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Unravelling the brain targets of γ -hydroxybutyric acid

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Abstract

γ -Hydroxybutyric acid (GHB) is a naturally occurring γ -aminobutyric acid (GABA) metabolite that has been proposed as a neurotransmitter/neuromodulator that acts via its own receptor (GHB_R). Its exogenous administration, however, elicits central nervous system-dependent effects (e.g. memory impairment, increase in sleep stages 3 and 4, dependence, seizures and coma) that are mostly mediated by GABA_B receptors. The past few years have seen important developments in our understanding of GHB neurobiology: a putative GHB_R has been cloned; a transgenic model of GHB aciduria has been developed; GABA_B receptor knockout mice and novel GHB analogs have helped to characterize the vast majority of exogenous GHB actions mediated by GABA_B receptors; and some of the cellular mechanisms underlying the dependence/abuse properties of GHB, and its ability to elicit absence seizures and an increase in sleep stages 3 and 4, have been clarified. Nevertheless, the physiological significance of a brain GHB signaling pathway is still unknown, and there is an urgent need for a well-validated functional assay for GHB_Rs. Moreover, as GHB can also be metabolized to GABA, it remains to be seen whether the many GABA_B receptor-mediated actions of GHB are caused by GHB itself acting directly on GABA_B receptors or by a GHB-derived GABA pool (or both).

Introduction

γ -hydroxybutyric acid (GHB) was originally synthesized as a γ -aminobutyric acid (GABA) analogue that would readily cross the blood–brain barrier [1], and was later found to occur naturally in the brain [2] as a metabolite and, under certain conditions, as a precursor of GABA [3] (Figure 1). Endogenous GHB has been suggested to act as a neurotransmitter/neuromodulator in a manner independent from that of the GABA systems [3]. Thus, GHB has its own high (and low) affinity binding sites in different species, including humans [3–5], the distribution and ontogeny of which differ from those of GABA_A and GABA_B receptors [3,4]; its own vesicular uptake system [6]; and a presynaptically located synthesizing enzyme [3,7].

Exogenously administered GHB induces a wide range of neuropharmacological actions, including sedation, memory impairment, an increase in sleep stages 3 and 4, seizures, dependence/abuse and coma [7]. Experimentally, GHB-elicited absence seizures in the rat [7] are the best pharmacological model of human typical absence seizures [8] whereas, clinically, GHB is successfully used for the treatment of cataplexy/narcolepsy [9,10] and has

a limited use as an anesthetic [7] and in alcohol dependence/withdrawal [11]. In addition, because of its euphorogenic, behavioural disinhibition and amnesic properties, coupled with simple administration (i.e. high solubility, colourless and tasteless drink), the abuse/misuse of GHB as a recreational substance and as a so-called 'date-rape drug' has risen sharply in recent years, leading to an increased number of overdoses and intoxications for which no specific antidote exists [7,12]. By the use of potent and selective GABA_B receptor antagonists, most of these actions of exogenous GHB have been shown to be mediated, either fully or in part, by GABA_B receptors [3,7,13,14].

Notwithstanding its endogenous presence in the brain, its few experimentally and clinically significant uses, and the toxicity resulting from its abuse/misuse or its pathological increase (e.g. in human GHB aciduria [7]), the physiological significance of a brain GHB signaling pathway and the detailed mechanisms of many actions of exogenous GHB remain unclear. Indeed, the uncertainty about the existence of a GHB receptor (GHBR), the potential conversion of GHB to GABA (Figure 1), its weak agonist activity at GABA_B receptors (Table 1) and the lack of potent and specific GHBR agonists/antagonists (including the 'putative' antagonist NCS-382, see Box 1) have often led to ambiguous and contradictory results, and thus to long-standing controversies. Here, in an attempt to focus future research, we review data from the past three years, highlighting major breakthroughs including the cloning of a putative GHBR, the development of novel GHB ligands, the elucidation of the component cellular mechanisms of some GHB actions, and the demonstration of its pathological increase in GHB aciduria (for recent reviews, see [7,13,14]).

