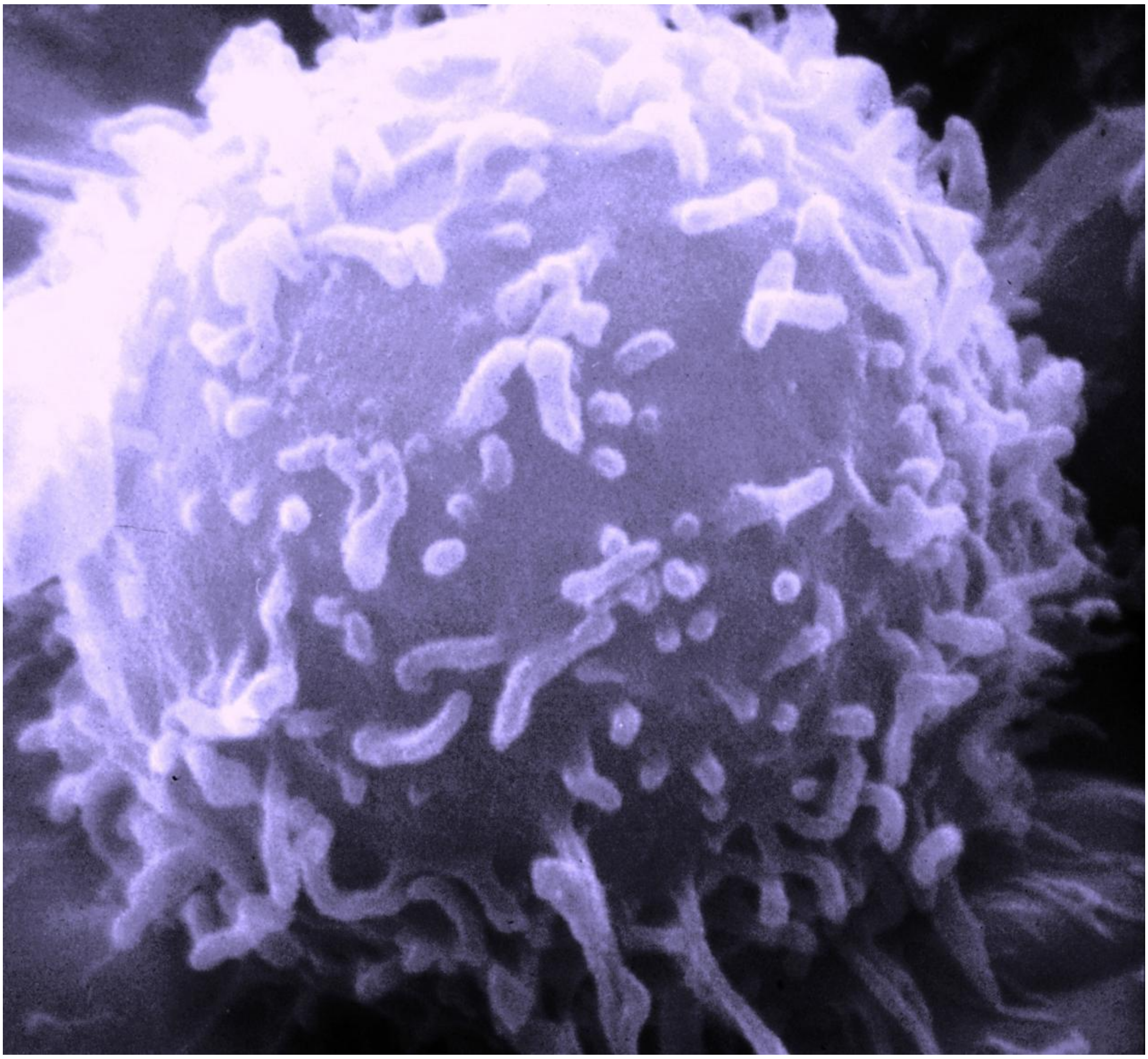
GABA receptors and the immune system



Arne Lucas ten Hoeve
3384004
Bachelor Biomedical Sciences 2011
Utrecht University
Supervisor: dr. P.A.J. Henricks

Contents

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Cover page picture: Electron microscopic image of a single human lymphocyte. National Cancer Institute USA

Abstract

Traditionally known as purely an inhibitory neurotransmitter, γ-aminobutyric acid (GABA), its' receptors and enzymes involved in GABA metabolism and catabolism have been shown to be widely distributed outside the brain and central nervous system. One of the organ systems expressing $GABA_A$ and $GABA_B$ receptors and other parts of the GABA system is the immune system. GABA and GABA analogous have primarily an inhibitory effect on the immune system, although the effect of activating $GABA_B$ receptors seems to be more complex and include immune stimulation. Molecular changes evoked by GABAergic compounds also translate to potential *in vivo* treatment of diseases associated with the immune system. Activation of immune cells leads in some cases to increased $GABA_B$ receptor expression, suggesting a 'natural' function of these receptors in immune system functioning.

PART 1. THE GABA SYSTEM

γ-Aminobutyric acid or γ-aminobutyrate (GABA) is, along with glycine, a major inhibitory neurotransmitter in the mammalian central nervous system (CNS). Together with the excitatory neurotransmitters glutamate and aspartate they form a large portion of the synaptic activity in the CNS. Although GABA had previously been reported in trace amounts in blood and urine, its' presence in the brain of a number of mammalian species was independently reported by both Awapara *et al.* and Roberts and Frankel in 1950 [1,2]. Interestingly, GABA and GABA receptors are also present in a number of other organisms, such as yeast, higher plants and several insect species [1,3,4].

GABA synthesis and transport

As the major inhibitory neurotransmitter, GABA is involved in a variety of CNS processes, which include anxiety-related behavior, cognitive processing, discrimination of information and sensorimotor gating [5]. Because it does not cross the blood-brain barrier, GABA is synthesized in the brain by a metabolic pathway known as the 'GABA shunt' (figure 1). The final step of this pathway yields the non-protein amino acid GABA through the conversion of the excitatory neurotransmitter and amino acid L-glutamate by glutamic acid decarboxylase (GAD). This enzyme exists in two isoforms with different localization and regulatory properties [6]. Both isoforms are products of distinct genes: the GAD_{65} gene (the number refers to the molecular mass of the enzyme) is located on chromosome 10, while the GAD_{67} gene is located on chromosome 2 [7]. The isoform GAD_{67} is localized in the neuronal body, while GAD_{65} is primarily expressed in nerve terminals. This distinction suggests a more important role for the latter in synaptic neurotransmission and for the former in regulating GABA synthesis [5]. Both GADs are also expressed outside of the CNS, such as in pancreatic β-cells, testis and epithelium of the oviduct [7].

As GABA is formed continuously in an amount exceeding usual requirements for neurotransmission, an enzyme that's also an important part of the 'GABA shunt' is γ-aminobutyric acid transaminase (GABA-T) [8]. Although GABA-T can synthesize GABA from succinic semialdehyde, its primary function seems to be degradation of GABA, as the anticonvulsant γ-vinyl GABA (vigabatrin), an irreversible inhibitor of GABA-T, causes GABA accumulation in glial cells of the retina [9]. In addition, other GABA-T inhibitors also produce a significant increase in GABA in the brain *in vivo* [8]. The presence of the cofactor pyridoxal-5'-phosphate is required as a carrier to yield succinic semialdehyde. The back reaction from succinic semialdehyde to GABA is also unlikely to happen to any important extent *in vivo* due to the co-localisation of high activity of succinic semialdehyde dehydrogenase (SSADH) and GABA-T [8]. In the brain, GABA-T is primarily expressed in glial and endothelial cells [7]. Therefor, it can be concluded that (in the CNS) GABA anabolism and catabolism occur in the neurons and glial cells, respectively. Although an integral part of GABAergic systems in the CNS, GABA-T is also expressed in a variety of other tissues, including liver, pancreas, kidneys, lungs, heart, stomach, hair follicles, the placenta and even platelets [8,10]. On a sub-cellular level expression seems to be concentrated to the inner mitochondrial membrane [8].

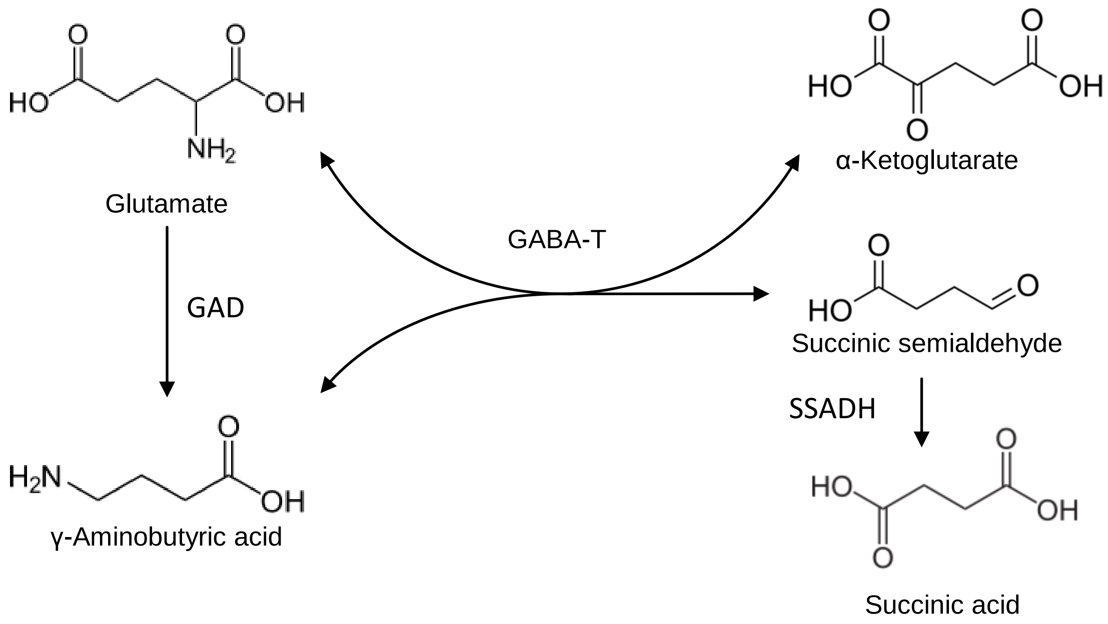


Figure 1. GABA shunt: synthesis and degradation. The neurotransmitter GABA is synthesized by GAD from glutamate, which is converted from α-ketoglutarate, an intermediate of the Krebs cycle, by GABA-T. GABA can be degraded by GABA-T to succinic semialdehyde and subsequently to succinic acid, also an intermediate in the Krebs cycle. γ-aminobutyric acid; GABA-T, γ-aminobutyric acid transaminase; GAD, glutamic acid decarboxylase; SSADH, succinic semialdehyde dehydrogenase.

