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GLUTAMATE-ENRICHED CHOLINERGIC SYNAPTIC TERMINALS IN THE ENTOPEDUNCULAR NUCLEUS AND SUBTHALAMIC NUCLEUS OF THE RAT

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Abstract—Several lines of evidence suggest that the cholinergic neurons of the mesopontine tegmentum contain elevated levels of glutamate and are the source of cholinergic terminals in the subthalamic nucleus and entopeduncular nucleus. The object of this study was to test whether cholinergic terminals in the entopeduncular nucleus and subthalamic nucleus, also express relatively high levels of glutamate. To address this, double immunocytochemistry was performed at the electron microscopic level. Perfuse-fixed sections of rat brain were immunolabelled to reveal choline acetyltransferase by the pre-embedding avidin–biotin–peroxidase method. Serial ultrathin sections of cholinergic terminals in both the entopeduncular nucleus and subthalamic nucleus were then subjected to post-embedding immunocytochemistry to reveal glutamate and GABA. Quantification of the immunogold labelling showed that choline acetyltransferase-immunopositive terminals and boutons in both regions were significantly enriched in glutamate immunoreactivity and had significantly lower levels of GABA immunoreactivity in comparison to identified GABAergic terminals. Furthermore, the presumed transmitter pool of glutamate i.e. that associated with synaptic vesicles, was significantly greater in the choline acetyltransferase-positive terminals than identified GABA terminals, albeit significantly lower than in established glutamatergic terminals. In the entopeduncular nucleus, a small proportion of cholinergic terminals displayed high levels of GABA immunoreactivity.

Taken together with other immunocytochemical and tracing data, the elevated levels of glutamate in cholinergic terminals in the entopeduncular nucleus and subthalamic nucleus, is further evidence adding weight to the suggestion that acetylcholine and glutamate may be co-localized in both the perikarya and terminals of at least a proportion of neurons of the mesopontine tegmentum. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: acetylcholine, basal ganglia, choline acetyltransferase, GABA, glutamate.

The entopeduncular nucleus (EP) together with the substantia nigra pars reticulata represent the major output nuclei of the basal ganglia in the rat. The activity of neurons in these nuclei is critical in the expression of basal ganglia function as they convey the final output signal of the basal ganglia to the thalamus and/or sub-cortical premotor regions. Neurons of the subthalamic nucleus also play a key role in the physiology and pathophysiology of the basal ganglia by virtue of their excitatory drive to virtually all nuclei of the basal ganglia.[9,11,39,49,61,62,68,74–76] The importance of the EP and subthalamic nucleus (STN) is exemplified by the recent findings that lesions of the internal segment of the globus pallidus, which is the primate equivalent of the EP,[3,29,51,57] and lesions or inactivation of the STN[2,6,7,17] are effective treatments for relieving the motor signs and symptoms of Parkinson's disease and of experimental models of Parkinson's disease. In view of the importance of the neurons in these nuclei in the expression of basal ganglia function, it is necessary to understand the nature of their afferent synaptic input that presumably influences their output.

The ascending and descending afferents of the EP and STN have been extensively studied at both light and electron microscopic levels.[9–12,15,18,80] One of their major afferent inputs arises in the mesopontine tegmentum[8,23] (MTg). This region is an interface for many neuronal circuits and has been reported to be involved in many functions including locomotion (see Ref. 36 for review), arousal and sleep-wake mechanisms (see Ref. 47 for review). The MTg has been shown to contain several neurochemically distinct populations of neurons including GABAergic, glutamatergic and cholinergic neurons.[24,25,26,46,47,53] It is clear from previous studies that the EP and STN

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Abbreviations: ChAT, choline acetyltransferase; DAB, diaminobenzidine; EP, entopeduncular nucleus; MTg, mesopontine tegmentum; PB, phosphate buffer; PBS, phosphate-buffered saline; STN, subthalamic nucleus; TBS, Tris-buffered saline.

receive cholinergic inputs[58,86–88] and inputs that are enriched in glutamate[8,23] from the MTg. Furthermore, it has been proposed that at least a subpopulation of the cholinergic neurons in the MTg also contain elevated levels of glutamate immunoreactivity.[25,26,53] These findings raise the possibility that the cholinergic neurons of the MTg that project to the EP and STN, and hence the cholinergic terminals in these regions, contain elevated levels of glutamate. The object of this study was therefore to address this question directly. The approach was to use a double immunocytochemical technique at the electron microscopic level to determine whether cholinergic terminals in the EP and STN, identified by choline acetyltransferase immunoreactivity, are also enriched in glutamate immunoreactivity.

Some of the findings of the present study have been published in abstract form.[21,22]

EXPERIMENTAL PROCEDURES

Preparation of tissue

All procedures were carried out on male Sprague–Dawley rats (250–500 g; Charles River, U.K.). Environmental conditions for the housing of the rats, and all procedures carried out on them were in accordance with the Animals (Scientific Procedures) Act 1986.

Five animals were deeply anaesthetized with pentobarbitone and then perfused with 200 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 over 20 min[60] and three animals were perfused with 300 ml of 2.5% glutaraldehyde and 2% paraformaldehyde in PB. Following fixation, the brains were removed from the cranium, divided into 5 mm-thick coronal slices and stored in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) at 4°C prior to further processing. Coronal sections (50 μm) of the entopeduncular nucleus and subthalamic nucleus were taken using a vibrating microtome and collected in PBS. Sections from the animals perfused with glutaraldehyde alone were then treated with 1% sodium borohydride in distilled water for 10 min and washed many times in PBS. The sections from both groups were freeze-thawed as described previously.[14]

Immunocytochemistry for choline acetyltransferase

Sections were incubated in monoclonal antibody to choline acetyltransferase (ChAT) derived from rat–mouse hybrid cells (mAb17, 2.5 μg/ml;[20,27,63,83]) overnight at room temperature or for 60 h at 4°C. After several washes in PBS the sections were incubated in a solution of biotinylated goat anti-mouse IgG (1:200; Vector Laboratories, Peterborough, U.K.) for 2 h at room temperature, followed by a 2 h incubation in an avidin–biotin–peroxidase complex (1:100 dilution; Vector Laboratories, Peterborough, U.K.) in PBS. The primary and secondary antibodies were diluted in PBS containing 1% bovine serum albumin and 10% normal goat serum. Peroxidase linked to the primary antibody by the avidin–biotin bridge was revealed by placing the sections in Tris buffer (0.05 M, pH 7.4) containing 0.025% diaminobenzidine (DAB) and 0.006% hydrogen peroxide for 10–15 min. The reaction was terminated by rinsing several times in Tris buffer and the sections were then placed in PBS until further processing.

Processing of sections for electron microscopy

All sections were placed flat at the bottom of a petri dish and postfixed in 1% osmium tetroxide (Oxkem, U.K.) in 0.1 M PB at pH 7.4 for 30 min. They were then washed in PB and dehydrated through a graded series of dilutions of alcohol. To enhance the contrast of the tissue in the electron microscope, the sections were stained with 1% uranyl acetate (Taab, U.K.) at the 70% alcohol phase. The sections were placed in two washes of propylene oxide for 10 min and then in resin (Durcupan, Fluka, U.K.) overnight. Finally, the sections were embedded in resin on microscope slides, placed in an oven and cured for 48 h at 60°C.

Post-embedding immunocytochemistry for glutamate and GABA

In order to test for the presence of fixed glutamate and GABA in ChAT-immunopositive and non-labelled terminals and boutons in the EP and STN, alternate ultrathin sections, cut on a Reichert–Jung Ultracut-E ultramicrotome, were collected on gold, Pioloform-coated single-slot grids and labelled by the post-embedding immunogold method. Glutamate-like and GABA-like immunoreactivity (hereafter referred to as glutamate and GABA immunoreactivity) were detected using polyclonal antisera directed against fixed amino acid–protein complexes.