The elusive GHBR

In 2003, exactly 40 years after its discovery in the brain [2], the cloning of a putative GHBR has been reported [15]. Using the high-affinity ligand NCS-400, a 512 amino acid protein (57 kDa) has been isolated that shows a predicted secondary structure indicative of seven-transmembrane-spanning regions (as for G-protein-coupled receptors [GPCRs]) and of several phosphorylation sites (Table 1). However, no sequence homology exists with any known GPCRs (including the GABA_B receptor), although there is a 65% homology (in a 175 amino acid sequence) with a member of the tetraspanin family, which contains four-transmembrane-spanning region proteins [16]. GHBR mRNA shows a brain distribution similar to that of native GHB binding sites, but with some differences, such as its high density in the cerebellum where no or very few native GHB sites have been detected (Table 1)[3,4,7]. The pharmacological profile of the recombinant protein expressed in Chinese hamster ovarian cells is also similar to that of native GHB sites (Table 1), but again with one major difference (i.e. the lack of NCS-382 binding); this and the presence of three mRNA bands are indicative of GHBR subtypes. The recombinant receptor elicits a transient inward cation current in response to low GHB concentrations (ED₅₀ of 4.0 μM), which is sensitive to GTPγS dialyzed into the cell [15], although the quality of these electrical recordings is debatable.

Box 1 The 'putative' GHB antagonist NCS-382

NCS-382 is the only antagonist within the limited array of pharmacological tools available to investigate the complex GHB neurobiology. Because inverse and partial agonist actions of NCS-382 have been reported in biochemical and electrophysiological studies, and its brain levels following systemic administration have never been measured, we previously discouraged uncritical interpretations of data that were based on the simplistic assumption that NCS-382 only behaves as a potent and specific GHB antagonist [13]. Indeed, a comprehensive analysis of NCS-382 effects [14], which also considered many behavioural investigations, has reached similar conclusions. Since then,

the first evidence of an NCS-382-sensitive, *in vitro* electrophysiological effect of GHB in native tissue has been reported [54]; however, many examples of inverse and/or partial agonist actions of NCS-382 have also been described for GHB effects as diverse as loss of righting reflex in mice [55], drug discrimination tests in rats [32], and ataxia and abdominal discomfort in baboons [34]. Moreover, the ability of NCS-382 to counteract GHB-elicited reductions in hippocampal glutamate release seems controversial [56,57], and partly in contrast to previous electrophysiological data [58]. Notwithstanding these issues, the usefulness of NCS-382 as a potent and specific ligand at native brain GHB binding sites [3,7] remains unquestionable. As such, the synthesis of [³H]-NCS-382 [59], together with the characterization of its binding in brain membrane homogenates and sections [60,61], has provided an invaluable tool for investigating the complex GHB neurobiology and the development of novel GHBR ligands [23,26].

In summary, although these data represent the first report of the cloning of a putative GHBR, it is surprising that its properties — its strong expression in the cerebellum, its lack of NCS-382 binding, and its link to a mixed cationic (Na⁺/K⁺) current (as opposed to a Ca²⁺ current [17,18]) — do not match those that would have been expected from the vast majority of *in vivo* and *in vitro* studies on native GHBRs [3,7].

Diversity of GHBR(s) and GABA_B receptors

Additional evidence that GHBR(s) and GABA_B receptors are distinctive entities has been provided by transgenic and molecular techniques. The two lines of GABA_{B1} receptor knockout (KO) mice, recently generated from a C57B16/j and a Balb/c background, lack the most prominent pharmacological actions of exogenously applied GHB (i.e. hypolocomotion, hypothermia, increase in striatal dopamine synthesis, and sleep delta wave induction) [19,20]. By contrast, [³H]-NCS-382 binding is not changed in both types of KO mice [19,21]. [³H]-GHB binding is unchanged in the Balb/c-derived KO line [19], but is reduced by 50% in the C57B16j-derived KO animals [21], indicating that in this latter line the action of GHB might be linked to GABA_B receptors. Furthermore, the study of Wu *et al.* [21] showed a lack of binding of ‘physiological’ concentrations (up to 100 nM) of [³H]-GHB and of [³H]-NCS382 to recombinant GABA_{B1b}, GABA_{B2} and GABA_{B1b+2} receptors expressed in HEK 293 cells, although high GHB concentrations (30 mM) do displace [³H]-CPG54626A binding, supporting previous data from COS cells (ED₅₀ of 5 mM) [22].

