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Selective modulators of α_5-containing $GABA_A$ receptors and their therapeutic significance

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Running title: Selective Modulators of α_5-containing $GABA_A$ Receptors

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ABSTRACT

$GABA_A$ receptors containing the α_5 subunit (α_5GABA_ARs) are found mainly in the hippocampus where they mediate a tonic chloride leak current and contribute a slow component to GABAergic inhibitory synaptic currents. Their inhibitory effect on the excitability of hippocampal neurons at least partly explains why changes in the level of activity of α_5GABA_ARs affect cognition, learning and memory. These receptors have been implicated as potential therapeutic targets for a range of clinical conditions including age-related dementia, stroke, schizophrenia, Down syndrome and anesthetic-induced amnesia. Accordingly, a range of pharmacological modulators that selectively target α_5GABA_ARs, as either inhibitors or allosteric enhancers, have been developed. Although many of these compounds show therapeutic effects in animal models of the above clinical disorders, none has been marketed yet due to unsuccessful clinical trials and toxicity in humans. These experiments have also revealed paradoxical effects of α_5GABA_AR modulation (e.g., cognitive impairments can be reversed by both positive and negative modulation), suggesting that our knowledge of the physiological roles of α_5GABA_ARs is incomplete. This review highlights the various positive and negative modulators for α_5GABAARs that have been developed, key findings concerning their effects in behavioral studies, and their importance across a number of therapeutic fields. It also highlights some of the gaps in our knowledge of the physiological and pathological roles of α_5GABA_ARs.

GRAPHICAL ABSTRACT (n.b. a high resolution eps file of this image is attached as supp info**)**

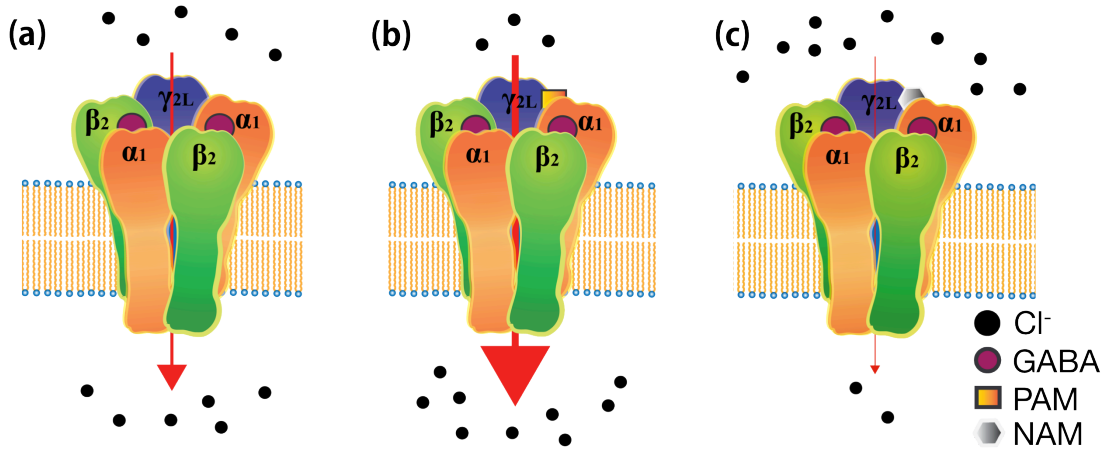

Fig. 1: Effects of positive allosteric modulator (PAM) and negative allosteric modulator (NAM) on GABA-induced Cl^- flow through $GABA_A$ receptors. (a) Binding of GABA to the receptor causes channel opening and Cl^- influx. (b) PAM binds to the allosteric site to increase Cl^- influx. (c) NAM reduces Cl^- influx.

KEYWORDS

allosteric modulator, alpha5 GABAA receptor, Alzheimer, amnesia, Down syndrome, memory impairment, nootropic, stroke.

1. INTRODUCTION

$GABA_A$ receptors ($GABA_ARs$) are the main inhibitory synaptic receptors in the central nervous system (CNS) [1]. As members of the pentameric ligand-gated ion channel family, functional $GABA_ARs$ comprise five subunits arranged symmetrically around a central ion-conducting pore. Each subunit consists of four α-helical transmembrane (TM) domains and a large extracellular amino-terminal domain that harbours the neurotransmitter binding sites and the signature 'cysteine-loop'. Receptors are constructed from a family of 19 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, π, θ, ε, δ, and ρ_{1-3}) plus a few splice variants. The most common stoichiometry *in vivo* is two α, two β and one γ subunit [2, 3]. Subunit distribution varies developmentally and regionally across the brain, and within each brain region it varies according to cell type and subcellular localisation [4]. This variability underlies a broad range of $GABA_AR$ stoichiometries which in turn leads to a broad variation in inhibitory postsynaptic current kinetics, pharmacological profiles, subcellular targeting mechanisms and plasticity mechanisms that together provide appropriately nuanced influences on CNS network behavior.

