kg). Using an operating microscope, the temporal lobe was gently retracted to expose the right oculomotor nerve in the basal cistern, proximal to the cavernous sinus. The nerve was completely transected, the cut ends inspected and then reapproximated. The cats were allowed to recover and were observed for 3 (cats 1-4) to 6 months (cat 5). Following this period of observation, five experimental and two control cats underwent another microsurgical procedure under nembutal anesthesia. A conjunctival peritomy was performed, and the right superior rectus muscle was exposed and isolated. To facilitate exposure of the muscle, the globe was collapsed by performing an anterior chamber paracentesis. The muscle was enveloped in absorbent cellulose sponges (Wek cells, Edward Weck Co, Research Triangle Park, NC) to prevent inadvertant leakage of HRP into the orbit. Using a Hamilton syringe, 2 /uL of 30% HRP (Type VI, Sigma, St. Louis, MO) in distilled water was injected into the superior rectus muscle sheath. After 48 hours, the cats were deeply reanesthetized. Intravenous heparin (5000 U) was administered and the cats were perfused through the ascending aorta with saline (1 liter) immediately followed by a 2 liter mixture of 1% paraformaldehyde, 1.25% gluteraldehyde in .1 M phosphate buffer (pH 7.4). The brainstem and third nerves were removed and immersed in 30% sucrose-phosphate buffered solution (pH 7.4) overnight at 4°C. The oculomotor nerve was grossly inspected for signs of reconnection. The pons and midbrain were cut into 50 μm transverse sections with a freezing microtome.
and the tissue reacted with the tetramethyl benzidine (TMB) method described by Mesulum, as modified by Itoh et al. Tissue sections were counterstained with neutral red. The oculomotor, trochlear and abducens nuclei were studied for intracellular HRP reaction product. HRP labelled cells from serial sections of the entire oculomotor complex were counted. Because the study focused on ipsilateral versus contralateral labelling, cell counts were confined to the somatic motor neurons of the extraocular muscles, excluding the central caudal nucleus. Photomicrographs were taken with phase optics on a Leitz microscope.

Cats in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

Oculomotor Function

All five cats who underwent third nerve section exhibited a complete oculomotor palsy OD. The pupil was fixed and dilated; the lid was ptotic; the eye was slightly exotropic and paretic (Fig. 1A). Ten to fourteen days later the lid began to return to its normal position in all cats, but pupillary and ocular motility remained unchanged. Over the subsequent 2–3 months cats 1–3 exhibited gradual but incomplete return of pupil and eye movements. In cat 3 recovery was noted 3–4 weeks after surgery.

"Incomplete" or "limited" functional recovery was manifested by a decrease in pupil size, sluggish constriction to light stimulation, and limited dysconjugate eye movements (Fig. 1B). Occasionally, transient widening of the ipsilateral lid fissure was observed. The development of limited oculomotor function 2–3 months after surgery was interpreted as evidence of oculomotor nerve regeneration. In cat 4 (Fig. 1C) there was occasional widening of the involved lid fissure; however, the pupil remained fixed and widely dilated; eye movements were absent 3 months after nerve transection. Cat 5 was observed for 6 months without return
of pupillary function or ocular motility. Thus cats 1–3 showed definite but incomplete signs of recovery, whereas cats 4 and 5 showed minimal signs of recovery, primarily confined to lid function.

Oculomotor Nerve

The intracranial third nerve was inspected for gross signs of anatomic reconnection. Union of the proximal and distal cut ends was evident in cats 1–4 with thickening and irregularity of the nerve at the transection site (Fig. 2). Unsuccessful regeneration was noted in cat 5, whose proximal nerve stump terminated in the sella. With the operating microscope one could, however, observe several small axon twigs entering the cavernous sinus.

HRP Cell Counts and Distribution

Following HRP injection into the right superior rectus, the two control animals showed heavy HRP labeling. Greater than 98% of the 836 and 657 HRP labelled cells respectively (Table 1) were found in the contralateral caudal-medial region of the oculomotor subnucleus (Fig. 3A). No HRP labelling was observed in the trochlear or abducens nuclei.

