Combined GABA-immunocytochemistry and TMB–HRP histochemistry of pretectal nuclei projecting to the inferior olive in rats, cats and monkeys

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Cells in the pretectal nucleus of the optic tract (NOT) of rats cats and monkeys were retrogradely labeled with horseradish peroxidase (HRP) stereotaxically injected into the inferior olive (IO) A procedure for stabilizing the tetramethylbenzidine (TMB)–HRP reaction product was used to visualize combined TMB–HRP and immunohistochemically localized γ-aminobutyric acid (GABA) in the same sections Positive GABAergic reaction product was found to be restricted to smaller-size intrinsic neurons Larger NOT cells projecting to the IO were consistently free of GABA reaction product and, in addition, appeared to be contacted by relatively few GABAergic terminals

A specific population of neurons in the nucleus of the optic tract (NOT) and the dorsal terminal nucleus of the accessory optic tract (DTN) in the pretectum of mammals is involved in the control of horizontal optokinetic nystagmus[4 6 10 12 13 16 23 30] These neurons receive a visual projection mainly from the contralateral eye[1 2 6] as well as from the ipsilateral visual cortex[27 34] and they in turn project to the ipsilateral dorsal cap of the inferior olive (IO)[14 28 33] The outstanding property of these NOT and DTN neurons is their directionally specific response to temporal to nasal movement of full-field visual stimuli presented to the contralateral eye[4 6 10 12 13] The results of pharmacological experiments suggest that γ-aminobutyric acid (GABA) plays an important role in the initiation of direction specificity in cells of the retina and visual cortex[29 36] Inhibitory GABAergic cells may also shape the preferred direction of directional cells in the NOT or mediate their inhibition caused by movement in the non-preferred direction Recent immunocytochemical studies have shown the presence of neurons within the NOT and DTN in a number of mammals that use GABA as a neurotransmitter[9 19 21 22 35] In the present study we investigated the possible structural basis for a direction selective mechanism in the NOT and DTN by determining whether neurons projecting to the IO are contacted by GABAergic terminals We used the retrograde axonal transport of horseradish peroxidase (HRP) to label NOT and DTN neurons, and combined HRP histochemistry with subsequent immunocytochemical methods for the localization of GABA

Four pigmented rats, two normally pigmented cats and two monkeys (*Macaca mulatta*) were used For the stereotaxic injection of HRP each animal was deeply anesthetized Rats were given a single dose of Nembutal (5 mg/100 g) and cats as well as monkeys were given an initial subcutaneous injection of ketamine (20 mg/kg) in combination with Rompun (0 05 ml/kg), followed by subsequent doses of ketamine (10 mg/kg) i v given as needed during surgery The cats and monkeys were intubated and artificially ventilated with a mixture of N_2O/O_2 (3 1) to maintain adequate anesthesia

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Each animal was subsequently placed in an appropriate stereotaxic instrument To approach the IO in rats and cats, the foramen magnum was enlarged and the dura excised to expose the medulla oblongata The caudal part of the cerebellum was gently elevated to visualize the forth ventricle and a Hamilton microliter syringe was advanced towards the IO For rats, the microliter syringe was inserted to a depth of 2 7 mm through the midline of the opening of the obex angled forward at an angle of 5° A similar approach was used for the first cat The syringe was inserted 1 mm lateral to the obex, angled 45° forward and advanced 7 mm below the brainstem surface A second cat received an HRP injection into the lateral geniculate nucleus The syringe was stereotaxically guided with reference to the atlas of Berman[3], and its final position was determined by electrophysiological recording of visually evoked activity In the monkey, the IO was approached stereotaxically according to the atlas of Snyder and Lee[31] with a syringe angled forward 45° The rats received 0 1 μl, the cats and monkeys received 0 2 μl of a 30% HRP solution in 2% dimethylsulfoxide (DMSO) in distilled water All injections were made slowly over a period of 20 min

Following a survival period of 72 h after an injection into the IO, and 45 h after the injection into the geniculate body, the animals were deeply anesthetized and perfused transcardially with the following solutions (1) 1 liter Ringer (0 9% NaCl) with 0 1% Novocain at 37 °C, (2) 3 liters of a fixative consisting of 1% paraformaldehyde and 1% glutaraldehyde in 0 1 M phosphate buffer (pH 7 4) at 4 °C and (3) 1 liter of a second fixative consisting of 2% paraformaldehyde and 0 1% glutaraldehyde at 4 °C The brains were immediately blocked and stored overnight in 0 1 M phosphate buffer The next day, 20–40 μm serial sections of the midbrain were cut on a vibratome and collected in 0 1 M phosphate buffer All sections were first processed for HRP-activity with tetramethylbenzidine (TMB) as a chromogen In order to demonstrate transported HRP and GABA-immunohistochemistry in the same section the following modification of the Mesulam[18] protocol for TMB-histochemistry was used