Due to the importance of $GABA_ARs$ as therapeutic targets, the effects of subunit composition on their physiological roles and pharmacological profiles have been investigated intensively. For instance, studies on genetically modified mice have demonstrated that the widely distributed α_1-containing $GABA_ARs$ mediate the sedative and amnesic effects of the benzodiazepines (BZDs) whereas α_2- and α_3- containing receptors, located mainly in the forebrain, mediate their anticonvulsant and anxiolytic effects [2, 5]. The high density of α_5-containing $GABA_ARs$ (α_5GABA_ARs) in the hippocampus suggests this subunit may be implicated in cognition, learning and memory [2, 6, 7]. In addition to the BZDs, other drugs or drug classes such as propofol, barbiturates and neurosteroids are also clinically important $GABA_AR$ modulators for indications including anaesthesia, anxiolysis, sedation or epilepsy. These drugs act mainly as positive modulators at the allosteric sites of $GABA_ARs$ to enhance the effect of GABA. As they non-selectively target many $GABA_AR$ subtypes resulting in undesired side effects (including tolerance, sedation, anxiety and convulsion), many researchers are now focusing on designing or discovering new drugs that selectively target specific $GABA_AR$ subtypes. A major subtype of interest in this respect is the α_5GABA_AR.

The α_5GABA_AR is of particular interest due to its interesting pharmacological benefits and its relatively sparse, compartmentalized CNS expression profile [4, 8]. For example, the locally high expression of α_5GABA_ARs within hippocampal dendrites suggests that this subtype may be a useful target for therapies aimed at this part of the brain. Therapeutic interest in the α_5GABA_AR has extended to various disorders including stroke, cognitive enhancement, schizophrenia and

dementia-related conditions. This review will discuss the physiological properties, the pharmacological properties and the clinical significance of α_5GABA$_A$Rs and will then consider recent progress in the development of α_5-selective compounds with different therapeutic utilities. The molecular structures, perceived clinical relevance and *in vivo* effects of all compounds discussed in this review are summarized in Table **1**, whereas the pharmacological profiles of the compounds at α_5GABA$_A$Rs *in vitro* are presented in Table **2**.

2. PHYSIOLOGY

Although generally sparsely distributed, α_5GABA$_A$Rs are highly expressed not only in the hippocampus, but also in the olfactory bulb, neocortex, subiculum and substantia gelatinosa [8]. They display a high sensitivity to GABA and a slow desensitization rate [9]: properties which are well suited to mediating tonic inhibition in the presence of the low ambient GABA concentrations that exist outside the synapse. Indeed, it is now well established that α_5GABA$_A$Rs mediate most of the tonic inhibition in hippocampal neurons [10]. However, the evidence to date that α_5GABA$_A$Rs contribute significantly to fast transient inhibitory neurotransmission is somewhat less compelling. Although immunofluorescence has provided evidence for α_5GABA$_A$R clusters in neurons, they did not appear to be located at postsynaptic densities [11, 12]. On the other hand, an immunogold study presented evidence for both synaptic and non-synaptic localization of α_5GABA$_A$Rs [13]. Electrophysiological investigations of α_5GABA$_A$R-mediated synaptic currents in the hippocampus suggest they contribute to a small, slow transient inhibitory component, but not to large, fast transient inhibition [9, 14-17], possibly implying a peri-synaptic localization of α_5GABA$_A$Rs [18].

3. SCHIZOPHRENIA

Antipsychotics are currently the first line treatment for schizophrenia. They are primarily used to treat the psychotic (or 'positive') symptoms, including hallucinations and delusions, experienced by schizophrenic patients. The negative symptoms, which include flattened expressions and lack of emotional responses, as well as the cognitive symptoms such as memory impairments, do not respond well to currently available antipsychotics. Moreover, debilitating side effects, such as agranulocytosis and seizures, can be caused by long-term use of the antipsychotics [19], highlighting the need for more effective treatments.

Neuronal gamma oscillations (30-100 Hz) are important for cognitive processes including attention, arousal and object recognition, whereas oscillations in the theta range (4–10Hz) serve complementary cognitive functions, especially in particular episodic memory formation [20].

Appropriate levels of tonic inhibition in the cortex and hippocampus are required to sustain these rhythms [21] and, as noted above, much of the tonic inhibition in these regions is provided by α_5GABA$_A$Rs. Disruptions in oscillatory activity (that could in principle be caused by any number of mechanisms) can lead to schizophrenia, and pharmacological manipulation of α_5GABA$_A$Rs is considered a promising means of normalizing these. Notably, a deficit in GABAergic neurotransmission has been shown to play a part in the pathology of schizophrenia, and several studies strongly indicate that expression levels of individual α subunits are altered in post-mortem brains of schizophrenic patients [22-25]. However, evidence implicating α_5GABA$_A$Rs has been elusive, with the expression of α_5 shown to increase significantly in one study [25], with no change in another [26], while a third study saw a decline in α_5 subunit expression [27, 28], suggesting the need for further clarification. Other studies have investigated the relationship between α_5GABA$_A$R expression and schizophrenia symptoms, and one, for instance, reported that the binding potential of [^{11}C] Ro 15-4513, a benzodiazepine partial inverse agonist with relatively higher affinity for α_5GABA$_A$Rs, was inversely correlated with negative symptom scores in medication-free schizophrenic subjects [29]. Another study noted similarities in behavioral abnormalities between those seen in schizophrenia and in α_5GABA$_A$R knockdown mice, in particular a deficit in prepulse inhibition (PPI) to startle, in which the hippocampus is believed to play a role [30].