All of these data strongly indicate that GHBRs and GABA_B receptors are separate molecular entities, highlight several GHB effects that are mediated by GABA_B receptors, and indicate the existence of GHBR subtypes. Interestingly, the NCS-382-insensitive GHBR subtype suggested by the work of Wu *et al.* [21] is linked to GABA_B receptors, and therefore differs from the cloned NCS-382-insensitive GHBR [15].

Novel GHB ligands

Another approach to differentiate between GHBR- and GABA_B receptor-mediated effects has been to use novel GHB ligands that are not metabolized to GABA and have no (or very little) activity at GABA_B receptors [23,24,25]. These compounds are unable to mimic discriminative stimulus effects, catalepsy and, in part, the loss of righting reflex elicited by GHB, effects which are blocked by GABA_B receptor antagonists.

In addition, the development of rigid, stereospecific GHB ligands that do not bind to GABA_B receptors has identified the R-isomer of HOCPCA (3-hydroxycyclopent-1-enecarboxylic acid) as the most potent ligand so far for native GHB binding sites (with a 39-fold higher affinity than GHB) [26]. This has helped elucidate, firstly, the conformational

structure of GHB when it binds to native GHB sites; secondly, the structural preference of the recognition site on the putative receptor complex; and thirdly, how a different GHB conformation is likely to be the one interacting with the GABA_B receptor. Some disagreement, however, still exists surrounding the precise role of the proton in the –OH group of the GHB molecule, with recent evidence indicating that it functions as either a hydrogen-bond acceptor [27] or a classical hydrogen-donor [26].

This rapid development of new pharmacological tools within the past three years has highlighted the urgent need for a well-validated functional assay for GHBR(s). In addition, knowledge of the *in vitro* and *in vivo* electro-physiological actions, as well as other biochemical and behavioural effects, of all these novel GHB ligands [23,26] would undoubtedly be of great help in understanding the physiological role of endogenous GHBRs.

A unifying cellular mechanism for the dependence/abuse and anti-craving properties of GHB

An elegant *in vitro* study [28] has recently demonstrated bi-directional effects of GABA_B receptor activation on the overall output of the ventral tegmental area (VTA), one of the key brain reward regions [29]. These data provide an interesting cellular mechanism to explain two opposing properties of GHB: dependence/abuse and anti-craving. Cruz *et al.* [28] showed that, owing to differences in the distribution of G-protein-dependent inward-rectifying K⁺ (GIRK) channel subunits (Figure 2), GABA_B receptor agonists hyperpolarize GABAergic neurons with a smaller ED₅₀ than they do dopaminergic neurons. Thus, at concentrations typically seen during its recreational use, GHB would preferentially inhibit GABAergic neurons, with a resulting disinhibition of dopaminergic neurons leading to the increased VTA output characteristic of drugs of abuse (Figure 2). At higher GHB concentrations, dopamine neurons would also be directly hyperpolarized, with a resulting decrease in VTA dopamine output (Figure 2). This scenario is supported by a recent *in vivo* investigation in the VTA [30] where intravenous administration of GHB was shown to either depress or increase firing in antidromically identified dopamine neurons that had a high or low baseline firing rate, respectively (for further details, see [30]). These bi-directional effects of GABA_B receptor activation in the VTA also explain why at therapeutic levels the classical high-affinity GABA_B receptor agonist baclofen blocks self-administration and displays potential anti-craving properties, but can also elicit euphoria [28].

Importantly, other GHB effects that are likely to contribute to its abuse potential and are shared by many drugs of abuse [29] are also exclusively mediated by GABA_B receptors; these include discriminative stimulus properties [23,25,31,32], physical dependence in baboons [33,34], depression of nucleus accumbens firing [30], an increase in activator protein 1 DNA binding [35], and the long-lasting inhibition of mitogen-activated protein kinase (MAPK) phosphorylation [36].

