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THE γ-HYDROXYBUTYRATE SIGNALLING SYSTEM IN BRAIN: ORGANIZATION AND FUNCTIONAL IMPLICATIONS

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Abstract—γ-Hydroxybutyrate is a metabolite of GABA which is synthesized and accumulated by neurons in brain. This substance is present in micromolar quantities in all brain regions investigated as well as in several peripheral organs. Neuronal depolarization releases γ-hydroxybutyrate into the extracellular space in a Ca^{2+}-dependent manner. Gamma-hydroxybutyrate high-affinity receptors are present only in neurons, with a restricted specific distribution in the hippocampus, cortex and dopaminergic structures of rat brain (the striatum in general, olfactory bulbs and tubercles, frontal cortex, dopaminergic nuclei A_9, A_{10} and A_{12}). Stimulation of these receptors with low amounts of γ-hydroxybutyrate induces in general hyperpolarizations in dopaminergic structures with a reduction of dopamine release. However, in the hippocampus and the frontal cortex, it seems that γ-hydroxybutyrate induces depolarization with an accumulation of cGMP and an increase in inositol phosphate turnover. Some of the electrophysiological effects of GHB are blocked by NCS-382, a γ-hydroxybutyrate receptor antagonist while some others are strongly attenuated by $GABA_B$ receptors antagonists.

Gamma-hydroxybutyrate penetrates freely into the brain when administered intravenously or intraperitoneally. This is a unique situation for a molecule with signalling properties in the brain. Thus, the γ-hydroxybutyrate concentration in brain easily can be increased more than 100 times. Under these conditions, γ-hydroxybutyrate receptors are saturated and probably desensitized and down-regulated. It is unlikely that $GABA_B$ receptors could be stimulated directly by GHB. Most probably, GABA is released in part under the control of GHB receptors in specific pathways expressing $GABA_B$ receptors. Alternatively, $GABA_B$ receptors might be specifically stimulated by the GABA formed via the metabolism of γ-hydroxybutyrate in brain. In animals and man, these GHBergic and GABAergic potentiations induce dopaminergic hyperactivity (which follows the first phase of dopaminergic terminal hyperpolarization), a strong sedation with anaesthesia and some EEG changes with epileptic spikes. It is presumed that, under pathological conditions (hepatic failure, alcoholic intoxication, succinic semialdehyde dehydrogenase defects), the rate of GHB synthesis or degradation in the peripheral organ is modified and induces increased GHB levels which could interfere with the normal brain mechanisms. This pathological status could benefit from treatments with γ-hydroxybutyric and/or $GABA_B$ receptors antagonists. Nevertheless, the regulating properties of the endogenous γ-hydroxybutyrate system on the dopaminergic pathways are a cause for the recent interest in synthetic ligands acting specifically at γ-hydroxybutyrate receptors and devoid of any role as metabolic precursor of GABA in brain.

CONTENTS

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ABBREVIATIONS

CHAPS	(3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
CSF	Cerebrospinal fluid
EEG	Electroencephalogram
GABA	γ-Aminobutyrate
GABA-T	GABA-transaminase
$GABA_A$	Class A GABA receptors
$GABA_B$	Class B GABA receptors
GBL	γ-Butyrolactone
GHB	γ-Hydroxybutyrate
GHB-DH	GHB-dehydrogenase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphatereduced form
NCS-382	Sodium salt of 6,7,8,9-tetrahydro-5-[H]benzocycloheptene-5-ol-4-ylidene acetic acid
SSA	Succinic-semialdehyde
SSADH	Succinic-semialdehyde dehydrogenase
SSR	Succinic-semialdehyde reductase
T-HCA	*Trans*-4-hydroxycrotonatesodium salt

1. INTRODUCTION

Since the early sixties, γ-hydroxybutyrate generally has been thought to be a drug which enters the brain easily and which possesses the general profile of a GABAergic ligand (Laborit, 1964). Up to now, the majority of the research devoted to this compound has focused on the neuropharmacological and neurophysiological aspects of systemic administration. However, GHB is primarily a naturally occurring substance in brain which was identified about 30 years ago and which is synthesized locally (Fishbein and Bessman, 1961; Bessman and Fishbein, 1963; Roth and Giarman, 1970; Roth, 1970). Like the biogenic amines, GABA is converted either by an oxidative pathway which produces succinate and enters the Krebs cycle or by a reductive pathway which gives rise to GHB in the neuronal cytosol. A large body of evidence favours a role for GHB as neuromodulator released by specific neuronal circuitry in the mammalian brain. This neuromodulation seems to occur mainly at dopaminergic, but also amino-acidergic synapses in the anterior part of the central nervous system. The GHB receptors seem to possess large functional (and probably structural) homologies with the $GABA_B$ receptors. However, despite the fact that the GHB system is not as well characterized as many other neurotransmitter/neuromodulator system, some results are now well established. The aim of this review is to focus on these results and to suggest some future directions in this area of research.

