Acetylcholinesterase-rich projections from the basal forebrain of the rhesus monkey to neocortex

MAREK-MARSEL MESULAM AND GARY W. VAN HOESEN

Bullard and Denny-Brown Laboratories, Harvard Neurological Unit, Beth Israel Hospital, Boston, Mass. 02215 and Aphasia Research Center, Department of Neurology, Boston University Medical School, Boston, Mass. 02130 (U.S.A.)

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In recent experiments with horseradish peroxidase (HRP) histochemical methods, the remarkable observation has been made that neurons in the basal forebrain of the monkey send axons directly and without a thalamic relay to neocortex. Although many basal forebrain neurons also contain large amounts of acetylcholinesterase (AChE), it is not known whether or not the projection to neocortex actually originates from these same AChE-rich cells. We tested and confirmed this hypothesis by combining HRP and AChE histochemistry within the same tissue section.

In 5 hemispheres of 4 rhesus monkeys, regions within cortical areas 4 and 6 were injected with a 20% solution of HRP (Sigma VI). After a 2-3-day survival period, the animal was perfused transcardially with 2 liters of a fixative containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer at a pH of 7.4. The brain was removed, postfixed in the same fixative for 3 h, placed in phosphate buffer containing 5% sucrose for 3 additional days and cut into 40 μm thick sections with a freezing microtome. Each of 4 sets of sections through the basal forebrain, consisting of sections collected at approximately 200 μm intervals, was processed according to 1 of 4 different histochemical procedures.

The first set was incubated in a medium containing 3,3’-diaminobenzidine (DAB) and hydrogen peroxide (H₂O₂) so that a brown reaction product was formed at sites containing HRP. These sections were counterstained with gallicyanin and examined microscopically under bright- and dark-field illumination. In the second set, benzidine dihydrochloride (Sigma) was substituted for DAB so that bright-blue granules were formed at sites containing AChE. Our method consists of preincubating sections for 5-10 min in 0.12% benzidine and 0.1% sodium nitro-ferricyanide (SNF) in 30% alcohol buffered with acetate at pH 5.0. Hydrogen peroxide is then added to this medium to reach a concentration of 0.015% and the sections are further incubated for 3-4 min. The tissue is then placed for 20 min into a stabilization bath of 9% SNF in 50% alcohol at 4 °C and buffered as above. These sections were
Fig. 1. A: three coronal sections from a case with HRP injections in areas 4 and 6. The cortical extent of the injected HRP is blackened. Areas where perikarya containing both AChE and HRP were seen are indicated by triangles. B: camera lucida drawing from section A2. Tissue stained for both HRP and AChE. The cellular profiles indicate AChE-rich perikarya in the nBSI and the nDB; the cells which are positive for both HRP and AChE are blackened. Abbreviations: ac, anterior commissure; amg, amygdala; cgs, cingulate sulcus; gp, globus pallidus; h, hypothalamus; ic, internal capsule; oc, optic chiasm; pu, putamen; rs, rhinal sulcus; spd, superior precentral dimple; Syl. f., Sylvian fissure.

counterstained in neutral red which produced sufficient contrast for satisfactory bright-field microscopy. The third set was processed in an acetylthiocholine–copper sulfate medium and further 'developed' so that a dense, black precipitate of silver sulfide was deposited at sites of AChE activity; ethopropazine was used to inhibit other cholinesterases. Lastly, the fourth set of sections was prepared so that the same tissue section simultaneously contained both coarse blue granules to indicate the presence of HRP as well as a finely granular reddish-brown precipitate at sites of AChE activity. In order to achieve this combination, free-floating sections were first incubated for 1 h at 21 °C in the acetylthiocholine medium of Geneser-Jensen and Blackstad; they were then washed and processed with benzidine and stabilized as described above; lastly, the tissue was washed and placed for 2 min in 10% potassium ferricyanide in order to produce a reddish-brown precipitate at sites of AChE activity.

This fourth set of sections was not counterstained. Therefore, a dense reddish-brown cellular profile, often including dendrites and the initial axonal segment,
indicated the presence of an AChE-rich cell. The additional presence of a cluster of blue granules within the reddish-brown profile was accepted as evidence that the AChE-rich cell under study had an axon extending into the area injected with HRP. Such cells were considered positive. Areas containing either blue granules or the reddish-brown outline alone were considered negative. When the blue granules were not completely contained within the reddish-brown cellular shape or when their quantity was negligible, the cell was classified as ‘questionable’. The tinctorial contrast among the two histochemical end-products and the background was satisfactory.
for the easy identification of the three cell types under bright-field illumination. The
distribution of positive cells was charted with the help of an x-y plotter coupled to
the mechanical stage of a microscope. Control procedures consisted of omitting the
substrate acetylthiocholine from the AChE incubation as well as processing a brain
without an HRP injection in a manner identical to the procedures described above;
in neither control procedure was either histochemical end-product detected within
the regions under study.

