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Anatomy and Embryology

Nucleus basalis Meynert neurons contain the vitamin D-induced calcium-binding protein (Calbindin-D 28k)*

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Summary. The neurons of the monkey basal nucleus of Meynert are shown to contain a protein indistinguishable from the chicken intestinal 28kd vitamin D-dependent calcium-binding protein (Calbindin-D 28k; CBP). CBP is thought to shuttle and buffer Ca^{++}-ions, thus regulating the intracellular calcium distribution and concentration. Our observation may engender interest in searching for the role of Vitamin D-metabolites, the CBP and calcium-ions in the physiology and pathology of nucleus basalis Meynert neurons.

Key words: Nucleus basalis Meynert – Calcium-ions – Vitamin D – Calbindin-D 28k – Neurodegenerative disorders

Introduction

The basal nucleus of Meynert (NBM) comprises clusters of large multipolar neurons in the basal forebrain (Meynert 1872; Kölliker 1896) which are known to provide the major cholinergic input to the cerebral cortex (Divac 1975; Mesulam et al. 1976; Lehmann et al. 1980; Mesulam et al. 1983). Central cholinergic pathways are thought to modulate behavioral states (Bartus et al. 1982) and a selective loss of cortical presynaptic cholinergic markers has been recognized in Alzheimer's and other neurodegenerative diseases (Tomlison et al. 1984).

We report in this paper the immunohistochemical detection of calbindin-D 28k [new denomination (Wassermann 1985) for the 28k vitamin D-induced calcium-binding protein (CBP) (Wassermann and Taylor 1966)] in the neurons of the basal nucleus of Meynert in primates. Calbindin-D 28k immunoreactivity in the brain has been reported previously by various groups (Taylor 1974; Roth et al. 1981a; Jande et al. 1981; Baimbridge et al. 1982, 1983; Feldman and Christakos 1983; Garcia-Segura et al. 1984) but no mention was made to the NBM.

The intracellular role of this protein has been related to the buffering or transport of calcium ions (Wassermann and Taylor 1966; Roth et al. 1981b). Together with the calcium-channels and the calcium-pumps of the plasma membrane, CBP may be involved in regulating the intracellular concentration of calcium-ions.

Our observations introduce a completely new element in the chemistry of the nucleus basalis Meynert and may

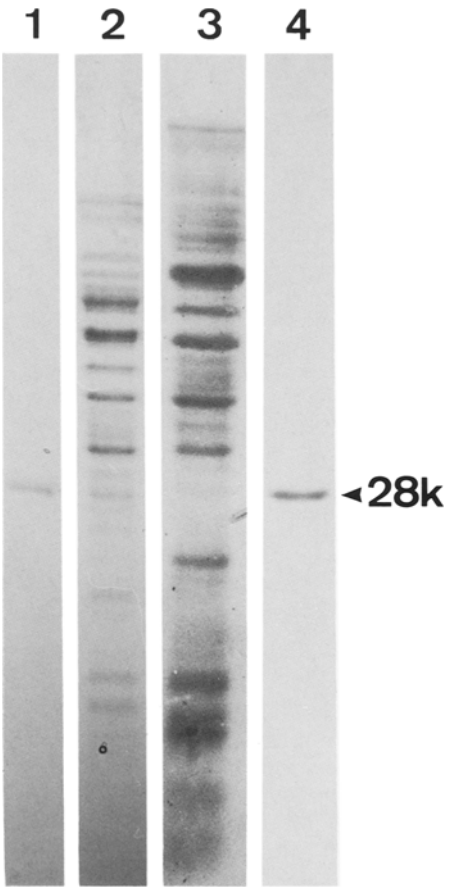

Fig. 1. Specificity-test for the antiserum against CBP used in this study. Purified chicken intestinal CBP (2 µg; *Lane 1*) and rhesus monkey NBM-extract (1.57 µg protein/ml; *Lane 2*) were run in parallel on an SDS-polyacrylamide-gel and blotted on nitrocellulose paper. The NBM-proteins transferred to the nitrocellulose were visualized in *Lane 3*. *Lane 4* shows a strip of the same nitrocellulose paper incubated with CBP antiserum. In the monkey NBM, the antiserum used in this study recognized a protein (*arrow*) with the same electrophoretic mobility as chicken CBP (28k)

