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Coexistence of carriers for dopamine and GABA uptake on a same nerve terminal in the rat brain

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1 The ability of γ-aminobutyric acid (GABA) to affect the release of [^{3}H]-dopamine in rat brain synaptosomes prepared from corpus striatum, frontal cortex and hypothalamus and prelabelled with the radioactive catecholamine in the presence of desipramine was examined.

2 GABA (10–300 μM) increased in a concentration-dependent way the basal release of [^{3}H]-dopamine from striatum and cortical synaptosomes; however, its effect was much less pronounced in hypothalamic nerve terminals. 2,4-Diaminobutyric acid (DABA) mimicked GABA although less potently.

3 Neutral amino acids such as leucine, valine or α-aminoisobutyric acid (100–300 μM) did not affect or increased minimally the release of [^{3}H]-dopamine.

4 The GABA-induced [^{3}H]-dopamine release was not prevented by the $GABA_A$-receptor antagonists, bicuculline or picrotoxin. The $GABA_A$-receptor agonist, muscimol (10–300 μM), displayed only a very weak, not significant, enhancing effect on [^{3}H]-dopamine release. The $GABA_B$-receptor agonist (−)-baclofen (100 or 300 μM) had no effect.

5 Three novel and selective inhibitors of GABA uptake, N-(4,4-diphenyl-3-butenyl)-nipecotic acid (SK&F 89976A), N-(4,4-diphenyl-3-butenyl)-guvacine (SK&F 100330A) and N-(4,4-diphenyl-3-butenyl)-homo-β-proline (SK&F 100561) potently counteracted the enhancing effect of GABA on [^{3}H]-dopamine release. Nipecotic acid also reduced the effect of GABA.

6 It is concluded that carriers for the uptake of dopamine and GABA may coexist on the same nerve terminal in the rat brain.

Introduction

It is well known that the release of neurotransmitters from presynaptic nerve terminals can be regulated by various modulators through the activation of receptors (presynaptic receptors) sited on the releasing nerve endings (for recent reviews see: de Belleroche, 1982; Chesselet, 1984; Raiteri *et al.*, 1984a; Vizi, 1984).

We have recently found, however, that γ-aminobutyric acid (GABA) enhanced the release of [^{3}H]-acetylcholine from rat hippocampus synaptosomes with no apparent involvement of GABA receptors. The effect of GABA was instead mediated by a carrier through which the amino acid entered into cholinergic nerve terminals. In fact, inhibitors of GABA uptake prevented the GABA-induced release of [^{3}H]-acetylcholine (Bonanno & Raiteri, 1986; 1987).

Reciprocally, the release of endogenous GABA from hippocampus synaptosomes was found to be enhanced by choline and the choline-induced GABA release was prevented by hemicholinium-3, a blocker of the high affinity choline uptake (Pittaluga & Raiteri, unpublished data).

Clearly these observations may imply coexistence of two carriers on a same nerve terminal. In other words, in rat hippocampus some nerve terminals appear to possess carriers for choline and GABA. Due to the novelty of the findings and to their implications (see Discussion) we thought that it would be relevant to explore other transmitter systems. Interestingly, in spite of the many studies available, results are still conflicting concerning the effects of GABA on dopamine release in the corpus striatum. Several groups have reported an increase of dopamine release by GABA in rat striatal slices (Giorguieff *et al.*, 1978; Starr, 1978; 1979; Stoof *et al.*, 1979; Ennis & Cox, 1981; Reimann *et al.*, 1982). However, discrepancies exist regarding the mechanisms underlying this effect and the main problem seems to be whether or not the

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GABA-induced dopamine release involves GABAergic receptors. The site of the GABA-dopamine interaction is also uncertain, due to the fact that the studies have essentially been carried out in brain slices or *in vivo*. In the present investigation we have re-examined the problem of the effect of GABA on dopamine release in the corpus striatum in the light of the above mentioned observations on the carrier-mediated effects of GABA on the release of [^{3}H]-acetylcholine. The study was extended to other brain areas receiving a dopaminergic innervation (frontal cortex and hypothalamus). The results suggest coexistence of carriers for GABA and dopamine on the same nerve endings in rat brain.

