

The Astrocytic GABA_A/Benzodiazepine-Like Receptor: The Joker Receptor for Benzodiazepine-Mimetic Drugs?

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Abstract: Long-term use of benzodiazepines as hypnotics, anxiolytics, anticonvulsants and muscle relaxing drugs is jeopardized by adverse effects on memory, addictive properties, and development of tolerance. Major efforts have gone into developing 'benzodiazepine-like' drugs that are more selective in their therapeutic effect, have additional uses and/or lack the adverse effects of benzodiazepines. The reviewed prototype patent exemplifies such efforts. Newer drugs are thought to act selectively on one of the two neuronal benzodiazepine receptors, on the astrocytic mitochondrial benzodiazepine receptor and/or on GABA_A/benzodiazepine receptor complexes displaying specific subunits. It is overlooked that astrocytes also express benzodiazepine receptors that enhance depolarization-mediated entry of Ca²⁺ by interacting with membrane-associated GABA_A-like receptors, *mediating depolarization* because of a high Cl⁻ concentration within astrocytes. The resulting increase in free cytosolic Ca²⁺, which stimulates glycogenolysis, is inhibited not only by the 'peripheral-type' benzodiazepine antagonist PK11195 but also by the 'neuronal' antagonist flumazenil. Increasing awareness of the role(s) of astrocytic Ca²⁺ homeostasis and energy metabolism for CNS function suggests that activation of this receptor might contribute to both therapeutic and adverse effects of benzodiazepine-like drugs. This receptor should be kept in mind when developing and testing new drugs; in turn these drugs may help elucidating its functional role.

Keywords: Anticonvulsant, anxiolytic, astrocyte, benzodiazepine, calcium channel, chloride channel, GABA, flumazenil, glycogen, hypnotic, PK 11195.

1. INTRODUCTION: WHY DEVELOP NEW BENZODIAZEPINE-LIKE DRUGS

Benzodiazepines are widely used clinically as anxiolytics, sedatives/hypnotics, anticonvulsants and muscle relaxants. However, the long-term use of benzodiazepines is jeopardized by adverse effects on memory, by addictive properties and by development of tolerance [1]. There is a consensus that most drugs that chemically are benzodiazepines act on different subtypes of benzodiazepine receptors on both neurons (BZ1 and BZ2 or omega1 and omega2) and astrocytes (omega3), and that they do not discriminate between GABA_A receptors displaying different combinations of α , β , and γ membrane spanning subunits. Major efforts have therefore gone into developing 'benzodiazepine-like' drugs that chemically are not benzodiazepines and therefore might i) be more selective in their therapeutic effect (e.g. mainly exert hypnotic effects, and ii) be without adverse effect displayed by the benzodiazepines. In contrast to the binding of most benzodiazepines proper to at least three sets of receptors and to GABA_A/benzodiazepine receptor complexes displaying different α , β , and γ subunit composition, some of the non-benzodiazepine benzodiazepine-like drugs act selectively on one of the two neuronal benzodiazepine receptors and show selectivity for receptor complexes where the GABA_A receptors display specific subunits. An example of such

drugs is the imidazopyridine-acetamide, zolpidem, which acts on only one of the two neuronal benzodiazepine receptors (omega1) and shows selectivity for GABA_A receptors displaying the $\alpha 1$ subunit [2]. These features may make it more selective as a hypnotic drug. Such drugs may or may not interact with the astrocytic omega3 benzodiazepine receptors, and another imidazopyridine-acetamide, the anxiolytic drug alpidem, binds with high affinity to the omega3 receptor [3].

Although a picture is only now beginning to emerge of correlations between chemical structure, binding characteristics, therapeutic effects and side effects of benzodiazepine-like drugs, the possibility to select for one of the many therapeutic effects of benzodiazepines with less expression of other effects exerted by these drugs has encouraged the invention of several new non-benzodiazepine benzodiazepine-like agents. The hope is that agonists or inverse agonists acting in this manner can be developed into drugs which can be used either alone against specific disorders or can be combined with other drugs to enhance the therapeutic effects of these drugs.

2. A PROTOTYPE PATENT AND THE JOKER RECEPTOR

2.1. The Patent

Scores of benzodiazepine-like drugs have been patented during recent years, indicating that this is an active and important field in drug development. We have chosen to discuss a very recent patent WO0501233306A2 from

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Neurogen Corporation [4]. The description of this patent is thorough and detailed, and a wide range of potential therapeutic indications is indicated. The inventions described in this patent provide pharmaceutical compositions that all are derivatives of imidazo-pyrimidines and triazolo-pyrimidines. Action of the new compounds as agonists at $\alpha_2\gamma_2$ and $\alpha_3\gamma_2$ receptors should be particularly useful for treatment of anxiety disorders (panic disorder, obsessive compulsive disorder, generalized anxiety disorder; post-traumatic stress disorder, and acute stress disorders). Agonist action at $\alpha_2\gamma_2$ and $\alpha_3\gamma_2$ receptor subtypes should also provide treatment for depressive or bipolar disorders, schizophrenia and sleep disorders, whereas agonist action at the $\alpha_5\gamma_2$ receptor subtype should induce amnesia. Action as agonists at the $\alpha_1\gamma_2$ receptor should be useful for treatment of sleep disorders and convulsive disorders such as epilepsy; conversely, action as inverse agonists at the $\alpha_5\gamma_2$ receptor subtype or both $\alpha_1\gamma_2$ and $\alpha_5\gamma_2$ receptor subtypes should provide treatment for cognitive disorders including those resulting from Down's Syndrome, Alzheimer's disease, Parkinson's disease and stroke related dementia. Action as inverse agonists at the $\alpha_5\gamma_2$ receptor subtype is thought to be useful for treatment of cognitive disorders through the enhancement of memory, particularly short-term memory, in memory-impaired patients, and action as antagonists at the benzodiazepine site reverses the effect of benzodiazepine overdose and may also be effective in the treatment of drug and alcohol addiction. In some of these situations the new compounds will be combined with other drugs acting on the central nervous system (CNS) in order to potentiate their effects.