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* Dedicated to Dr. Ennio Rossetti, Ambrì, on occasion of his 66th birthday

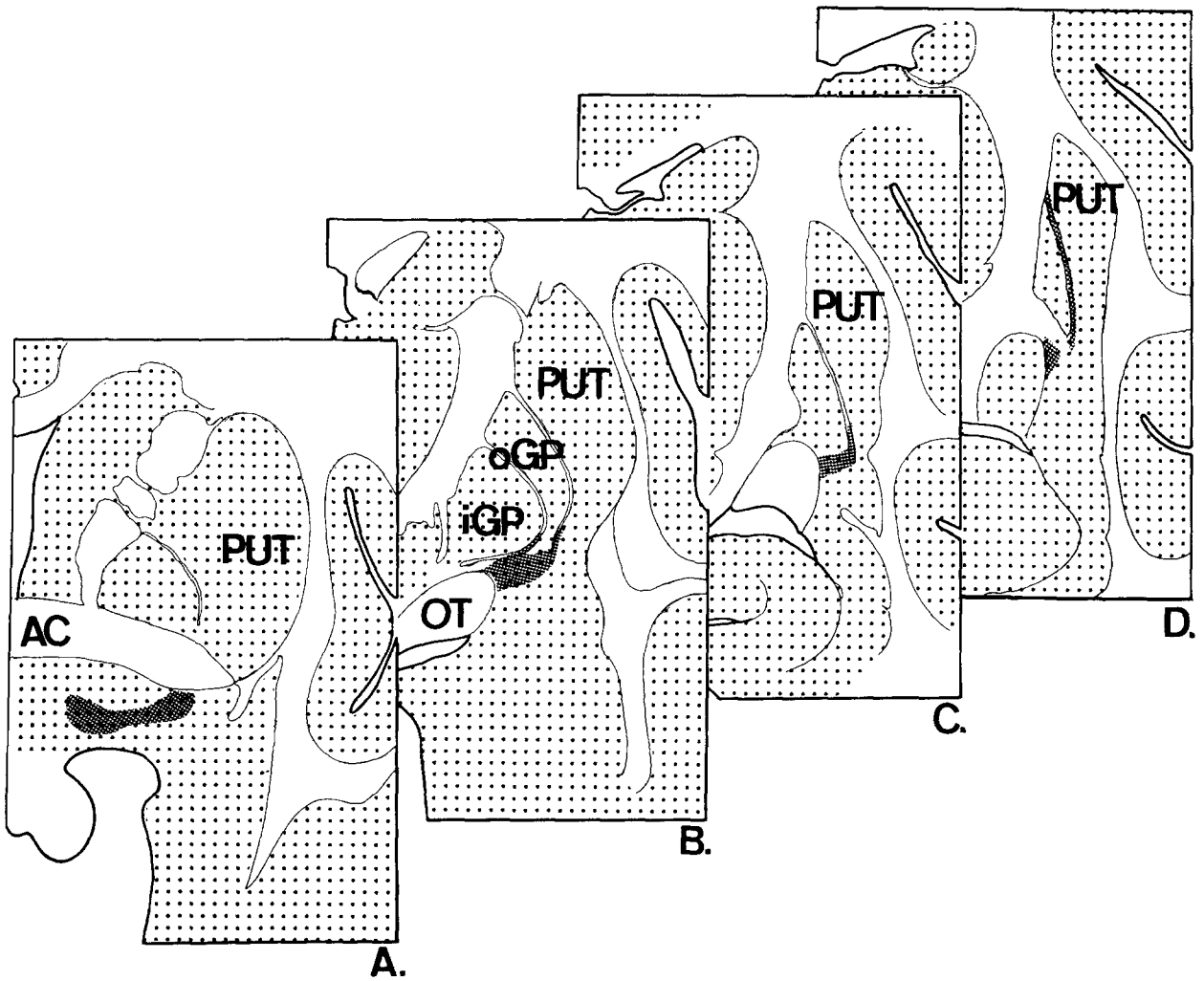

Fig. 2. Distribution of CBP-immunoreactive magnocellular elements (dark) in 4 coronal sections of the basal forebrain of the squirrel monkey (*Saimiri sciureus*). The figure is redrawn from the stereotactic atlas of Emmers and Akert (1963) and corresponds to the levels: A = 12.5, B = 10, C = 8 and D = 7. *AC* anterior commissure. *OT* optic tract. *PUT* putamen. *oGP* outer pallidal segment. *iGP* inner pallidal segment. The NBM region at level A corresponds approximately to the tissue dissected out from a fresh rhesus monkey brain and used for the "immunoblotting" of Fig. 1. The NBM regions approximately at level B and D are enlarged in Fig. 3, respectively Fig. 4

indicate a new direction for research in the field of neurodegenerative disorders.