2. MOLECULAR AND CELLULAR ORGANIZATION OF THE γ-HYDROXYBUTYRATE SYSTEM IN BRAIN

2.1. γ-Hydroxybutyrate is Present in Small Quantities in Mammalian Brain

γ-Hydroxybutyrate real concentration in brain has been a matter of debate because it needs gas chromatography with preferably mass spectrometric detection to measure actual levels (Doherty *et al.*, 1975a, 1978; Ehrhardt *et al.*, 1988). In addition, endogenous GHB concentrations fluctuate rapidly in the ischaemic brain, so that brain dissection must be carried out rapidly after death (Snead and Morley, 1981; Eli and Cattabeni, 1983; Vayer *et al.*, 1988). The lowest levels of GHB have been found in brain of animals killed by microwave irradiation or in brain rapidly frozen after extraction. Probably for this technical reason, the concentrations of GHB in the brain of small laboratory animals (guinea-pig or rat brains) have been found, in general, to be lower than in the brain of larger animals (bovine and monkey brains). Human brains obtained after autopsy also present higher GHB values (Doherty *et al.*, 1978; Snead and Morley, 1981).

The GHB is present in all of the brain regions investigated. In the adult rat brain, GHB levels range from about 0.4 μM in the frontal cortex, 1.2 μM in the hippocampus, 1.8 μM in the striatum to 4.6 μM in the substantia nigra. The GHB concentrations are highest in human brain and in monkey brain, reaching about 11–25 μM in the striatum, but the values found for guinea-pig brain are similar to those found for rat brain. In developing brain, the concentrations of GHB have been found to be higher than in adult brain (rat, monkey and human). In the rat, the highest concentration is found in the immature hypothalamus and cortex with a decrease occurring between postnatal days 12 and 14.

Gamma-hydroxybutyrate also has been found in rat peripheral organs (Nelson *et al.*, 1981) such as heart (12.4 μM), kidney (28.4 μM), liver (1.4 μM), muscle (10.2 μM) and brown fat (37 μM). Studies of the apparent subcellular distribution of GHB have been carried out in the rat brain: GHB appeared to be concentrated in cytosolic and synaptosomal fractions (Snead, 1987), which most probably implies a mechanism for its presynaptic accumulation.

2.2. γ-Hydroxybutyrate Synthesis in Brain

Gamma-aminobutyrate is the major precursor of GHB in brain (Roth and Giarman, 1968). Labelled GABA (^{13}C or ^{3}H-GABA) administered into the lateral ventricles of awake rats rapidly gave rise to labelled GHB, with a maximum concentration after 20 min (Gold and Roth, 1977). Radioactive glutamate, the precursor of GABA in brain, also rapidly induced the formation of radioactive GHB (Santaniello *et al.*, 1978). The GABA-transaminase inhibitors (γ-vinylGABA or aminooxyacetic acid) blocked the metabolism of GABA to GHB, which implicates GABA-T in this transformation (Snead *et al.*, 1989; Eli and Cattabeni, 1983). This enzyme, classically located in the mitochondria of brain cells

(Schousboe *et al.*, 1980), synthesizes succinic semialdehyde (SSA) (Matsuda and Hoshino, 1977), which could give rise to GHB after its reduction. Thus GABA, like the biogenic amines, possesses two types of catabolite: oxidation of SSA by the mitochondrial enzyme semialdehyde succinic dehydrogenase (SSADH) produces succinic acid which enters the Krebs cycle of brain cells; reduction of SSA gives rise to GHB present in the cytosol of some neurons (Fig. 1).