Cellular mechanism of GHB-elicited absence seizures

Absence seizures elicited by systemically administered GHB are the best pharmacological model of typical absence seizures [7,8], and can also be induced by *in vivo* application of GHB directly into the rat ventrobasal (VB) thalamus [37]. Gervasi *et al.* [38] investigated the effects of GHB on GABA_A inhibitory postsynaptic currents (IPSCs) (from the GABAergic thalamic reticular neurons) and glutamatergic excitatory postsynaptic currents (EPSCs) (from the cortex) recorded in thalamocortical neurons of the rat VB. A concentration of GHB similar to the threshold brain concentration for the induction of absence seizures (i.e. 250 μM) produces a 20% decrease in the amplitude of all EPSCs but in almost none of the GABA_A receptor IPSCs (Figure 3). At 500 μM and 1 mM, GHB

reduces (by 35%) all EPSCs and about 45% of IPSPs; at 10 mM, all IPSCs and EPSCs are reduced by 55% (Figure 3). All of these effects of GHB are abolished by GABA_B receptor antagonists, but are not blocked by NCS-382 nor mimicked by 4-hydroxycrotonic acid or NCS-356, two GHBR agonists. The 'excessive' inhibitory input resulting from this presynaptic GABA_B receptor-mediated imbalance between excitatory and inhibitory drive to thalamocortical neurons seen with 250 μ M GHB was also shown to lead to an increase in neuronal bursting and synchronization (Figure 3), both of which are believed to underlie the development of absence seizures [8]. In addition, because baclofen depressed all EPSCs and IPSCs even at concentrations as low as 0.1 μ M [38], this study also provides the first evidence for the existence of presynaptic GABA_B autoreceptors, which are less sensitive to GHB than presynaptic GABA_B heteroreceptors.

Cellular mechanism of GHB-elicited increase in sleep stages 3 and 4

GHB administered to healthy volunteers [39] and during the treatment of human cataplexy/narcolepsy [9,10] leads to an increase in stages 3 and 4 of non-REM sleep (which are characterized by a progressive contribution of delta waves in the electroencephalogram [EEG]), an action mirrored by the GHB-elicited induction of EEG delta waves in normal mice [19], rats [40] and cats [41]. These and other sleep-related EEG waves are critically set by cellular activities within the different components of the thalamo-cortical loop [42], including dorsal thalamic nuclei such as the VB. Thus, the presynaptic and post-synaptic GABA_B receptor-mediated actions of GHB on thalamocortical [38,43,44] and cortical [45] neurons provide a solid cellular platform to explain GHB effects on sleep waves. As indicated in the previous section, at GHB concentrations >250 μ M and up to 1 mM, the extent of the GHB-elicited decrease in EPSPs and IPSPs becomes stronger and, more importantly, the postsynaptic GABA_B receptor-mediated activation of GIRK channels increases [38,43]. These combined effects lead to a progressively larger hyperpolarization of thalamocortical neurons and the instating of delta oscillations [46] (Figure 3), which are the cellular thalamic counterpart of sleep delta waves [42]. Higher doses of GHB, however, bring about a much stronger hyperpolarization (via massive GIRK activation), which will force the membrane potential of thalamocortical neurons below the narrow voltage range in which delta oscillations occur [46] (Figure 3). Eventually, the massive thalamic [38,43] and cortical [45] hyperpolarization, together with the strong (>55%) decrease in EPSPs and IPSPs both in thalamic [38,44] and cortical [45] neurons, leads to a condition where cellular and network excitability is almost irredeemably compromised, as experienced during overdose/coma (Figure 3).

Is GHB-derived GABA underlying the actions of GHB?