Glutamate and GABA immunoreactivity were revealed on alternate grids using the method described previously.[8,23] Briefly, the grids were first washed in 0.05 M Tris buffer (pH 7.6) containing 0.9% NaCl (Tris-buffered saline; TBS) and 0.01% Triton X-100 (TBS–Triton) and then incubated overnight at room temperature on drops of a 1:500–5000 dilution of rabbit anti-glutamate antiserum (Arnel Products Co., New York, U.S.A.[1,40,66]) or a 1:5000–15000 dilution of rabbit anti-GABA antiserum (code 9[41,78,79]) in TBS–Triton. After several washes in TBS–Triton and one wash in TBS at pH 8.2, the grids were incubated for 1–1.5 h at room temperature in a 1:25 dilution of 15 nm gold-conjugated goat anti-rabbit IgG (BioCell, Cardiff, U.K.) in TBS at pH 8.2. The grids were washed in TBS at pH 8.2 and then in water, stained with 1% aqueous uranyl acetate for 1–1.5 h and then with lead citrate for 1–2 min. The sections were then examined in a Philips 410 electron microscope.

Analysis and quantification of glutamate- and GABA-immunolabelled material

Immunoreactivity for glutamate or GABA was detected by the presence of the electron-dense immunogold particles overlying peroxidase-labelled and unlabelled structures. In order to quantify the immunoreactivity, the cross-sectional areas of terminals and blood vessels in micrographs were calculated with the aid of a digitizing pad and MacStereology software. These values were then used to calculate the density (particles/μm^2) of immunogold particles overlying ChAT-immunopositive and non-immunopositive boutons or terminals. The values were then corrected for non-specific binding of the antibodies to tissue-free resin by subtracting the density of gold particles overlying the lumen of capillaries in the same ultrathin section. The corrected density of immunogold particles overlying terminals and boutons in glutamate-labelled sections in the EP was normalized and referred to as the index of glutamate immunoreactivity, by expressing it as a ratio of that overlying striatal-like terminals (average of nine terminals/section from three animals), i.e. terminals that have the morphology of striatal terminals,[11,15] form symmetrical synapses and have previously been shown to be GABAergic.[15,16] In the STN the labelling was normalized by expressing it as a ratio of that overlying terminals forming symmetrical synaptic specializations that have previously been shown to be GABAergic[12,74] (average of 30 terminals/section from three animals).

The density of immunogold particles overlying terminals and boutons in GABA-labelled sections in the EP was also normalized (index of GABA immunoreactivity), by expressing it as the ratio of the density overlying terminals forming asymmetrical synapses that have previously been shown to be glutamate-positive (average of seven terminals/section from three animals).[9,15,16,23,62,75] In the STN the corrected

density was normalized by expressing it as the ratio of the density overlying GABA-negative terminals forming asymmetrical synapses[8] (average of 14 terminals/section from two animals). Issues concerning the quantification of immunolabelling have been discussed extensively on a previous occasion.[12]

Choline acetyltransferase-immunopositive and non-labelled boutons or synaptic terminals randomly encountered during systematic scans of the glutamate-immunolabelled ultrathin sections were photographed and the density of immunogold particles overlying them calculated and normalized as described above. Most of the ChAT-immunoreactive structures and some of the non-labelled structures were also examined and photographed in the next serial section that was GABA-labelled.

Since glutamate in presynaptic terminals may subserve both a metabolic and a transmitter role, the density of immunogold particles overlying vesicles in each of the categories of boutons and synaptic terminals in the glutamate-immunolabelled sections was also calculated.[44,73] The area of bouton occupied by grouped synaptic vesicles (excluding single outlying vesicles) was measured; any area occupied by mitochondria was excluded. The values were expressed as the density of immunogold particles overlying the vesicle-rich areas of the boutons after subtraction of the background labelling overlying tissue-free resin. These values were normalized as described above.

The glutamate and GABA immunoreactivity of populations of boutons and the glutamate immunoreactivity associated with the vesicles in different populations of boutons was compared statistically using the Mann–Whitney *U*-test. P-values that were less than 0.01 following Bonferoni's correction for multiple testing were considered to be significant.

RESULTS

In confirmation of previous findings with antibodies against ChAT, cholinergic axons and terminals were observed at both the light and electron microscopic levels in the EP and STN.[8,23,86–88] In addition to the relatively sparse terminal labelling in the EP (in relation to the STN), a few ChAT-immunopositive perikarya and dendrites were observed along its medial and lateral borders that were continuous with the cholinergic (Ch4) cells of the nucleus basalis of Meynert. In the STN a dense network of cholinergic fibres and boutons was observed throughout the nucleus.

In the electron microscope, the characteristics of the ChAT-positive terminals were similar in the EP and STN. They were of small to medium size, contained variable densities of round or pleomorphic synaptic vesicles. All boutons that were seen to form synaptic contacts had asymmetrical membrane specializations (Figs 1A,B,D,F, 2C,D) and occasionally possessed subjunctional dense bodies (Fig. 2C,D). Synaptic targets were dendritic shafts and spines (Figs 1, 2).