Material and methods

For this study tissue from the following animals were used: 2 adult female squirrel monkeys (approx. 5 years old; Shamrock Farm, UK) and 3 adult male rhesus monkeys (approx. 9 years old; 2 for immunohistochemistry and one for the "immunoblotting"-experiment).

The method used in this study has been amply described in other publications (Celio and Heizmann 1981; Celio 1984) and only minor modifications were introduced. In principle the animals were anesthetized and perfused with cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer PH 7.3 or 4% formalin in the same buffer). After 15 min perfusion the brain was dissected out and postfixed for 12 h in the same fixative. After various washes, the tissue was sliced with a vibrating microtome into 50 µm thick coronal or horizontal sections and pre-incubated for 4 h with 0.1% Triton-X 100 in 0.1 M Tris-HCl buffer pH 7.3. The sections were then incubated with the antiserum against chicken intestinal 28k CBP, diluted 1:5,000 in Tris-buffer. After 72 h incubation on a shaker at 4° C, the sections were rinsed and incubated for 2 h with goat-anti-rabbit IgG (diluted 1:500, Miles) and afterwards for 4 h with the peroxydase-anti-peroxydase complex (diluted 1:500, Sternberger-Meyer Inc., Jarretsville Pike, Jarretsville Md USA; PAP-Method (Sternberger 1979)). The sections were incubated for 1 h in the developing solution consisting of DAB-HCl and H_2O_2 to reveal the peroxydase.

Some figures were obtained with the technique of "histography". By this method, the sections on the slide are directly projected on photographic paper which is subsequently developed and fixed. The image is therefore a negative of the original and the immunostained structures (brown in the original) appear white.

The antiserum used in this study has been used in various immunohistological and RIA studies (Roth et al. 1981; Christakos et al. 1979; Feldman and Christakos 1983; Garcia Segura et al. 1984; Celio 1984) and found to satisfy various criteria of specificity. Controls included fluid phase preadsorption of the antiserum with purified 28k chicken intestinal calcium-binding protein. No crossreactions with