The patent also describes a variety of *in vitro* uses for the novel GABA_A receptor modulators. These compounds may be used as probes for the detection and localization of GABA_A receptors in tissue sections or fractions thereof or in cultured cells, as positive controls in assays for receptor activity, and as standards and reagents for determining the ability of a candidate agent to bind to GABA_A receptors with specific subtype compositions. They may also be useful as radiotracers for positron emission tomography (PET) imaging or for single photon emission computerized tomography (SPECT) in order to characterize GABA_A receptors in living subjects.

What is not discussed in the prototype patent is the use of assays for demonstrating (or excluding) that some of the novel drugs (and perhaps also some presently prescribed drugs) may mainly or partly owe their effect to an action exerted on GABA_A/benzodiazepine-like receptor complexes in glial cells, especially astrocytes (the GABA_A receptors which have been demonstrated on cultured oligodendrocytes disappear during cell maturation [5]); the neglect of this receptor is, however, not special for this patent. Few, if any, patent applications or pharmacology textbooks acknowledge the existence of the GABA_A/benzodiazepine-like receptor complex in astrocytes in spite of the facts that (i) it is activated by presently used benzodiazepines with high potency, and (ii) flumazenil (previously known as RO 15-1788), which generally is regarded a typical 'neuronal-type' ligand (because it does *not* interact potently with the 'peripheral-type' omega3 receptor present in astrocytic

mitochondria), is an effective antagonist. This receptor is accordingly the Joker among the benzodiazepine recognition sites. The present review describes (i) its distinction from other benzodiazepine receptors, (ii) its expression on astrocytes, as indicated by binding characteristics and 'classical' physiological effects of GABA and/or benzodiazepines, (iii) the consequences for astrocytic function and CNS function of its activation by these drugs, (iv) the pharmacological relevance of the Joker receptor, and (v) its possible participation in a GABA-mediated neuronal-astrocytic inhibitory network.

2.2. Distinction of the Joker Receptor from other Benzodiazepine Receptors

Astrocytes are highly specialized cells, which in gray matter of the mammalian brain account for ~25% of the volume [6]. They have long been known to constitute the major brain site for a mitochondrial omega 3 receptor. This receptor is not associated with any GABA recognition site, and it is distinctly different from the astrocytic GABA_A/benzodiazepine receptor complex. The omega3 receptor, which is mentioned in the patent, is widespread in many organs, with high activity in adrenal glands [7]. The omega3 receptor regulates the transport of cholesterol across the inner mitochondrial membrane into the mitochondrial matrix, where it is used for synthesis of neurosteroids, many of which are neuromodulatory and exert a positive allosteric effect on neuronal GABA_A receptors. In experimental animals administration of these neurosteroids has anxiolytic, sedative/hypnotic, anticonvulsant and anesthetic effects (typical benzodiazepine-like effects), whereas a reduction in their concentration produces anxiety-like behavior and aggression [8]. The mitochondrial receptor is not inhibited by the 'neuron-specific' benzodiazepine antagonist flumazenil but generally by the 'peripheral-type' antagonist PK 11195 [7, 9].

In addition to mitochondrial omega3 receptors astrocytes express benzodiazepine binding sites that are part of a complex with membrane-associated GABA_A-like receptors and with Cl⁻ channels, which are opened by the binding of GABA to the complex. Like the neuronal GABA_A receptors the astrocytic GABA_A-like receptor is a pentameric structure made up by α , β , and γ subunits. Although these receptors are very similar to the neuronal GABA_A receptors, a few benzodiazepines affect receptor function differently than in neurons [10, 11]. This distinction is not important for the general effects of benzodiazepines on the astrocytic GABA_A-like receptor, although it affects the response to some specific ligands. *In contrast, it is extremely important that GABA-mediated opening of Cl⁻ channels in astrocytes causes an exit of Cl⁻, because of a high intracellular Cl⁻ concentration*, established by the operation of two inwardly directed Cl⁻ transport mechanisms, a Na⁺,K⁺,2Cl⁻ cotransporter [12-14] and a Cl⁻/HCO₃⁻ exchanger [15, 16]. Therefore, in contrast to the neuronal hyperpolarization following exposure to benzodiazepine agonists, astrocytes will become slightly depolarized by these drugs.

During normal brain activity the extracellular K⁺ concentration increases, which also causes a modest depolarization. Summation of the benzodiazepine-provoked depolarization and the K⁺-mediated depolarization leads to a

depolarization that is larger than that evoked by elevated K⁺ concentrations alone. One of the major consequences of astrocytic depolarization is an opening of voltage-sensitive L-channels for Ca²⁺, which in turn causes an increase in free cytosolic Ca²⁺ concentration ([Ca²⁺]_i) [17, 18], an important signaling mechanism in astrocytes. In the absence of any GABA- or benzodiazepine-provoked depolarization the K⁺-mediated Ca²⁺ uptake reaches its maximum at 40-50 mM K⁺ [19]. However, in the presence of GABA/benzodiazepine agonists, submaximal K⁺-mediated increase in [Ca²⁺]_i is potently enhanced, an effect that is directly inhibited not only by the 'peripheral-type' benzodiazepine antagonist PK11195 but also by the 'neuron-specific' flumazenil, which has no effect on the mitochondrial, peripheral-type omega 3 receptor. This will be discussed in detail below. However, first the expression of GABA_A/benzodiazepine receptor complexes on astrocytes will be briefly described.

3. EXPRESSION OF ASTROCYTIC GABA_A/ BENZODIAZEPINE RECEPTOR COMPLEXES

3.1. Evidence for GABA_A Receptor Expression

3.1.1. Immunocytochemistry

Antibodies that specifically recognize the affinity-purified GABA_A receptor of rat brain, the α_1 subunit C-terminus, or the α_1 subunit N-terminus all stain the cell body and/or processes of freshly isolated hippocampal rat astrocytes, as shown by dual labeling for GFAP (glial fibrillary acidic protein) and each of these antibodies [18]. No label was seen following substitution of the antibodies with the preimmunization sera from the animal which subsequently was used for antibody production. Immunogold studies have indicated the preponderance of α_2 and α_1 expressing GABA_A receptors in the astrocytic Bergmann fibers of the cerebellum [20], but it is unknown whether this is a cell type characteristic or special for this type of astrocyte processes. Astrocytic heterogeneity is indicated by the observation that GABA_A-expressing astrocytes in the supraoptic nucleus lack glutamate transporters [21].