After release into the synaptic cleft, GABA is rapidly removed from the intercellular space by specific transporters to prevent GABA spillover to neighboring synapses and tonic activation of GABA receptors [11]. The majority of the released GABA is transported back into the synapse, while a smaller fraction is taken up by astrocytes surrounding the synapse [12]. Currently, four of these GABA transporters (GATs) have been described: GAT-1 through GAT-3 (rat nomenclature) and the Betain/GABA transporter type 1 (BGT-1), a receptor that uses both GABA and betain as substrates. GATs are GABA Na^+/Cl^- coupled transporters, with the Na^+ gradient being the primary driving force for GABA uptake, while Cl^- can significantly enhance uptake [10]. GAT-1 seems to be conserved in mammals displaying a high degree of amino acid sequence homology in rat, mouse and human and essentially identical pharmacological properties. GAT-2 and GAT-3 show a higher degree of sequence identity with each other and BGT-1 than with GAT-1. In the adult rat brain GATs are mainly localized in astrocytes and some neuronal terminals, hypothesized to be GABAergic synapses. Expression of GAT-1 differs strongly between brain regions, with high expression in the forebrain, intermediate expression in the cerebellum and lower expression in the majority of the hindbrain [13].

GABA$_A$ receptors

In the brain, 17-20% of all neurons are GABAergic and most of the physiological activities of GABA are generated through GABA$_A$ receptors (GABA$_A$-Rs) [14]. These ionotropic receptors or ligand-gated ion channel (LGIC) are chloride anion (Cl^-) channels that can be opened and activated by the endogenous neurotransmitter GABA and several drug classes, including benzodiazepines, barbiturates, steroids, anesthetics and convulsants. As the primary receptor for GABA in the CNS, GABA$_A$ receptors are involved in a variety of behavioral and cognitive processes [14].

Structure

GABA$_A$ receptors are composed of 5 protein subunits, which all have a large extracellular N-terminal domain, four transmembrane (TM) domains, a large intracellular loop between TM3 and TM4 and a relatively short C-terminal domain (figure 2A). The number of different subunits is 19, not including different splice variants, and that makes this set the largest of any among the mammalian ion channel receptors [14]. These subunits can be divided into 2 categories: 16 (α1-6, β1-3, γ1-3, δ, ε, θ and π) which can be combined to form the traditional GABA$_A$-R and 3 ρ (rho) subunits (Greek letters signify >70% sequence identity). Receptors containing ρ subunits are sometimes referred to as GABA$_C$ receptors, though that is recommended against by the International Union of Basic and Clinical Pharmacology (IUPHAR) [15]. The most prevalent GABA$_A$-Rs contain α, β and γ subunits, but native receptors lacking a γ subunit do exist. In the CNS approximately 75-80% of the receptors contain γ2, while γ1 and γ3 are rarer. The most common α subunit is α1, often colocalized with β2 and γ2. Among the β subunits β2 is the most abundant, while β1 is the least common [15]. Interestingly, birds express also β4 and γ4 subunits, but seems to lack both ε and θ subunits [16].

The protein subunits are combined in different ways and arranged around a central pore, which allows Cl^- to pass through (figure 2B). The receptors roughly share their structure with other

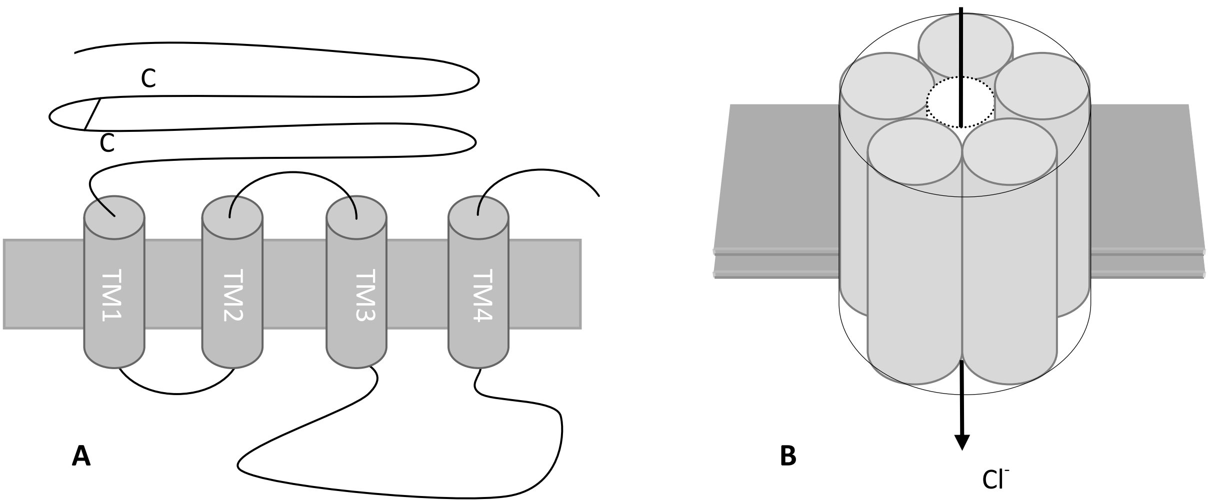


Figure 2. GABA$_A$ receptor and subunit structure. A) Each subunit consists of a large N-terminal domain, 4 transmembrane domains (TMs), a large loop between TM 3 and 4 and a relatively short C-terminal domain. The characteristic 'cys-loop' is located in the N-terminal domain. B) Five subunits are grouped around a central pore to form a GABA$_A$ receptor.

closely related Cys-loop pentameric LGICs. This superfamily also includes nicotinic acetylcholine receptors (nAChR), inhibitory glycine receptors, ionotropic 5-HT_3 (serotonin) receptors and a Zn^{2+}-activated ion channel [16]. All 44 subunit members of the superfamily show around 30% sequence homology and greater secondary and tertiary structure similarity [15]. They all use similar sequences and functional domains in ion channel structure, endogenous ligand binding sites and membrane topology. In 2005 the structure of the nAChR in a species of electric ray (*Torpedo marmorata*) was resolved to a resolution of 4 Å, through cryo-electron microscopy and image reconstruction [17]. More recently the structure of a toxin-bound murine nAChR-subunit was determined in 2007 [18]. Also two high resolution X-ray studies of prokaryotic pentameric LGICs have provided significant insight into structure [19,20]. And, although the structure of the $GABA_A$-R has not been determined, it can therefore be approximated through homology modeling. The resulting models of the majority of $GABA_A$ receptors (those containing 1 γ, 2 α and 2 β subunits), show not only the arrangement of subunits within the receptor, but also several structural features in the extracellular domain and their binding pockets [15]. The benzodiazepine binding site (Bz BS) is located at the interface of an α and γ-subunit. Classic benzodiazepines, such as diazepam, exhibit a high affinity for specific subunit combinations. These diazepam sensitive (DS) receptors include those composed of α1βγ2, α2βγ2, α3βγ2 and α5βγ2, as well as less well known analogues containing the γ3-subunit. Some Bz BS ligands are also able to interact with receptors composed of α4βγ2 and α6βγ2, while diazepam is not. These can therefore be referred to as diazepam insensitive (DI) receptors [21].

Function and distribution

Interestingly, the large diversity of $GABA_A$ receptors that can be created by combining different subunits seems to be directly linked to their function, as their expression In the CNS and other organs differs considerably. In addition, some subtypes have a prominent role in seizure susceptibility, while others are involved in anxiety or memory and learning (Table 1).

Table 1. Action of benzodiazepines at α1-6β3γ2 receptor subtypes [21]

Subtype	Associated effect
α1	Sedation, anterograde amnesia, some anticonvulsant action and ataxia.
α2	Anxiolytic, hypnotic (EEG) and some muscle relaxation.
α3	Some anxiolytic action, some anticonvulsant action and possibly some muscle relaxation.
α4	Diazepam insensitive (DI) receptor
α5	Negative effect on cognition and temporal and spatial memory. May also be involved in the memory component of anxiety.
α6	Diazepam insensitive (DI) receptor

The combination of specific subunit combinations and distinct effects allows the development of subtype selective ligands in order to elicit a specific respons [21]. Also other non-α subunits can be important in neuropharmacology: γ2-subunits are involved in anxiety, while δ-subunits have a role in learning and memory [22]. Akinci and Schofield provide a comprehensive summary on receptor subunits and associated effects [23]. Although the α5 subunit is relatively rare in the brain, its expression is high in the hippocampus, which corresponds with its link to temporal and spatial memory. The α4 and α6 subunits are highly expressed in the forebrain and cerebellum [15].

Outside of the mammalian CNS and peripheral nervous sytem (PNS), $GABA_A$ receptor subunits are also expressed in a variety of other tissues. In cell lines subunits are also expressed. For example, mRNA for the α2, α3, β2, β3, γ2 and ε subunits can be found in the NCI-H295R adrenocortical carcinoma cell line, which could be sufficient to form functional receptors [24]. In mice TM3 Leydig cells, mRNA of subunits α1, α2 β1, β3 and γ1 was present and cell proliferation was significantly increased by selective $GABA_A$ receptor agonists [25]. In an article on experiments in rats, Akinci and Schofield report mRNA detection of many $GABA_A$-R subunits in a number of peripheral tissues [23]. Placenta, ovaries and testis all have a large repertoire of subunits, while the repertoires of the small intestine and uterus are smaller. Interestingly, in the endocrine tissues in these experiments, ρ3 mRNA was only present in the testis. In the testis, mRNA of α1-5, β1-3, γ1 and γ2, δ, ε, ρ1 and ρ2 was also reported, while in the uterus α1, α2 and α6, β3, γ1, ε and ρ1 mRNA was detected [23]. $GABA_A$-Rs are also present in other parts of the female reproductive system such as the oviduct mucosa and fallopian tubes [26].

Healthy human hepatic tissue expresses a number of $GABA_A$ receptor subunits: β3, ε, π. In addition to these, in some hepatocellular carcinoma cells also α3 mRNA can be found [27]. Literature on the effects of administration of $GABA_A$-R agonists on hepatocyte proliferative activity is conflicting [27-29]. Both a decrease and increase on hepatocellular carcinoma cells has been reported, while on nonmalignant cells a decrease in proliferation was reported. Biju *et al.* proposed $GABA_A$ receptors on hepatocytes have an important role in maintaining normal liver mass by reducing DNA synthesis [29]. In other malignancies such as pancreatic cancer, $GABA_A$-R activation has been reported to be associated with increased growth [30]. In both healthy and malignant thyroid tissue, also a number of subunits are expressed. For example, β2 mRNA and protein could be found in thyroid vasculature, while α2 was expressed in malignant thyrocytes [31]. In chondrocytes isolated from the rat tibial growth plate, also mRNA of $GABA_A$-R subunits can be detected, including α1-4, α6, β1-3 and δ. In the same experiment, murine embryonal carcinoma-derived ATDC5 cells, which undergo chondrogenic differentiation *in vitro*, showed an increase in proliferation in response to the $GABA_A$-R agonist muscimol [32].