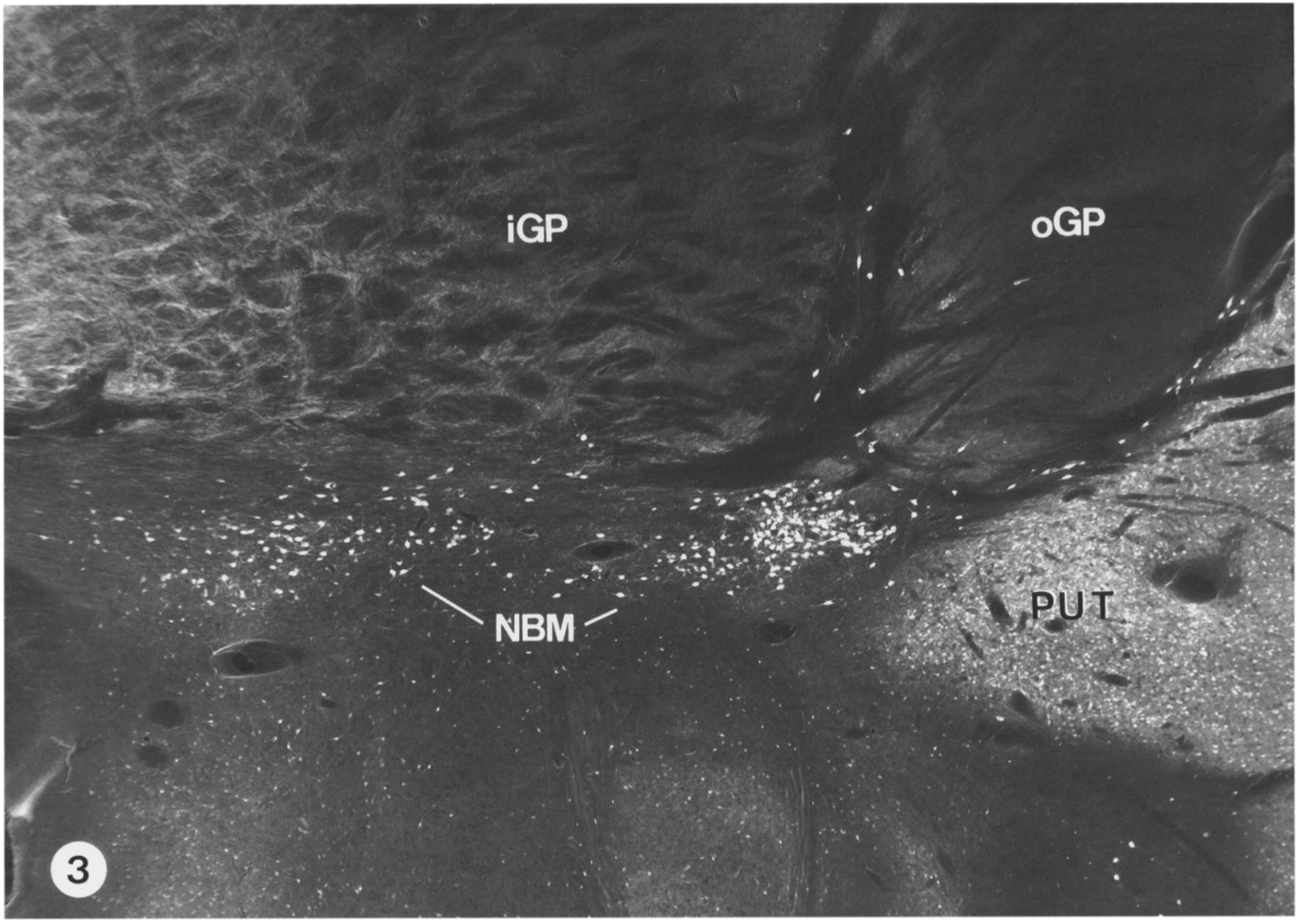

Fig. 3. Immunohistochemical detection of CBP in the neurons of the basal nucleus of Meynert (*NBM*) of a Rhesus monkey. The level depicted here corresponds approximately to the NBM region in the coronal section of Fig. 2C. The picture visualizes groups of CBP-positive, large, multipolar neurons and few single neurons infiltrating the medullary laminae between Putamen (*PUT*) and Globus Pallidus (*GP*) and between outer (*oGP*) and inner (*iGP*) pallidal segments. The contingent of CBP positive cells located between Putamen and Globus Pallidus increased at more caudal levels (see Figs. 2D, 4). Note the diffuse, but specific immunostaining in the Putamen. For abbreviations see Fig. 2. × 25