The respective importance of these two pathways is very unequal. From about 0.05% (*in vitro*, Rumigny *et al.*, 1981a) to 0.16% (*in vivo*, Gold and Roth, 1977) of the metabolic flux coming from GABA takes the reductive route in order to form GHB. As GHB is formed in the cytosol of a restricted population of neurons (see below), the amount of SSA transported from the mitochondria to the cytoplasm is critical for GHB formation and probably is strictly regulated. Assuming an average concentration of about 2 mM for GABA in brain, the level of GHB in the whole brain represents a value not far from 0.1% of the GABA concentration.

Other precursors of GHB in brain have been postulated. 1,4-butanediol and γ-butyrolacetone are present in rat brain, at concentrations of about 1/10 of those of GHB (Barker *et al.*, 1985; Doherty *et al.*, 1975a). The former compound is transformed rapidly into GHB when introduced directly into the brain *in vivo*, the transformation being catalysed by a pyrazole-insensitive dehydrogenase (Maxwell and Roth, 1972; Snead *et al.*, 1982). The role of γ-butyrolactone is more obscure, since no lactonase activity has been described in the brain cell (Fishbein

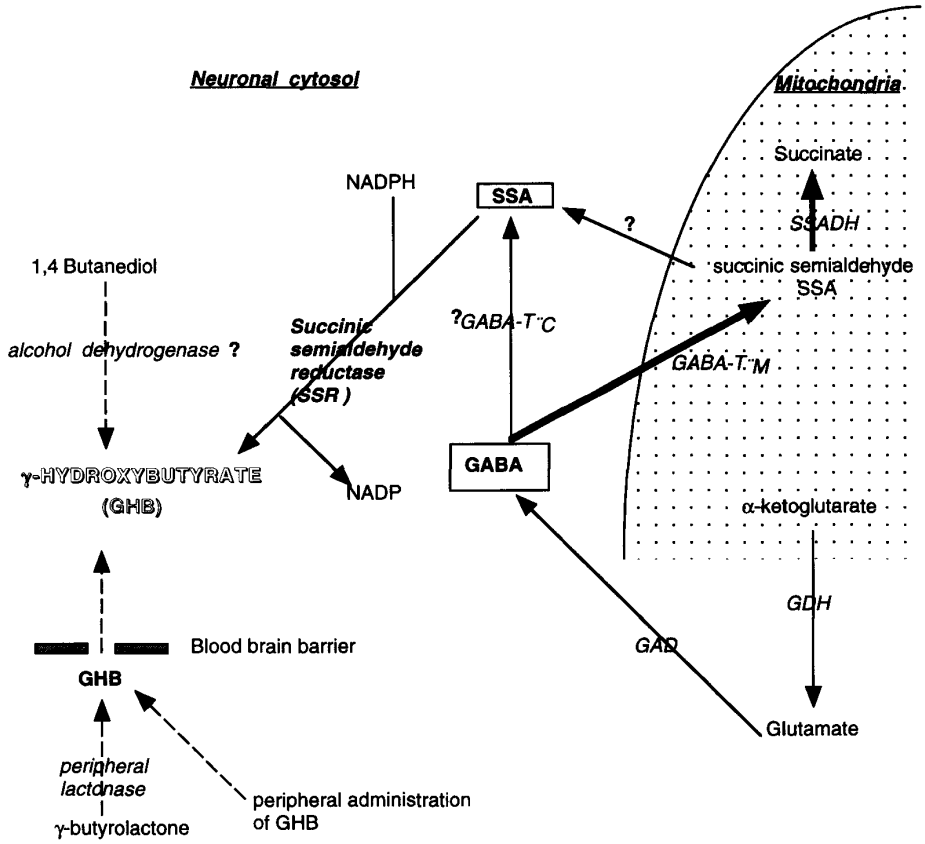

Fig. 1. Biosynthesis of GHB in brain. The GABA is metabolized in brain by a mitochondrial GABA-T (GABA-T_M) which gives rise to a succinic semialdehyde (SSA) pool in the mitochondria. The SSA then is oxidized by succinic semialdehyde dehydrogenase (SSADH) to succinate. This oxidative pathway is the main pathway of GABA degradation. The SSA is reduced by succinic semialdehyde reductase (SSR), present exclusively in the neurons cytoplasm. This enzyme is fairly specific for SSA ($K_m = 30$ μM) and is not inhibited by valproate, ethosuximide or barbiturates, but only by phthalaldehydic acid (competitive inhibition) and 4-*n*-propylheptanoic acid (non-competitive inhibition). An alternative pathway for SSA synthesis directly in the cytosol is the possible degradation of GABA via a supposed cytoplasmic GABA-T (GABA-T_C). A minor route for GHB synthesis is the reduction, possibly by alcohol dehydrogenase, of 1,4-butanediol which is present in low amount in brain. The concentration of GHB in brain could be increased easily by peripheral administration of GHB which penetrates freely into the brain. Gamma-butyrolactone (GBL) is sometimes used as a GHB precursor because it is transformed by peripheral tissue into GHB.

and Bessman, 1966). However, the equilibrium between γ-butyrolactone and GHB in brain could be the result of a chemical rather than an enzymatic process.