Methods

Preparation of synaptosomes

Adult male Sprague Dawley rats weighing 200–250 g were used. Rats were killed by decapitation and the brain was rapidly removed. The brain regions used were dissected according to Glowinski & Iversen (1966). Crude synaptosomes were prepared as previously described (Gray & Whittaker, 1962; Raiteri *et al.*, 1984b). Briefly, the tissue was homogenized in 40 vol of 0.32 M sucrose buffered at pH 7.4 with phosphate. The homogenate was centrifuged (5 min, 1000 *g*) to remove nuclei and debris and synaptosomes were isolated from the supernatant by centrifugation at 12000 *g* for 20 min. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (mM): NaCl 125, KCl 3, $CaCl_2$ 1.2, $MgSO_4$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 22 and glucose 10 (aeration with 95% O_2 and 5% CO_2 at 37°C) pH 7.2–7.4. Protein was measured by a modification of the method of Lowry (Petersen, 1977).

Uptake studies

Uptake of [^{3}H]-GABA (final concentration: 1 μM), [^{3}H]-noradrenaline ([^{3}H]-NA; 0.1 μM) and [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT; 0.03 μM) was studied in aliquots (about 0.15 mg protein) of cortical synaptosomes. Identical aliquots of striatal synaptosomes were used in experiments on [^{3}H]-dopamine (0.1 μM) uptake. Synaptosomes were preincubated 5 min at 37°C in physiological medium with or without the uptake inhibitor SK&F 89976A. The radioactive substrates were then added and incubation was continued for 2 min. The tissue was collected by vacuum filtration on GF/B Whatman filters which were washed rapidly with 3 × 4 ml of medium and counted for radioactivity. Blank values were determined at 0°C.

Release studies

Synaptosomes were incubated with [^{3}H]-dopamine (final concentration 0.04 μM to label striatal synaptosomes and 0.06 μM to label cortical and hypothalamic synaptosomes) for 15 min at 37°C in presence of 1 μM desipramine to minimize labelling of noradrenaline and 5-HT nerve terminals and aliquots of the suspension were layered on 0.65 μm Millipore filters (0.20–0.50 mg of protein per filter, depending on the brain area studied) at the bottom of several parallel superfusion chambers (Raiteri *et al.*, 1974; Raiteri & Levi, 1978, for technical details).

Superfusion was started with standard medium at a rate of 0.6 ml min^{-1} and, after 34 min to equilibrate the system, 8 separate 2 min fractions were collected. Synaptosomes were exposed to GABA, DABA, leucine, valine, α-aminoisobutyric acid, nipecotic acid (when used as a carrier substrate), muscimol or (−)-baclofen at the end of the second fraction collected; bicuculline, picrotoxin, (N-4,4-diphenyl-3-butenyl)-nipecotic acid (SK&F 89976A), N-(4,4-diphenyl-3-butenyl)-homo-β-proline (SK&F 100561), N-(4,4-diphenyl-3-butenyl)-guvacine (SK & F 10330A), nipecotic acid (when used as an uptake inhibitor) and β-alanine were added 8 min before GABA.

Superfusate fractions were collected into vials containing 100 μl of a protective solution (1.5% EDTA, 1% ascorbic acid and 0.001% unlabelled dopamine) and the [^{3}H]-dopamine present in each fraction and that remaining in the filters at the end of superfusion was separated from ^{3}H-deaminated metabolites on Bio Rex 70 columns according to the method of Smith *et al.* (1975).

The release of [^{3}H]-dopamine in the superfusate samples was calculated as the percentage of [^{3}H]-dopamine content of synaptosomes at the onset of the respective collection period. The effects of the drugs tested on [^{3}H]-dopamine release were evaluated by obtaining the ratio between the percentage efflux in the fraction corresponding to the maximal effect of GABA (in general the 7th fraction collected) and that in the 2nd fraction. This ratio was compared to the corresponding ratio obtained under control conditions.