Glutamate and GABA immunoreactivity in the entopeduncular nucleus

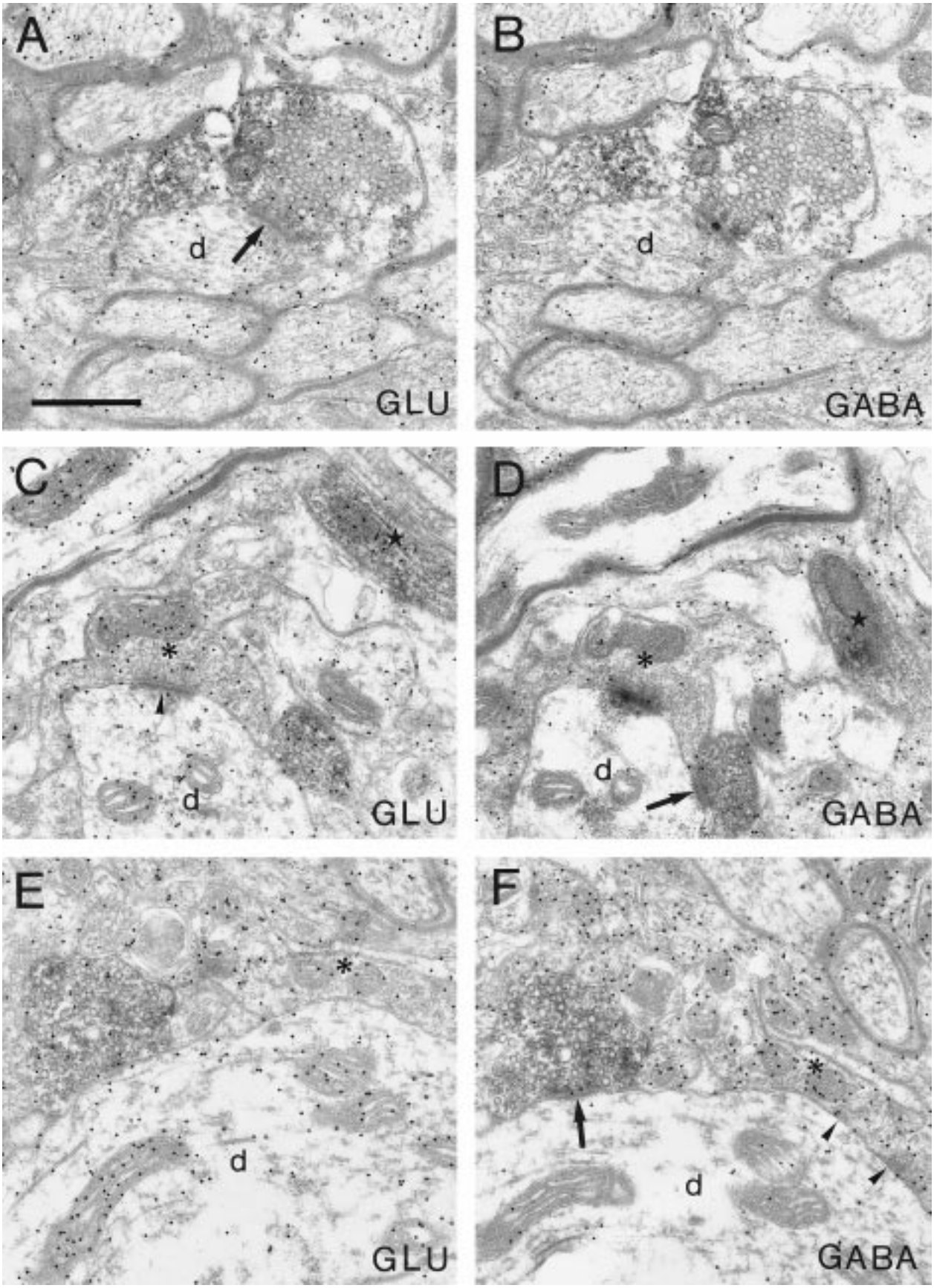
Post-embedding immunocytochemistry for glutamate and GABA was carried out on sections from three animals that were perfused with the paraformaldehyde/glutaraldehyde mixture. The pattern of glutamate and GABA immunolabelling in the EP was similar to that observed previously[15,23] and was similar for all three animals. Boutons forming asymmetrical synaptic contacts in the EP had levels of glutamate associated with them that were consistently higher than the levels associated with boutons forming symmetrical synaptic contacts (Fig. 1C,D). Boutons forming symmetrical synaptic contacts on the other hand, exhibited levels of GABA immunoreactivity greater than the levels associated with any other structure, including cell bodies, dendritic structures and the majority of terminals forming asymmetrical synaptic contacts (Fig. 1E,F).

The immunolabelling associated with four populations of terminals and boutons, defined on the basis of their origin or morphology was analysed quantitatively (Figs 1, 3, 4, 7; Table 1). (1) Striatal-like terminals were defined as irregularly-shaped terminals containing numerous round and ovoid synaptic vesicles, few mitochondria and forming symmetrical synaptic contacts (Fig. 1E,F).[9,15] (2) Pallidal-like terminals were defined as larger terminals containing vesicles that were usually congregated close to the active zone, several mitochondria and forming symmetrical synaptic contacts.[10,15] (3) Terminals forming asymmetrical synaptic contacts were classified only according to their synaptic specialization (Fig. 1C,D). (4) ChAT-immunopositive terminals were identified by the presence of peroxidase reaction product (Fig. 1A–F).

The results of the quantitative analysis of the levels of GABA and glutamate immunoreactivity in unlabelled terminals and boutons in the EP (Figs 3, 4; Table 1) were consistent with the results of previous studies on the amino acid content of the different populations of terminals and boutons.[15,23] Striatal-like terminals had significantly greater levels of GABA immunoreactivity than the levels associated with terminals forming asymmetrical synaptic contacts or ChAT-immunopositive terminals (Mann–Whitney *U*-test; $P<0.0001$ in each case). Terminals forming asymmetrical synaptic contacts, on the other hand, had levels of glutamate immunoreactivity that were significantly greater than the levels associated with striatal-like, pallidal-like or ChAT-immunopositive terminals ($P<0.0001$ in each case).

The terminals and boutons immunopositive for ChAT had high levels of glutamate immunoreactivity that were significantly greater than the levels associated with striatal-like terminals ($P<0.0001$) and levels of GABA immunoreactivity that were significantly lower than the levels associated with striatal-like or pallidal-like terminals ($P<0.0001$). Analysis of the level of GABA immunoreactivity in individual ChAT-immunopositive boutons revealed a small number of boutons with indices of immunoreactivity greater than five (Fig. 4; Table 1); these were considered to be GABA-immunopositive.

Analysis of the glutamate immunolabelling associated with synaptic vesicles in the EP (Fig. 7) revealed


A
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that the ChAT-positive terminals had a significantly greater density of immunogold particles associated with their vesicles (mean ± S.E.M.; 1.93 ± 0.14, n=64) than did the striatal-like (1.00 ± 0.05, n=92; P<0.0001) or pallidal-like (1.37 ± 0.19, n=28; P<0.001) terminals but was significantly less than that associated with terminals forming asymmetrical synapses (4.34 ± 0.18, n=59; P<0.0001). There was no significant difference between the density associated with striatal-like and pallidal-like terminals.

Glutamate and GABA immunoreactivity in the subthalamic nucleus

Post-embedding immunocytochemistry for glutamate and GABA was carried out on sections from three animals that were perfused with glutaraldehyde alone. The pattern of glutamate and GABA immunolabelling in the STN was similar to that observed previously[8,12] and was similar for all three animals (Figs 5, 6; Table 2). A small population of boutons forming asymmetrical synaptic contacts in the STN were GABA-immunopositive. Those that were immunonegative for GABA had levels of glutamate associated with them that were consistently higher than the levels associated with boutons forming symmetrical synaptic contacts, i.e. GABAergic terminals. Boutons forming symmetrical synaptic contacts, i.e. terminals probably derived from the globus pallidus, on the other hand, exhibited levels of GABA immunoreactivity greater than the levels associated with any other structure, including cell bodies, dendritic structures and the majority of terminals forming asymmetrical synaptic contacts.

The terminals and boutons that were immunopositive for ChAT had levels of glutamate immunoreactivity that were significantly greater than the levels associated with the pallidal-like boutons, but significantly less than the levels associated with terminals that formed asymmetrical synaptic contacts (P<0.0001 in each case). The levels of GABA immunoreactivity were significantly lower than those associated with pallidal-like boutons (P<0.0001) as previously reported.[8]

Analysis of the glutamate immunolabelling associated with synaptic vesicles in the STN (Fig. 8) revealed that the ChAT-positive terminals had a significantly greater density of immunogold particles associated with their vesicles (mean ± S.E.M.; 4.99 ± 0.37, n=137) than did the terminals forming symmetrical synaptic contacts i.e. pallidal-like (1.06 ± 0.14, n=118; P<0.0001) but was significantly less than that associated with terminals forming asymmetrical synapses (9.64 ± 0.58, n=78; P<0.0001).