GABA$_B$ receptors

In addition to acting on the ionotropic GABA$_A$ receptor, GABA is also an endogenous agonist of the GABA$_B$ receptor (GABA$_B$-R). The GABA$_B$-R is a member of the large metabotropic G-protein coupled receptor (GPCR) superfamily, with 367 known members in humans as of 2003, a major target of pharmaceutical drugs and involved in for example taste, smell, metabolism, reproduction, development, hormonal homeostasis, and behavior [33]. It can be found in the brain, at both excitatory and inhibitory synapses, and in several other organs. By interacting with multiple downstream signaling cascades, GABA$_B$-Rs have many physiological roles [34].

Structure and signaling

A functional GABA$_B$-R consists of two distinct subunits, known as GABA$_{B1}$ (also GABBR1 or GABA$_B$R1) and GABA$_{B2}$ (GABBR2), which form heterodimers (figure 3). Interestingly, unlike other GPCRs such as metabotropic glutamate (mGlu) receptors, GABA$_B$-R dimers can also form large complexes with other dimers [35]. Both subunits comprise seven transmembrane (7TM) domains. The GABA$_{B1}$ subunit interacts with external ligands, while the GABA$_{B2}$ subunit is needed for membrane targeting and signal transduction [34]. Gene splicing gives rise to two predominant isoforms of GABA$_{B1}$ subunit: GABA$_{B1a}$ and GABA$_{B1b}$, which show highly similar pharmacological and biophysical properties *in vitro*, but other splicing variants do exist. Not surprisingly these isoforms also have a high structural similarity, differing only in the N-terminal ectodomain. The sushi repeats, only present in the GABA$_{B1a}$ variety, may mediate association with distinct auxiliary proteins and cell adhesion molecules. Expression of the GABA$_{B1a}$ isoform results from the presence of a second transcription initiation site within the fifth GABA$_{B1b}$ gene. In neurons, both isoforms are possibly always co-expressed and, until 2006, no solid evidence existed for differential subcellular localization [36]. In 2006, two papers reported on the inhibition of synaptic transmission, between layer 1 (L1) and 5 (L5) neurons in the hippocampus, by GABAergic interneurons. GABA$_B$-Rs located on the interneuron's presynaptic terminal contained the GABA$_{B1a}$ isoform, while receptors on the postsynaptic terminal on the L5 pyramidal neuron contained the GABA$_{B1b}$ isoform. Through these receptors, release of GABA from the interneuron, could cause long-lasting inhibition of glutamatergic transmission between the L1 and L5 neurons [37,38].

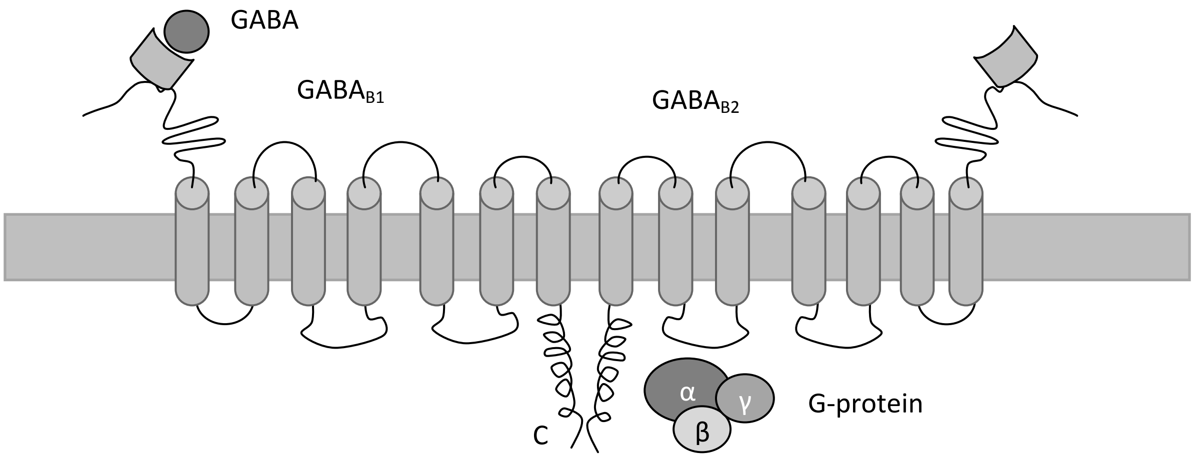


Figure 3. General GABA$_B$ receptor heterodimer structure. The functional GABA$_B$ receptor is composed of the 2 subunits and associates with heterotrimeric G proteins upon GABA binding.

As a type of GPCR, $GABA_B$-Rs transduce extracellular signals via heterotrimeric G proteins, particularly the $G_i\alpha$- and $G_o\alpha$-types. Through binding and activating G protein subunits, $GABA_B$-Rs are coupled to a variety of effectors, including enzymes and ion channels. For example, $GABA_B$-Rs couple to several types of voltage-gated Ca^{2+} channels. High-voltage activated Ca^{2+} channels of the N- or P/Q-type or intermediate-voltage-activated R-type can be potently inhibited by Gβγ complexes, that bind specific channel subunits upon activation and liberation from G_o proteins [39,40]. L-type Ca^{2+} channels ("Long-Lasting") can be both inhibited and augmented by $GABA_B$-R activation. The latter effect may be partly mediated by protein kinase A (PKA) or, more effectively, protein kinase C (PKC) activity [41]. More recently, it has also been reported that the $GABA_{B2}$ subunit can interact directly with L-type Ca^{2+} channels. Through the formation of a protein complex, this interaction modulates Ca^{2+} influx [42]. In addition to Ca^{2+} channels, $GABA_B$-Rs are also coupled to K^+ channels. $K_{ir}3$ (or GIRK) is a subfamily of 'inwardly rectifying potassium channels'. Activation of $K_{ir}3$ channels results *in situ* in a K^+ efflux, thereby hyperpolarizing the cell and inhibiting excitability [39]. It is likely $GABA_B$-Rs are also coupled to other types of K^+ channels. Specifically blocking rapidly inactivating A-type K^+ channels, for example, inhibits a $GABA_B$ agonist-induced current in neurons [39]. Small-conductance Ca^{2+}-activated K^+ (SK) channels, important in regulating synaptic plasticity, memory and learning, are activated by GABA [43]. This activation may be due to inhibition of cyclic adenosine monophosphate (cAMP) production through $GABA_B$-Rs [39].

$GABA_B$-Rs influence levels of cAMP, a major component in signal transduction, by indirectly inhibiting and enhancing different adenylate cyclases. One soluble and nine transmembrane isoforms of adenylate cyclase have been identified, all of which catalyze the conversion of adenosine triphosphate (ATP) to cAMP. Isoforms I, III, V and VI are inhibited by $G_i\alpha$ and $G_o\alpha$ proteins, while Gβγ protein complexes enhance I, IV and VII and inhibit II. The enhancement of adenylate cyclases by $GABA_B$-Rs is dependent on activation of other GPCRs [39,44]. Modification of the intracellular cAMP levels reduces or enhances the activity of protein kinase A (PKA). PKA is composed of four subunits that dissociate upon binding of cAMP to the two regulatory subunits [45]. Following dissociation, the two catalytic subunits can enter the nucleus, where they activate the cAMP response element-binding (CREB) protein. Activated CREB recruits its co-activator CREB-binding protein (CPB) and binds to a specific DNA sequence called cAMP response element (CRE). When bound to CRE, gene transcription is enhanced through association of CPB with RNA polymerase II (Pol II) complexes. Genes with CREs in the promoter include neuropeptides, neurotransmitters and growth factors, and factors involved in metabolism, immune regulation, cell survival and cycle, cell structure and transport [46].

Another signaling pathway induced by $GABA_B$-Rs is the PI-3-kinase–Akt pathway [47]. Akt, also known as protein kinase B (PKB), regulates key cellular functions, such as nutrient metabolism, cell growth, apoptosis and survival by several mechanisms, including inactivating the pro-apoptotic proteins Bad and caspase-9 [48]. Activation of $GABA_B$-Rs, for example, leads to neuroprotection via PI-3-kinase, independent of cAMP levels [49]. The PI-3-kinase binds the activated GPCR, which actives it to convert membrane-bound $PI(4,5)P_2$ to $PI(3,4,5)P_3$. The PIP_3 recruits the two kinases Akt and phosphoinositidine-dependent protein kinase 1 (PDK1) and the latter activates Akt in conjunction with a third kinase. The activated Akt dissociates from PIP_3 and phosphorylates target proteins [50]. Interestingly, activation of Akt by $GABA_B$-Rs may also be direct, since $GABA_{B2}$ subunits can interact and complex with Akt [47].

Also an important target of $GABA_B$-Rs is the protein kinase C (PKC) family, which shares a significant sequence homology with PKB [50,51]. The PKC family has 12 members in mammals and other eukaryotes. All PKC isoforms share a highly conserved carboxy-terminal kinase domain [52]. Analogous to other GPCRs coupled to $G_i\alpha$- and $G_o\alpha$-type G proteins, when Gβγ complexes are released following G protein activation by the $GABA_B$-R, they are able to activate the phospholipase Cβ (PLCβ) [51]. Subsequently, activation of PLCβ leads to the formation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). The latter product acts as an agonist on IP3-gated Ca^{2+} channels, opening them to release Ca^{2+} into the cytosol from stores in the endoplasmatic reticulum (ER). Combined, the free Ca^{2+} and DAG recruit PKC to the plasma membrane and activate it. An important downstream of PKC is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor which regulates genes involved in cell growth and differentiation and inflammatory response. Another target of PKC is the protein kinase D (PKD) family, which can also be activated by DAG [53]. It's likely, that pathway allows $GABA_B$-Rs to activate ERK, a mitogen-activated protein kinase (MAPK) also involved in proliferation and differentiation and a downstream target of several growth factor receptors [54,55].

Distribution

Like $GABA_A$ receptors and other parts of the GABA system, $GABA_B$-Rs are expressed in a wide range of tissues and organs. In the CNS, $GABA_B$-Rs are expressed in the thalamus, cerebellum, hippocampus, cerebral cortex, dentate gyrus, interpenduncular nucleus and dorsal root ganglia [56,57]. $GABA_B$-Rs can also be found in peripheral organs, such as the esophagus. In rats, GABBR1 is present in the adrenals, pituitary, spleen, kidney, liver and prostate [57]. Functional receptors have also been reported in airway epithelial cells and smooth muscle, islets of Langerhans, the placenta and fallopian tubes [55,58]. Expression of $GABA_{B1a}$, $GABA_{B1e}$ (a truncated subunit) and $GABA_{B2}$ has been detected in the NCI-H295R adrenocortical carcinoma cell line [24]. Interestingly, $GABA_B$-Rs are also expressed in cancer cells. In human prostate cancer cells, the $GABA_B$-R agonist baclofen promotes migration, while suppression has also been reported, for example in hepatocellular carcinoma cells [59].