A two tailed Student's *t* test was used for comparison of mean values. The data in Figure 1 and the effect of muscimol (Figure 2) were evaluated by use of the one-factor ANOVA test.

Drugs

[^{3}H]-dopamine (49.6 Ci mmol^{-1}), [^{3}H]-NA (35.0 Ci mmol^{-1}) and [^{3}H]-GABA (57.0 Ci mmol^{-1}) were obtained from Amersham Radiochemical Centre (U.K.); [^{3}H]-5-HT (23.0 Ci mmol^{-1}) from New England Nuclear (Boston, MA, U.S.A.); GABA, β-

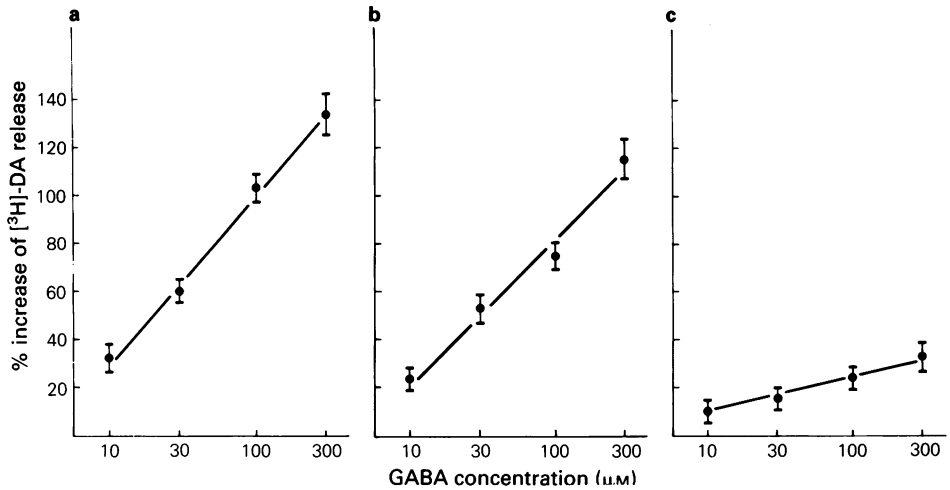

Figure 1 γ-Aminobutyric acid (GABA) enhances [^{3}H]-dopamine ([^{3}H]-DA) release in (a) rat striatum, (b) frontal cortex and (c) hypothalamus. Synaptosomes were labelled and superfused as described in the Methods section. Fractions were collected and the [^{3}H]-dopamine present was determined by chromatographic separation (Smith *et al.*, 1975). The GABA-induced tritium release was totally accounted for by unmetabolized [^{3}H]-dopamine. The percentage of [^{3}H]-dopamine present in the second fraction collected (basal release) was 0.615 ± 0.026 ($n = 15$) (striatum); 0.646 ± 0.035 ($n = 13$) (frontal cortex); 0.685 ± 0.071 ($n = 8$) (hypothalamus). The data presented in the figure are means of 4–10 experiments run in duplicate; s.e.mean shown by vertical lines. Panels (a) and (b): $P < 0.005$; panel (c): $P < 0.05$, using one-factor ANOVA test.

alanine, L-leucine, L-valine and α-aminoisobutyric acid from Serva (Heidelberg, FRG); (+)-bicuculline, picrotoxin, nipecotic acid and L-2,4-diamino-*n*-butyric acid from Sigma (St. Louis, MO, U.S.A.). The following drugs were generous gift by the companies indicated: (−)-baclofen (Ciba Geigy, Basel, Switzerland); muscimol (Zambon Farmaceutici, Milan, Italy); SK&F 89976A, SK&F 100330A and SK&F 100561 (Smith Kline & French, Welwyn, England).