DISCUSSION

The major findings of the present study confirm and extend our knowledge and understanding of the synaptology and chemical anatomy of the EP and STN. First, they show, in confirmation of previous findings, that the EP and the STN contain distinct populations of axonal boutons and terminals that are enriched in either glutamate or GABA immunoreactivity and that these differences correlate with differences in morphology and the type of synaptic specialization. Secondly, the present work confirms the presence of cholinergic terminals in the EP and STN. Thirdly, the most important finding is that the majority of cholinergic boutons and their synaptic vesicles in both the EP and STN are significantly enriched in glutamate immunoreactivity and are GABA-immunonegative. Additionally, in the EP, a small proportion of cholinergic terminals are GABA-immunopositive. The present findings therefore demonstrate directly that the majority of cholinergic axonal boutons in the EP and STN, which are probably derived from the MTg (see Introduction for references), are enriched in glutamate immunoreactivity compared to identified GABAergic

Fig. 1. Pairs of electron micrographs of serial sections of synaptic terminals in the entopeduncular nucleus that were immunolabelled to reveal choline acetyltransferase and GABA or glutamate. The ChAT was revealed by the avidin–biotin–peroxidase method using DAB as the chromogen. One of each pair was immunolabelled to reveal glutamate (A,C,E; GLU) and the other to reveal GABA (B,D,F; GABA), using the post-embedding immunogold method. The ChAT-immunopositive terminals form asymmetrical synaptic contacts (arrows) with the dendrites (d) of entopeduncular neurons. Two of the ChAT-immunopositive synaptic terminals are associated with relatively high levels of glutamate immunoreactivity (A=3.264, C=3.709) compared to terminals forming symmetrical synapses whereas the third has a level of glutamate immunoreactivity similar to terminals forming symmetrical synapses (E=1.255). The ChAT-immunopositive synaptic terminals have low levels of GABA immunoreactivity (B=1.299, D=0.713, F=0.362) compared to terminals forming asymmetrical synapses. In C and D the dendrite postsynaptic to the ChAT-immunopositive terminal also receives input from a terminal (asterisk) forming an asymmetrical synaptic contact (arrowhead). This terminal is rich in glutamate immunoreactivity (3.878) but GABA-immunonegative (0.555). A second ChAT-immunopositive axonal profile (star) which does not make a synapse in these micrographs is associated with a high level of glutamate immunoreactivity (2.258) and is GABA immunonegative (0.425). In E and F the dendrite postsynaptic to the ChAT-immunopositive terminal probably receives symmetrical synaptic input (arrowhead) from a striatal-like terminal (asterisk) which is associated with a low level of glutamate immunoreactivity (1.528) and a high level of GABA immunoreactivity (4.49). Adjacent to this terminal another striatal-like terminal (visible in micrograph F) makes symmetrical synaptic contact (arrowhead) with the same dendrite and has a low level of glutamate immunoreactivity (1.11) and a high level of GABA immunoreactivity (3.23). All micrographs are at the same magnification; Scale bar=0.5 μm.

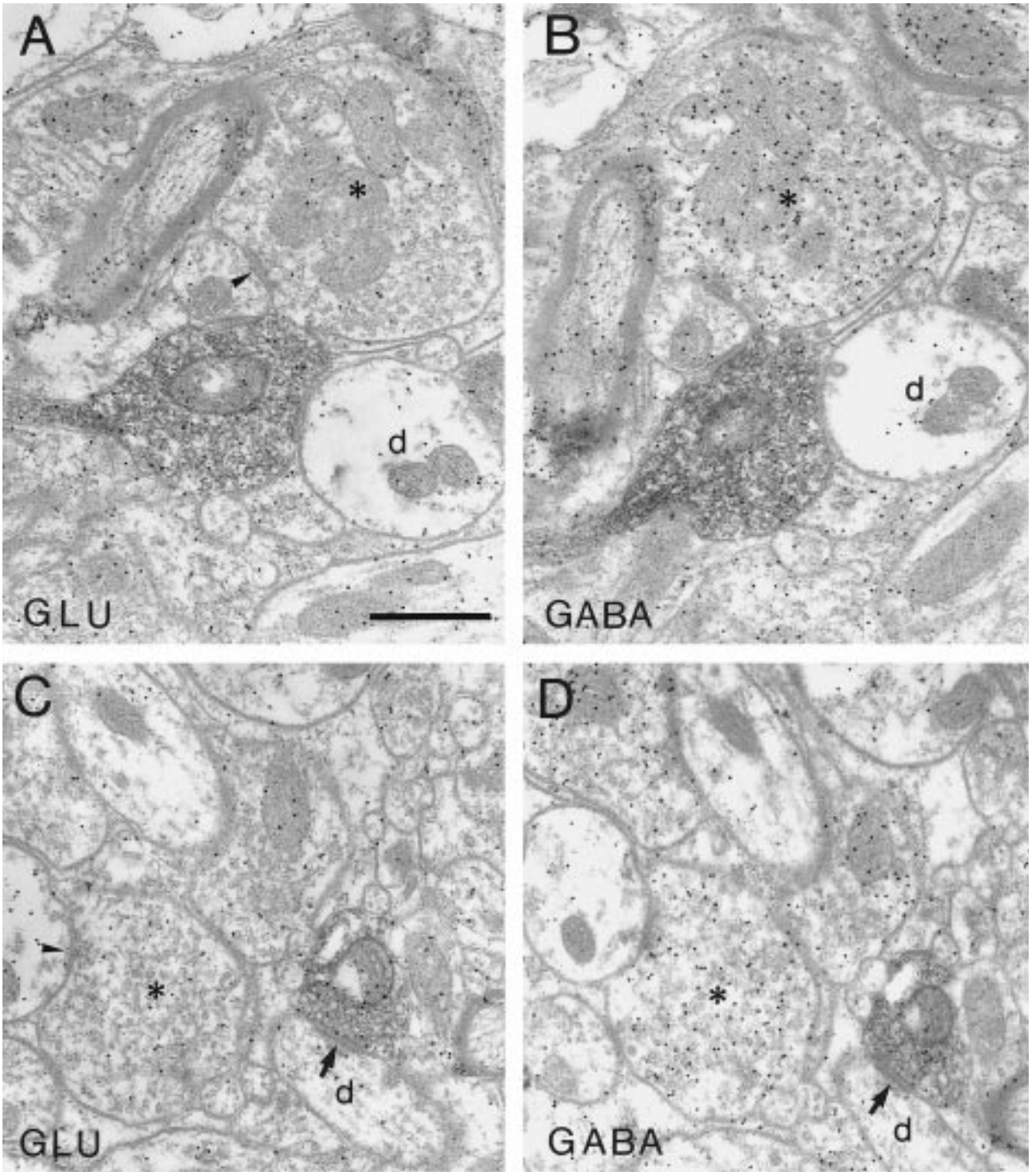

Fig. 2. Pairs of electron micrographs of serial sections of synaptic terminals in the subthalamic nucleus that were immunolabelled to reveal choline acetyltransferase and GABA or glutamate. The ChAT was revealed by the avidin–biotin–peroxidase method using DAB as the chromogen. One of each pair was immunolabelled to reveal glutamate (A,C; GLU) and the other to reveal GABA (B,D; GABA), using the post-embedding immunogold method. The ChAT-immunopositive terminals form asymmetrical synaptic contacts (arrows) with the dendrites (d) of subthalamic neurons. The ChAT-immunopositive synaptic terminals are associated with relatively high levels of glutamate immunoreactivity (A=2.701, C=5.266) and are GABA immunonegative (B=1.685, D=1.529). Additional terminals are present in both sets of micrographs (asterisks) that form symmetrical synaptic contacts (arrowheads). These pallidal-like terminals are glutamate-immunonegative (A=0.624, C=0.588), but are rich in GABA immunoreactivity (B=20.39, D=16.56). All micrographs are at the same magnification; Scale bar=0.5 μm.