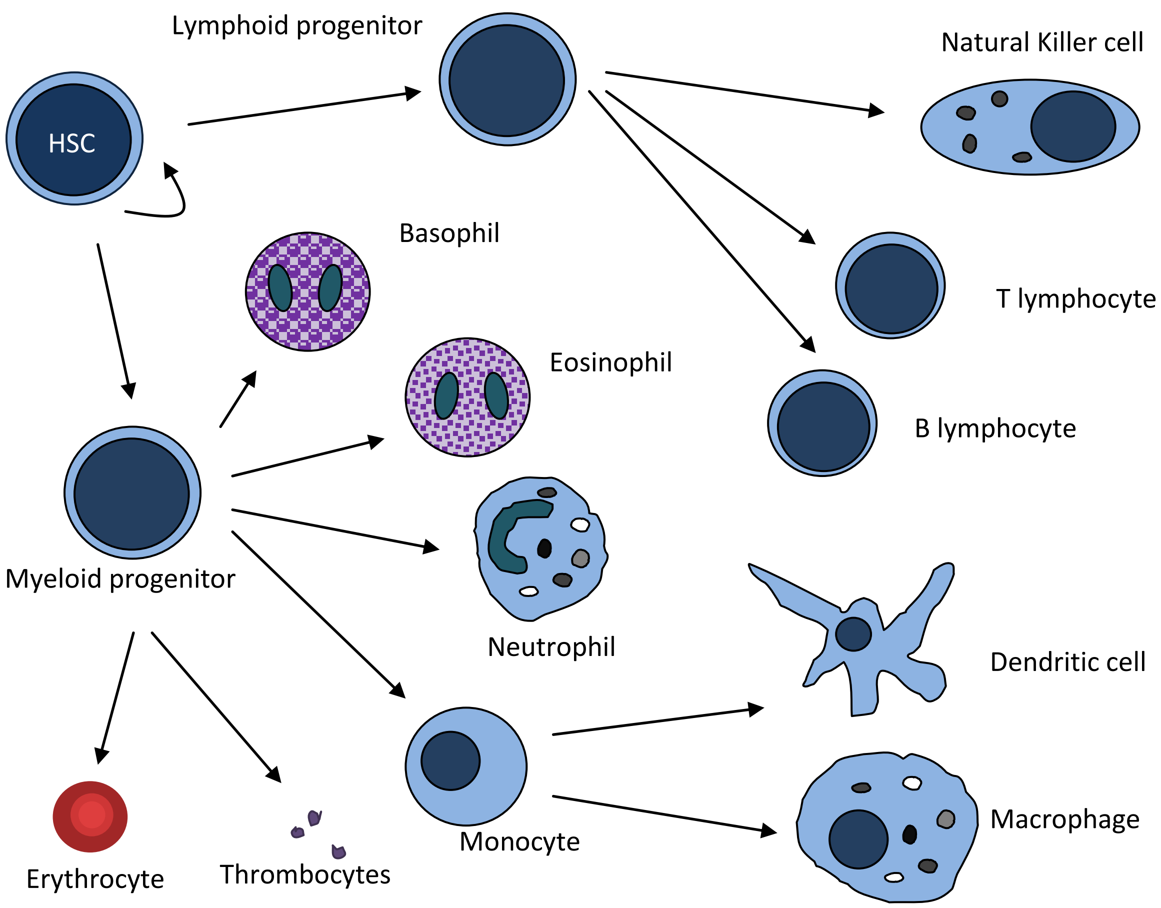
GABA and the immune system

The components of the immune system can be categorized in various ways, one of which is the distinction between 'humoral' and 'cellular' immunity. The former focuses primarily on antibodies, but the complement system and other antimicrobial substances may also be categorized as part of the humoral immune system. The cellular immune system includes a diverse repertoire of cell types and is extensively intertwined with the humoral part.

Cells of the immune system

The cell types of the human immune system (figure 4) can be divided into two main categories: cells which are part of the 'innate' immune system and those which are part of the 'adaptive' immunity. The most prominent cells of the innate immunity are phagocytes; cells that engulf foreign substances, such as bacteria and fungi, and the remnants of dead cells. Phagocytes are also mainly responsible for secretion of pro- and anti-inflammatory cytokines and antigen presentation. Examples of mononuclear phagocytes are macrophages, monocytes and dendritic cells. Chow *et al.* extensively reviewed mononuclear phagocyte functioning [60]. Neutrophil granulocytes, also referred to as polymorphonuclear neutrophils (PMNs), are another class of phagocytes and account for approximately 70% of all leukocytes (white blood cells). Other granulocytes include eosinophils and basophils. Natural killer (NK) cell are also considered to be part of the innate immune system, but are in origin more closely related to other lymphocytes, such as T and B cells. NK cells recognize

Figure 4. Basic hematopoiesis in humans. Hematopoietic Stem Cells (HSCs) in the bone marrow give rise to all lymphoid and myeloid lineages.



and eliminate virus-infected and cancer cells. Sun and Lanier recently provided valuable insight in NK cell development and function [61].

Cells considered part of the adaptive immunity consist of T and B lymphocytes. The latter are the producers of all classes of antibodies, also known as immunoglobulins. Some antibodies are produced in response to infection, while IgM, for example, is also secreted 'naturally' by B cells [62]. T lymphocytes can be further divided into a number of types, which include $CD8^+$ (cytotoxic) T cells, which can be activated by $CD4^+$ T (helper) cells. Zhang and Bevan recently reviewed $CD8^+$ T cell activation and functioning [63]. Other types include Regulatory T (T_{reg}) cells, which can suppress immune activation, and more recently discovered and not completely understood γδ T cells and Natural killer T (NKT) cells. Turchinovich and Pennington [64] summarize current research on γδ T cells, while Godfrey and Rossjohn have published a review on NKT cells [65].

GABA metabolism and the immune system

Like in the CNS and other peripheral organs and tissues, components of the GABA system are also present in the immune system. Although not strictly a part of the immune system, platelets are closely related. Also known as thrombocytes, platelets are cytoplasm-containing cell fragments pinched-off from megakaryocytes. Early in the megakaryocyte-lineage megakaryocytes, and therefore platelets, share progenitors with all other hematopoietic cells (figure 4). Megakaryocytes are most closely related to erythrocytes and granulocytes than to monocytes and lymphocytes. More recently, it has also become clear the role of thrombocytes is not confined to thrombosis and also extends to immune response [66]. Platelets express a number of neurotransmitter receptors. GABA seems to be present in cultured platelets, but in much lower concentrations than in cultured hippocampal and cortical tissue [67]. Addition of gabaculine, an irreversible GABA-T and GABA reuptake inhibitor, increased GABA concentrations significantly suggesting the expression of functional GABA-T in platelets. This increase was largely abolished by the removal of calcium from the medium.

GABA has been reported in cultured resting murine macrophages, and was also found in extract of macrophages cultured from peripheral blood monocytes [68]. Macrophages and lymphocytes in skin of psoriasis patients are positive for GABA [69]. The enzyme GAD_{65} was present in significant amounts in dendritic cells (DCs) and in lower concentrations also in peritoneal macrophages, suggesting these cells posses functional synthetic machinery to produce GABA [70]. Macrophages, DCs and T lymphocytes also secrete GABA. Stimulation of macrophages and DCs with lipopolysaccharide (LPS) increased GAD_{65} expression, while the amount of secreted GABA wasn't influenced significantly. Stimulation of $CD4^+$ T cells with anti-CD3 and anti-CD28 antibodies also had no effect on the concentration of GABA in the medium. The presence of GABA-T was reported in macrophages and T lymphocytes. Stimulation increased the expression of GABA-T in T cells, but did not significantly alter expression in macrophages [70]. Expression of GAD67 mRNA was reported in 70-80% of resting and 100% of activated lymphocytes [71]. Both B cells and T cells were present in this lymphocyte isolate. It is likely, that the studied population was mainly formed by T cells since a specific T lymphocyte mitogen (phytohemagglutinin; PHA) was used for stimulation and the T:B cell ratio was about 3:1,. B-T cell interactions however, may have influenced expression. The vesicular inhibitory amino acid transporter (VIAAT) protein, detected in most resting and activated isolated lymphocytes, was clustered, suggesting this transporter of GABA and glycine may be associated with vesicular compartments that store GABA in these cells. GAT-1 mRNA was present in 50% of resting lymphocyte samples, while in activated samples, both GAT-1 and GAT-2 mRNA expression was reported. Stimulated lymphocytes showed significantly higher GABA uptake than resting cells. Depletion of Na^+ largely diminished this increase [71]. Intact GAD_{65} and GAD_{67} are also present in neutrophil granulocytes, indicating neutrophils may also produce GABA [47]. Surprisingly, neither GAD_{65} nor GAD_{67} are expressed by microglia, resident macrophages of the CNS, but these cells do express GABA-T [72].

Table 2. $GABA_A$ receptor subunits expression by mammalian immune cells

Cell type	mRNA	Protein
Neutrophil granulocyte	?	α1 [73]
Monocyte	β2 [77]	Possibly α1, α4, β2, β2, γ1 and/or δ [76]
Macrophage	α1, α2, β2, β3 and δ [76]	α1 [76]
Dendritic cell	?	?
Peripheral lymphocytes	α1, α3, α6, γ2, δ and ρ2 (resting) [71] α1, α3, α6, β3, γ2, δ and ρ2 (activated)	?
B lymphocyte	α1, α3, β2 [73]	α1 [73]
$CD4^+$ T lymphocyte	α1, α2, α3, β1, β2 and δ (naïve) [75] α1, α2, α3, β1, β2, δ and γ3 (stimulated)	Possibly α1 [73]
$CD8^+$ T lymphocyte	α1, β2 [73]	Possibly α1 [73]
PBMCs	α1, α3, α4, β2, β3, γ2, δ and ε [73]	α1 [73]
Peripheral blood leukocytes	α3, β3, θ [74]	?
Jurkat cells	α1, α3, α4, α6, β1-3, γ2, ε and θ [73]	α1 [73]
HL-60 cells	?	α1 [73]
THP-1	α4, β2, γ1 and δ [77]	?