Results

Figure 1 shows that GABA increased in a concentration-dependent way (10–300 μM) the basal release of [^{3}H]-dopamine from synaptosomes prelabelled with the radioactive catecholamine. The effect was present in the corpus striatum (Figure 1a) and in the frontal cortex (Figure 1b) but was much less pronounced in hypothalamic nerve endings (Figure 1c). Concentrations of GABA higher than 300 μM were not tested in order to avoid possible non specific effects.

Neutral amino acids like leucine, valine and α-aminoisobutyric acid, tested at 100–300 μM, produced only a very modest enhancement of [^{3}H]-dopamine release. In particular, at 300 μM, their effect was

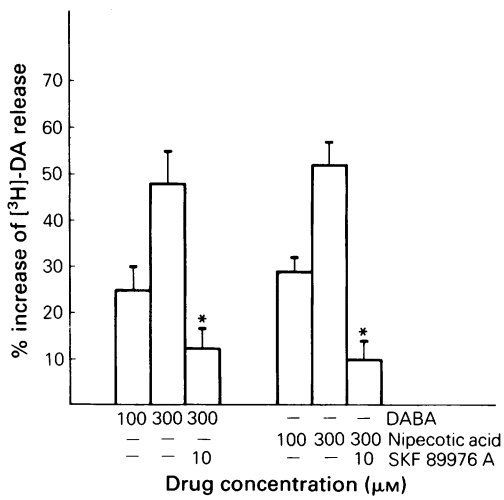

Figure 2 Effect of 2,4-diaminobutyric acid (DABA), nipecotic acid and SK&F 89976A on the basal release of [^{3}H]-dopamine ([^{3}H]-DA) in rat striatal synaptosomes. For experimental details see legend to Figure 1 and Methods. Values are means of 3 experiments run in triplicate; vertical lines indicate s.e.mean. * $P < 0.02$ when compared to the respective control.

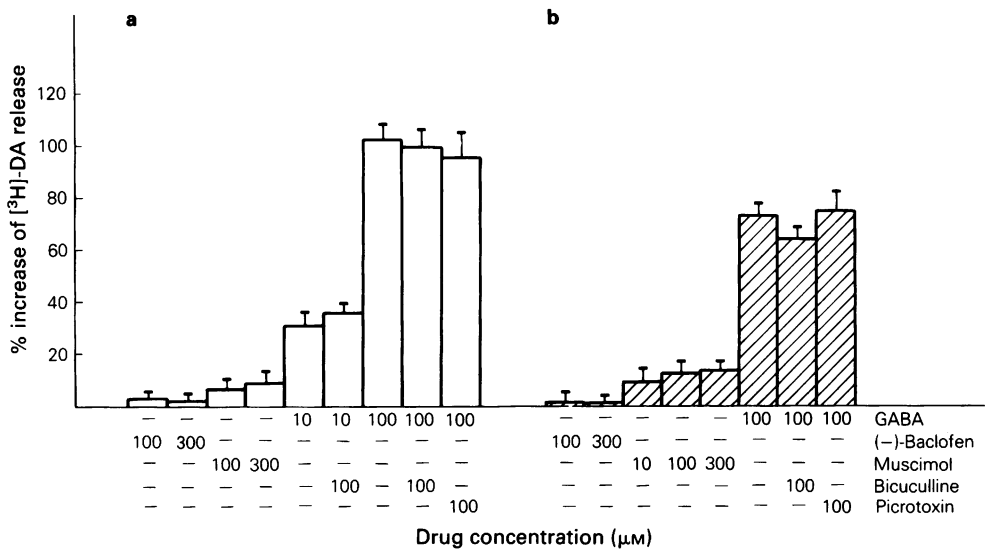

Figure 3 Study of the γ-aminobutyric acid (GABA)-induced [^{3}H]-dopamine ([^{3}H]-DA) release in striatum (a) and frontal cortex (b) using drugs selective for GABA receptors. For experimental details see Methods. Means of 4–6 experiments run in triplicate are presented. Vertical lines indicate s.e.mean.