3.1.2. mRNA Expression

Much lower levels of the total amount of mRNA for the different subunits of the GABA_A receptor were found in cultured cerebellar astrocytes than in cultured cerebellar granule neurons [22]. Nevertheless, almost all the subunits were expressed in the astrocytes, the α_1 and α_2 , the β_1 and β_3 and the γ_1 mRNAs abundantly, which is consistent with the expression of GABA_A receptors expressing α_2 and α_1 in Bergmann glia.

3.1.3. Muscimol Binding to Glial Cells Isolated by Gradient Centrifugation

A high-affinity (K_D 14.5 nM) binding of [³H]muscimol with a B_{max} of ~10 pmol/mg protein has been observed in brain membrane preparations from rat glial cells obtained by gradient centrifugation [2]. However, for unknown reasons early autoradiographic and biochemical studies failed to show GABA_A receptors on cultured astrocytes [24-26].

3.2. Evidence for Benzodiazepine Receptor Expression

3.2.1. Membrane-Associated Benzodiazepine Binding

A fluorescent benzodiazepine derivative of RO 7-1986, which has high affinity and selectivity for 'central-type' GABA_A receptors [27] has been used to demonstrate membrane-bound receptors on freshly isolated astrocytes (Fig. 1). The membrane-bound receptors occur in discrete patches on both cell bodies and distal processes [18]. This is similar to what had previously been described after staining with three different monoclonal antibodies towards the GABA_A/benzodiazepine receptor complex on cultured astrocytes after mild fixation, whereas unfixed cultured cells

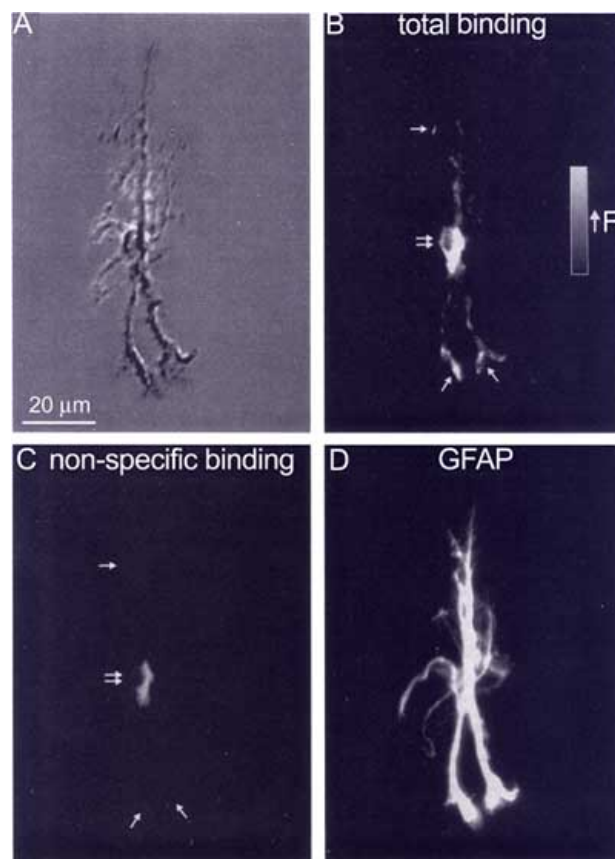


Fig. (1). Localization of 'central-type' benzodiazepine receptors reacting with a fluorescent derivative of RO 7-1986 (5 nM) on an acutely isolated astrocyte from the rat hippocampus. A: Digital photomicrograph of the isolated cell. B: Areas of intense fluorescence indicate the presence of GABA_A/benzodiazepine receptor complexes on both the cell body (double arrow) and on several larger processes (single arrows). The black/white scale bar represents increasing fluorescence intensity. C: Non-specific binding in the presence of 500 nM diazepam. D: Immunostaining of the astrocyte for the astrocyte-specific protein GFAP following fixation. From [18].

failed to stain, suggesting that the epitope reacting with the antibody was located within the membrane or on its cytoplasmic side [28]. When these antibodies were reacted

with Western blots of proteins extracted from the astrocytic cultures they recognized a single protein of approximately 63 kDa, a molecular weight which is significantly higher than those of the mass for neuronal and GABA_A receptor subunits (~50 and ~55 kDa, respectively), suggesting that the antibodies may recognize a GABA_A/benzodiazepine receptor protein that is related to, but biochemically distinct from the neuronal receptor.

Photoaffinity labeling of flunitrazepam, which otherwise only occurs to the 'central-type' benzodiazepine receptor [29] could be demonstrated to membranes of glial cells isolated by gradient centrifugation, but not to membranes from other tissues known to express "peripheral-type" (omega 3) receptors (liver and kidney), suggesting that a different receptor may be involved [23].

3.2.2. Benzodiazepine Binding to Glial Cells Isolated by Gradient Centrifugation and Cultured Astrocytes

Binding of [³H]flunitrazepam to glial cells fractions isolated by gradient centrifugation is of high affinity (K_D 1.3 nM) with a B_{max} of ~2 pmol/mg protein [23]. Flunitrazepam is potently displaced by clonazepam and the 'peripheral-type' ligand RO 5-4864, whereas flumazenil is a weak displacer. Primary cultures of astrocytes also display a typical 'peripheral-type pattern' for displacement of diazepam binding by other benzodiazepines [30], i.e. very low IC_{50} values for RO 5-4864 and PK 11195 (an antagonist in most cell types, but an agonist in some [9]), but similar to the observation in freshly isolated cells a high value for displacement by flumazenil (IC_{50} : 73 μ M). The reason for this 'peripheral-type' binding pattern is likely to be a high total binding density (a B_{max} for flunitrazepam binding of ~6 pmol/mg protein [31]), most of which reflects binding to omega3 receptors, obscuring binding characteristics for the GABA_A/benzodiazepine-like receptor. This is one of the reasons this receptor so frequently is overlooked.