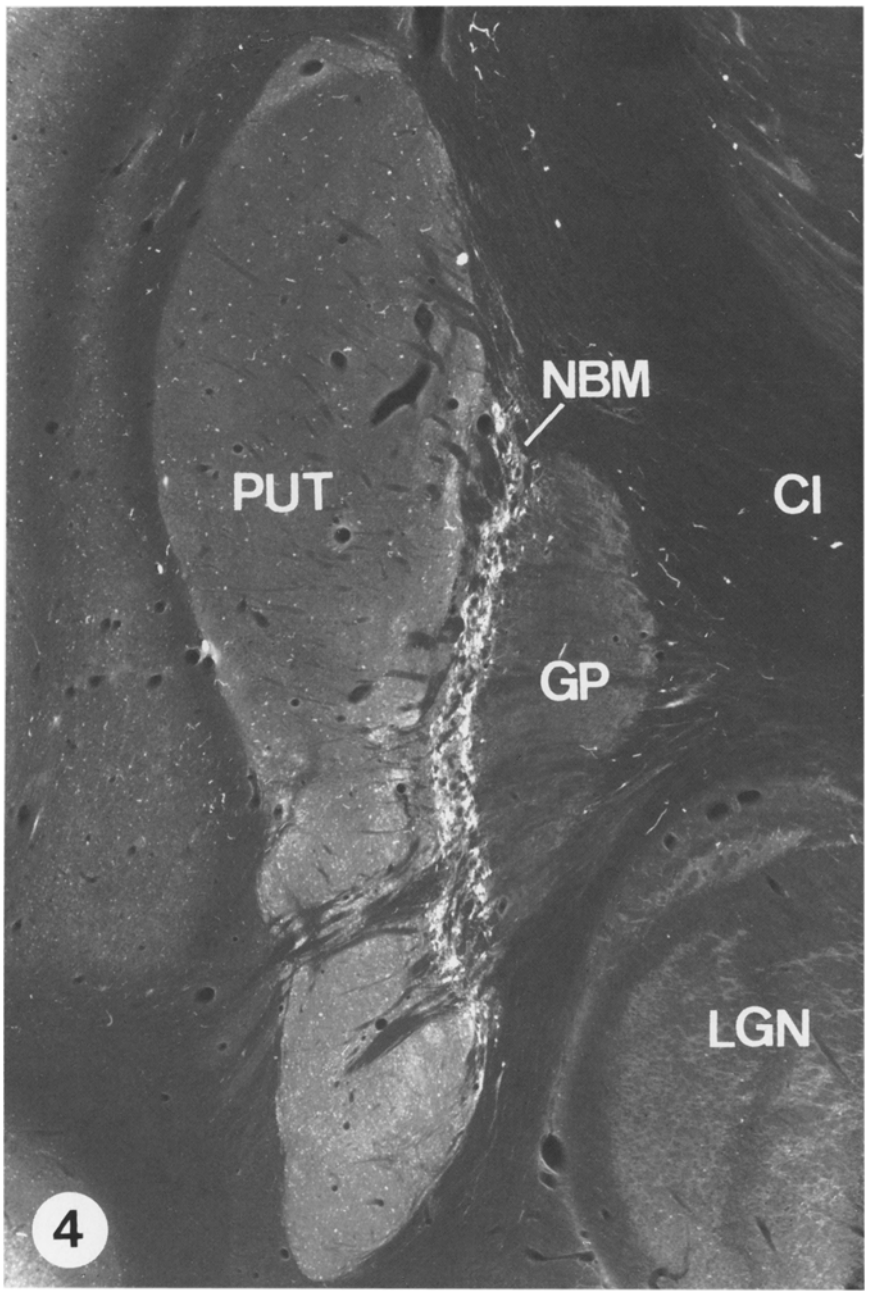

Fig. 4. Histography of the detection of CBP in a caudal section of the NBM corresponding to Fig. 2D. The CBP-positive neurons invade the medullary lamina between putamen (*PUT*) and globus pallidus (*GP*). The perfusion of this animal was not optimal and the endogenous peroxydase of erythrocytes show up in some blood vessels. *LGN* dorsal lateral geniculate nucleus. *CI* capsula interna. ×12

parvalbumin and calmodulin were detected in a dot-immunobinding assay.

To further characterize the protein recognized by the CBP antiserum in the NBM, an "immunoblot" after SDS-gel electrophoresis of a simian NBM extract was performed. For this, the subcommissural portion of the NBM (Fig. 2A) was dissected out from the brain of a Rhesus monkey killed by a overdose of anaesthetics. 10 µl of the homogenized tissue sample was subjected to SDS-gel-electrophoresis in parallel with purified chicken intestinal CBP (1 ug) and subsequently transferred to a sheet of nitrocellulose paper (Berchtold et al. 1984). The NBM-proteins transferred to the nitrocellulose were visualized with AuroDye^R (Janssen Pharmaceutica) and parallel strips of nitrocellulose were incubated with the CBP-antiserum diluted 1:2,000. The bound antibody was revealed by an indirect peroxydase method with 4-chlor-naphthol as a substrate.

Results

The "immunoblot" in Fig. 1 demonstrates that the antiserum utilized in this study recognizes in the NBM a protein which displays the same exact electrophoretic mobility as chicken 28k CBP. This "immunoblot" indirectly demonstrates that the antiserum used here does not cross-react with the cholinergic markers acetylcholinesterase (MW of the catalytic subunit ca. 80,000) and choline-acetyl-transferase (MW ca. 70,000) known to occur in NBM neurons.