The reduction of SSA to GHB is carried out by an aldehyde reductase which possesses a low K_m for SSA. Enzymes purified from several species (human, rat, pig, bovine) have been described which are able to reduce SSA (Cash *et al.*, 1979; Rumigny *et al.*, 1980, 1981a; Rivett *et al.*, 1981; Rivett and Tipton, 1981; Cromlish and Flynn, 1985; Cromlish *et al.*, 1985; Hearl and Churchich, 1985; Cho *et al.*, 1993). Generally, two SSA reductases have been characterized in each species: one with a "high" K_m for SSA (50–200 μM), the other with a lower K_m (20–30 μM), the co-factor being in each case NADPH. The high K_m SSA reductase (ALR_1) exhibits a broad substrate specificity, reducing a wide range of aldehydes (including *p*-nitrobenzaldehyde, 4-carboxybenzaldehyde, DL-glyceraldehyde and 3-pyridine carboxaldehyde), whereas the low K_m SSA reductase shows a fairly high degree of specificity for SSA and structural analogues of SSA.

The high K_m SSA reductase of human and rat brain have been reported to be inhibited by several compounds including anti-epileptic drugs (valproate, ethosuximide, barbiturates) and some branched chain fatty acids (Cash *et al.*, 1979; Vayer *et al.*, 1985c). When administered *in vivo*, most of these compounds induce an increase in brain GHB levels (Snead *et al.*, 1980). It is thus difficult to implicate the high K_m SSA reductase in the *in vivo* synthesis of cerebral GHB.

From a theoretical point of view, the SSA reductase which synthezises GHB in brain must meet certain criteria: (1) it must possess a high affinity and a high specificity for SSA; (2) the enzyme must be preferably localized in the cell compartment which has the highest concentration of GHB (cytosolic and synaptosomal fractions); (3) finally, the GHB synthesizing enzyme must not be inhibited by valproate and related compounds (short-chain fatty acids and anti-epileptics) which lead to the accumulation of GHB in brain. In rat brain, these criteria lead to the selection of what has been called SSR_2 and which is now designated as succinic semi-aldehyde reductase (SSR). This enzyme is mainly cytosolic but is present also in the synaptosomal fraction (Rumigny *et al.*, 1981b, 1982; Weissmann-Nanopoulos *et al.*, 1982). It is a monomeric protein of molecular weight of about 43 000–45 000 kDa with a pH optimum of 5.0. The NADPH is the co-substrate of the SSA reduction; the activity is five times less with NADH. The role of rat brain SSR has been characterized by selective inhibition of the enzyme in brain slices incubated under physiological conditions. Incorporation of [^{3}H]-GABA into [^{3}H]-GHB is reduced only when the activity of SSR is inhibited (Rumigny *et al.*, 1981a). On the contrary, blockade of high K_m SSA reductase increases the radioactivity in the GHB pool which indicates that this enzyme has no role in GHB synthesis but could be implicated in GHB degradation.

The regional and cellular distribution of SSR has been studied using a specific polyclonal antibody produced against the pure enzyme (Weissmann-Nanopoulos *et al.*, 1982). The SSR is present only in the cytoplasm of numerous neurons of various sizes; glial cells appear not to be labelled. At the light microscopic level, the large majority of SSR immunoreactive neurons are also labelled with an antibody directed against glutamate decarboxylase, the enzyme that synthesize GABA (Weissmann-Nanopoulos *et al.*, 1984). Thus, GHB formation occurs in GABAergic neurons or in neurons which are able to synthesize GABA. At the electron microscopic level, SSR staining appears in the somata of neurons and in fibres or axonal terminals. In the hippocampus (results not published), SSR immunoreactivity is associated closely with pyramidal cells (CA1, CA2 and CA3). Regional distribution studies of SSR activity in rat brain shows that the enzyme is present in all regions investigated, with a maximum in cerebellum, colliculi and median hypothalamus (Rumigny *et al.*, 1981b, 1982).