The distribution of HRP-containing cells in the basal forebrain was essentially
identical in the three sets of sections incubated with DAB or benzidine. Furthermore,
the distribution of AChE was also identical in the third and fourth sets. Within
the basal forebrain, cells containing the HRP reaction product were seen pre-
dominantly in the septum, substantia innominata (SI), internal and external medulla-
ry laminae of the globus pallidus and the lateral hypothalamus. These findings are in
good agreement with those of Kievit and Kuypers. Furthermore, AChE histoche-
mistry revealed intensely positive cells in the medial septum, nucleus of the diagonal
band (nDB), nucleus basalis of the SI, the medullary laminae of the globus pallidus
and the hypothalamus. The definitive demonstration that the projection to the
motor belt actually originated from some of these AChE-rich cells was confirmed as a
result of combined AChE and HRP histochemistry performed on the same tissue in
the fourth set of sections.

This method revealed that many cells with a high AChE content also contained
the HRP reaction product (Figs. 1 and 2). Such positive cells were encountered, al-
most exclusively ipsilaterally, in the nDB, nucleus basalis of the SI (nBSI) and the
cells of the medullary laminae of the globus pallidus; there were questionably
positive cells in the lateral hypothalamus. The majority of positive cells were found
among the large and hyperchromic multipolar cells of the nBSI; similarly, almost all
of the HRP clusters in the nBSI were within AChE-rich cells. Especially striking were
positive cells in the medullary laminae of the globus pallidus. Their AChE-rich,
HRP-positive large perikarya and spider-like multipolar dendritic processes could be
appreciated with ease on the background of the AChE-free white matter. In fact,
on the basis of morphological, embryological and physiological criteria, these cells
in the medullary laminae of the primate globus pallidus have been considered as
aberrant cells of the SI (refs. 1 and 3); our results would be consistent with this inter-
pretation. Although the basal forebrain is a complex region where different cellular
groups are intricately juxtaposed, the nBSI where most of the positive cells were
seen forms a conspicuous and discrete formation in both monkey and man.

It is well known that the presence of AChE is not sufficient to prove the cholin-
ergic nature of a central nervous system (CNS) pathway. Nevertheless, with some
exceptions, several independent lines of investigation converge upon the generally
accepted notion that perikarya and axons in the CNS with a strong histochemical
reaction for AChE are likely to be 'cholinergic' whereas presumptively 'cholinocep-
tive' cells have significantly less AChE in the perikaryon and negligible amounts along
their axons. It is of interest, therefore, that the perikarya of our 'positive' cells gave a strong AChE reaction. In non-primate mammals, there is also physiologi-
neurochemical, embryological and histochemical evidence suggesting a 'cholinergic' input into neocortex. However, in contrast to the fast, 'detonator' effect of acetylcholine upon the post-synaptic membrane in the peripheral nervous system, the acetylcholine released by these central 'cholinergic' exons may have a rather slow and modulating effect on the post-synaptic potential. Furthermore, in the monkey as well as in the human brain the precentral cortex is particularly rich in axons containing AChE.

In conclusion, when considered in light of this evidence, our observations with combined HRP and AChE histochemistry prove that an AChE-rich and possibly 'cholinergic' projection arising from the basal forebrain, mostly from the nucleus basalis of the substantia innominata, innervates the precentral neocortex of the rhesus monkey. On a morphological basis, the SI is a rostral extension of the reticular formation. Accordingly, it receives a heterogeneous set of afferents from the limbic system, olfactory cortex, the pontine taste area and the hypothalamus, thus becoming a likely candidate for influencing the motor output of the organism according to the prevailing physiological and motivational state. Furthermore, cholinergic agonists are known to have complex effects on sleep, aggressiveness, appetitive behavior and learning, and this possibly 'cholinergic' projection from the SI may therefore modulate the motor sequences which underlie these behavioral effects. Moreover, in counterbalance to the considerable emphasis placed on noradrenergic projections of subcortical origin to the cerebral cortex, the data presented here suggests that there may be a corresponding 'cholinergic' system arising in the basal forebrain which also projects widely to neocortical areas.

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