HRP protocol

(1) Rinse sections 6 times in ice-cold distilled water

(2) Preincubate in a mixture of solution A 100 mg sodium nitroferricyanide, 92 5 ml distilled water and 5 ml 0 2 M citrate buffer (pH 3 3), and B 5 mg TMB dissolved in 2 5 ml of reagent grade absolute alcohol

(3) Incubate by adding 200 mg β-D-glucose, 40 mg ammonium chloride and 0 5 mg glucose oxidase (GOD) (instead of H_2O_2)[15], to 100 ml of preincubation bath for about 40 min until the desired degree of staining is obtained

(4) Stabilize TMB in an ice-cooled 5% ammonium heptamolybdate (AHM) solution[8,20] for 20 min (note without this initial stabilization, TMB reaction product may be lost in the second stabilization step.)

(5) Rinse sections 30 s in 0 1 M phosphate buffer (pH 7 4)

(6) Further stabilize TMB for 5–8 min with DAB-Co as described by Rye et al [25] prepare a solution of 50 mg DAB in 100 ml 0 1 M phosphate buffer (pH 7 4), and while stirring slowly add 2 5 ml of a 1% cobalt acetate solution dropwise, followed by addition of H_2O_2 (for a final dilution of 0 01% H_2O_2)

(7) Rinse 5 times in cold 0 01 M phosphate-buffered saline (PBS), pH 7 3

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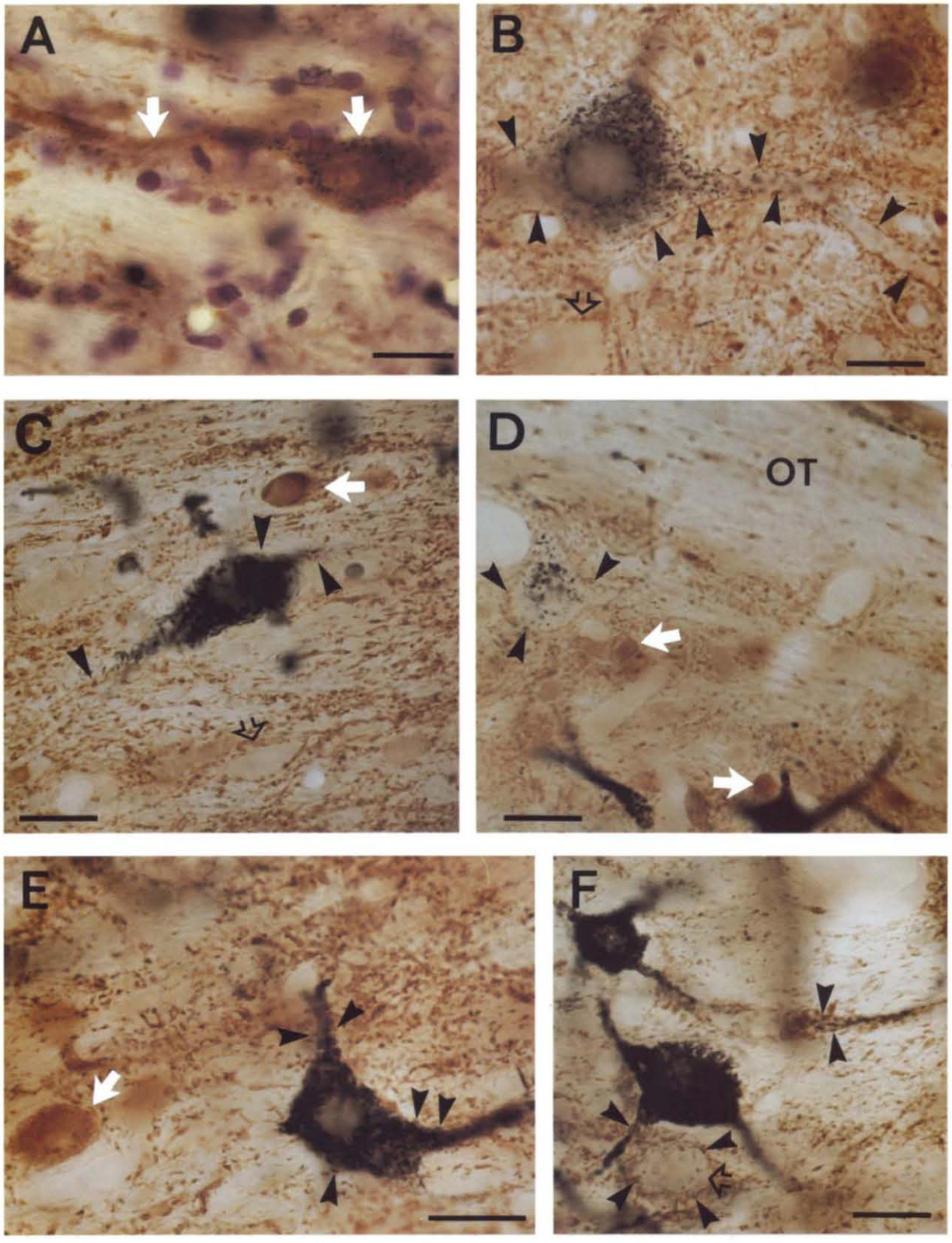
Fig 1 Micrographs showing the black tetramethylbenzidine (TMB) reaction product of retrogradely transported horseradish peroxidase (HRP) and the brown label of γ-aminobutyric acid (GABA) from an immunoreaction with GABA-antibody (Immuno Nuclear) in the same section Solid black arrowheads point to GABAergic terminals (puncta) on somata and proximal dendrites of retrogradely labeled cells Open black arrows point to cells not retrogradely labeled but outlined by dense clustering of GABAergic terminals White arrows point to GABAergic neurons A GABAergic neuron in the perigeniculate nucleus of a cat The brown soma and dendrite are clearly visible due to their high content of GABA In addition, the soma contains the granular black TMB reaction product due to the retrogradely transported HRP from an injection into the lateral geniculate nucleus B cell in the intermediate layers of the superior colliculus in the cat The cell was retrogradely labeled by an HRP injection into the inferior olive and GABAergic puncta (marked by black arrows) outline soma and dendrites These terminals probably stem from the nigrotectal projection[17] C–F cells in the nucleus of the optic tract retrogradely labeled by HRP injections into the inferior olive are not GABAergic C monkey (*Macaca mulatta*) D pigmented rat E,F cat In contrast