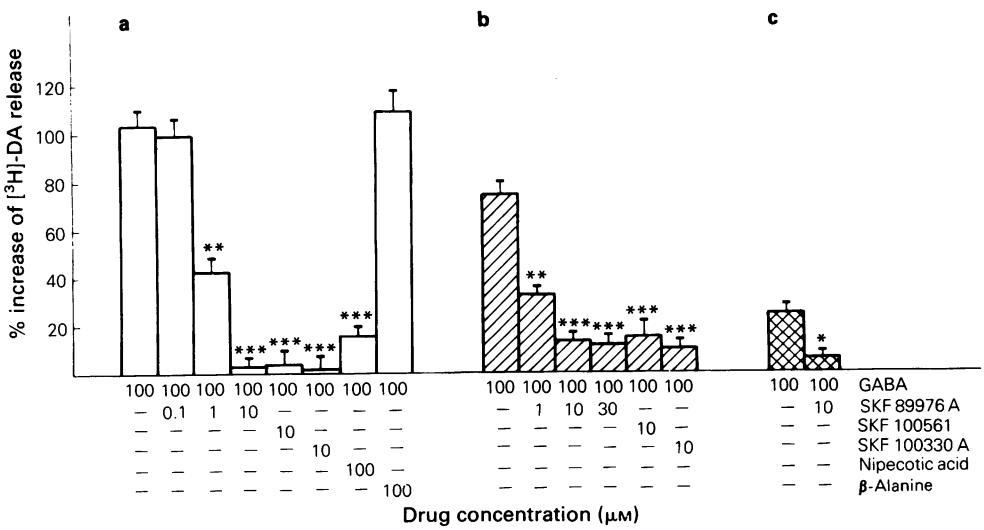

Figure 4 The γ-aminobutyric acid (GABA)-induced [^{3}H]-dopamine ([^{3}H]-DA) release in striatum (a), frontal cortex (b) and hypothalamus (c) is prevented by inhibitors of GABA uptake. Experimental details are given in the Methods section. The data are means of 4–6 experiments run in triplicate; s.e.mean shown by vertical lines. $*P<0.05$; $**P<0.01$; $***P<0.001$ when compared to the appropriate control obtained with GABA alone.

significantly lower than that produced by 10 μM GABA.

Figure 2 shows that DABA, considered to be a substrate of GABA transport (Martin, 1976) stimulated the release of [^{3}H]-dopamine from rat striatal synaptosomes. Similar results were obtained with nipecotic acid, a substrate-inhibitor of GABA uptake (Storm-Mathisen *et al.*, 1976). Both DABA and nipecotic acid were less potent than GABA.

The GABA-induced [^{3}H]-dopamine release in both striatum and cortex was insensitive to bicuculline and to picrotoxin (Figure 3). Muscimol was almost ineffective in the striatum and caused a slight but non-significant release of [^{3}H]-dopamine, in the cerebral cortex. (−)-Baclofen, up to 300 μM, was totally inactive on the basal release of [^{3}H]-dopamine.

Figure 4 shows that the effect of GABA in corpus striatum and cortex was potently countereacted by SK&F 89976A, SK&F 100561 or SK&F 100330A, three novel inhibitors of GABA uptake (Yunger *et al.*, 1984). The effect of 100 μM GABA was halved by 1 μM SK&F 89976A. All three compounds (at 10 μM) totally abolished the [^{3}H]-dopamine releasing effect of GABA in the corpus striatum while a small portion of the effect present in the cerebral cortex was insensitive to the SK&F compounds tested at 30 μM. Also the slight effect observed in hypothalamic synaptosomes with 100 μM GABA was counteracted by 10 μM SK&F 89976A. When used as a GABA uptake inhibitor, nipecotic acid also prevented the effect of GABA on [^{3}H]-dopamine release (Figure 4). On the other hand, [^{3}H]-dopamine releasing effects of nipecotic acid (used as a substrate of the GABA carrier) and of DABA were blocked by SK&F 89976A (Figure 2). The effect of GABA on [^{3}H]-dopamine release was not reduced by β-alanine, a blocker of GABA-uptake into glial cells (Martin, 1976; Figure 4).