In both squirrel and rhesus monkeys, the magnocellular components of the basal nucleus of Meynert displayed a strong immunoreactivity with the antiserum against CBP (Figs. 2, 3, 4). In primates the mapping of CBP immunoreactive NBM neurons coincided almost exactly with the NBM subdivision of Emmers and Akert (1963) in the squirrel monkey (Fig. 2) and with the Ch4 sector of Mesulam

et al. (1983) in the rhesus monkey. The cell bodies and the diverging thick stem dendrites were homogeneously stained. The dendrites intermingled together, and some cell processes penetrated into adjacent structures such as the Globus pallidus and the putamen. The immunolabeled perikarya were mostly ordered in compact collections and had an approximate diameter of 25–30 μm (Fig. 3). Large, CBP-positive interstitial neurons were also observed around the anterior commissure and in the basal portions of the medullary laminae separating the putamen from the pallidum and the inner from the outer pallidal segment (Fig. 3). At more caudal levels (Figs. 2, 4) CBP positive neurons occupied the whole medullary lamina between putamen and outer pallidal segment.

Discussion

This paper demonstrates the presence of the calcium binding protein calbindin-D 28k in the simian basal nucleus of Meynert by both immunohistological and biochemical methods.

From this study, we cannot definitely delineate if the axons and the terminals of NBM neurons in the cortex contain the CBP. In fact, other CBP-positive massive projections from the intralaminar thalamic nuclei obscured the picture in the cortical mantle. Nevertheless, in those cortical layers known to receive NBM projections (Saper 1984), CBP immunoreactivity was indeed present. It is also not as yet known if all, or only a subpopulation of, cholinergic neurons in the NBM contain CBP.

Antisera against some other known calcium binding proteins (Calmodulin, Parvalbumin, S-100) were tested on consecutive sections of the same material but none gave positive results in the NBM.

The NBM is not the only brain region which contains CBP and CBP is not restricted to cholinergic neurons in the brain. Many other neurons of the central and peripheral nervous system harbor this protein (Taylor 1974; Roth et al. 1981; Baimbridge et al. 1982, 1983; Jande et al. 1981; Feldmann and Christakos 1983; Garcia Segura et al. 1984; Celio 1984). Particularly worthy of note in this connection is the striking immunostaining of the adjacent putamen (Fig. 2) and caudate nucleus, of intralaminar thalamic nuclei, of a cortical interneuron subpopulation concentrated in layers II–III and V–VI, and of the substantia nigra (Celio 1984). It is important to realize that the NBM is also not the only CBP-positive subcortical system supplying the cerebral cortex. Among the other highly divergent pathways, the dorsal raphe nucleus and the locus coeruleus also contain CBP-immunoreactive neurons (Garcia-Segura et al. 1984).

Interestingly, several of the systems containing CBP have been implicated in the pathology of neurodegenerative disorders. Thus, seemingly disparate elements of neurodegenerative pathology all share an important chemical marker, that of CBP, a protein which is presumably critically important in these cells for intraneuronal homeostatic mechanisms involving calcium.

The suggestive name attributed by the discoverer of this chicken "intestinal" protein (Wassermann and Taylor 1966) lead to the spontaneous question if its synthesis in the brain is also Vitamin D-dependent. In the chick intestine 1,25-(OH_2)-D_3 acts as a steroid hormone regulating the synthesis of CBP at the nuclear level. There seem to be "no exceptions" to the rule that CBP occurs only in cells with specific receptors for 1,25 $(OH)_2$ D_3 (Norman et al. 1982). In the brain 1,25 $(OH)_2$ D_3 receptors are indeed present in nuclei of neurons of various hypothalamic regions (Stumpf 1982) but nothing is known about the NBM. Differences in the concentration of CBP in the brain of rachitic and vitamin-D repleted animals were not detected (Christakos et al. 1979). Notwithstanding, the brain CBP-concentration could be raised by long-term cholecalciferol administration in chronically rachitic chicks (Taylor 1977).

Calcium-ions are used as a transmembrane information transfer system. Their intracellular concentration is strictly controlled by a variety of mechanisms, of which the calcium-binding proteins may represent an essential component. It is well known that the uncontrolled elevation of the intracellular calcium concentration leads to protein denaturation and cell death (Campbell 1983). Malfunctioning or interference with the function of CBP in NBM neurons may render these cells more susceptible to intermittent calcium fluctuations and perhaps more prone to accumulate intolerable quantities of calcium.

Future studies will be necessary in order to determine if CBP is implicated in the pathogenesis of the degeneration of NBM and other neurons seen in various mental illnesses (Tomlison et al. 1984).

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