The DNA-methylating agent, methylaxozymethanol acetate (MAM), induces a developmental model of schizophrenia whereby affected animals exhibit both the structural and behavioral abnormalities that are normally seen in schizophrenia [31]. These animals were found to exhibit an increase in dopaminergic activity within the ventral tegmental area (VTA) that was thought to be due to hippocampal hyperactivity [32]. In a separate study, selective reduction of hippocampal tonic inhibition by knocking out α_5 subunits in mice led to hyperactivity in the hippocampal network [10], suggesting that α_5GABA$_A$R deficiency could be one of the factors underlying the hyperdopaminergic activity seen in the schizophrenic mouse model. Hence, it was proposed that reducing hippocampal hyperactivity by using an α_5GABA$_A$R-selective positive modulator could be a novel therapeutic approach for schizophrenia [33]. This was investigated by monitoring of VTA neurons in MAM rats in the presence of SH-053-2'F-R-CH3 (Table **1, 2**), a benzodiazepine α_5-selective positive modulator [31]. This compound successfully diminished the number of spontaneously active dopaminergic neurons in the VTA in addition to reversing the heightened locomotor response to low dose of D-amphetamine in MAM but not in control rats [31], supporting the possible use of a similar treatment in schizophrenia. Given that inhibition of α_5GABA$_A$Rs enhances learning and memory [14, 34], it is feasible that an enhancer like SH-053-

2'F-R-CH3 could produce anterograde memory impairment; however, no such memory impairment has been observed [35].

Paradoxically, however, the selective negative modulation of α_5GABA$_A$Rs by RO4938581 (Table **1, 2**) has also proved promising in reversing cognitive impairments in the phenylcyclidine-induced schizophrenic rat model as well as in attenuating amphetamine-induced hyperactivity in rats [36]. This prompts the need for mechanistic studies to define the role of positive and negative α_5GABA$_A$R-selective modulators on neuron oscillatory behavior.

4. NOOTROPIC

A role for α_5GABA$_A$Rs in learning and memory has long been speculated due to its localization within the hippocampus. This hypothesis was first supported by a behavioral study, which saw significant improvement in the cognitive performance of α_5 subunit-deficient mice compared to wild-type mice in the Morris water maze, a test of hippocampal-dependent spatial working memory [14]. Furthermore, reduced expression of α_5 subunits in the mouse hippocampus was found to facilitate trace fear conditioning, a hippocampal-dependent associative learning paradigm [37, 38]. In light of this, it was hypothesized that selective negative modulators of α_5GABA$_A$Rs should enhance cognition while being devoid of side effects such as sedation and convulsion that result from modulation of other GABA$_A$R subtypes.

Among a series of novel benzodiazepine site ligands developed by Merck, Sharp and Dohme, the 6,7-dihydro-2-benzothiophen-4 (5*H*)-ones were found to exhibit high selectivity for α_5, but low oral bioavailability. One compound of this class, Compound 43 (Table **1, 2**), enhanced the cognitive performance of rats in the delayed matching-to-position (DMTP) version of the Morris water maze model without the anxiogenic or convulsive side effects typical of non-selective benzodiazepine receptor inverse agonists such as methyl-6, 7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM) [39-42]. A similar result was observed with an orally administered α_5GABA$_A$R-selective pyrazolotriazine compound, MRK-016 (Table **1, 2**), although this drug was discontinued because it was poorly tolerated in elderly subjects and exhibited unpredictable pharmacokinetics [43, 44]. Two structurally similar triazolophthalazines, α_5IA and α_5IA-II (Table **1, 2**), were also developed to be orally bioavailable and selective for α_5GABA$_A$Rs. These negative allosteric modulators that bind at the benzodiazepine site enhanced the performance of rodents in the DMTP water maze test without showing anxiogenic, sedative or convulsant effects [41, 45-47]. The cognition enhancing effect of α_5IA-II was further substantiated in a behavioral study demonstrating a positive effect on the encoding and retrieval phases of memory and learning in rats [34]. Despite the promising outcomes of these trials, the development of these prototypes has been

discontinued. The nootropic effect of orally administered α_5IA did not successfully translate into human clinical trials, as it not only failed to improve cognitive function in elderly subjects, but also significantly impaired their performance in a paired-associate learning task [46]. This was exacerbated by renal toxicity resulting from the accumulation of crystalline metabolites in the kidney [46]. Although both triazolophthalazines were devoid of convulsant side effects, α_5IA-II, but not α_5IA, was found to possess proconvulsant efficacy at high receptor occupancy owing to an observed potentiating effect on pentylenetetrazole-induced seizures in mice [41].

Prior to the elucidation of the role of α_5GABA$_A$Rs in memory and learning, an imidazobenzodiazepine compound, L-655 708 (Table **1, 2**), a negative allosteric modulator with high binding selectivity for α_5GABA$_A$Rs, had been developed for the purpose of investigating α_5GABA$_A$R structure and function [48, 49]. A behavioral study using the elevated plus-maze model later demonstrated that L-655 708 promoted an anxiogenic-like profile at doses required for efficacy [50], hence limiting the use of the drug as nootropic in humans even though it improved performance of mice in the Morris water maze [51]. Although L-655 708 binds with higher affinity to α_5GABA$_A$Rs, it also produces inverse agonist activity at other GABA$_A$R subtypes (Table **2**), and this is thought to contribute to its anxiogenic and possibly other side effects [49].