Table 1. Glutamate and GABA immunoreactivity associated with different types of terminal in the entopeduncular nucleus

Terminal type	Glutamate immunoreactivity	GABA immunoreactivity
ChAT-immunopositive terminals	1.615 ± 0.08* (64) range: 0.24–3.709	1.916 ± 0.24** (46) range: 0–7.9
Striatal-like terminals	0.999 ± 0.05 (92) range: 0–2.236	7.06 ± 0.34 (71) range: 2.05–13.38
Pallidal-like terminals	1.434 ± 0.1 (28) range: 0.547–3.175	7.57 ± 0.62 (23) range: 2.623–13.97
Asymmetrical synaptic contacts	3.325 ± 0.12 (59) range: 1.271–7.15	1.0 ± 0.07 (59) range: 0–2.883

Values indicate the mean ± S.E.M. of the number of boutons shown in brackets and range of the indices of glutamate and GABA immunoreactivity in ChAT-immunopositive terminals and unlabelled terminals in the entopeduncular nucleus. The single asterisk indicates that ChAT-immunopositive terminals and boutons had significantly greater levels of glutamate immunoreactivity than striatal-like terminals but significantly less than the levels associated with terminals that formed asymmetrical synaptic contacts (Mann–Whitney *U*-test; $P<0.0001$ in each case). The double asterisk indicates that ChAT-immunopositive terminals and boutons had levels of GABA immunoreactivity that were significantly lower than the levels associated with striatal-like or pallidal-like terminals ($P<0.0001$).
The frequency distribution of this data is illustrated graphically in Figs 3 and 4.

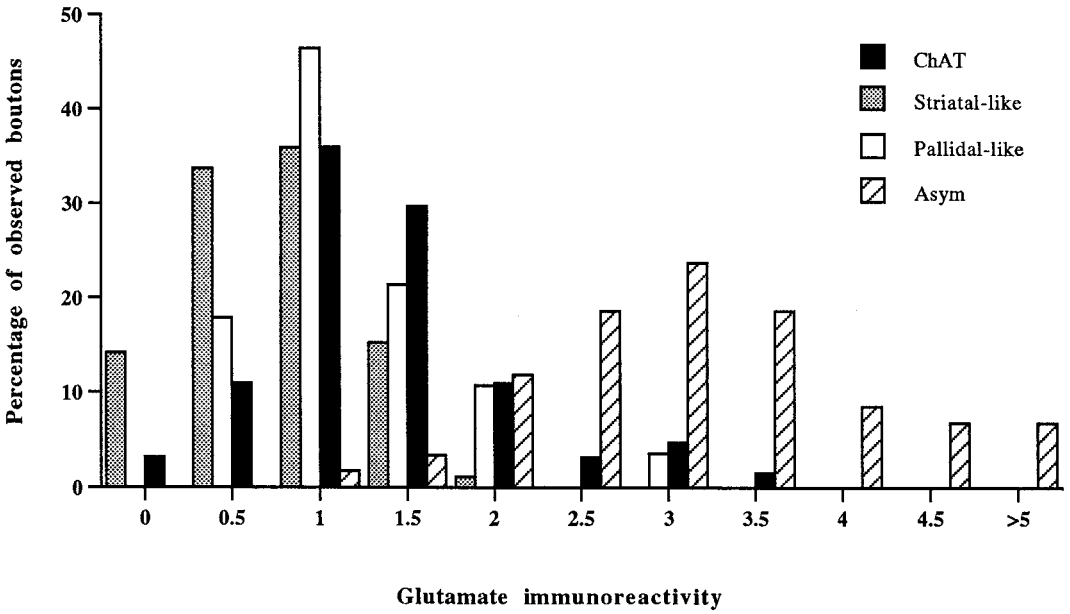

Fig. 3. Frequency distribution of the level of glutamate immunoreactivity associated with ChAT-positive and unlabelled synaptic terminals and boutons in the entopeduncular nucleus. The unlabelled terminals were divided on morphological grounds into striatal-like, pallidal-like and terminals forming asymmetrical synaptic contacts (Asym). Glutamate immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals or boutons to that overlying striatal-like terminals forming symmetrical synapses in the same section. The ChAT-immunopositive, pallidal-like and terminals forming asymmetrical synaptic contacts are significantly enriched in glutamate compared to that found in striatal-like synaptic terminals (Mann–Whitney *U*-test; $P<0.0001$).

terminals. This, together with findings from previous studies,[25,26,53] adds weight to the suggestion that a population of terminals in the EP and STN may use both acetylcholine and glutamate as neurotransmitters.

Technical considerations

Technical issues concerning the localization and quantification of immunolabelling using the post-embedding immunogold method have been discussed extensively on previous occasions[12,64,78] and will not be discussed further here. However three points should be noted. First, due to differential fixation of the EP and STN, which presumably relates to differences in vascularization, it was necessary to use different fixatives to obtain optimal immunolabelling for ChAT and the amino acids. Indeed, in the STN, in which fixation was presumably better, there were clearer differences in the indices of glutamate immunoreactivity between putative glutamatergic terminals and GABAergic terminals. For this reason it is not possible to directly compare the levels of immunoreactivity for the amino acids obtained in the two nuclei. Secondly, the levels of amino acid immunoreactivity observed in the cholinergic boutons are likely to be an underestimate of the true values as the immunoperoxidase reaction product

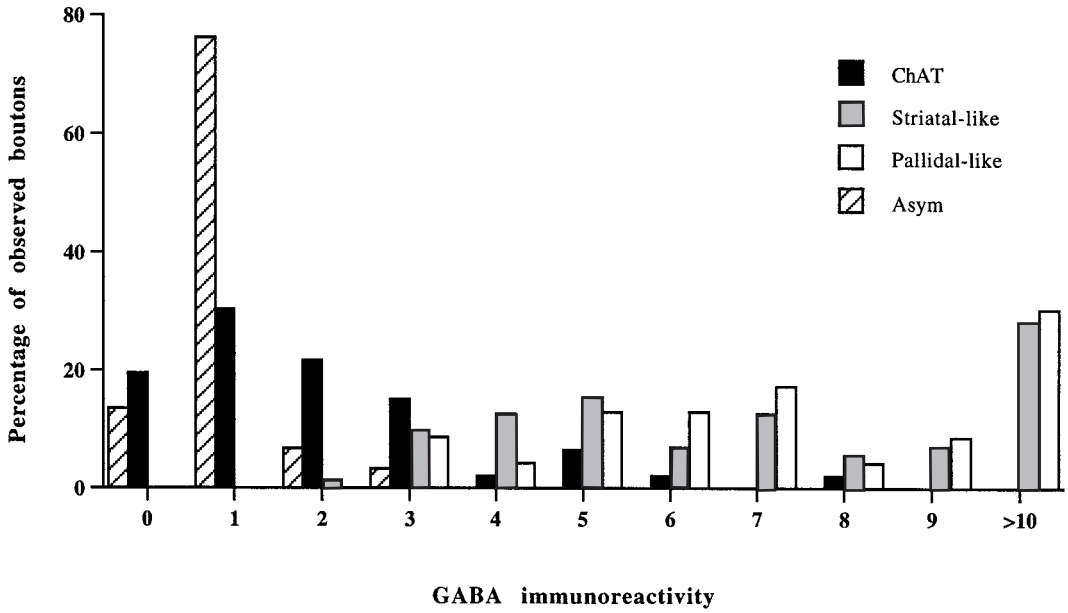

Fig. 4. Frequency distribution of the level of GABA immunoreactivity associated with ChAT-positive and unlabelled synaptic terminals and boutons in the entopeduncular nucleus. The unlabelled terminals were divided on morphological grounds into striatal-like, pallidal-like and terminals forming asymmetrical synaptic contacts (Asym). GABA immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals or boutons to that overlying terminals forming asymmetrical synapses in the same section. ChAT-immunopositive terminals and terminals forming asymmetrical synapses have significantly lower levels of GABA immunoreactivity compared to that associated with striatal-like and pallidal-like terminals (Mann–Whitney *U*-test; $P<0.0001$).