In human and pig brain, the same SSR activity has been isolated with about the same characteristics (Cash *et al.*, 1979; Cromlish and Flynn, 1985). However, the enzyme appears to be a dimer of molecular weight of about 80 000 kDa. Beside this SSA reductase activity, a mitochondrial SSA reductase has been described in pig brain which possesses high activity for malonic semialdehyde and *p*-nitrobenzaldehyde (Hearl and Churchich, 1985). Evidence that this enzyme is implicated in the synthesis of a GHB pool involved in interneuronal signalling is very poor, mainly due to its subcellular localization. An SSA reductase from bovine brain has been purified more recently but its high K_m for SSA (67 μM), its high activity with *p*-nitrobenzaldehyde and the absence of any inhibition profile make its identification difficult (Cho *et al.*, 1993). No immunocytochemical localization of SSA reductase isolated from the brain of species other than the rat has been performed.

2.3. γ-Hydroxybutyrate Degradation

The disappearance of ^{14}C-GHB after intraventricular administration appears to be very rapid, one-half of the isotope being eliminated in less than 5 min (Doherty *et al.*, 1975b). As finally most of the radioactivity is found in succinic acid and in the Krebs cycle, the general opinion favours the transformation of GHB first into SSA (Möhler *et al.*, 1976; Doherty and Roth, 1978). The reaction is most probably catalysed by the low K_m SSA reductase (ALR_1) in the presence of NADP (Vayer *et al.*, 1985c). As already mentioned, this enzyme is present in the brain of most of the species investigated (bovine, human, rat and pig brain) and is located in the cytosol, but no precise immunocytochemical distribution has so far been carried out. The low K_m SSA reductase is now referred to as GHB dehydrogenase (GHB-DH) because of the following properties (Vayer *et al.*, 1985c; Kaufman and Nelson, 1979; Kaufman *et al.*, 1983; Kaufman and Nelson, 1987). Firstly, as already quoted, the enzyme is strongly inhibited by various anti-epileptic drugs, short-chain fatty acids (K_i for valproate = 60–80 μM). When tested *in vivo*, these compounds induce a significant increase in brain GHB levels, most probably by inhibiting GHB catabolism (Snead

et al., 1980). In brain slices, inhibitors of GHB dehydrogenase lead to the accumulation of radioactive GHB after incubation of the tissue with radioactive GABA (Rumigny *et al.*, 1981a). In brain homogenates or when isolated *in vitro*, the enzyme behaves like a non-specific SSA reductase, strongly inhibited by low concentrations of valproate, and present in all the rat brain regions investigated (Rumigny *et al.*, 1981b). Its specific activity in these brain regions is about 15-fold higher than the specific activity of SSR (Rumigny *et al.*, 1982). However, in the presence of NADP, purified GHB-DH has a very high apparent K_m for GHB ($K_m = 2$ mM) which is the result of the competitive inhibition by both SSA ($K_i = 14$ μM) and NADPH (K_i about 7–21 μM) for the random binding of GHB to the enzyme (Vayer *et al.*, 1985c). The SSA and NADPH are the products formed by GHB-DH from GHB and NADP, therefore GHB-DH activity appear to be strictly controlled by the negative feedback activity of the reaction products. This phenomenon could play a role in the regulation of GHB concentrations in brain. *In vitro*, the problem of SSA and NADPH accumulation can be avoided by coupling GHB-DH activity to the reduction of D-glucuronate which releases NADPH accumulation (Kaufman and Nelson, 1981, 1991). This result occurs mainly because, as already described, GHB-DH can actively catalyse the reduction of glucuronate and make the pentose phosphate pathway more active (a property attributed to GHB administration; Taberner *et al.*, 1972). In addition, *in vitro* accumulation of SSA can be reduced by its being metabolized by GABA-T, e.g. (Vayer *et al.*, 1985b, 1985c). Under these conditions, when SSA and NADPH concentrations remain low, the apparent K_m GHB and K_m NADP for GHB-DH have been measured at 175 and 1.4 μM, respectively. Hence, physiological concentrations of GHB (2–60 μM) could be rapidly catabolized under these conditions *in vitro* (Vayer *et al.*, 1985c) (Fig. 2).