As has been highlighted in the previous sections, solid and independent approaches have clearly demonstrated that the vast majority of exogenous GHB-elicited effects, including an increase in neurosteroids [47], are mediated by GABA_B receptor activation. As up to 0.36% of GHB can be metabolized into GABA, at least *in vitro* [48] (Figure 1), it is of critical importance to know whether these GABA_B receptor-mediated actions of GHB are either fully or in part caused by GHB itself acting directly on GABA_B receptors, or by a GHB-derived GABA pool that then activates GABA_B receptors. Recent evidence, however, has not helped to resolve this long-standing controversy [3,7,12], as data supporting either view have been reported. Thus, on the one hand, Ren and Mody [36] found no difference between the GHB-elicited inhibition of MAPK activity in the presence of valproate, which blocks over 93% of the conversion of GHB to GABA [48](Figure 1), and that in its absence. Conversely, Wu *et al.*[21] did not detect any binding of up to 100 nM GHB to different combinations of recombinant GABA_B receptors, although higher GHB concentrations, similar to those achieved during its exogenous administration [7], should have been tested.

GHB aciduria

Succinic semialdehyde dehydrogenase (SSADH) KO mice (SSADH^{-/-}) [49] have been developed in which a null mutation of this enzyme results in higher GABA and GHB levels in brain, liver, blood and urine, leading to a phenotype similar to the complex spectrum of non-specific neurological symptoms observed in humans suffering from the rare autosomal, recessively inherited disorder called GHB aciduria (or SSADH deficiency). SSADH^{-/-} mice have already proved useful for assessing novel avenues of treatment (e.g. taurine) for the human disorder [49], and are also a highly suitable model to study the pathophysiological mechanisms of this disease and those underlying the toxicity arising from GHB abuse/misuse. In this respect, it is worth noting that NCS-382 shows a stronger protection (62%) against lethality in these KO mice than does vigabatrin (50%) or CGP35348 (27%) [50], although more potent GABA_B receptor antagonists fully protect normal rats against a lethal GHB dose [51]. The high protection provided by NCS-382 suggests a potentially critical role for GHBR(s) in the mortality of these mice, a view that is supported by the developmental appearance (in the 3rd postnatal week) of GHB binding sites in normal animals [4] and of critical symptoms in the SSADH^{-/-} mice [49], and by the protection achieved with vigabatrin [50], which blocks the conversion of GABA to GHB [3] (Figure 1).

Finally, because the postnatal development of the EEG and behavioural phenotype of SSADH^{-/-} mice (from absence seizures to myoclonic jerks and generalized convulsive seizures) [52] is similar to that often observed in juvenile absence epilepsy [8,53], these mice might offer the first model for this human disorder and could become an invaluable tool to investigate the pathophysiological mechanisms of the transitions among these different epileptic states, and the relative contribution of GHBR(s) and GABA receptors.

Conclusions

The past few years have seen important developments in our understanding of GHB neurobiology: a putative GHBR has been cloned and some of the key cellular mechanisms of its exogenous actions have been unravelled. Full characterization of the cloned GHBR must now follow in order to clarify the inconsistencies between its properties and those of the native GHB sites, and the field urgently needs a well-validated functional assay for GHBR(s). The vast majority of the actions elicited by exogenous GHB has been shown to be mediated by GABA_B receptors, but it is still debatable whether GHB itself or a GHB-derived GABA pool activates these GABA_B receptors. Moreover, the physiological significance of the endogenous GHB signaling pathway in the brain remains unknown. Future studies using structurally rigid, potent and specific GHB analogues that are insensitive to metabolic degradation, as well as transgenic models and novel ligands, will undoubtedly help to resolve these critical issues.

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Figure 1.

GHB synthesis and metabolism. Schematic diagram of the pathways responsible for the synthesis and metabolism of GHB (the enzymes involved are indicated by numbers, see below). GHB activates GHB receptors, but also GABA_B receptors as a weak agonist. Because GHB can be converted to GABA, some of the GABA_B receptor-mediated actions of GHB could be elicited by the GHB-derived GABA pool interacting with GABA_B receptors. 1: alcohol dehydrogenase and acetaldehyde dehydrogenase; 2: β -oxidation pathway; 3: NADPH-dependent cytoplasmic aldehyde reductase, ALR-1; 4: mitochondrial pyridine nucleotide-independent oxido-reductase; 5: NADPH-dependent cytoplasmic aldehyde reductase, ALR-2; 6: specific succinic semialdehyde reductase; 7: γ -aminobutyric acid transaminase; 8, succinic semialdehyde dehydrogenase; TCA: tricarboxylic acid.