may suppress immunolabelling for the amino acids by the post-embedding immunogold method.[5,35] This may account, at least in part, for the significantly lower levels of glutamate immunoreactivity in the ChAT-immunopositive terminals compared to terminals forming asymmetrical synapses, although the degree of quenching is not known. Furthermore, other factors may account for, or contribute to, this difference and it is not necessarily the case that different populations of glutamatergic terminals should contain similar levels of glutamate immunoreactivity. Thirdly, due to the inherent variability of the post-embedding immunogold technique, it is preferable to normalize the density of immunolabelling with respect to known immunonegative structures. This procedure in itself may cause problems because of the ubiquitous nature of the glutamate that subserves metabolic roles and because glutamate is the precursor of GABA. There is, however, debate in the literature as to the method of analysis most suitable for establishing whether the glutamate present in a presynaptic terminal solely subserves a metabolic role or also has a transmitter function. Some authors favour whole bouton analysis[8,12,13,23,32,64,67,77] whereas others have suggested that metabolic and transmitter pools of glutamate can be distinguished by analysis of the density of glutamate immunolabelling associated with the synaptic vesicles.[44,73] An additional refinement of the technique, where the density of glutamate immunolabelling overlying vesicles is expressed as a ratio of that overlying the axoplasmic matrix in the same bouton, was not possible in this study due to the high packing density of vesicles in all the types of terminal we analysed. Consequently we examined the density of glutamate immunolabelling overlying the boutons as a whole and that overlying the vesicles in both the EP and STN.

Glutamate and GABA immunoreactivity in cholinergic terminals in the entopeduncular nucleus

The post-embedding immunocytochemistry for glutamate and GABA revealed that ChAT-immunopositive terminals in the EP are significantly enriched in glutamate immunoreactivity compared to a population of known GABAergic terminals i.e. those terminals we characterized as neostriatal in origin. This was not, however, the case for those with the features of pallidal terminals when the density in the terminals as a whole was considered. High levels of glutamate immunolabelling have been observed previously in terminals derived from the globus pallidus.[23,72] The neurons of the globus pallidus are known to be GABAergic and to have a high basal activity.[28,33,50,59,65] The relatively high levels of glutamate may thus be due to a high metabolic requirement for glutamate and/or due to a high turnover rate of GABA. The fact that the levels of glutamate are not different from those of the ChAT-positive

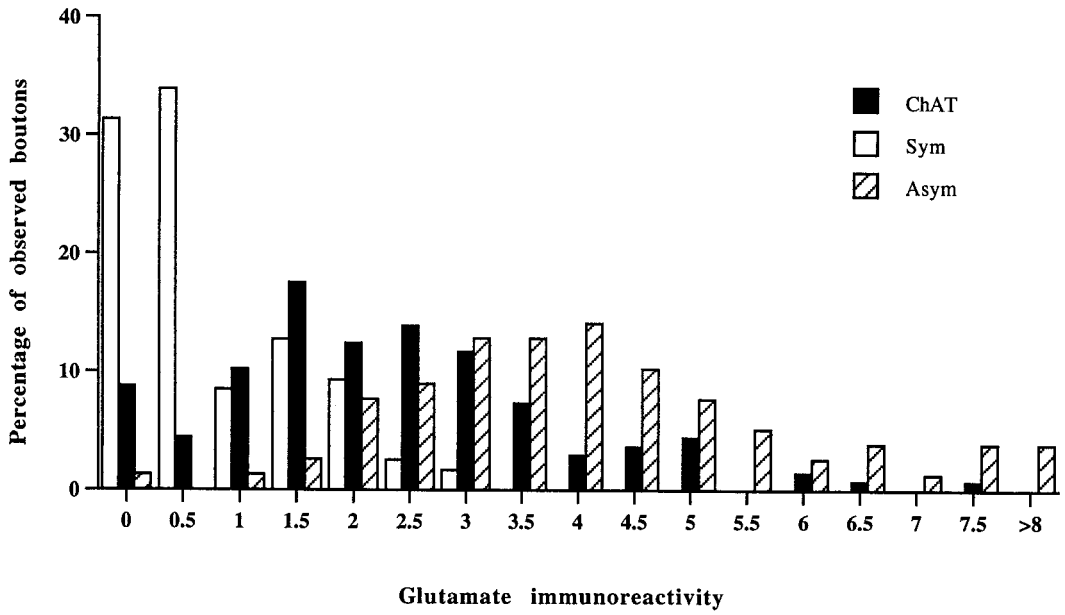

Fig. 5. Frequency distribution of the level of glutamate immunoreactivity associated with ChAT-positive and unlabelled synaptic terminals and boutons in the subthalamic nucleus. The unlabelled terminals were divided on the basis of their synaptic specializations into those forming symmetrical (Sym) and those forming asymmetrical synaptic contacts (Asym). Glutamate immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals or boutons to that overlying terminals forming symmetrical synapses in the same section. The ChAT-immunopositive terminals and terminals forming asymmetrical synaptic contacts are significantly enriched in glutamate compared to that found in terminals forming symmetrical synaptic contacts (Mann–Whitney *U*-test; $P<0.0001$).

terminals raises the possibility that the glutamate immunolabelling in cholinergic terminals may also be due to a high metabolic requirement for glutamate. However, when the presumed transmitter pool of glutamate was determined, i.e. that associated with vesicles, ChAT-positive terminals had a significantly higher level of glutamate immunoreactivity compared to both striatal-like and pallidal-like terminals. Furthermore, the levels associated with the vesicles of pallidal-like terminals were not significantly different from those associated with the vesicles of striatal-like terminals.

The ChAT-immunopositive terminals are enriched in glutamate immunoreactivity when considered as a population, however, there are important features that should be noted. The levels of glutamate in the ChAT-positive terminals are significantly lower than the levels in terminals that form asymmetrical synaptic specializations. These terminals are mainly derived from the subthalamic nucleus and are therefore likely to glutamatergic.[11,49,62,75] The lower levels in ChAT-immunopositive terminals may represent a real difference in the concentration of glutamate in the two populations of terminals, but may equally be due to the quenching effect of the peroxidase reaction product or, alternatively, it may be that the population of ChAT-containing terminals is heterogeneous. Indeed, it is clear from the frequency distributions (Figs 3, 7) that a number of ChAT-positive terminals have low levels of glutamate immunoreactivity that are comparable to known non-glutamatergic terminals i.e. striatal terminals. The data imply therefore, that cholinergic terminals in the EP are heterogeneous with respect to their content of glutamate. This heterogeneity is consistent with the findings that only a sub-population of the cholinergic neurons in the pedunculopontine region are glutamate-immunoreactive.[24,26,53] Statistical tests to determine the presence of two populations in a mixture could not be applied to our data since the tests require the assumptions that both sub-populations are normally distributed and that they have a common variance.[45]

It was not possible to determine the origin of the population of cholinergic terminals that possessed elevated levels of glutamate immunoreactivity but the weight of evidence suggests that they are derived from cholinergic neurons of the MTg. Thus, (1) the MTg contains populations of cholinergic neurons.[58,81,86–88] (2) This region projects to the EP.[19,23,30,38,43,54,55,69,70,82,88] (3) Tracing studies combined with ChAT immunocytochemistry have shown that it is the cholinergic neurons of the MTg that project to the EP.[19,55,88] (4) No other sources of cholinergic terminals in the EP have been identified.[86,87] (5) Tracing studies combined with immunolabelling for glutamate have demonstrated