$GABA_A$ receptors and the immune system

Transcripts of $GABA_A$ receptor subunits have been reported in a number of immune cell types (Table 2). Jurkat T cell leukemia cells for example, express α1, α3, α4, α6, β1-3, γ2, ε and θ subunit mRNAs [73]. Human peripheral blood mononuclear cells (PBMCs), which include lymphocytes, monocytes and macrophages, expressed a more limited repertoire comprising of α1, α3, α4, β3, δ and ε mRNA. Human B and T cells and Human promyelocytic leukemia (HL-60) cells also seemed to express α1 subunit protein, while neutrophils did not. Irradiated B cells and $CD4^+$ T cells expressed α3 subunit mRNA, while $CD8^+$ T cells and macrophages did not. In dendritic cells (DCs) neither α1 nor β2 mRNA was present. Unfractionated human peripheral blood leukocytes expressed α3, β3 and θ subunit RNA [74]. Tian *et al.* studied the expression of $CD4^+$ T lymphocytes [75]. $GABA_A$-R α1, α2, β1, β2 and δ subunit RNA was present in these in naïve $CD4^+$ T cells. Furthermore, when activated with anti-CD3 and anti-CD28 antibodies, β1 expression increased and γ3 RNA, not present in naïve cells, became detectable. In some resting lymphocytes from healthy humans, α1, α3, α6, γ2, δ and ρ subunit mRNA was present, while activated lymphocytes also expressed β3 [71].Reyes-García *et al.* reported on the expression of subunits in murine peritoneal macrophages [76]. $GABA_A$-R α1 protein was present in these macrophages and α1, α2, β3 and δ subunit mRNA was also detected. Notable is the increase of immunoreactivity of α1 protein after stimulation with lipopolysaccharide (LPS). Recently the subunit expression profiles of human myleomonocytic cell line (THP-1) cells and human monocytes were researched [77]. Of all the 19 known subunits, only β2 mRNA was present in freshly prepared monocytes, while THP-1 cells also expressed α4, γ1 and δ subunits.

Although several types of immune cells do express $GABA_A$ receptor subunit mRNA and protein, it can not be deduced from that, that these cells also posses fully functional receptors. Bath *et al.* studied the effects of $GABA_A$-R ligands on cytokine production and intracellular signaling [70]. The anti-convulsant topiramate, a drug with $GABA_A$-R agonist properties, inhibited the cytokine production of antigen challenged murine splenocytes in dose-dependent manner. Both pro-inflammatory tumor necrosis factor (TNF), interleukin 17 (IL-17) and IL-6 and anti-inflammatory IL-10 showed significant reduced production. Activated purified T lymphocytes on the other hand, showed no significant influence of $GABA_A$-R agonists on proliferation and production of interferon γ (IFNγ), TNF, IL-17 or IL-6. Surprisingly, Tian *et al.* did report inhibition of antigen-induced T cell proliferation by the $GABA_A$-R agonist muscimol [78]. Peritoneal macrophages isolated from mice treated with either the GABA-T inhibitor vigabatrin or topiramate, produced after LPS stimulation significantly lower quantities of pro-inflammatory IL-1β [70]. The same diminished production was also seen in dendritic cells. Interestingly, macrophages from animals treated with topiramate reduced the production of pro-inflammatory IFNγ by T cells when co-cultured. The reverse of this setting did not have a comparable effect. Production of other cytokines by macrophages was also modified by $GABA_A$-R activation [76]. GABA added to cultured murine peritoneal macrophages decreased IL-6 and IL-12 production, while the non-competitive $GABA_A$-R antagonist picrotoxin, reversed this inhibition in a dose-dependent manner, suggesting the effect was mediated by $GABA_A$-Rs.

$GABA_B$ receptors and the immune system

Although the presence of $GABA_A$ receptors on immune cells has been studied extensively, less is known about $GABA_B$ receptors. Thrombocytes do seem to have functional $GABA_B$ receptors in addition to the GABA metabolizing enzyme GABA-T [67]. Adding of GABA alone to platelet rich plasma and washed platelet suspension did not significantly influence platelet aggregation, while addition of both GABA and the calcium ionophore A23187 markedly increased aggregation over both compounds alone [67]. Increasing concentrations of the PI-3-kinase inhibitor Wortmannin decreased this aggregation concentration-dependent, therefore indicating functional $GABA_B$-Rs may be present on platelets. Expression of functional $GABA_B$-Rs seems to start early in immune cells development [79]. $GABA_{B1}$ gene products (both mRNA and protein) were present in $CD34^+$ hematopoietic progenitor and stem cells (HSPCs). Interestingly, expression was found to be higher in immature cells than more mature progenitors. Seidel *et al.* also confirmed $GABA_B$-Rs on $CD133^+$ HSPCs isolated from mobilized peripheral blood [80]. Neutrophil granulocytes seem to retain expression of $GABA_B$-Rs [47]. Both immunoblotting and confocal microscopy showed the presence of $GABA_{B2}$ protein in neutrophils. Kuhn *et al.* reported the expression of both $GABA_{B1(a,b,e)}$ and $GABA_{B2}$ protein and receptors responding to the $GABA_B$-R agonist baclofen in macrophage-like microglial cells [81]. The functional approach was also employed by Duthey *et al.* and suggested the presence of $GABA_B$-Rs in PBMCs [82].

Functional research on $GABA_B$ receptors and the immune system is relatively scarce compared to $GABA_A$ receptors. Pretreating neutrophils with 10 μM of the $GABA_B$-R agonist baclofen significantly induced chemotaxis, measured as migration across a polyethylene membrane, although less effective than chemoattractant fMLP (N-Formylmethionine leucyl-phenylalanine) [47]. Furthermore, the

$GABA_B$-R antagonist CGP52432 significantly inhibited baclofen-stimulated neutrophil chemotaxis. Reduction of chemotaxis by the reversible PI-3-K inhibitor LY294002 was partly reverted by baclofen, suggesting induction of chemotaxis in neutrophils is mediated by PI-3 kinase. Functional $GABA_B$-Rs expressed by PBMCs were also reported to have a notable effect on cytokine production [82]. PBMCs activated with PHA showed a significant 40% decrease in TNFα and a slight (non-significant) increase in IL-4 production in response to baclofen, while IFNγ, IL-2, IL-5, IL-6 and IL-10 showed no significant changes. Microglia stimulated with LPS secreted a significant amount of pro-inflammatory IL-6 and IL-12p40 compared to controls [81]. Co-incubation of LPS and baclofen caused a considerable concentration-dependent reduction in secretion of these cytokines. Baclofen had no notable effect on IL-6 and IL-12 in unstimulated controls. Migration of $CD133^+$ HSPCs is primarily regulated by stromal cell-derived factor-1α (SDF-1α) [80]. Adding SDF-1α increase locomotion of these cells by approximately 50% and co-incubation with baclofen normalizes migration. Treatment with baclofen alone results in a slight decrease of migration compared to untreated controls. The reduction by this $GABA_B$-R agonist is comparable to GABA itself in the same concentration, indicating the effect is primarily mediated by $GABA_B$-Rs. In addition to mentioned molecular effects, $GABA_B$-R agonists potentially influence various other cellular functions (figure 5).

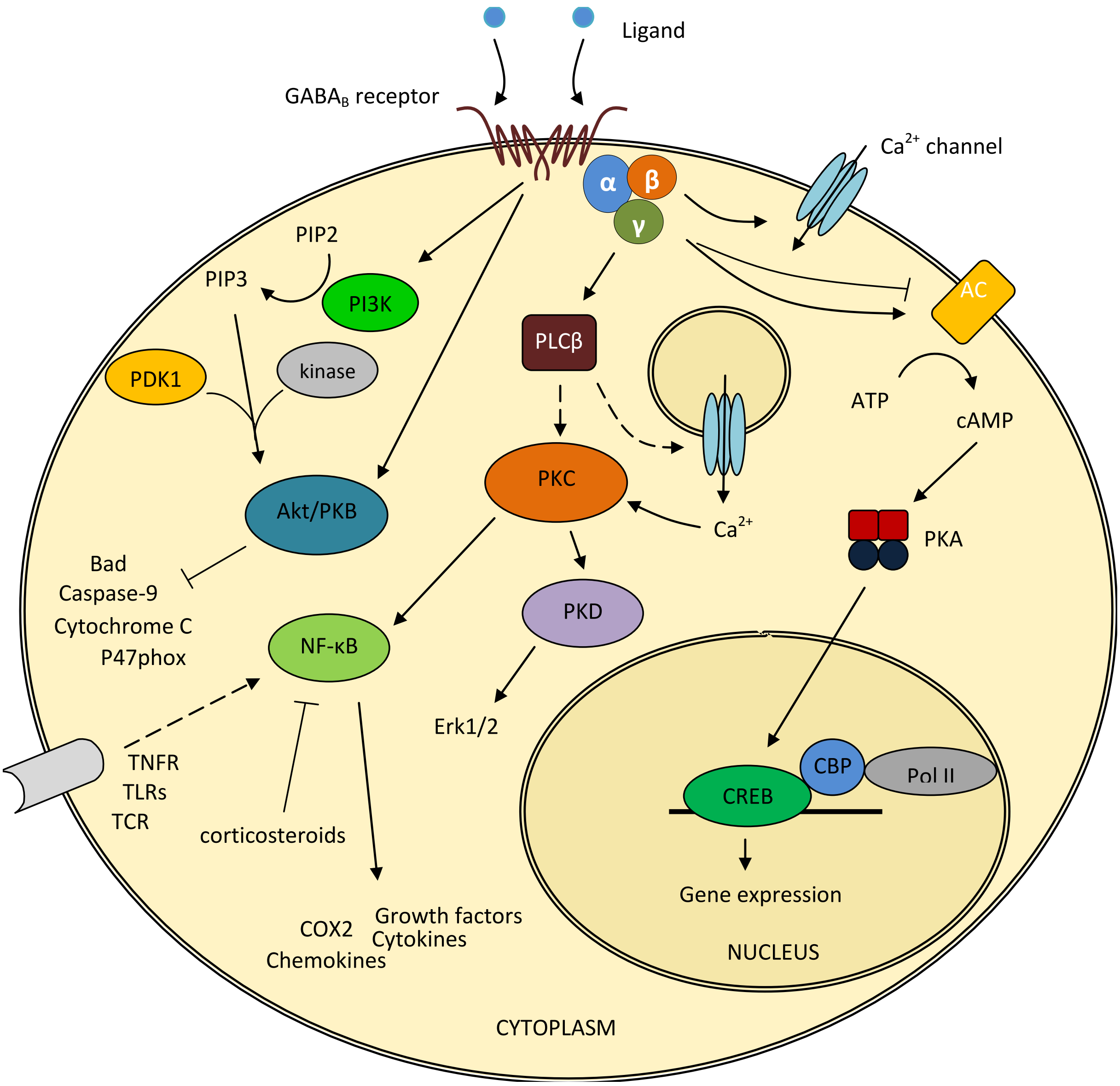


Figure 5. Important signaling pathways associated with the $GABA_B$ receptor and the immune system. AC, Adenylate cyclase; Bad, Bcl-2-associated death promoter; COX2, cyclooxygenase-2; Erk1/2, Extracellular signal-regulated kinase 1/2; TCR, T cell receptor; TLR, Toll-like receptor; TNFR, tumor necrosis factor receptor