3.3. Physiological Evidence for Astrocytic GABA_A/Benzodiazepine Receptors

A GABA-induced depolarization of astrocytes in primary cultures was first described by Kettenmann and coworkers [32, 33] and it has been confirmed in explant cultures [34]. All astrocytes from cerebral hemispheres responded to GABA, the underlying mechanism was an increase in Cl⁻ conductance, and picrotoxin and bicuculline blocked the response, indicating similarity to neuronal GABA_A receptors [35]. In the presence of GABA concentrations between 10 μ M and 1 mM, flunitrazepam at a concentration of 1 μ M augmented the depolarization (Fig. 2), and the response was inhibited by the 'neuronal-type' benzodiazepine antagonist flumazenil, but not by the 'peripheral-type' antagonist RO 5-4864 [10]. PK 11195 was not tested, but the α -carboline DMCM (methyl-4-ethyl-6,7-dimethoxy- α -carboline-3-carboxylate), which in neurons is an inverse agonist, in most cells functioned as an agonist, i.e. increased the depolarization, rather than counteracting it, an observation confirmed by Bovolin *et al.* [22]. The inhibition by flumazenil is especially important because this suggests a possible contribution of this receptor to the therapeutic and adverse effects of benzodiazepines, most of which are counteracted by flumazenil, generally considered to act only

on the neuronal omega1 and omega 2 receptors and therefore used as a tool to distinguish between benzodiazepine effects on neurons and astrocytes *in vivo*.

GABA-activated inward currents have been confirmed in astrocytes in brain slices [36] and in freshly isolated hippocampal astrocytes, where a reversal potential of 0 mV is consistent with the expected Cl⁻ equilibrium potential as derived by the Nernst equation [11]. Moreover a decrease of the extracellular Cl⁻ concentration shifted the reversal potential in the expected positive direction, confirming that GABA activates a Cl⁻ conductance. Bicuculline and picrotoxin depressed the response to GABA by three quarter or more, whereas it was more than doubled by 10 μ M diazepam. In 20% of the cells DMCM potentiated the response, whereas it reduced it in the remaining 80%, indicating receptor heterogeneity. GABA_A receptors in cultured astrocytes from the spinal cord also react differently to DMCM depending upon whether they show a 'fibrous' or a 'protoplasmic' morphology [37].

Response of astrocytic GABA receptors to neuronally released GABA in an intact CNS structure has been demonstrated in the whole rat pituitary, which can be isolated and maintained *in vitro*. Stellate astrocytes in the pars intermedia, whose identity was confirmed after the experiment by immunostaining for GFAP, reacted to stimulation of K⁺-stimulated the infundibular stalk with an initial depolarization, which could be blocked by the GABA_A antagonist bicuculline [11].

4. SECONDARY EFFECTS OF GABA_A/BENZODIAZEPINE RECEPTOR ACTIVATION

4.1. Opening of Ca²⁺ Channels and Modulation of K⁺-Mediated Depolarization

4.1.1. Astrocytes Express Voltage-Sensitive L-Channels for Ca²⁺

Astrocytes in primary cultures display voltage sensitive Ca²⁺ channels of the L-type as indicated by potent inhibition of K⁺-stimulated ⁴⁵Ca uptake by the dihydropyridine L-channel blocker nifedipine, although only provided that they have been differentiated by culturing in the presence of dibutyryl cyclic AMP [38]. GABA has also been found to cause an increase in [Ca²⁺]_i in one third of acutely isolated astrocytes tested [18], in all probability a result of the opening of such channels.

4.2.2. Benzodiazepine-Mediated Augmentation of K⁺-Induced [Ca²⁺]_i Increase

In the presence of a K⁺ concentration (20 mM) causing submaximum depolarization and increase in [Ca²⁺]_i, the benzodiazepines midazolam (20 nM) (Fig. 3) and diazepam (100-500 nM) greatly enhance the effect of 20 mM K⁺ alone control on [Ca²⁺]_i in cultured astrocytes [19, 39, 40]. The dihydropyridine L-channel blocker nimodipine abolishes both the effect of elevated K⁺ and that of the benzodiazepine, whereas the benzodiazepine-mediated augmentation of the response is selectively prevented in the presence of 1 μ M of the 'peripheral-type' benzodiazepine antagonist PK 11195; 0.3 μ M PK 11195 causes only partial inhibition [40]. Since PK 11195 has very low affinity for the neuronal benzodiazepine receptor [30] and has little effect on GABA-