Other notable α_5GABA$_A$R-selective negative modulators include RO4938581 and PWZ-029 (Table **1, 2**). Like other α_5GABA$_A$R negative modulators, the imidazo-triazolo-benzodiazepine RO4938581 managed to improve the cognitive performance of rats in the DMTP and Morris water maze models. In addition, RO4938581 enhanced the prefrontal executive function of cynomolgus monkeys in an object retrieval task without having any adverse effects on anxiety, convulsion, motor coordination or muscle strength [52, 53]. RO4938581 also significantly improved the performance of scopolamine- and diazepam-induced memory-impaired rats in the DMTP and Morris water maze, respectively [52, 53]. PWZ-029 is unusual in that it inhibits α_5GABA$_A$Rs at nanomolar concentrations but potentiates other GABA$_A$R isoforms at much lower potencies [54, 55]. In terms of memory-enhancing properties, orally administered PWZ-029 successfully improved the task learning of rats in a hippocampal-dependent passive avoidance test without producing anxiety or convulsions, although hypo-locomotion was observed at higher doses [55]. In a Pavlovian fear conditioning study, PWZ-029 notably reversed the scopolamine-induced impairment of contextual memory [54], in addition to improving the performance in novel object recognition test [56]. However, in contrast to most tested α_5GABA$_A$R negative modulators, PWZ-029 failed to improve cognitive performance in the Morris water maze model, either alone or in countering scopolamine-induced cognitive impairment in rats, prompting the need for further investigations to validate the cognition-enhancing properties of PWZ-029 [56].

5. NEURODEGENERATIVE CONDITIONS: ALZHEIMER'S DISEASE AND HUNTINGTON'S DISEASE

Neurodegenerative diseases are characterized by progressive deficits in the structure and function of neurons leading to a combination of motor and cognitive decline. There is no effective cure for these diseases, which include Alzheimer's and Huntington's diseases, with currently available treatments being symptomatic and aimed at improving the quality of life of those affected.

Post-mortem brains of Alzheimer's disease (AD) patients have shown that, in addition to β-amyloid plaques, neuronal network function in brain areas such as the cerebral cortex, brainstem and hippocampus is debilitated [57]. The loss of neurons in the hippocampus is partly due to the excessive stimulation of the excitatory synaptic glutamatergic receptors, which induces neuronal death [57]. Moreover, in both animal studies and neuroimaging of elderly individuals, age-related memory impairment has been shown to be associated with increased neural activity in the hippocampus [58-61]. On top of that, the number of inhibitory GABAergic interneurons in the hippocampus was found to be reduced in a mouse model that over-expresses apolipoprotein E4 (apoE4), a well-known genetic risk factor for AD that leads to learning and memory deficits [62]. Initial interventions to counter the net excessive hippocampal activity included treatment with $GABA_AR$ non-selective positive modulator, pentobarbital, which successfully rescued spatial learning and memory deficits in apoE4-knock-in mice without affecting the number of hippocampal GABAergic interneurons [62]. However, chronic administration of pentobarbital led to numerous side effects, presumably due to non-specific effects on multiple $GABA_AR$ subtypes.

Interestingly, various assays quantitating changes in protein, mRNA and ligand binding all showed that α_5GABA_AR expression in the hippocampus of human subjects with severe AD progression was distinctively lower compared to normal or mild AD individuals, although the precise mechanisms were poorly comprehended [63, 64]. As such, despite previous findings that negative modulators improve cognitive function, positive modulators that target α_5GABA_ARs selectively are being investigated to restore hippocampal activity in aged brains. Using a rat model of age-related memory impairment, two distinct, but non-orally bioavailable, positive modulators of α_5GABA_ARs, a benzothiophenone (compound 44) and a pyridazine (compound 6) (Table **1, 2**), considerably improved hippocampal-dependent performance tasks, while a negative allosteric modulator had no effect in any of the tasks, further supporting the use of α_5GABA_AR positive modulators in age-related memory impairment [61].

As with AD, patients with Huntington's disease (HD) suffer from progressive cognitive decline. Experiments employing the R6/1 HD transgenic mouse model revealed that the disease's hippocampal-dependent cognitive impairment could be partially due to an imbalance in the cholinergic/GABAergic septohippocampal (SH) neuronal projection [65, 66]. The SH projection is known to modulate the hippocampal theta oscillation important for memory formation and learning [67, 68], and abnormal hippocampal theta oscillation has indeed been demonstrated in humans with HD [69]. Therefore, restoring normal SH and oscillatory activities could be crucial for alleviating cognitive dysfunction in HD. As modulating α_5GABA$_A$R activity has been shown to restore hippocampal dysfunction, the same theory could possibly be extended to HD, although more studies are warranted to establish the role of α_5GABA$_A$Rs in HD.

6. COGNITIVE DYSFUNCTION IN DOWN SYNDROME AND AUTISM

A majority of individuals with Down syndrome, or trisomy 21, exhibit mild to moderate cognitive dysfunction [70]. Exaggerated GABAergic activity in the hippocampus has been proposed to contribute to the memory and learning impairment in this disorder [21, 60, 71]. Consistent with this, the non-competitive GABA$_A$R antagonist, pentylenetetrazole, improved cognitive performance in the segmentally trisomic Ts65Dn Down syndrome mice model [72], whereas picrotoxin effectively restored hippocampal long-term potentiation by non-selectively blocking GABA$_A$Rs in the same animal model [71]. Nonetheless, as both pentylenetetrazole and picrotoxin have convulsant effects, their use has not translated into human clinical use. As an alternative, since α_5GABA$_A$Rs are well documented to be involved in cognition, it was thought that negative modulators targeting these receptors may reverse the cognitive dysfunction associated with this syndrome. The cognitive ability of Ts65Dn mice in both novel object recognition and Morris water maze tasks was indeed rescued by intraperitoneal injection of the inhibitor, α_5IA [73]. The same compound was also reported to restore the expression of immediate early genes, namely the *c-Fos* and *Arc* genes, which regulate cognitive function in Ts65Dn mice [74]. Unfortunately, renal toxicity associated with the use of α_5IA prevented its progression into clinical trials [46]. Nonetheless, inspired by the success of this finding, a separate study chronically treated the Ts65Dn mice with another α_5GABA$_A$R-selective negative modulator, RO4938581, and found a similar improvement in cognitive function, in addition to improvements in the hippocampal synaptic plasticity and adult neurogenesis. Furthermore, the hyperactive Ts65Dn mice were successfully calmed by RO4938581 without producing any adverse convulsant, motor or anxiety effects [75]. At present, Hoffman-La Roche is sponsoring several Phase 1 clinical trials of RG 1662, an analogue of RO4938581, in young healthy and Down syndrome adults.