GABA, GABA receptors and the immune system *in vivo*

The influence of $GABA_A$ and $GABA_B$-R agonists and other GABAergic agents on cytokine production and cellular signaling results in various *in vivo* effects. Treatment of mice with experimental autoimmune encephalomyelitis (EAE), a model used to mimic inflammatory demyelinating diseases of the CNS such as multiple sclerosis, with GABA-T inhibitor vigabatrin or anti-convulsant topiramate significantly reduces the severity of symptoms [70]. Non-obese diabetic (NOD) mice are prone to developing autoimmune insulin dependent diabetes mellitus. Splenic T cells respond spontaneously to β cell antigens by secreting the pro-inflammatory cytokine IFNγ [75]. Adding GABA inhibits this spontaneous response *ex vivo*. Consequently, NOD/scid (severe combined immunodeficiency) mice implanted with pallets releasing GABA over a period of 21 or 60 days, showed a much slower progression to diabetes. NOD mice implanted two times consecutively with a similar pallet, releasing GABA over a total period of 180 days, showed a similar effect. While placebo treated controls developed diabetes from 20 weeks of age on, the GABA treated group showed no diabetes prevalence until after 40 weeks. Collagen-induced arthritis (CIA) is a mouse model sharing many immunological and pathological features with human rheumatoid arthritis. Mice treated orally with GABA had a lower disease severity than controls [83]. Splenic T lymphocytes *in vivo* primed with bovine collagen type II (bCII) peptide showed reduced proliferation *ex vivo* in a GABA-containing medium. Levels of total immunoglobulin G (IgG) and subtype IgG2a specific for bCII in serum of collagen immunized mice, were lower in those treated with GABA. Mice suffering from allergic contact dermatitis benefited of treatment with $GABA_B$-R agonist baclofen [82]. The ear swelling response for example, was markedly reduced by intraperitoneal baclofen injection. Histology also showed a reduction of inflammatory infiltrate, visible as a reduced number of macrophages, monocytes, $CD45^+$ lymphocytes and neutrophils. A study on the $GABA_B$-R agonist phenibut demonstrated the immunomodulating effects in immune hyperactivation [84]. Mice injected with Pseudomonas aeruginosa-derived LPS showed characteristics of immune stress, including increased delayed-type hypersensitivity and phagocytic activity of neutrophils. These parameters normalized after phenibut intra-abdominal injection. In contrast to these immunosuppressive effects, another study on phenibut showed it to be a potent immunostimulant [85]. Mice intraperitoneally injected with the immunosuppressant cyclophosphamide had a reduced spleen weight, thymus weight, delayed-type hypersensitivity response, antibody response and reduced numbers of nucleated cells in the spleen and thymus. Treatment with different phenibut salts normalized one or more of these parameters.

The mentioned *in vivo* and *in vitro* research both point to the potential of modifying the peripheral GABA system in the clinic. Pharmaceuticals that inhibit GABA degradation could be used to reduce inflammation in autoimmune diseases. $GABA_A$ receptor agonists could serve the same purpose, especially when designed to hinder activation in the CNS. Both local (topical) and systemic use is possible. $GABA_A$-R antagonists and compounds which reduce GABA synthesis may be useful in treating drug-induced immune suppression, for example in patients treated with cytostatics. $GABA_B$ receptor agonists, especially those recently designed to act primarily on peripheral receptors [86], have potential in treating autoimmune disease. Its potential seems more restricted, but may be successful in diseases dominated by TNF-α, such as rheumatoid arthritis, asthma and inflammatory bowel disease [87-89].

PART 2. MINI INTERNSHIP

Materials and methods

Cell isolation. Peripheral blood mononuclear cells (PBMCs), kindly provided by S. de Kivit (Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands), were isolated from human donors and stored as previously described [90].

Stimulation. When appropriate, PBMCs were stimulated with either LPS or anti-CD3 (1:10,000, Sanquin, Netherlands) and anti-CD28 (1:10,000, Sanquin, Netherlands) antibodies or both, through 24 h incubation.

Staining. Before staining 2 x 10^5 PBMCs were transferred to 96-well plates and washed in FACS buffer (PBS/2% FCS). Cells were stained with rabbit anti-GABBR2 (EP2411Y) (1:100, Abcam, Cambridge, UK), goat anti-rabbit FITC (F0112) (1:50, R&D Systems, Minneapolis, USA), anti-CD4-PerCP-Cy5.5 (1:50, BD Biosciences) and/or anti-CD14-PerCP-Cy5.5 (1:50, BD Biosciences). PBMCs were incubated in FACS buffer supplemented with antibodies for 30 minutes for staining. Cells were fixed in 150µL FACS buffer supplemented with 50µL 2% paraformaldehyde (PFA). After staining and fixing, cells were analyzed with the FACS Canto II (BD Biosciences).

Results

Using forward scatter (FSC) and GABBR2 FITC fluorescence, it can be determined that human blood contains several mononuclear cell populations, some of which are weakly or strongly express $GABA_{B2}$ receptors (figure 6). To further identify the different cell types present and the extent of their GABBR2 expression, samples were labeled for CD4, a surface marker primarily expressed by T helper lymphocytes and monocyte/macrophage-marker CD14. In addition, cells were stimulated with either LPS or anti-CD3/28 to explore the effect of activation on GABBR2 expression.

Most of unstimulated $CD14^+$ monocytes expressed $GABA_{B2}$ receptors on their membranes (figure 7B, *red*). Also a number of $CD14^-$ cells expressed $GABA_{B2}$ receptors. Interestingly, after stimulation of the PBMCs with LPS, the number of cells expressing membrane $GABA_{B2}$ receptors increased in both $CD14^+$ (figure 7C, *red*) and $CD14^-$ cells. Stimulation of unfractionated PBMCs with anti-CD3 and anti-CD28 antibodies seems to have increased membrane-bound CD14 and slightly increased both CD14 and GABBR2 expressing cells (figure 8A, *blue*). Surprisingly, co-stimulation with both anti-CD3 and

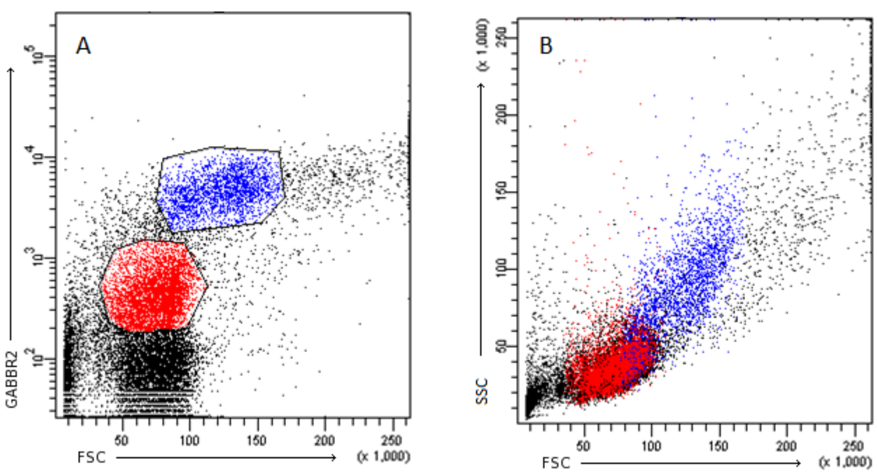


Figure 6. $GABA_{B2}$ receptor expression on unfractionated PBMCs. A number of cell populations differing in size express the GABBR2 *(A)*. Gating of two clear-cut populations *(B)* indicates these comprise of lymphocytes *(red)* and monocytes *(blue)*.

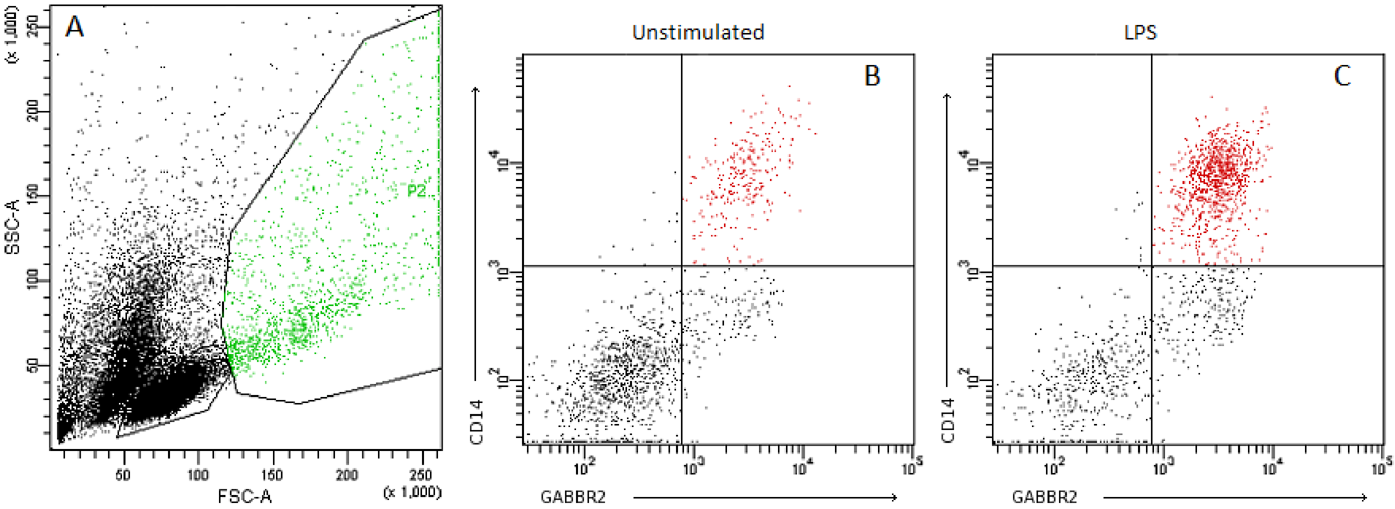


Figure 7. GABA$_{B2}$ receptor expression by monocyte CD14$^+$ cells. Based on FSC and SSC monocyte populations were selected (*A*, *P2 green*) and CD14 expression was plotted against GABBR2 unstimulated (*B*) and LPS-stimulated (*C*).

anti-CD28 antibodies in addition to LPS ameliorated the increase in CD14$^+$GABBR2$^+$ cell caused by LPS (resp. figures 8B, *blue* and 7C, *red*).