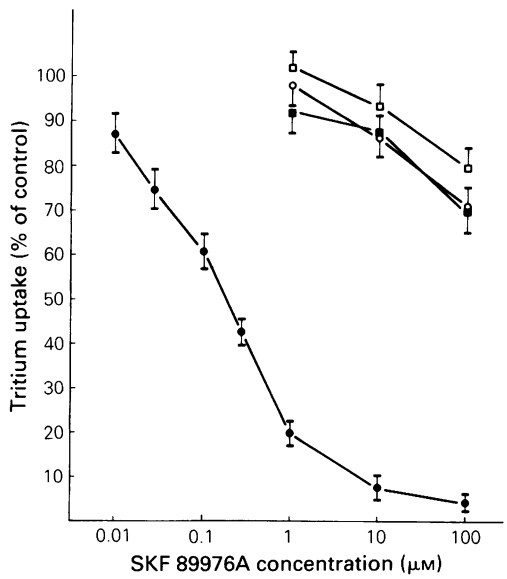

Figure 5 Effect of SK&F 89976A on the uptake of [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA), [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT), [^{3}H]-noradrenaline ([^{3}H]-NA), (cortical synaptosomes) and of [^{3}H]-dopamine ([^{3}H]-DA) (striatal synaptosomes). Experimental details are described in the Methods section. The uptake of [^{3}H]-GABA, [^{3}H]-dopamine, [^{3}H]-5-HT and [^{3}H]-NA at the concentrations used, in the absence of SK&F 89976A was 426.7 ± 25.4, 59.87 ± 12.77, 3.90 ± 0.94, 5.45 ± 1.62 pmol mg^{-1} protein 2 min^{-1}, respectively. The data are means of 3 experiments in duplicate. Vertical lines indicate s.e.mean. (●) [^{3}H]-GABA (1 μM); (○) [^{3}H]-5-HT (0.03 μM); (■) [^{3}H]-DA (0.1 μM); (□) [^{3}H]-NA (0.1 μM).

Since the SK&F compounds used are relatively new and little studied GABA-uptake inhibitors (Yunger *et al.*, 1984) we have investigated the selectivity of one of them, SK&F 89976A by measuring its ability to inhibit the uptake of [^{3}H]-GABA, [^{3}H]-NA, [^{3}H]-dopamine and [^{3}H]-5-HT. The results of this study are shown in Figure 5 and demonstrate that the compound inhibited [^{3}H]-GABA uptake with an IC_{50} of 0.2 μM whereas the IC_{50} values for the uptake of the biogenic amines were higher than 100 μM. Thus, at the concentrations used in this study, SK&F 89976A probably acts as a potent and selective inhibitor of [^{3}H]-GABA uptake.

Discussion

The experiments described have been carried out with rat brain synaptosomes prelabelled with nanomolar concentrations of [^{3}H]-dopamine in the presence of the noradrenaline (and 5-HT) uptake inhibitor, desipramine. Under these conditions, most of the [^{3}H]-dopamine should be taken up by the dopamine carrier sited on dopamine nerve terminals. Moreover, the release was studied by superfusing a thin layer of synaptosomes, i.e. in conditions in which the indirect effects occurring in more complex brain tissue preparations should be minimized (see Raiteri & Levi, 1978 for technical details). Finally, the GABA-induced release of radioactivity was totally accounted for by unmetabolized [^{3}H]-dopamine. Thus, the first conclusion of this work is that GABA can enhance the basal release of [^{3}H]-dopamine by acting directly on dopaminergic nerve terminals.

It is well accepted that GABA can activate two types of receptors: $GABA_A$ and $GABA_B$ (Bowery *et al.*, 1984; Enna, 1984). The findings that the effect of GABA was insensitive to bicuculline or to picrotoxin and was not mimicked by muscimol tend to exclude an involvement of receptors of the $GABA_A$ subtype.