As with Down syndrome, children diagnosed with autism spectrum disorder (ASD) may also experience impairments in hippocampal-dependent learning and memory [76]. However, unlike Down syndrome, it is the excessive neural activity, leading to excitatory/inhibitory imbalance in the brain, that has been implicated with the neuropathological characteristics of ASD [21, 77]. This was further reinforced when antiepileptic drugs used in ASD patients meant to relieve partial seizures inexplicably improved cognitive function in some individuals [60]. Also, low doses of the non-selective benzodiazepine, clonazepam, managed to rescue abnormal social behaviours and cognitive deficits in the $Scn1a^{+/-}$ mice model of Dravet's syndrome, a syndromic form of ASD [78]. Considering that selective α_5GABA_AR negative modulators successfully reduced excessive hippocampal tonic inhibition in animal models to regain cognitive ability, perhaps the same principle can be applied to ASD in future studies.

7. STROKE

Stroke is consistently ranked as one of the leading causes of death. Long-term functional and cognitive disabilities often persist following stroke, which in turn negatively affect the patient's employability and quality of life. The area adjacent to the stroke site, the peri-infarct area, has been demonstrated to undergo poorly understood mechanisms of neuronal repair by means of neurogenesis, axonal sprouting and remapping of cognitive functions in order to regain functional and cognitive abilities [18, 79-81]. These changes are not only slow, but currently available medications are mainly preventatives for recurrent stroke (e.g., anticoagulants, antihypertensives) and do not facilitate brain repair or recovery following the stroke, prompting the need for more effective stroke treatments.

There is evidence for augmented GABAergic tonic inhibition in the peri-infarct region of cortical pyramidal neurons at a time delay of 3 to 14 days post-stroke [18, 82]. It has been proposed that although enhanced tonic inhibition at the time of stroke is necessary to limit further neuronal injury, a persistent increase in tonic inhibition may deter proper neuronal repair and functional recovery [18]. Hence, it was thought that attenuating GABAergic tonic inhibition, starting at day 3 after the onset of stroke, could help in promoting neuronal and functional recovery. Additionally, post-stroke brain recovery is drastically improved by stimulating the learning and memory pathway, in which the α_5GABA_AR plays an important role [82, 83]. Recent evidence showed that chronic treatment with the α_5-selective negative modulator, L-655 708, starting from the third day following stroke in a mouse model, significantly improved functional recovery that was evident from the seventh day post-stroke whereas acute treatment had negligible effect on recovery [82]. Not only

that, knockout of the α_5GABA$_A$R also boosted the rate of motor recovery in post-stroke mice, the effect comparable to the group that was administered with L-655 708 [14, 82]. On a cellular level, nanomolar L-655 708 successfully diminished GABAergic tonic inhibitory currents both in control and post-stroke neurons, albeit more conspicuously in post-stroke neurons, further substantiating the implication of α_5GABA$_A$Rs in stroke and possibly other types of brain injuries [82]. It is noteworthy that best outcomes were achieved several days after the stroke because it implies it may be feasible to develop treatments that work at delayed time points when options for early intervention have been missed.

8. PREVENTION OF AMNESIA

Postoperative Cognitive Dysfunction (POCD), or memory deficits occurring post-surgery, is common especially in elderly patients after major surgery and is caused by the after-effects of anesthetic administration. POCD has been reported to persist for up to three months following surgery, and in addition POCD patients are predisposed to increased risk of death in the first year after surgery [84, 85]. The mechanisms underlying POCD remain controversial, although the high expression level of α_5GABA$_A$Rs in the hippocampus is thought to play a part in mediating the amnesic side effects of anesthetics [86, 87]. This notion is supported by several electrophysiological and behavioral studies showing that low concentrations of isoflurane and etomidate selectively potentiate α_5GABA$_A$Rs in hippocampal pyramidal neurons to mediate the memory-blocking effect of anesthetics [9, 86-88]. In a separate study, α_5GABA$_A$R-knockout mice were resistant to the memory-blocking effect of inhaled isoflurane and sevoflurane, further substantiating the role of α_5GABA$_A$Rs in POCD [89]. Thus, it was hypothesized that administering a negative modulator of α_5GABA$_A$Rs pre-surgery might mitigate the post-anesthesia amnesic side effect. Consistent with this idea, animal behavioral studies demonstrated that pre-treatment with the α_5GABA$_A$R negative modulator, L-655 708, was indeed able to reverse short- and long-term memory impairment in mice anesthetized with isoflurane [87, 89]. Furthermore, L-655 708 and another α_5GABA$_A$R-selective negative allosteric modulator, MRK-016 (Table **1, 2**), significantly inhibited isoflurane and sevoflurane-potentiated GABA currents in hippocampal neurons of wild-type mice, whereas the GABA response in α_5GABA$_A$R-knockout mice was not affected by the anesthetics [90]. On a related matter, pre-treatment with α_5IA in human subjects managed to selectively counter the deterioration in the ability to recall a word list following alcohol consumption [46, 91]. Hence, it seems possible that the applicability of α_5GABA$_A$R-selective negative modulators may extend to other amnestic disorders, especially in light of a study which showed that hippocampal GABA$_A$Rs

also play a role in Wernicke-Korsakoff syndrome, a type of diencephalic amnesia attributed to vitamin B_1 deficiency [92].