The experimental cats demonstrated HRP labelling qualitatively and quantitatively different from controls. HRP cell counts correlated with functional recovery and nerve re-union. Those cats with definite but incomplete signs of recovery (#1–3) and reunion of the third nerve displayed an 80% reduction in the total number of HRP labelled cells (Table 1); the number ranged from 138–148 (mean 142). Among individual cells, the density of reaction product was less than that exhibited in the control cats. Moreover, HRP labelled cells in experimental animals were located in both the ipsilateral and contralateral oculomotor subnucleus (Figs. 3B, 4A, B). Of the total HRP labelled cells in cats 1–3, 34–45% were contralateral and 55–66% were ipsilateral (Table 1, Fig. 4A). Cat 4, which showed minimal signs of recovery and third nerve reconnection, had 52 HRP labelled cells again distributed in both the contralateral (25%) and ipsilateral (75%) oculomotor subnucleus (Table 1, Fig. 4B). Cat 5, which showed no recovery and minimal anatomic reconnection, had only 14 HRP labelled cells in both the ipsilateral and contralateral halves of the oculomotor nucleus (Table 1, Fig. 4B). Minimal signs of recovery in these last two cats were associated with a 96% reduction in the number of HRP labelled cells compared to controls. With the exception of a single HRP labelled cell in the trochlear nucleus of cat 3, labelled cells were not found in the trochlear and abducens nuclei of the experimental animals. There was no labelling observed in the ventral tegmental area, the central gray, or the anteromedian nucleus.

Table 1. Horseradish peroxidase labelled cell counts in control and experimental cats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total HRP count</th>
<th>Ipsilateral HRP count</th>
<th>Contralateral HRP count</th>
<th>I:C Ratio*</th>
</tr>
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<tbody>
<tr>
<td>Controls</td>
<td>836</td>
<td>12 (2%)</td>
<td>824 (98%)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>657</td>
<td>4 (1%)</td>
<td>653 (99%)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>746</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incomplete functional recovery</td>
<td>1</td>
<td>139</td>
<td>76 (55%)</td>
<td>63 (45%)</td>
</tr>
<tr>
<td>2</td>
<td>138</td>
<td>80 (58%)</td>
<td>58 (42%)</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>148</td>
<td>97 (66%)</td>
<td>51 (34%)</td>
<td>1.9</td>
</tr>
<tr>
<td>Mean</td>
<td>142</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No functional recovery</td>
<td>4</td>
<td>52</td>
<td>39 (75%)</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>6 (43%)</td>
<td>8 (57%)</td>
<td>.8</td>
</tr>
<tr>
<td>Mean</td>
<td>33</td>
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* I:C = number of ipsilateral HRP labelled cells / number of contralateral HRP labelled cells

Discussion

Using a variety of techniques, studies have consistently shown that superior rectus motoneurons occupy the contralateral caudal-medial two-thirds of the oculomotor nucleus, and the motoneurons of the inferior oblique, medial, and inferior recti are always ipsilateral.16–18 Because the motoneurons of the superior rectus are contraterally localized from the somatic motoneurons of the other extraocular muscles which are ipsilateral, we chose to inject the superior rectus HRP following regeneration. Consistent with prior reports,17,18 the control cats in the present study demonstrated contralateral labelling after superior rectus HRP injection. The rare labelling in the ipsilateral nucleus (usually near the midline) in controls, and the absence of labelling within the trochlear and abducens nuclei in nearly all cats indicates that leakage of HRP did not appreciably contaminate the orbit.

Within 3 months of injury, functional recovery in the experimental cats was evident. Recovery was manifested by limited return of lid and ocular motility, a decrease in the size of the pupil, limited reactivity of the pupil, and, in one case, signs of paradoxical lid elevation. Other investigators have reported similar signs and rates of recovery in cat models.19,20 To a limited extent, the observations made in cats mimic those in human cases of acquired oculomotor synkinesis5,6 as well. Functional recovery appeared to correlate with anatomical reconnection of the severed nerve.