Activation of $GABA_B$ receptors by GABA is unlikely since the $GABA_B$ agonist, (−)-baclofen, was totally inactive on the basal release of [^{3}H]-dopamine.

The GABA-induced [^{3}H]-dopamine release was prevented by drugs (SK&F 89976A, SK&F 100330A, SK&F 100561 and nipecotic acid) that are potent and selective inhibitors of the uptake of GABA into rat brain synaptosomes (Yunger *et al.*, 1984; Figure 5). Although the possibility that GABA activates a novel non-$GABA_A$, non-$GABA_B$ receptor subtype (at which the above compounds would be potent antagonists) cannot be ruled out, the findings that the three GABA uptake blockers had strong *in vivo* anticonvulsant activity and did not displace [^{3}H]-muscimol (Yunger *et al.*, 1984) or [^{3}H]-GABA (Pittaluga & Raiteri, unpublished data) from their binding sites make it unlikely that they also possess GABA receptor antagonist activity. Thus, on the basis of the present results, GABA appears to enhance the spontaneous release of [^{3}H]-dopamine by a mechanism involving GABA uptake by a transporter located on dopamine nerve terminals. In other words, in rat corpus striatum and frontal cortex (much less so in the hypothalamus), at least a proportion of dopamine nerve endings would possess not only a carrier for dopamine uptake, but also a carrier for the uptake of GABA. The releasing effect of DABA and nipecotic acid strengthens this idea. The possibility that a non-specific amino acid carrier is involved is unlikely since a number of neutral amino acids such as leucine, valine or α-aminoisobutyric acid do not evoke [^{3}H]-dopamine release. Similarly, it seems improbable that GABA penetrates into 'gliosomes' which may be present in our synaptosomal preparations (Henn *et al.*, 1976) since the effect of GABA was not prevented by β-alanine, an inhibitor of glial GABA uptake (Martin, 1976). As mentioned in the Introduction, facilitation by GABA of tritium release from rat striatal slices prelabelled with [^{3}H]-dopamine had been previously observed by several workers. Some of them found the effect of GABA to be resistant to bicuculline and picrotoxin (Starr, 1978; 1979; Stoof *et al.*, 1979; Ennis & Cox, 1981) whereas others obtained some antagonism (Giorguieff *et al.*, 1978). The enhancing effect of GABA on basal tritium release was found to be converted into reduction by the presence of nipecotate (Reimann *et al.*, 1982), an inhibitor-substrate of the GABA uptake (but see Ennis & Cox, 1981), suggesting penetration of GABA into dopamine axon terminals.

In conclusion, the phenomenon of carrier coexistence on the same nerve terminal already observed in rat hippocampus for choline and GABA (Bonanno & Raiteri, 1986; 1987) appears to exist also for dopamine and GABA, particularly in rat corpus striatum and frontal cortex.

Why a carrier for GABA, which has been thought of as a characteristic of GABAergic nerve endings, should also be present on cholinergic and dopaminergic nerve terminals, respectively, can only be a matter of speculation.

Carriers for a given transmitter are thought to be present on the nerve endings releasing that transmitter in order to remove it from the synaptic gap (Iversen, 1973). Receptors sensitive to the neurone's own transmitter (autoreceptors) are also present on the terminals to regulate release. Some nerve endings may possess heteroreceptors through which modulators can regulate the release of the transmitter. Similarly, the presence of a *heterocarrier* on a given nerve terminal might be evidence for a hitherto unknown route of presynaptic modulation of neurotransmitter release via penetration of the modulator into the nerve ending rather than activation of presynaptic heteroreceptors.

This interpretation of the phenomenon of carrier coexistence would be compatible with a second hypothesis, implying coexistence of GABA with dopamine or acetylcholine in some nerve terminals. It is well accepted that transmitter substances can coexist in the same neurone (Hökfelt *et al.*, 1980; Iversen, 1984). If this were the case, the coexistence of carriers would allow reciprocal modulation of release between the hypothetical cotransmitters.

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