9. DRUG DISCOVERY STRATEGIES

This review has highlighted the need for new drugs that specifically modulate α_5GABA$_A$Rs as therapeutic leads for a variety of clinical indications. What strategies can be used to discover such drugs? Here we briefly consider two points related to identifying new lead candidates. The first point relates to the chemical diversity to be screened. Over the last 20 years, major pharmaceutical companies have focused more on probing the artificial chemical diversity that can be generated via combinatorial chemistry, and less on the natural chemical biodiversity found in natural products [93]. If the number of drugs reaching the clinic is the yardstick, it must be concluded that this approach has not worked. This is considered to be largely due to the limited structural diversity inherent in conventional combinatorial libraries, and indeed has prompted renewed interest in the development of drugs from natural sources [93-95]. However, natural product drug discovery has its limitations too, including difficulties with the resupply of raw materials, difficulties with the repeated isolation of known compounds, and difficulties with synthesizing natural compounds of interest [94]. More recently, hybrid approaches have been developed whereby combinatorial libraries incorporate the broader chemical diversity of natural products [96, 97]. When coupled with complementary approaches such as fragment-based discovery [98], diversity-orientated synthesis [99], and dynamic combinatorial chemistry [100], the potential for generating new generations of α_5GABA$_A$R-specific modulators looks promising.

The second issue relates to the choice of methods of screening compound libraries against GABA$_A$R subtypes. This involves a trade-off between the low cost and high throughput of fluorescent assays (notably voltage-sensitive dyes or yellow fluorescent protein) and the precision and temporal resolution of patch-clamp electrophysiology [101, 102]. Although automated patch-clamp technologies are advancing steadily in throughput and cost, electrophysiology is not yet a viable means of first round high-throughput screening. The approach we recommend is to perform initial screening via a fluorescence assay, with confirmatory screening and validation of successful hits by high throughput electrophysiology [103, 104].

CONCLUSION

Compounds that selectively target α_5GABA$_A$R, as either positive or negative modulators, are of utmost importance clinically as they have the potential for treating a range of CNS disorders.

For example, positive allosteric modulators for α_5GABA$_A$Rs hold promise as new generation treatments for schizophrenia and age-related cognitive impairments, whereas negative allosteric modulators may be useful as nootropics and as treatments for conditions like Down syndrome, stroke and amnestic disorders. Although a number of α_5GABA$_A$R-selective compounds have been identified, none have yet reached the clinic due to toxicity, lack of efficacy, side effect profiles and unpredictable pharmacokinetics in humans. This highlights the need for further research to identify new α_5-selective ligands with better efficacy as well as safer pharmacological and pharmacokinetic profiles in humans. Subunit specific compounds can also be used as pharmacological probes to understand the basic neural mechanisms of the aforementioned diseases.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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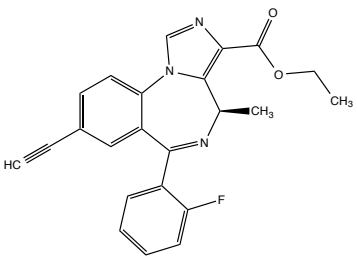
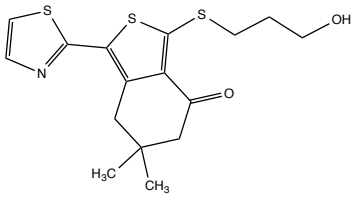
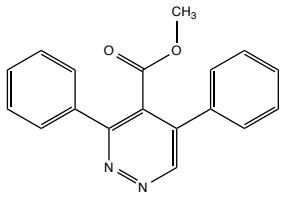
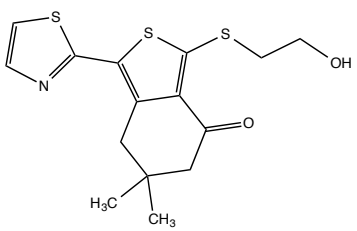
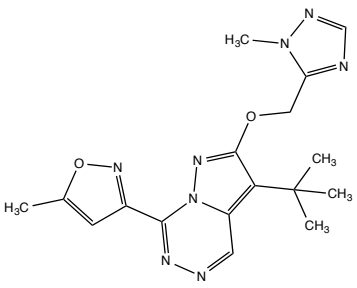
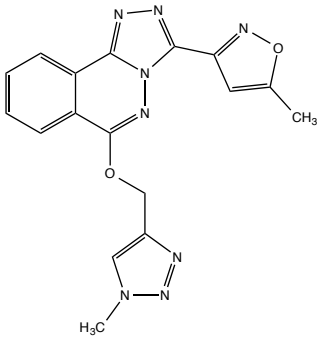
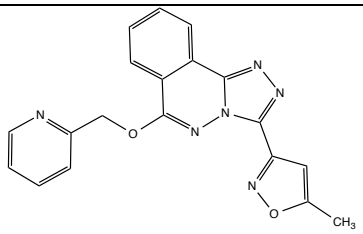
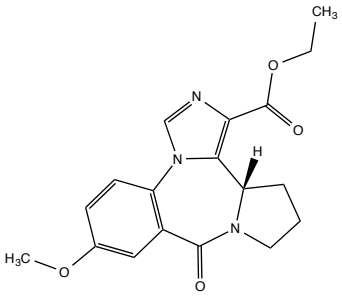
Table 1: Structure of α_5GABA$_A$R-selective positive and negative modulators, their functional relevance and summary of the key findings from previous studies.