Based on cell size and complexity, the cell cluster most likely to contain CD4$^+$ T cells was gated (figure 9A, *red*). As shown in figure 9B, this cluster includes both CD4$^+$ (upper quadrants), which are most likely T helper cells, and CD4$^-$ cells, which include other lymphocytes such as B cells, CD8$^+$ T cells and NK cells. Large numbers of CD4$^-$ lymphocytes expressed GABA$_{B2}$ receptors (figure 9B, *green)*, while practically no GABBR2$^+$CD4$^+$ lymphocytes were present (figure 9B, *purple*). Stimulation with LPS did not significantly alter the number of GABBR2$^+$ lymphocytes (figure 9C). Incubation of the PBMCs with anti-CD3 and anti-CD28 antibodies did also not notably influence GABBR2 expressing numbers (figure 9D). Not surprisingly, stimulation with both antibodies and LPS did not increase the number of GABBR2$^+$ cells (figure 9E).

Discussion

Although not as pronounced as GABA$_A$ receptors, GABA$_B$ receptors do have immunomodulatory effects [82]. Analogous to several other neurotransmitter receptors [86], including GABA$_A$ receptor subunits, membrane GABA$_{B2}$ levels are influenced by T cell and monocyte stimulation. It is highly likely that immune cells expressing GABBR2 also express GABBR1, as functional GABA$_B$ receptors have been identified on several immune cell types (page 14). Direct activation of monocytes with LPS

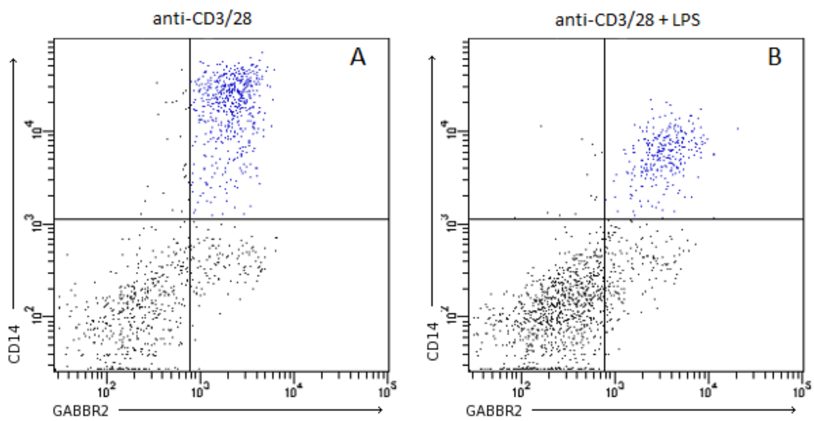


Figure 8. GABBR2 expression on CD14$^+$ monocytes. The CD14$^+$ monocyte and macrophage-like cell populations selected as in fig. 7A were plotted against GABBR2 after stimulation with anti-CD3/28 (*A*) or anti-CD3/28 and LPS (*B*).

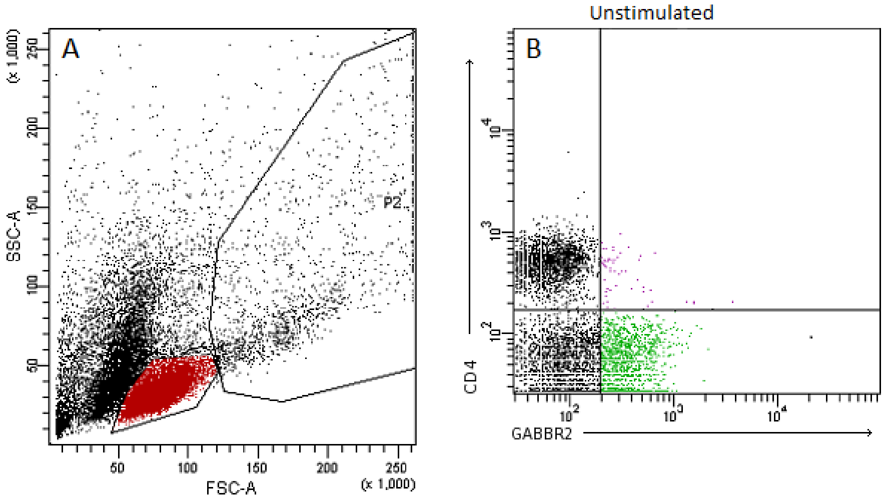
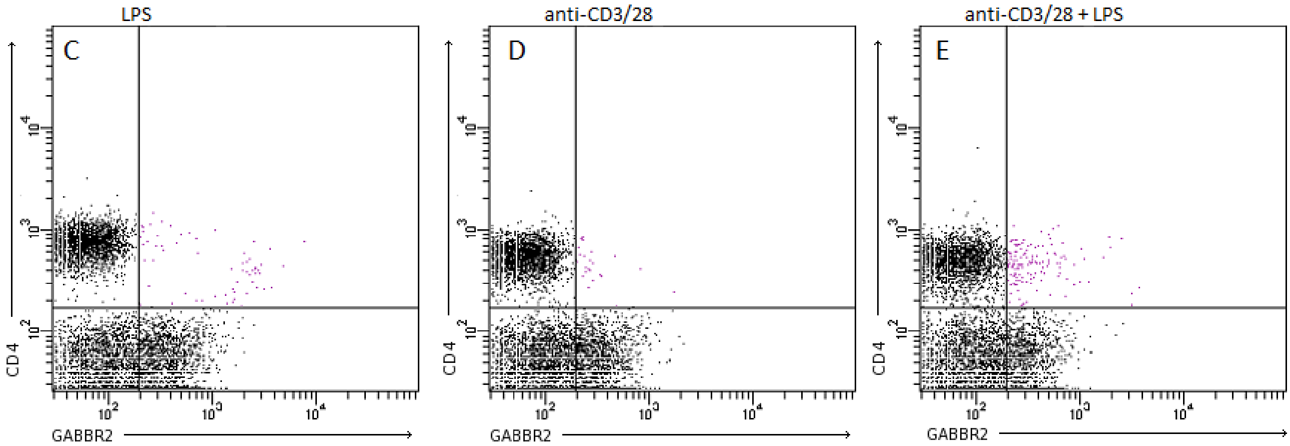


Figure 9. GABBR2 expression on lymphocytes. Based on FSC and SSC the cell population most likely to contain CD4$^+$ T cells and other small lymphocytes was selected (*A*) and membrane CD4$^+$ expression was plotted against GABBR2 in unstimulated gated cells (*B*), LPS (*C*), anti-CD3/28 (*D*) and anti-CD3/28 + LPS stimulated selected PBMCs (*E*).



led to an increase of membrane GABBR2 expressing monocytes. Specific T cell stimulation did also increase the number of GABBR2$^+$ monocytes and slightly increased the amount of GABBR2 expression on monocytes. This indicates GABBR2 expression in monocytes can be induced via T cells. As $GABA_B$-R agonists have anti-inflammatory properties [82], this increased receptor expression may possibly temper monocyte activation and proliferation *in vivo*. Treatment with both antibodies and LPS does not increase or induce GABBR2 expression in monocytes. These effects may be mediated by cell-cell contact or cytokines, as cytokine production in response to anti-CD3/28 and LPS differs slightly and may compensate for or complement each other. Treatment with different cytokines can elucidate the specific mechanism. None of the activation strategies induced GABBR2 expression in T lymphocytes, suggesting functional $GABA_B$-Rs may not have any significant function in T cell activation and proliferation.

Activation of monocytes and T lymphocytes with both anti-CD3/28 and LPS did not modify GABBR2 expression on monocytes. Anti-CD3 and anti-CD28 antibodies mimic T cell receptor (TCR) binding to an antigen presented in a major histocompatibility complex (MHC), combined with a costimulatory signal. LPS mimics the presence of gram-negative bacteria. Since treatment with the antibodies and LPS did not influence GABBR2 expression on monocytes, while treatment with either separately did, it stands to reason that *in vivo* immune activation by both peptide antigens and non-peptide microbial products also does not influence GABBR2 expression on monocytes. This allows immune activation to proceed in the presence of both types of foreign substances, while immune activation is reduced or blocked by peripheral GABA when only one type is present.

References

1. Awapara J, Landua AJ, Fuerst R, Seal B. Free γ-aminobutyric acid in brain. J Biol Chem. 1950; 187:35-9
2. Roberts E, Frankel S. γ-Aminobutyric acid in brain. Fed Proc. 1950; 9: 219
3. Lummis SC, McGonigle I, Ashby JA, Dougherty DA. Two Amino Acid Residues Contribute to a Cation-{pi} Binding Interaction in the Binding Site of an Insect GABA Receptor. J Neurosci. 2011; 31:12371-6.
4. Shelp BJ, Bown AW, McLean MD. Metabolism and functions of gamma-aminobutyric acid. Trends in Plant Science. 1999; 4:446-452.
5. Vinkers CH, Mirza NR, Olivier B, Kahn RS. The inhibitory GABA system as a therapeutic target for cognitive symptoms in schizophrenia: investigational agents in the pipeline. Expert. Opin. Investig. Drugs. 2010; 19:1217-33
6. Nasreen Z, Jameel T, Hasan A, Parveen N, Sadasivudu B. Glutamate Decarboxylase and GABA Aminotransferase Levels in Different Regions of Rat Brain on the Onset of Leptazol Induced Convulsions. Neurochem. Res. 2011 Sep 21. [Epub ahead of print]
7. Watanabe M, Maemura K, Kanbara K, Tamayama T, Hayasaki H. GABA and GABA receptors in the central nervous system and other organs. Int Rev Cytol. 2002; 213:1-47.
8. Sherif FM. GABA-transaminase in brain and blood platelets: basic and clinical aspects. Prog. Neuropsychopharmacol. Biol Psychiatry. 1994; 18:1219-33.
9. Neal MJ, Cunningham JR, Shah MA, Yazulla S. Immunocytochemical evidence that vigabatrin in rats causes GABA accumulation in glial cells of the retina. Neurosci Lett. 1989; 98:29-32.
10. De Biase D, Barra D, Simmaco M, John RA, Bossa F. Primary structure and tissue distribution of human 4-aminobutyrate aminotransferase. Eur J Biochem. 1995; 227:476-80.
11. Jin XT, Galvan A, Wichmann T, Smith Y. Localization and Function of GABA Transporters GAT-1 and GAT-3 in the Basal Ganglia. Front Syst Neurosci. 2011; 5:63
12. Schousboe A, Sarup A, Larsson OM, White HS. GABA transporters as drug targets for modulation of GABAergic activity. Biochem. Pharmacol. 2004; 68:1557-63.
13. Pow DV, Sullivan RK, Williams SM, Scott HL, Dodd PR, Finkelstein D. Differential expression of the GABA transporters GAT-1 and GAT-3 in brains of rats, cats, monkeys and humans. Cell Tissue Res. 2005; 320:379-92.
14. Sieghart W. Structure, pharmacology, and function of GABAA receptor subtypes. Adv Pharmacol. 2006; 54:231-63.
15. Olsen RW, Sieghart W. GABAA receptors: Subtypes provide diversity of function and pharmacology. Neuropharmacology. 2009; 56:141-8.
16. Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of γ-Aminobutyric AcidA Receptors: Classification on the Basis of Subunit Composition, Pharmacology, and Function. Update. Pharmacol Rev. 2008; 60:243-60.
17. Unwin N. Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J Mol Biol. 2005; 346:967–89.
18. Dellisanti CD, Yao Y, Stroud JC, Wang ZZ, Chen L. Crystal structure of the extracellular domain of nAChR alpha1 bound to alpha-bungarotoxin at 1.94 A resolution. Nat Neurosci. 2007; 10:953-62.
19. Hilf RJ, Dutzler R. X-ray structure of a prokaryotic pentameric ligand-gated ion channel. Nature. 2008; 452:375-9.
20. Zimmermann I, Dutzler R. Ligand activation of the prokaryotic pentameric ligand-gated ion channel ELIC. PLoS Biol. 2011; 9:e1001101
21. Clayton T, Chen JL, Ernst M, Richter L, Cromer BA, Morton CJ, Ng H, Kaczorowski CC, Helmstetter FJ, Furtmüller R, Ecker G, Parker MW, Sieghart W, Cook JM. An updated unified pharmacophore model of the benzodiazepine binding site on gamma-aminobutyric acid(a) receptors: correlation with comparative models. Curr Med Chem. 2007; 14:2755-75.
22. Nutt D. GABAA receptors: subtypes, regional distribution, and function. J Clin Sleep Med. 2006; 2:S7-11.
23. Akinci MK, Schofield PR. Widespread expression of GABAA receptor subunits in peripheral tissues. Neurosci Res. 1999; 35:145-53.
24. Metzeler K, Agoston A, Gratzl M. An Intrinsic gamma-aminobutyric acid (GABA)ergic system in the adrenal cortex: findings from human and rat adrenal glands and the NCI-H295R cell line. Endocrinology. 2004; 145:2402-11.
25. Geigerseder C, Doepner RF, Thalhammer A, Krieger A, Mayerhofer A. Stimulation of TM3 Leydig cell proliferation via GABA(A) receptors: a new role for testicular GABA. Reprod Biol Endocrinol. 2004; 2:13.
26. Ong J, Kerr DIB. GABA-receptors in peripheral tissues. Life Sci. 1990; 46:1489-1501.