In vivo, NADPH concentrations in GHBergic neurons could be maintained low by the reduction of glucuronate and the cytosolic pool of SSA, always very low, could be rapidly transported to the mitochondria and transformed into succinic acid. In addition, a direct transport of GHB itself to the mitochondria cannot actually be ruled out. A GHB-oxoacid-transhydrogenase, capable of reducing GHB to SSA is located in the mitochondria (Kaufman *et al.*, 1988; Kaufman and Nelson, 1991). However, this enzyme does not appear to be involved in GHB catabolism since it is not inhibited by valproate and is absent from foetal and neonatal brain (Nelson and Kaufman, 1994).

Several authors have suggested that at least a part of the cytosolic pool of SSA coming from GHB degradation is transformed into GABA. *In vivo*, labelled GABA is formed from labelled GHB with no increase in the brain GABA concentration (Mitoma and Neubauer, 1968; Margolis, 1969; Doherty *et al.*, 1975b), although one group has reported a GABA increase in rat brain 120 min after i.p. administration of 500 mg/kg of GHB (Della Pietra *et al.*, 1966). De Feudis and Collier (1970) also reported an increase in GABA radioactivity 60 and 120 min after 1-[^{14}C]-GHB injected i.p. Others studies show very little incorporation of [^{14}C]-GHB into [^{14}C]-GABA, however these studies measured the brain radioactive amino acid pool less than 20 min after [^{14}C]-GHB administration (Doherty and Roth, 1978; Möhler *et al.*, 1976).

In vitro, radioactive GHB is consistently transformed by brain extract into radioactive GABA (Vayer *et al.*, 1985b). Semicarbazide, a GAD inhibitor, reduced radioactive GABA production when [^{14}C]-glutamate was the precursor but not when [^{14}C]-GHB was the precursor, indicating that GHB is converted directly to GABA by the brain homogenate without passing through glutamic acid (Mitoma and Neubauer, 1968). In our hands, *in vitro* experiments carried out on brain homogenates or on brain slices incubated under physiological conditions always gave rise to significant amounts of radioactive GABA (Vayer *et al.*, 1985b). In the presence of brain slices, 30 min incubation of labelled glutamate and non-radioactive GHB generated labelled 2-oxoglutarate, suggesting that GABA-T is involved in catalysing GABA synthesis. Furthermore, specific inhibitors of GABA-T (ethanolamine-O-sulphate, gabaculine or aminooxyacetic acid) strongly reduced the production of labelled GABA from labelled GHB and of labelled 2-oxoglutarate from labelled glutamate. Under these conditions, transformation of GHB into GABA was not inhibited by malonate, demonstrating that the succinate-linked pathway is not involved in the generation of GABA. With 2–70 μM GHB in the medium, the apparent K_m for the transformation of GHB into GABA by the multienzymatic system (GHB-DH + GABA-T) was found to vary from 55 to 145 μM, which is compatible with the brain GHB concentrations that exist *in vivo* (Vayer *et al.*, 1985b, 1985c).

When administered *in vivo*, the effects of GABA-T inhibitors on GHB levels were found to be apparently contradictory. Eli and Cattabeni (1983) report a decrease of brain GHB levels after i.p. administration of γ-acetylenicGABA or aminooxyacetic acid to rats 120 or 60 min respectively before being sacrificed. In apparent contradiction to these results is the report of Snead (1987) that indicates an increase in GHB concentrations, *in vitro* and *in vivo*, in presence of GABA-T inhibitors (γ-vinylGABA, γ-acetylenic-GABA or aminooxyacetic acid). Even if another source of GHB exists in brain (other than GABA), the increase in brain GHB levels (seen particularly in the synaptosomal fraction) seems to indicate that a GABA-T activity is involved in the degradation of GHB. The discrepancy observed with the results of Eli and Cattabeni (1983) is in favour of two different GABA-T activities (two different protein species and/or with different cellular locations) participating in both the synthesis and degradation of GHB. The different subcellular localization of these two GABA-T activities and perhaps their respective sensitivities to the inhibitors make the kinetics of inhibition of these two GABA-T pools somewhat different. In a particulate fraction of rat brain, the cytosolic GABA-T pool is more rapidly exposed to the inhibitors than the mitochondrial pool. In the *in vivo* experiments, differences between the times of sacrifice after administration of the various inhibitors

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