Figure 2.

GABA_B receptor-mediated cellular actions of GHB in the VTA. The lower EC₅₀ (0.9 mM) for the coupling of postsynaptic GABA_B receptors to GIRK channels in GABAergic compared to that of dopaminergic neurons (27 mM) is determined by the different GIRK subunit compositions (GIRK1, 2 and 3) in the two neuronal types [28]. At concentrations seen in its recreational use (<1 mM), GHB preferentially activates postsynaptic GABA_B receptors on GABAergic neurons, hyperpolarizing them and decreasing their firing rate (middle panel). This effect would in turn disinhibit the dopaminergic neurons, increasing their firing rate and thus leading to enhanced dopamine output from the VTA, which is typical of drugs of abuse (middle panel). Higher GHB concentrations, however, would also decrease the firing rate of dopaminergic neurons by directly hyperpolarizing these neurons, thus giving rise to an overall reduction in dopamine output from the VTA (right panel), which explains the anti-craving properties of GHB that are exploited therapeutically in the treatment of alcohol dependence/withdrawal [11]. No information is available on the action of GHB on presynaptic GABA_B receptors of GABAergic neurons (dashed line) and other nerve terminals of extrinsic origin to the VTA. Schematic representation of changes in firing under different conditions is represented on the right hand side of each neuronal type.

Figure 3.

GABA_B receptor-mediated cellular effects of GHB in the thalamus. Increasing GHB concentrations (left to right in the figure) progressively affect a greater number of presynaptic GABA_B receptors on GABAergic terminals of the nucleus reticularis (NRT) neurons, whereas all presynaptic GABA_B receptors on the glutamatergic cortical and sensory afferents are activated, even by 250 μM GHB [38]. The intensity of this effect (i.e. depression of synaptic potentials), as well as the amplitude of the hyperpolarization resulting from the activation of postsynaptic GABA_B receptors on thalamocortical neurons, becomes stronger with increasing GHB concentrations (indicated by an increased thickness of curved arrows). The enhanced inhibition of thalamocortical neurons arising from the imbalance between excitatory and inhibitory synaptic drive with 250 μM GHB increases the number of active neurons, their bursting characteristics and synchronization (as shown by the left-hand electrical records), as it occurs during absence seizure paroxysms. Importantly, 240 μM GHB is the threshold brain concentration for the EEG appearance of EEG spike and wave discharge [7], the hallmark of absence seizures [8]. At higher GHB concentrations, the GIRK-mediated hyperpolarization [38,43] and the increased depression of synaptic inputs [38,44] hyperpolarize thalamocortical neurons (middle electrical record) into the membrane potential region where delta oscillations (enlarged section) might occur. At much higher GHB concentrations (right-hand electrical record), delta oscillations will only momentarily occur as the membrane potential crosses their voltage region of existence; eventually, a very hyperpolarized membrane potential will be instated, as is likely to occur during overdose/coma.

Table 1
Comparison of the cloned GHB receptor, native GHB sites and native GABA_B receptors.

	Cloned GHB receptor	Native GHB sites	Native GABA _B receptors
GHB binding	High affinity	High and low affinity	Low/no affinity
NCS-382 binding	None	High affinity (mainly)	None
GABA binding	Almost absent	None	High affinity
Baclofen binding	None	None	High affinity
Secondary structure	Seven-transmembrane regions (predicted)	Unknown	Seven-transmembrane regions
Effector system	Cation current	VACC (in NCB-20 cells only)	GIRK and VACC ^a
Ontogeny	Unknown	Appear at postnatal week 3	Present at birth
Regions with high density of binding sites	Cerebellum, hippocampus (dentate gyrus), olfactory bulb	Hippocampus, septum, prefrontal cortex, amygdala	Cerebellum, thalamus, interpeduncular nucleus, frontal cortex
Regions with low density of binding sites	Caudate/putamen, thalamus, amygdala	Cerebellum, brain stem	Striatum, basal forebrain

VACC, voltage-activated calcium current.