Positive modulator	Compound structure	Function/ disease relevance	Key findings
SH-053-2'F-R-CH3 ((R)-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4H-2,5,10b-triaza-benzo[e]azulene-3-carboxylic acid ethyl ester)		Schizophrenia	**Advantages** - Reduced spontaneously active dopaminergic neurons in VTA [31] - Reduced locomotor response to low dose of D-amphetamine in MAM-induced schizophrenic rat model [31] - No effect on visual recognition and spatial working memory in rhesus monkeys [35] - Non-sedating in primates [105] **Disadvantages** - Not orally bioavailable, dosed intravenously [105] or intraperitoneally [106] - No data on toxicity
Compound 44 (6,6-dimethyl-3-(3-hydroxypropyl)thio1-(thiazol-2-yl)-6,7-dihydro-2-benzothiophen-4(5*H*)-one)		Alzheimer's disease	**Advantages** - Improved escape performance of aged rats in water maze task [61] **Disadvantages** - Not orally bioavailable, drug was administered by intracerebroventricular infusion [61] - No data on toxicity
Compound 6 (methyl 3,5-diphenylpyridazine-4-carboxylate)		Alzheimer's disease	**Advantages** - Improved spatial memory of aged rats in radial arm maze test [61] - Treatment in young rats had no effect on cognitive performance [61] **Disadvantages** - Not orally bioavailable, drug was injected intraperitoneally [61] - Toxicity data not available
Negative modulator	**Compound structure**	**Function/ disease relevance**	**Key findings**
Compound 43 (6,6-Dimethyl-3-(2-hydroxyethyl)thio1-(thiazol-2-yl)-6,7-dihydro-2-benzothiophen-4(5*H*)-one)		Nootropic	**Advantages** - Enhanced cognitive performance of normal rats in DMTP water maze [39] - Not anxiogenic and convulsant in animal models [39]
MRK-016 (3-tert-		Nootropic	- Improved performance of normal rats in

butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1H-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-d]-[1,2,4]triazine)			DMTP water maze [40] - Neither anxiogenic, proconvulsant nor produce kindling in mice [44] - Orally bioavailable [44] - Well tolerated in healthy young subjects [44] **Disadvantages** - Effect on improving cognitive function in human subjects not evaluated [44] - Poorly tolerated in elderly subjects with unpredictable pharmacokinetics [44]
		Postoperative cognitive dysfunction	**Advantages** - Inhibited isoflurane and sevoflurane-potentiated GABA currents in wild-type mice hippocampal neurons [90]
α5IA (1,2,4-Triazolo[3,4-a]phthalazine, 3-(5-methyl-3-isoxazolyl)-6-[(1-methyl-1H-1,2,3-triazol-4-yl)methoxy]-, 3-(5-Methylisoxazol-3-yl)-6-(1-methyl-1,2,3-triazol-4-yl)methoxy-1,2,4-triazolo[3,4-a]phthalazine)		Nootropic	**Advantages** - Enhanced performance of rodents in DMTP water maze at 40% receptor occupancy [41] - Not anxiogenic, sedative and convulsant in animal models [41] - Orally bioavailable [41, 46] **Disadvantages** - Impairs performance of elderly human subjects in paired-associate learning task [46] - Short half-life [46]
		Down syndrome	**Advantages** - Rescued cognitive ability of Ts65Dn Down syndrome mice model in novel object recognition and Morris water maze model [73] - Restored immediate early gene expression related to cognitive function in Down syndrome mice [74] **Disadvantages** - Formation of crystalline metabolite with low solubility resulted in renal toxicity in human subjects [46] - Long-term dosing necessary for clinical efficacy cannot be achieved [46]
		Alcohol-induced amnesia	**Advantages** - Oral pre-treatment prevented negative effect of alcohol on the ability to recall word list in human subjects [46, 91]
α5IA-II (3-(5-Methylisoxazol-3-yl) -6-(2- pyridyl) methyloxy- 1,2,4-triazolo [3,4-a]		Nootropic	**Advantages** - Oral dosing available [47] - Enhanced performance of rodents in DMTP water maze [47] - Not anxiogenic, sedative and convulsant