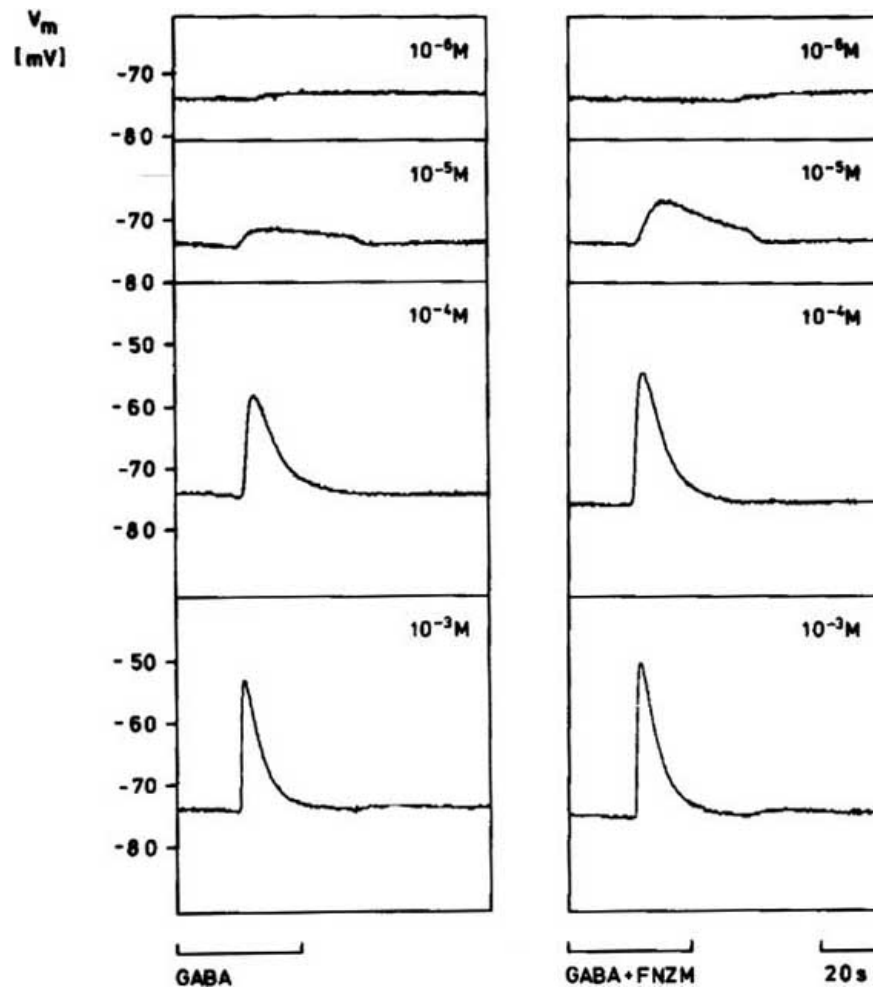


Fig. (2). Effect of flunitrazepam on depolarization by different concentrations of GABA (indicated in right upper corner of each trace) applied to the same cultured astrocyte during continuous intracellular recording of membrane potential (V_m); concentration changes were made at 10 min intervals, and membrane potential is shown during application of increasing concentrations of GABA alone (left) and of GABA in the presence of $1\mu\text{M}$ flunitrazepam (right). GABA was applied during the time periods indicated by the bars, and flunitrazepam was added 2 min before and during the exposure to GABA. From [10].

mediated Cl^- currents in neurons [41] the interaction of the 'neuronal-like' receptor with PK 11195 represents another difference between the neuronal and the astrocytic GABA_A/benzodiazepine complex.

Since the 'neuronal-type' benzodiazepine antagonist flumazenil inhibits GABA-mediated astrocytic depolarization [10], it becomes important to demonstrate whether the response of $[\text{Ca}^{2+}]_i$ to benzodiazepines is also inhibited by this drug. We therefore carried out experiments similar to those shown in Fig. 3, using flumazenil instead of PK 11195. Results for diazepam are shown in Fig. 4, demonstrating that the benzodiazepine effect was abrogated by $1\mu\text{M}$ flumazenil, whereas the K^+ effect was untouched; again $0.3\mu\text{M}$ flumazenil caused an incomplete inhibition [40]. The requirement for a minimum amount of GABA for the benzodiazepine-mediated depolarization of cultured astrocytes (Fig. 2) suggests that endogenous GABA must have been present. That this is the case is shown by the observations that cultured astrocytes even in the absence of neurons do produce small amounts of GABA [42, 43]. It is

also known that retinal Müller cells release GABA during exposure to elevated K^+ [44], and expression of GABA immunoreactivity has been shown in reactive astrocytes after ischemia [45].

4.2. Enhancement of K^+ -Mediated Glycogenolysis

Slightly elevated K^+ concentrations similar to those occurring in the extracellular space during neuronal excitation cause glycogenolysis in brain slices [46]. At the normal medium K^+ concentration of 5.4mM no glycogenolysis occurs in primary cultures of astrocytes, but when the extracellular K^+ concentration is increased to 10mM , previously incorporated glycosyl units are released from glycogen (an indication of glycogenolysis), an effect which increases with higher K^+ concentrations. This effect is almost abolished by 100nM nifedipine [47], a potent inhibitor of L-channels for Ca^{2+} . On the other hand, the effect of 10mM K^+ (causing on its own a submaximal depolarization and Ca^{2+} entry), is enhanced by 20nM midazolam, as indicated by the observation that

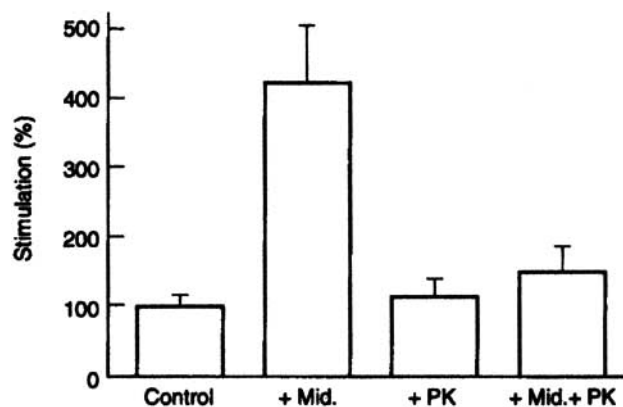


Fig. (3). Effects of 10 nM midazolam (+ Mid.), of 1 μM of the 'mitochondrial benzodiazepine antagonist' PK 11195 (+ PK), and of midazolam plus PK 11195 (+ Mid. + PK) on the increase in $[\text{Ca}^{2+}]_i$ evoked by an increase in the extracellular K^+ concentration to 20 mM. The control value (100%) represents the increase by the elevated K^+ concentration alone, which amounted to approximately a doubling of the resting $[\text{Ca}^{2+}]_i$ at 5.4 mM K^+ (about 100 nM). Midazolam almost quadruples the response to the increase in extracellular K^+ and this effect is abrogated by PK 11195. The value in the presence of midazolam is statistically significantly different from all other values, none of which differs from any of the other. From [39].

glycogenolysis in the presence of 10 mM K^+ during 30 sec is increased by 20 nM midazolam to become similar to that occurring during 1 min in the presence of 20 mM K^+ without midazolam (Fig. 5). Thus, benzodiazepines acting on the astrocytic GABA_A /benzodiazepine receptor complex enhances activity-induced glycogenolysis.

5. CNS CONSEQUENCES OF GABA_A /BENZODIAZEPINE RECEPTOR ACTIVATION

5.1. Consequences for the Extracellular Milieu

Early studies emphasized the role of the astrocytic GABA_A /benzodiazepine receptor complex for regulation of ion concentrations and pH of the extracellular space, where activation of Cl^- efflux from astrocytes increases the ambient Cl^- concentration [48, 49]. Since the astrocytic Cl^- channel is permeable to bicarbonate ions, activation will also induce extracellular alkalosis [50]. Because the intracellular volume is large compared to the narrow extracellular spaces, a decrease in extracellular Ca^{2+} concentration might also occur as a consequence of the Ca^{2+} entry into the cells.

5.2. Consequences for Astrocytic Development and Function

5.2.1. GABA Effects on Astrocytic Development

GABA is known to affect the morphological organization of astrocytes in the forebrain, with administration of muscimol increasing GFA content, number of astrocytic processes and branching of these processes [51]. Similar effects have been observed in neuronal-astrocytic co-cultures [52]. It is not known whether this effect is potentiated by benzodiazepines. If it is, use of benzodiazepine antagonists and inverse agonists during pregnancy might have severe adverse effects on brain development *in utero*.