After regeneration, there were alterations in the quantity and distribution of oculomotor neurons that...
innervated the superior rectus (Table 1). Retrograde HRP transport correlated with functional recovery and reconnection of the third nerve. HRP labelling was significantly different from controls. Almost all cell labelling was contralateral to the HRP injected muscle (Fig. 3A) in the control cats; whereas experimental cats consistently showed HRP labelling in both the ipsilateral and contralateral oculomotor subnucleus (Fig. 4A, B). Under these experimental conditions, bilateral HRP labelling indicates that the neurons terminating on the superior rectus muscle after regeneration originate from those regions of the oculomotor nucleus that previously innervated the inferior rectus, medial rectus, and inferior oblique, as well as the superior rectus. These findings directly support the misdirection hypothesis.

This study confirms the studies of Brushart and Mesulum. Working with a different model, they studied the distribution of HRP labelled motor neurons of the tibial and peroneal nerves after sciatic nerve regeneration in rats. The distribution of peroneal motoneurons
normally peaks at the L4 level; after regeneration, the pool shifts caudally to the L5 and L6 level, an area normally innervated by motoneurons of the tibial nerve. In contrast to our 80% reduction in labelled cells, they found a 30% reduction compared to controls. In addition to using a different animal, they resutured the nerve after sectioning, which we did not do. Regeneration in their model may therefore have been more successful than ours.

The present findings are also consistent with earlier work suggesting an absence of neurotropism in peripheral regeneration of adult mammals. Weiss and Hoag have shown that, regardless of their origin, axons behave as “equals” during the process of reinnervation. These results were confirmed by Bernstein and Guth using a different experimental model. The lack of reinnervational specificity in mammals is characteristic.

It has been suggested that the clinical features of acquired oculomotor synkinesis in humans might be a consequence of ephaptic transmission. The present findings appear to be incompatible with this hypothesis. HRP can be retrogradely transported from the proximal stump of an acutely transected nerve, however, that such transport occurs 3 months after injury is unlikely, since axons which fail to reestablish peripheral connections degenerate. Moreover, while axons can alter the electrical activity of adjacent axons, cross diffusion of HRP would presumably require an anatomic junction. To our knowledge, there has been no study documenting the morphology of an acquired ephapse permeable to HRP long after nerve injury.

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**Fig. 4.** A, Schematic summary of the number and distribution of HRP labelled cells in those cats which showed definite but incomplete functional recovery (Cats 1-3 are a, b, and c, respectively) 3 months after right oculomotor nerve transection. B, The distribution of HRP labelled cells in two cats that showed minimal or no functional recovery (a) 3 months and (b) 6 months after nerve transection. Each dot represents a single HRP labelled cell. Right third nerve was sectioned; right superior rectus was injected with HRP. Labelling is located in the ipsilateral and medial contralateral oculomotor nucleus.
The anatomic basis for naturally occurring ephaptic transmission appears to be the gap junction; that gap junctions form among adjacent axons in regenerating nerves has never been shown. Where gap junctions normally exist, they are impervious to HRP. Thus, the ephaptic hypothesis seems inadequate to explain the experimental observations made on oculomotor nerve regeneration.

The central theory of acquired oculomotor synkinetics states that peripheral nerve injury induces retrograde neuronal changes that result in synaptic reorganization of the oculomotor nucleus. It is argued that peripheral nerve injury “unmasks” or “releases” encoded connections that result in “en masse” nuclear discharges. While central changes after peripheral nerve injury are well known, the present study showing transport of HRP from the superior rectus to the ipsilateral oculomotor nucleus cannot be explained solely by retrograde central synaptic reorganization. Thus, the functional effects of peripheral nerve injury are well known, the present study showing transport of HRP from the superior rectus to the ipsilateral oculomotor nucleus cannot be explained solely by retrograde central synaptic reorganization. The central hypothesis fails to explain how morphologically reorganized neuronal processes that central reorganization must be mediated through the injured nerve and not through another peripheral pathway. Furthermore, it is difficult to explain the universal absence of acquired oculomotor synkinesis after diabetic oculomotor nerve palsies. The central hypothesis appears to be inconsistent with both the clinical and experimental findings.

The present study has traced misdirected oculomotor axons from their destination in the superior rectus muscle to their origin in the ipsilateral and contralateral oculomotor subnucleus. These observations provide an anatomic and physiological foundation for the misdirection hypothesis.

Key words: oculomotor nerve, nerve regeneration, synkinesis, aberrant regeneration, palsy, horseradish peroxidase

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References