phthalazine)			in animal models[47] - Improved encoding and retrieval phases of memory and learning in rats [34] **Disadvantages** - Proconvulsant at high doses [47] - No data on toxicity
L-655 708 (ethyl (13aS)-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-imidazo [1,5-a]pyrrolo[2,1 c][1,4]benzodiazepine-1-carboxylate)		Nootropic	**Advantages** - Improved performance of mice during acquisition and consolidation phases in Morris water maze model [51] **Disadvantages** - Not orally bioavailable - Anxiogenic at doses required to enhance cognition [50] - Although with high affinity for α_5GABA$_A$R, negative modulation at other GABA$_A$R subtypes may lead to unwanted side effects [51] - Toxicity data unknown
		Recovery from stroke	**Advantages** - Chronic treatment a delay after stroke onset improved functional recovery from stroke [82] - Diminished GABAergic tonic inhibitory currents more conspicuously in post-stroke neurons [82] **Disadvantages** - Drug was implanted (chronic) or administered intraperitoneally (acute) [82]
		Postoperative cognitive dysfunction	**Advantages** - Blocked short- and long-term memory impairment induced by anaesthetics in mice [87, 89] - Inhibited GABA responses enhanced by isoflurane and sevoflurane in mice hippocampal neurons [89]
RO4938581 ((3-Bromo-10-difluoromethyl-9H-imidazo[1,5-a] [1,2,4] triazolo[1,5-d] [1,4] benzodiazepine)		Schizophrenia	**Advantages** - Improved cognitive impairment in PCP-induced schizophrenic rat model [36] - Reduced amphetamine-induced hyperactivity in rats [36] - Has both binding and functional selectivity [52, 53] **Disadvantages** - No data on toxicity
		Nootropic	**Advantages** - Enhanced cognitive performance of normal rats in DMTP task and Morris

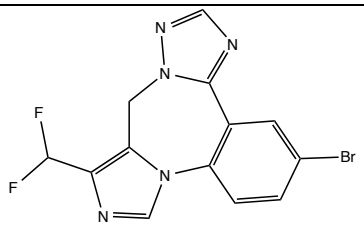
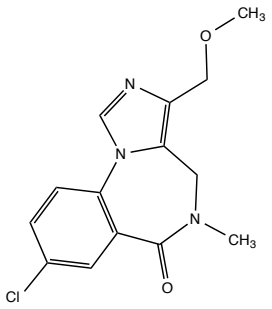
			water maze model [52, 53] - Enhanced prefrontal executive function of cynomolgus monkeys in object retrieval task [52] - Only 30% receptor occupancy needed to enhance cognition in animal model [52] - Orally bioavailable [36, 52] - No effect on anxiety, convulsion, motor coordination or muscle strength [53] - Reversed scopolamine- and diazepam-induced memory impairment of rats in DMTP and Morris water maze [52, 53]
		Down syndrome	**Advantages** - Long-term treatment improved cognitive impairment in Ts65Dn Down syndrome mice model [75] - Improved hippocampal synaptic plasticity and neurogenesis [75] - Reduced hyperactivity tendency in Ts65Dn mice [75] - A structurally related compound, RG 1662, is currently in clinical trials to treat cognitive impairments associated with Down syndrome
PWZ-029 (8-chloro-3-(methoxymethyl)-5-methyl-4H-imidazo[1,5-a][1,4]benzodiazepin-6-one)		Nootropic	**Advantages** - Improved the task learning of rats in passive avoidance test [55] - No effect on anxiety or convulsions [55] - Reversed scopolamine-induced memory impairment in Pavlovian fear conditioning in mice model [54] - Improved performance of rodents in novel object recognition test [56] **Disadvantages** - Hypo-locomotion observed in rodents at higher receptor occupancy [55] - Failed to improve cognitive performance of rats in Morris water maze [56]

Table 2: In vitro selectivity profile (affinity vs efficacy) of modulators at $\alpha_x\beta_3\gamma_2$ $GABA_AR$ subtypes.

Modulator	Binding affinity (K_i, nM); % modulation[a]				Reference
	α_1	α_2	α_3	α_5	
Diazepam	14.0; 239 (at 100 nM), 314 (at 1 μM)	7.8; 426 (at 100 nM), 536 (at 1 μM)	13.9; 437 (at 100 nM), 752 (at 1 μM)	13.4; 274 (at 100 nM), 342 (at 1 μM)	[105]
SH-053-2'F-R-CH3	759.1; 111 (at 100 nM), 154 (at 1 μM)	948.2; 124 (at 100 nM), 185 (at 1 μM)	768.8; 125 (at 100 nM), 220 (at 1 μM)	95.2; 183 (at 100 nM), 387 (at 1 μM)	[105, 106]
Compound 44	79; NA	48; NA	48; NA	4.7; 25[b]	[40]

Compound 6	154; NA	NA; NA	64; NA	12; 27 (at 300 nM)	[107]
Compound 43	20; NA	16; NA	20; NA	1.6; -38[b]	[40]
MRK-016	0.83; -16[b]	0.85; 6[b]	0.77; -9[b]	1.36; -55[b]	[44]
α5IA	0.88; -4[b]	0.58; 12[b]	0.61; 4[b]	0.66; -29[b]	[41, 45]
α5IA-II	0.93; -2[b]	1.5; 15[b]	0.96; -4[b]	0.62; -46[b]	[41, 47]
L-655 708	70; -18[b]	48; -23[b]	31; -11[b]	1.0; -17[b]	[51]
RO4938581	174; -3[c]	185; -4[c]	80; 2[c]	4.6; -35[c]	[52, 53]
PWZ-029	>300; 114 (at 1 μM), 120 (at 10 μM)	>300; 105 (at 1 μM), 115 (at 10 μM)	>300; 118 (at 1 μM), 145 (at 10 μM)	38.8; -20 (at 1 μM), -20 (at 10 μM)	[55]

[a] Efficacy is determined as % of control currents from electrophysiology experiments.

[b] Value represents % modulation of GABA EC_{20} concentration.

[c] Value represents % modulation of GABA EC_{10} concentration.

NA Data not available.