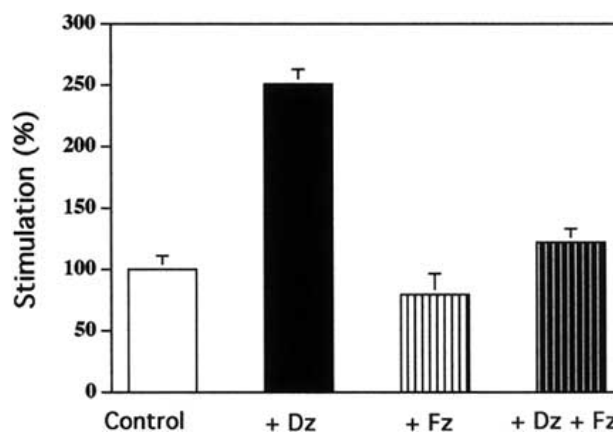


Fig. (4). Effects of 500 nM diazepam (+ Dz.), of 1 μM of the 'neuronal-type benzodiazepine antagonist' flumazenil (+ Fz), and of diazepam plus flumazenil (+ Dz + Fz) on the increase in $[\text{Ca}^{2+}]_i$ evoked by an increase in the extracellular K^+ concentration to 20 mM. The control value (100%) represents the increase by the elevated K^+ concentration alone, which amounted to a 126% increase of the resting $[\text{Ca}^{2+}]_i$ (about 100 nM). Diazepam more than doubles the response to the increase in extracellular K^+ and this effect is abrogated by flumazenil. The value in the presence of diazepam is statistically significantly different from all other values, none of which differs from any of the other. Modified from [40].

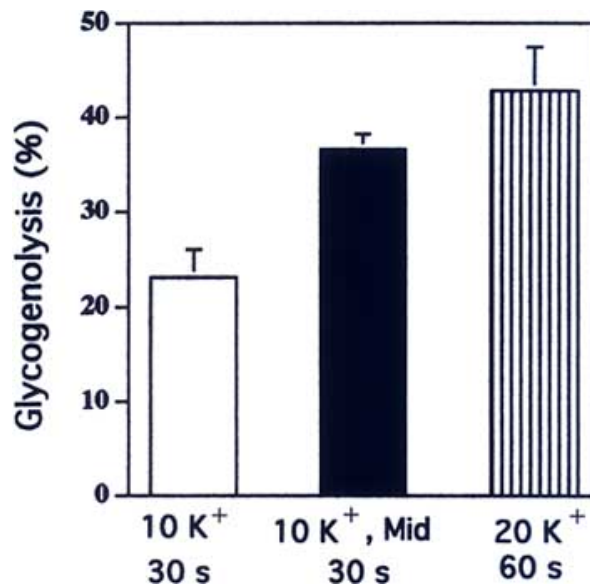


Fig. (5). Release of previously incorporated label in glycogen during exposure to 10 mM extracellular K^+ for 30 sec in the presence and absence of 20 nM midazolam and to 20 mM K^+ without midazolam for 60 sec. In the presence of midazolam glycogenolysis during exposure to 10 mM K^+ for 30 sec is significantly increased and its magnitude becomes similar to that seen after 1 min of exposure to 20 mM K^+ alone. From [47].

5.2.2. Consequence of Reduction of Intracellular Cl^- Concentration

The GABA-mediated decrease in intracellular Cl^- concentration causes secondarily a decrease in K^+

conductance, which will have important consequences for such phenomena as spreading depression and astrocytic swelling by passive uptake of K⁺ and Cl⁻ at pathologically high extracellular K⁺ concentrations [53].

5.2.3. Effects on Gap Junction-Mediated Astrocytic Coupling

Astrocytes constitute a functional syncytium of individual cell coupled by gap junctions, and prolonged exposure (24 hrs) to bicuculline, an inhibitor of GABA_A-mediated effects reduces gap-junctional coupling in neuronal-astrocytic co-cultures, but has no effect in isolated astrocytic cultures [54]. This observation suggests an increased astrocytic coupling in response to neuronally released GABA, and its potential enhancement by benzodiazepines could have pronounced influence on astrocytic signaling.

Gap junction permeability is also increased by high extracellular K⁺ concentrations [55] in a Ca²⁺-dependent, nifedipine-inhibited manner, indicating that the response to excess K⁺ is triggered by opening of L-channels for Ca²⁺ [56]. This response is prevented by an inhibitor of Ca²⁺-calmodulin (CaM) protein-kinases, known to phosphorylate and thereby regulate a variety of cellular processes following depolarization- or transmitter-mediated increase of [Ca²⁺]_i. It is consistent with such a 'down-stream' event that the response to elevated K⁺ was delayed and on the other hand maintained for 1 hr after re-exposure to a normal extracellular K⁺ concentration.

5.2.4. Effects on the Astrocytic Na⁺,K⁺,2Cl⁻ co-Transporter

Ca²⁺ uptake in astrocytes through L-channels activates in a nifedipine-inhibited manner the previously mentioned astrocytic co-transporter which in concert accumulates Na⁺, K⁺ and 2Cl⁻ [57]. Since the accumulated Na⁺ subsequently is extruded by the Na⁺,K⁺-ATPase in exchange with K⁺, this process in essence mediates an uptake of K⁺ together with Cl⁻, and it leads to vasopressin inhibitable swelling due to osmotically induced water uptake [58]. Astrocytes are important for regulation of the extracellular K⁺ concentration in brain [59], and this is one of the two active processes by which they accumulate K⁺, the other being K⁺-stimulated Na⁺,K⁺-ATPase activity [60, 61].

5.2.5. Effects on Neuronal-Astrocytic Signaling

Increases in [Ca²⁺]_i modulate signaling between astrocytes and neurons, although these effects generally have been studied in connection with transmitter-mediated or mechanically induced elevations of [Ca²⁺]_i. Astrocytes surrounding a synapse respond to neuronally released glutamate, acting on metabotropic glutamate receptors, with an increase in [Ca²⁺]_i, which can remain locally and by a resulting release of astrocytic glutamate reinforce and extend synaptic function in the initially activated synapse and/or its immediate neighbors [62-64]. Alternatively, the increase in [Ca²⁺]_i can propagate through the astrocytic syncytium as Ca²⁺ waves [65], driven by gap junction-mediated transcellular transport of inositol trisphosphate (IP₃) and/or release of ATP and its action on adjacent P1 receptors, and eventually interact with distant neurons. The activity of these neurons may either be stimulated by release of astrocytic

glutamate [66] or inhibited by release of astrocytic ATP, which is degraded by ecto-ATPases to adenosine, acting on inhibitory P1 receptors [67, 68]. It is unknown whether increases in [Ca²⁺]_i evoked by opening of L-channels might also induce Ca²⁺ waves. However, the mere fact that gap junction coupling is altered suggests that at propagation of Ca²⁺ waves may be modulated, since it partly occurs by gap junction-mediated transport of IP₃.