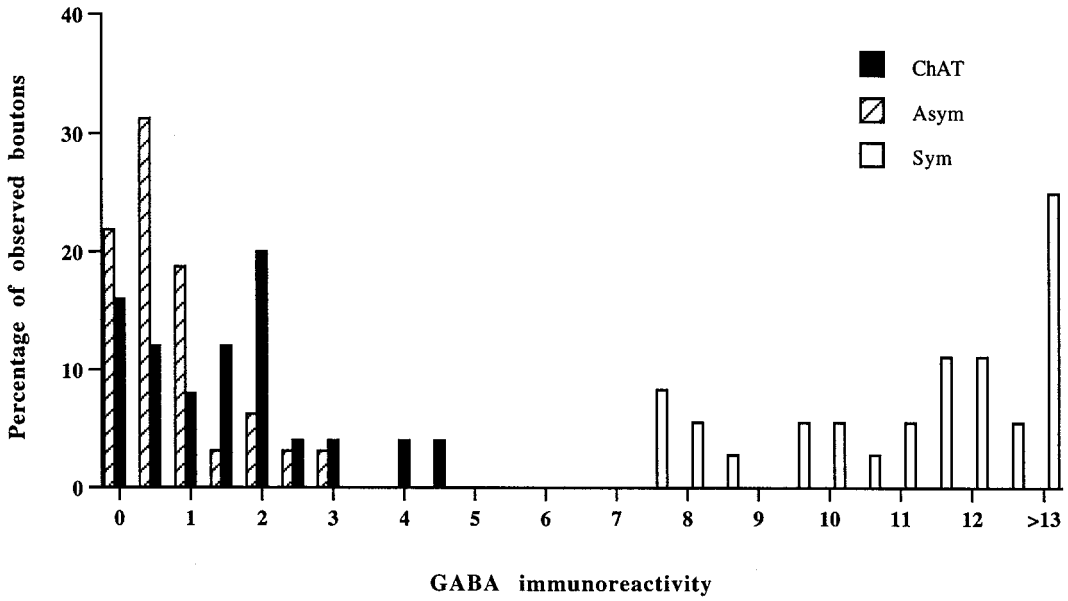

Fig. 6. Frequency distribution of the level of GABA immunoreactivity associated with ChAT-positive and unlabelled synaptic terminals and boutons in the subthalamic nucleus. The unlabelled terminals were divided on the basis of their synaptic specializations into those forming symmetrical (Sym) and those forming asymmetrical synaptic contacts (Asym). GABA immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals or boutons to that overlying terminals forming asymmetrical synapses in the same section. ChAT-immunopositive terminals and terminals forming asymmetrical synapses have significantly lower levels of GABA immunoreactivity compared to that associated with terminals forming symmetrical synaptic contacts (Mann–Whitney *U*-test; $P<0.0001$).

Table 2. Glutamate and GABA immunoreactivity associated with different types of terminal in the subthalamic nucleus

Terminal type	Glutamate immunoreactivity	GABA immunoreactivity
ChAT-immunopositive terminals	2.517 ± 0.13* (137) range: 0–7.734	1.69 ± 0.27** (21) range: 0–4.704
Symmetrical synaptic contacts	0.936 ± 0.07 (118) range: 0–3.34	13.106 ± 0.8 (32) range: 7.64–23.608
Asymmetrical synaptic contacts	4.342 ± 0.19 (78) range: 0.49–9.15	1.0 ± 0.15 (28) range: 0–3.329

Values indicate the mean ± S.E.M. of the number of boutons shown in brackets and range of the indices of glutamate and GABA immunoreactivity in ChAT-immunopositive terminals and unlabelled terminals in the subthalamic nucleus. The single asterisk indicates that the ChAT-immunopositive terminals and boutons had levels of glutamate immunoreactivity that were significantly greater than those associated with terminals that formed symmetrical synaptic contacts but significantly less than the levels associated with terminals that formed asymmetrical synaptic contacts (Mann–Whitney *U*-test; $P<0.0001$ in each case). The double asterisk indicates that the levels of GABA immunoreactivity were significantly lower than the levels associated with terminals forming symmetrical synaptic contacts ($P<0.0001$).
The frequency distribution of this data is illustrated graphically in Figs 5 and 6.

that at least part of the projection from the MTg to the EP is glutamate enriched.[23]

The distribution of GABA immunoreactivity in ChAT-immunopositive terminals in the EP (Fig. 4) showed that as a population they are GABA-negative, having a level of GABA immunoreactivity not significantly different from that in terminals forming asymmetrical synaptic contacts and significantly less than that in striatal-like and pallidal-like terminals. However, a number of terminals with indices of GABA immunoreactivity greater than five were observed. This level of immunoreactivity is generally associated with terminals that use GABA as a neurotransmitter. The origin of these terminals, however, is unclear. They are unlikely to be derived from the MTg as cholinergic neurons in this region do not express glutamic acid decarboxylase.[34,46,52] One possibility is that they are local collaterals of the adjacent cholinergic neurons of the basal forebrain, that sometimes lie within the EP, as a small proportion of these neurons have been shown to contain glutamate decarboxylase-like immunoreactivity[52] and they have been suggested to be the origin of synaptic terminals in the striate cortex of the cat that are immunoreactive for both GABA and ChAT.[5]

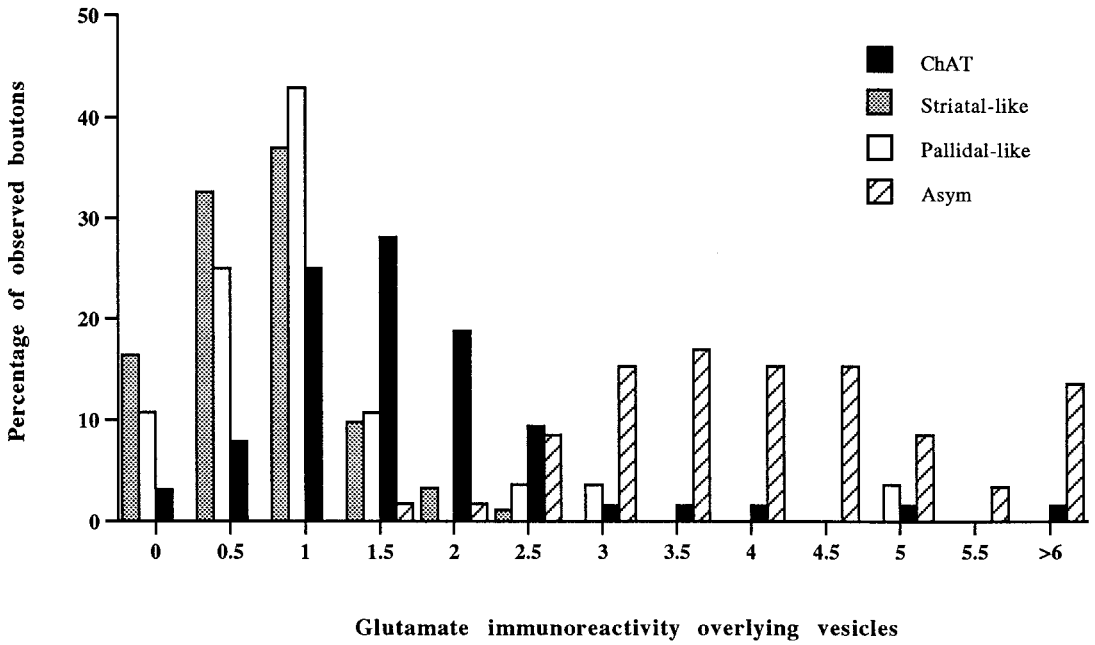

Fig. 7. Frequency distribution of the density of immunogold particles overlying vesicles in ChAT-positive and unlabelled synaptic terminals and boutons in glutamate-immunolabelled sections in the entopeduncular nucleus. The unlabelled terminals were divided on morphological grounds into striatal-like, pallidal-like and terminals forming asymmetrical synaptic contacts (Asym). Glutamate immunoreactivity is represented as the ratio of the density of immunogold particles overlying the vesicles in synaptic terminals or boutons to that overlying the vesicles in striatal-like terminals forming symmetrical synapses in the same section. The ChAT-immunopositive terminals and terminals forming asymmetrical synaptic contacts have significantly greater levels of vesicle-associated glutamate immunolabelling than striatal-like and pallidal-like synaptic terminals (Mann–Whitney *U*-test; $P<0.001$).