to the collicular neuron (B) the pretectal neurons are contacted by only a few GABAergic terminals Bars = 25 μm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After completion of the HRP reaction, the free-floating sections were processed for GABA-immunocytochemistry using the unlabeled antibody peroxidase-antiperoxidase (PAP) method[32]. Sections were first incubated in 10% methanol/3% H_2O_2 to destroy the remaining activity of HRP and endogenous peroxidases. Non-specific background staining was suppressed incubating the sections for 30 min at room temperature in 10% normal swine serum (NSS) with 0.5% Triton X-100 in PBS. Then the sections were incubated for 12–16 h in a solution at 4 °C containing rabbit anti-GABA serum (Immuno Nuclear Corp) diluted 1:3000 in PBS containing 5% NSS and 0.5% Triton X-100. The specific antigen was labeled by subsequently incubating the sections in swine anti-rabbit IgG (Dakopatts) diluted 1:100 in the same solution for 1 h at room temperature (RT), and in rabbit PAP (Dakopatts) diluted 1:50 in PBS for 1 h at RT. The PAP complexes were visualized by a 5–10 min incubation in the freshly prepared solution consisting of 0.05% DAB in 0.05 M Tris-buffered saline (TBS), pH 7.6 and 0.01% H_2O_2 or by 20–30 min incubation using the DAB-glucose oxidase method of Itoh et al.[15] without cobalt. As a control, some sections were incubated in a solution in which the primary antibody was replaced with normal rabbit serum. After rinsing, mounting and drying, alternate sections were counterstained with 0.1% Cresyl violet before dehydrating and coverslipping.

The double-label method resulted in homogeneous brown labeled GABA-positive cell structures and granular black-labeled retrogradely transported HRP contained in neurons. The two labels were clearly distinguishable from one another by color and appearance of the reaction product (Fig. 1). GABA-immunoreactivity was found primarily in cell bodies and punctate profiles determined from electron microscopic studies to be axon terminals[24]. Due to limited ability of the antibody to penetrate the tissue GABA-positive labeling is restricted to the outer 8 μm of the section's surface. As a control, we first verified two critical points: (1) both markers can be independently demonstrated in the same cell, and (2) GABAergic terminals can be distinguished on retrogradely labeled cells. For the first point we performed the control experiment described by Fitzpatrick et al.[7]. The neurons of the perigeniculate nucleus are known to be GABAergic and they project