5.2.6. Stimulation of Energy Metabolism

Transmitter-induced increases in [Ca²⁺]_i stimulate oxidative metabolism [69-71], secondary to an increase in intramitochondrial Ca²⁺, which exerts a direct stimulatory effect on mitochondrial dehydrogenases (reviewed in [6, 72]). Again it is unknown if similar effects can be evoked by channel-mediated Ca²⁺ uptake. However, glycogenolysis, the process that was directly shown to be influenced by benzodiazepines (Fig. 5) is stimulated both by K⁺-mediated opening of L-channels and by transmitters increasing intramitochondrial Ca²⁺ [73, 74].

Glycogenolysis in the CNS is almost exclusively an astrocytic process (reviewed in 72). In contrast to prevailing concepts glycogen does not primarily serve as an emergency fuel, securing metabolic substrate to astrocytes and neurons, when glucose supply is failing. Rather glycogenolysis and glycogen synthesis are both enhanced during activity within the nervous system [74-77]. The main purpose of glycogenolysis may be to provide an immediately accessible metabolic substrate to the most peripheral astrocytic extensions ('peripheral astrocytic processes', or 'PAPs'), which are too tiny to contain mitochondria, but on the other hand may carry out most of the energy-requiring uptake processes (e.g., of K⁺ and of glutamate) into astrocytes. Slightly later ATP and creatine phosphate synthesized in the more proximal processes and cell body by oxidative metabolism may provide fuel not only for the continuing uptake processes but also for re-synthesis of glycogen [6, 74, 78].

6. IS THE JOKER RECEPTOR PHARMACOLOGICALLY RELEVANT?

6.1. The Concentration Dependence is Right

The high potency of benzodiazepines in increasing L-channel opening in astrocytes is an indication that the response may be relevant for clinically observed benzodiazepine effects, because the effective concentrations are similar to those obtained clinically [79, 80]. Maximum stimulation of Cl⁻ influx in cortical neurons (70% stimulation) on the other hand requires a low micromolar diazepam concentration, and 100-500 nM causes only a 15-30% increase [81], although the response could be more potent in intact tissue [82]. The observed difference in potency between diazepam and midazolam observed in cultured astrocytes has also been observed *in vivo* where midazolam is about twice as potent as diazepam in inducing amnesia [83].

6.2. Cotransporter Activation and Glycogenolysis may Decrease CNS Activity

At first sight it might seem peculiar that an action potentiating physiological and biochemical effects of

elevated K^+ concentrations, characteristic of neuronal excitation, should exert sedative/hypnotic/anti-convulsant/anesthetic effects. However, this might be a compensatory, counter-regulatory action, when extracellular K^+ concentrations reach levels high enough to cause partial astrocytic depolarization. Activation of cotransporter-mediated uptake of K^+ serves the purpose of clearing extracellular fluid off excess K^+ , and glycogenolysis may at least initially contribute to providing the metabolic fuel for K^+ uptake and perhaps also for uptake of extracellular glutamate in the peripheral astrocytic processes [6, 74, 78]. Reduction of K^+ conductance may contribute to lowering of the extracellular K^+ concentration.

Whether the augmentation of the K^+ -mediated increase in cell coupling within the astrocytic syncytium is of importance for any of the therapeutic or adverse effects of benzodiazepines and benzodiazepine-like drugs is unknown. However, the delayed onset and subsequent persistence of this effect might be of considerable interest.

6. 3. Are there Endogenous Ligand(s) for the Joker Receptor

Astrocytes synthesize the so-called endozepines, which act as endogenous ligands of benzodiazepine receptors. One of these is triacontatetrapeptide (TTN), a peptide made up by the C-terminal 34 amino acids of diazepam binding inhibitor (DBI), a 9 kDa polypeptide found in brain and other organs [84]. At concentrations from 0.1 nM, TTN causes an increase in $[Ca^{2+}]_i$ in cultured rat astrocytes, which is significantly reduced by 100 nM nifedipine [85]; moreover TTN induces membrane depolarization, and change in the Cl concentration in the incubation medium shifts the reversal potential; however, in addition to activating the Joker receptor TTN also stimulates steroidogenesis by an effect on the mitochondrial omega3 receptor.

7. CURRENT AND FUTURE DEVELOPMENTS

7. 1. Drug Development

Development of non-benzodiazepine benzodiazepine-like drugs interacting with $GABA_A$ /benzodiazepine receptor complexes expressing many different combinations of α , β , and γ subunits of the receptor complex is actively pursued in many laboratories, including Neurogen Corporation, the present patentee [4, 86]. Various combinations of subunits are screened for functional efficiency and selectivity, for example using electrophysiological recordings carried out on *Xenopus* oocytes expressing appropriate constructs, although there is also evidence that many agonists are more potent in brain cortical preparations than in oocytes [82]. The purpose of the present review is far from discouraging such studies but rather to emphasize the possible involvement of the astrocytic $GABA_A$ /benzodiazepine receptor complex, to encourage the inclusion of preparations that allow studies specifically of this receptor (e.g., determination of drug effects on $[Ca^{2+}]_i$ in appropriately prepared astrocytic cultures and appropriate staining of astrocytes). In studies using intact tissues it would be important to include testing of antagonists that suggest participation of effects exerted on astrocytic receptor complexes. The demonstration in this review that both flumazenil, a prototypically 'neuronal-type' drug and PK 11195, a prototypical 'peripheral-type' drug

inhibit benzodiazepine effects on the astrocytic $GABA_A$ -like receptor shows that the constellation of inhibition by flumazenil and by PK11195 might be a useful tool to identify *in vivo* effects exerted on the neuronal-like $GABA_A$ receptor in astrocytes by either classical benzodiazepines or newer benzodiazepine-like drugs (Fig. 6). Possible regional and subregional heterogeneity of the astrocytic receptor complex should be kept in mind.