Glutamate and GABA immunoreactivity in cholinergic terminals in the subthalamic nucleus

The post-embedding immunocytochemistry for glutamate revealed that ChAT-immunopositive terminals in the STN are significantly enriched in glutamate immunoreactivity compared to identified GABAergic terminals, i.e. terminals forming symmetrical synaptic contacts and presumably derived mainly from the globus pallidus. Furthermore, the immunolabelling associated with vesicles in ChAT-positive terminals was significantly greater than that associated with the pallidal-like terminals although significantly lower than the terminals forming asymmetrical synapses. The present studies also confirm that ChAT-immunopositive terminals in the STN are GABA-immunonegative.[8] As was the case with the EP, the origin of the cholinergic terminals in the STN was not identified but the weight of evidence also suggests they are derived from the MTg.[8,86–88]

The frequency distributions (Figs 5, 8) reveal that in addition to the main body of the population exhibiting high levels of glutamate immunoreactivity, there are a number of ChAT-immunopositive terminals with low levels of glutamate immunoreactivity. The levels of glutamate immunoreactivity in these terminals is in the range of those obtained for known GABAergic terminals. As is the case in the EP, these findings suggest that in the STN there may be more than one population of cholinergic terminals with respect to their levels of glutamate, although statistical analysis to test this could not be applied.[45] This suggestion is consistent with the presence of glutamate immunoreactivity in only a sub-population of cholinergic neurons in the MTg.[25,26,53]

Functional considerations

Elevated levels of a putative transmitter in terminals fulfils one of the criteria that the substance acts as a neurotransmitter. The detection of elevated levels of glutamate in synaptic terminals by immunocytochemical means, is consistently observed in known or putative glutamatergic terminals.[8,12,13,23,32,48,56,64,67,68,77,84,85] Our findings that cholinergic terminals in the EP and STN, that are probably derived from the cholinergic neurons of the MTg, contain elevated levels of glutamate implies that they may use glutamate as a transmitter. The fact that they also have the ability to synthesize acetylcholine implies that there is co-existence of the neurotransmitters, acetylcholine and glutamate, in a sub-population of terminals in the entopeduncular nucleus and subthalamic nucleus, that are derived from the mesopontine tegmentum. Thus our data, other immunocytochemical and tracing

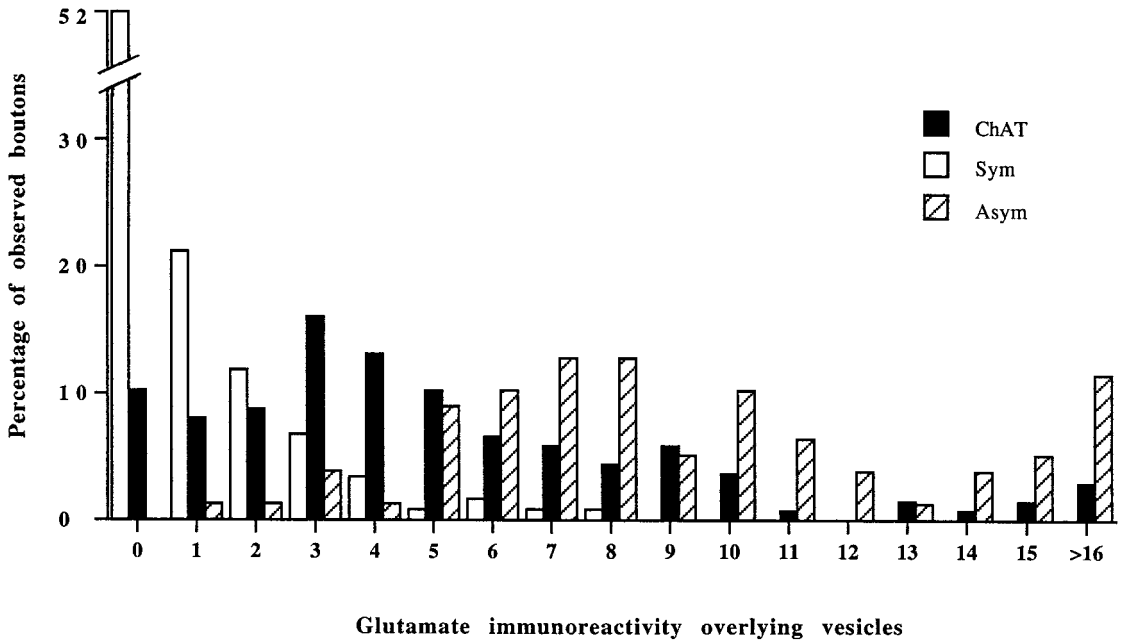

Fig. 8. Frequency distribution of the density of immunogold particles overlying vesicles in ChAT-positive and unlabelled synaptic terminals and boutons in glutamate-immunolabelled sections in the subthalamic nucleus. The unlabelled terminals were divided on the basis of their synaptic specializations into those forming symmetrical (Sym) and those forming asymmetrical synaptic contacts (Asym). Glutamate immunoreactivity is represented as the ratio of the density of immunogold particles overlying the vesicles in synaptic terminals or boutons to that overlying vesicles in terminals forming symmetrical synapses in the same section. The ChAT-immunopositive terminals and terminals forming asymmetrical synaptic contacts have significantly greater levels of vesicle-associated glutamate immunolabelling than terminals forming symmetrical synaptic contacts (Mann–Whitney *U*-test; $P < 0.0001$).

data,[19,24–26,53] together with the findings of physiological studies[37,71] raise the possibility that glutamate and acetylcholine act as co-transmitters in at least some of the terminals of mesopontine tegmental neurons in the entopeduncular nucleus and subthalamic nucleus.

Although our data only address one of the criteria that a substance is a neurotransmitter, i.e. its presence in terminals, there are precedents for the co-release of glutamate and acetylcholine in other systems. Thus the depolarization-induced co-release of glutamate and acetylcholine has been demonstrated from synaptosomal preparations of rat cerebral cortex.[31,42] The study by Israel and co-workers[42] demonstrated that acetylcholine was released in preference to glutamate at low concentrations of extracellular calcium (0.1 mM) whereas they were co-released at high concentrations (3–10 mM). Furthermore, Bartfai and co-workers have suggested that different stimulus frequencies and different presynaptic receptors are important factors in controlling their release.[4] Obviously the precise functional significance of the co-localization of acetylcholine and glutamate described in the present study depends on whether the two putative transmitters are co-released and on the location and types of receptors expressed by the pre- and postsynaptic structures. Studies are in progress to determine whether excitatory amino acid and acetylcholine receptors co-localize at synapses in the STN and EP.

CONCLUSIONS

The main finding of this study clarifies and extends previous analysis on the projections from the mesopontine tegmentum to the EP[23] and the STN.[8] Taken together, they demonstrate the synaptic convergence of cholinergic terminals, some of which are enriched in glutamate, with terminals that mediate the direct and indirect pathways of information flow through the basal ganglia, i.e. terminals derived from the striatum, globus pallidus and subthalamic nucleus. Finally, the present study adds data to the converging evidence for multiple transmitter systems arising from neurons in the MTg and emphasizes the need to elucidate the circuitry involving these systems.

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