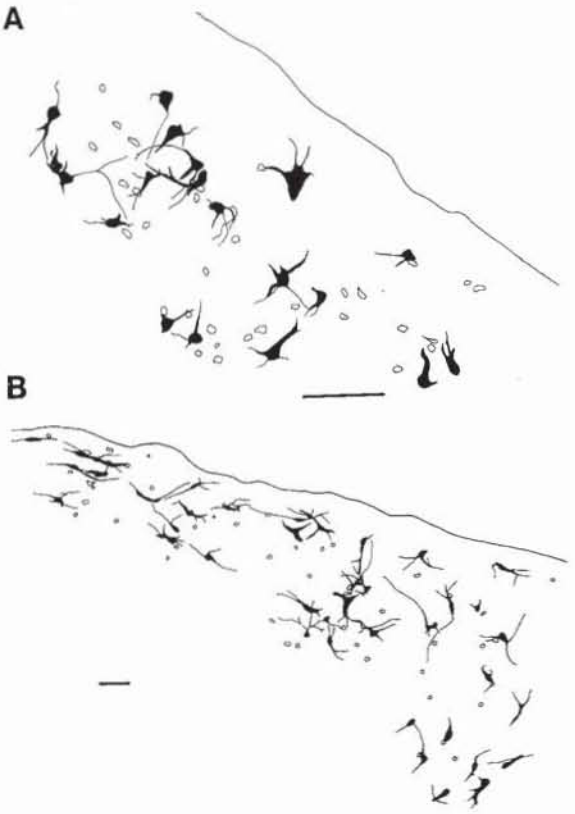

Fig. 2. Camera lucida drawings showing close proximity and size difference between cells in the nucleus of the optic tract projecting to the inferior olive (black) and GABAergic somata (open outlines). A: rat. B: cat. Bars = 100 μm.

to the lateral geniculate body. Following injections of HRP into the lateral geniculate body of a cat, we were able to demonstrate GABA-immunoreactivity in retrogradely labeled cells of the perigeniculate nucleus (Fig. 1A). To prove the second point, we examined the large cells in the intermediate layers of the superior colliculus which were retrogradely filled with HRP after IO injections. These neurons receive a substantial input from the substantia nigra pars reticulata[5,11]. Lu et al.[17] have demonstrated that these terminations are glutamate decarboxylase (GAD) immunoreactive. Fig. 1B shows a retrogradely labeled neuron in the intermediate layer of the cat's superior colliculus outlined by numerous GABA-immunoreactive terminals. These technical controls verify the validity of our observations concerning cells in the pretectum. In our material, in rat, cat and monkey the distribution of GABA-immunoreactive neuronal somata and terminals in the vicinity of retrogradely labeled pretectal cells resembles that

observed in previous studies using antibodies to GABA[21] or to the synthesizing enzyme glutamate decarboxylase (GAD)[9 19 35] (Fig 1D–F) In no case did we find any NOT or DTN neuron labeled for both the presence of transported HRP and GABA-immunoreactivity Measurements of cell body areas in the NOT revealed that in rat as well as cat, GABA-positive neurons are significantly smaller than retrogradely labeled cells in the same section (U-test, $P < 0.01$) The line drawings shown in Fig 2 illustrate the relative size and positions of the GABAergic intrinsic and non-GABAergic projection neurons in the NOT of rat and cat Similar relations were found in the monkey By counting every GABA-immunoreactive and Nissl-stained neuron in samples taken from the NOT, we estimated the relative proportion of GABA-positive neurons in this nucleus In both rat and cat, GABA-positive neurons made up about 30% of the total neuronal population in the NOT We have also examined the relative proportion of GABA-immunoreactive neurons in the superficial layer (SGS) of the superior colliculus, a structure that also receives and processes visual information from the retina and visual cortex The proportion of GABA-positive neurons in the SGS of the cat (47%) is significantly higher than in the corresponding area of the rat (30%)

Using the double-label method, it was possible to demonstrate in some cases retrogradely labeled neurons with their somata and/or proximal dendrites closely associated with GABA-positive puncta resembling presumed nerve terminals (Fig 1C–F) These projection neurons, however, were contacted by only a few GABA-positive terminals, suggesting that GABAergic interactions are weak or restricted to the distal part of the dendrites Since the appearance of stained GABA-positive terminals is restricted to the surface of the section and therefore the examination, too, no attempt has been made to systematically quantify the number of GABA-positive terminals associated with NOT neurons projecting to the IO

These results were critically dependent upon the formulation of the new two-step procedure for stabilizing the TMB–HRP reaction product Since the TMB-reaction product is normally unstable in neutral buffers, subsequent immunocytochemical staining usually causes a marked loss of reaction product This difficulty was overcome using the stabilization protocol of Rye et al [25], combining the sensitivity of TMB with the stability of the DAB-reaction product Furthermore, we found that the preceding incubation step with 5% AHM[8 20] prevented the loss of TMB-reaction product previously observed during the short DAB reaction

Our results specifically disprove the hypothesis[19] that the NOT-DTN cells send a GABAergic projection to the IO Similar double labeling studies with HRP injections into the MTN or contralateral NOT should be performed to point out whether the reciprocal connections between the NOT and other pretectal and accessory optic nuclei are GABAergic Such inhibitory interactions are assumed to contribute to the distinct direction selectivity of neuronal populations in these nuclei

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