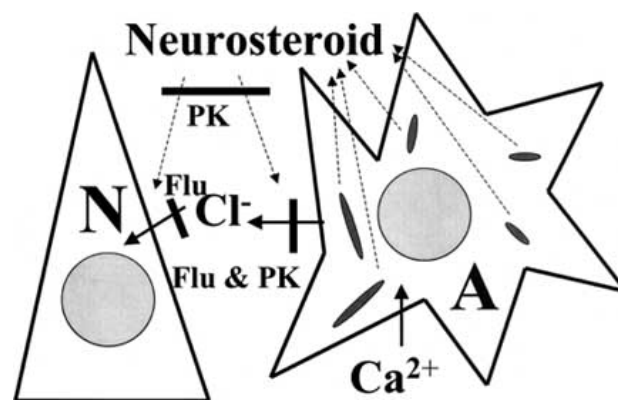


Fig. (6). Benzodiazepine effects on the neuronal-astrocytic inhibitory network. In the presence of GABA, a benzodiazepine agonist opens Cl⁻ channels in both neurons (acting on omega1 and 2 receptors) and astrocytes (acting on the astrocytic $GABA_A$ /benzodiazepine-like Joker receptor, thereby mediating Cl⁻ influx and hyperpolarization in neurons (N), and Cl⁻ efflux and depolarization in astrocytes (A)). Neuronal hyperpolarization causes neuronal inhibition, and astrocytic depolarization facilitates K^+ -mediated Ca^{2+} entry through L-channels. Established effects of opening of L-channels for Ca^{2+} include stimulation of glycogenolysis and stimulation of cotransporter-mediated uptake of K^+ , both of which may serve the purpose of clearing extracellular fluid off stimulatory agents by energy-requiring uptake into astrocytes. However, it is likely that other astrocytic parameters may also be affected by the influx of Ca^{2+} and ensuing increase in $[Ca^{2+}]_i$. In addition benzodiazepines act on the astrocytic mitochondria (shown as elongated dark gray structures in A) acting on mitochondrial omega 3 benzodiazepine receptor and stimulating the formation of neurosteroids, which can enhance the effect of omega 1 and 2 receptor activation as well as that of stimulation of the Joker receptor. Distinction of benzodiazepine effects on the different benzodiazepine receptors *in vivo* may be facilitated by the pattern of inhibition, with omega 1 and 2 receptor effects being inhibited by flumazenil (Flu), but not by PK 11195 (PK), omega3 receptor effects by PK 11195, but not by flumazenil, and effects exerted *via* the Joker receptor by both flumazenil and PK 11195.

Among the many proposed uses of the benzodiazepine-mimetic drugs, use of agonists (or partial agonists) as hypnotics and anxiolytics stand out simply on account of the number of patients in need of such drugs. In spite of the success of the hypnotics zolpidem and zaleplon, which act specifically on receptor complexes containing the α_1 subunit [2], these drugs can still cause memory impairment and rebound insomnia [86]. Cleaner drugs without these potentials would present major advantages. Similarly, newer anxiolytics are needed, which do not cause sedation [87].

The proposed use of inverse agonists as cognition-enhancing drugs might present a major break-through. The feasibility of such an approach has been demonstrated in animal experiments [88]. It is presently unknown whether the neuronal-like GABA_A receptor in astrocytes is involved in any of these effects.

7.2. Which Subunits are Expressed by Astrocytic GABA_A Receptors?

With the emphasis on the functional importance of the combination of α , β , and γ subunit expression for the type(s) of drug effects which are exerted by benzodiazepine-like drugs it would be important to determine subunit composition of GABA_A receptors on astrocytes at different locations. The findings that cultured Bergmann glia express the α subunit of the GABA_A receptor [20] and that cultured cerebellar astrocytes preferentially express mRNA for this subunit [22] are consistent with function of DMCM as an agonist in some, but not all, astrocytes; however expression of the α subunit could also be expected to convert flumazenil from an antagonist to an agonist and be associated with low potency of benzodiazepines [89], none of which was observed in the presently used cells. More information about combinations of α , β , and γ subunits expressed in the brain *in vivo* in different types of astrocytes at different locations, combined with emerging evidence of the role of subtype expression for determination of the type of drug effects evoked would go a long way to establish the possible importance of the astrocytic GABA_A/benzodiazepine receptor complex.

7.3. Deciphering a Neuronal-Astrocytic Inhibitory Network

The multifunctional capabilities of benzodiazepine-mimetic drugs are, at best, poorly understood. Although it is likely that both the hyperpolarization of the neuronal membrane evoked by GABA acting at omega 1 and omega 2 receptors and the modulation of these receptors by neurosteroids synthesized by the action of benzodiazepines on astrocytic omega3 receptors play major roles (which vary with the location and subtype of the receptor and with the drug administered), it is almost inconceivable that the effect of benzodiazepines and, perhaps, of a smaller or larger number of benzodiazepine-like drugs on the astrocytic GABA_A/benzodiazepine-like receptor, modulating key astrocytic functions should be therapeutically and toxicologically irrelevant.

Similar to the modulation of omega1 and omega2 receptors by omega3-mediated neurosteroid synthesis, the effects of benzodiazepine-mimetic drugs on the astrocytic GABA_A/benzodiazepine-like receptor might act in concert with GABA effects on the omega 1 and omega2 receptors. It has also been demonstrated that neurosteroids like those generated following activation of the mitochondrial astrocytic omega3 receptor affect the astrocytic GABA_A/benzodiazepine receptor complex [90]. In the case of excitatory transmission by glutamate a network of neuronal-astrocytic interactions has been established (reviewed in 63). An analogous network, using different biochemical and biophysical parameters, might exist in the case of inhibitory transmission by GABA, its modulation by

benzodiazepine-like agonists and its reversal by inverse agonists of the benzodiazepine receptor (Fig. 6). Emerging understanding of the operation of such a network using all GABA receptors, whether neuronal or astrocytic, would be greatly facilitated by the invention of new drugs capable of separating individual therapeutic effects as well as reducing unwanted side effects. The presently reviewed patent may help elucidating its functional role.

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