27. Liu Y, Li YH, Guo FJ, Wang JJ, Sun RL, Hu JY, Li GC. Gamma-aminobutyric acid promotes human hepatocellular carcinoma growth through overexpressed gamma-aminobutyric acid A receptor alpha 3 subunit. World J Gastroenterol. 2008; 14:7175-82.
28. Zhang M, Gong Y, Assy N, Minuk GY. Increased GABAergic activity inhibits alpha-fetoprotein mRNA expression and the proliferative activity of the HepG2 human hepatocellular carcinoma cell line. J Hepatol. 2000; 32:85-91.
29. Biju MP, Pyroja S, Rajeshkumar NV, Paulose CS. Hepatic GABA(A) receptor functional regulation during rat liver cell proliferation. Hepatol Res. 2001; 21:136-146.
30. Takehara A, Hosokawa M, Eguchi H, Ohigashi H, Ishikawa O, Nakamura Y, Nakagawa H. Gamma-aminobutyric acid (GABA) stimulates pancreatic cancer growth through overexpressing GABAA receptor pi subunit. Cancer Res. 2007; 67:9704-12.
31. Roberts SS, Mendonça-Torres MC, Jensen K, Francis GL, Vasko V. GABA receptor expression in benign and malignant thyroid tumors. Pathol Oncol Res. 2009; 15:645-50.
32. Tamayama T, Maemura K, Kanbara K, Hayasaki H, Yabumoto Y, Yuasa M, Watanabe M. Expression of GABA(A) and GABA(B) receptors in rat growth plate chondrocytes: activation of the GABA receptors promotes proliferation of mouse chondrogenic ATDC5 cells. Mol Cell Biochem. 2005; 273:117-26.
33. Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA. The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A. 2003; 100:4903-8.
34. Chalifoux JR, Carter AG. GABAB receptor modulation of synaptic function. Curr Opin Neurobiol. 2011; 21:339-44.
35. Pin JP, Comps-Agrar L, Maurel D, Monnier C, Rives ML, Trinquet E, Kniazeff J, Rondard P, Prézeau L. G-protein-coupled receptor oligomers: two or more for what? Lessons from mGlu and GABAB receptors. J Physiol. 2009; 587:5337-44.
36. Huang ZJ. GABAB receptor isoforms caught in action at the scene. Neuron. 2006; 50:521-4.
37. Pérez-Garci E, Gassmann M, Bettler B, Larkum ME. The GABAB1b isoform mediates long-lasting inhibition of dendritic Ca2+ spikes in layer 5 somatosensory pyramidal neurons. Neuron. 2006; 50:603-16.
38. Vigot R, Barbieri S, Bräuner-Osborne H, Turecek R, Shigemoto R, Zhang YP, Luján R, Jacobson LH, Biermann B, Fritschy JM, Vacher CM, Müller M, Sansig G, Guetg N, Cryan JF, Kaupmann K, Gassmann M, Oertner TG, Bettler B. Differential compartmentalization and distinct functions of GABAB receptor variants. Neuron. 2006; 50:589-601.
39. Bettler B, Kaupmann K, Mosbacher J, Gassmann M. Molecular structure and physiological functions of GABA(B) receptors. Physiol Rev. 2004; 84:835-67.
40. Takahashi T. Dynamic aspects of presynaptic calcium currents mediating synaptic transmission. Cell Calcium. 2005; 37:507-11.
41. Shen W, Slaughter MM. Metabotropic GABA receptors facilitate L-type and inhibit N-type calcium channels in single salamander retinal neurons. J Physiol. 1999; 516:711-8.
42. Park HW, Jung H, Choi KH, Baik JH, Rhim H. Direct interaction and functional coupling between voltage-gated CaV1.3 Ca2+ channel and GABAB receptor subunit 2. FEBS Lett. 2010; 584:3317-22.
43. Hammond RS, Bond CT, Strassmaier T, Ngo-Anh TJ, Adelman JP, Maylie J, Stackman RW. Small-conductance Ca2+-activated K+ channel type 2 (SK2) modulates hippocampal learning, memory, and synaptic plasticity. J Neurosci. 2006; 26:1844-53.
44. Simonds WF. G protein regulation of adenylate cyclase. Trends Pharmacol Sci. 1999; 20:66-73.
45. Skalhegg BS, Tasken K. Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA. Front Biosci. 2000; 5:D678-93.
46. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol. 2001; 2:599-609.
47. Rane MJ, Gozal D, Butt W, Gozal E, Pierce WM Jr, Guo SZ, Wu R, Goldbart AD, Thongboonkerd V, McLeish KR, Klein JB. Gamma-amino butyric acid type B receptors stimulate neutrophil chemotaxis during ischemia-reperfusion. J Immunol. 2005; 174:7242-9.
48. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med. 2005; 9:59-71.
49. Tu H, Xu C, Zhang W, Liu Q, Rondard P, Pin JP, Liu J. GABAB receptor activation protects neurons from apoptosis via IGF-1 receptor transactivation. J Neurosci. 2010; 30:749-59.
50. Newton HB. Molecular neuro-oncology and development of targeted therapeutic strategies for brain tumors. Part 2: PI3K/Akt/PTEN, mTOR, SHH/PTCH and angiogenesis. Expert Rev Anticancer Ther. 2004; 4:105-28.

51. Kuczewski N, Fuchs C, Ferrand N, Jovanovic JN, Gaiarsa JL, Porcher C. Mechanism of GABAB receptor-induced BDNF secretion and promotion of GABAA receptor membrane expression. J Neurochem. 2011; 118:533-45.
52. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol. 2010; 11:103-12.
53. New DC, Wong YH. Molecular mechanisms mediating the G protein-coupled receptor regulation of cell cycle progression. J Mol Signal. 2007; 2:2.
54. Rozengurt E. Protein kinase D signaling: multiple biological functions in health and disease. Physiology (Bethesda). 2011; 26:23-33.
55. Mizuta K, Osawa Y, Mizuta F, Xu D, Emala CW. Functional expression of GABAB receptors in airway epithelium. Am J Respir Cell Mol Biol. 2008; 39:296-304.
56. Fatemi SH, Folsom TD, Reutiman TJ, Thuras PD. Expression of GABA(B) receptors is altered in brains of subjects with autism. Cerebellum. 2009; 8:64-9.
57. Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ. International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. Pharmacol Rev. 2002; 54:247-64.
58. Zhou Z, Sun H, Li X, Li Y, Zhao S, Zhang D, Yao Z, Li J. A local GABAergic system is functionally expressed in human fallopian tube. Biochem Biophys Res Commun. 2010; 398:237-41.
59. Wang T, Huang W, Chen F. Baclofen, a GABAB receptor agonist, inhibits human hepatocellular carcinoma cell growth in vitro and in vivo. Life Sci. 2008; 82:536-41.
60. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. Nat Rev Immunol. 2011; 11:788-98.
61. Sun JC, Lanier LL. NK cell development, homeostasis and function: parallels with $CD8^+$ T cells. Nat Rev Immunol. 2011; 11:645-57.
62. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. Eur J Immunol. 2011 Oct 18. [Epub ahead of print]
63. Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. Immunity. 2011; 35:161-8.
64. Turchinovich G, Pennington DJ. T cell receptor signalling in γδ cell development: strength isn't everything. Trends Immunol. 2011; 32:567-73.
65. Godfrey DI, Rossjohn J. New ways to turn on NKT cells. J Exp Med. 2011; 208:1121-5.
66. Li Z, Yang F, Dunn S, Gross AK, Smyth SS. Platelets as immune mediators: their role in host defense responses and sepsis. Thromb Res. 2011; 127:184-8.
67. Kaneez FS, Saeed SA. Investigating GABA and its function in platelets as compared to neurons. Platelets; 20: 328–333.
68. Stuckey DJ, Anthony DC, Lowe JP, Miller J, Palm WM, Styles P, Perry VH, Blamire AM, Sibson NR. Detection of the inhibitory neurotransmitter GABA in macrophages by magnetic resonance spectroscopy. J Leukoc Biol. 2005; 78:393-400.
69. Nigam R, El-Nour H, Amatya B, Nordlind K. GABA and GABA(A) receptor expression on immune cells in psoriasis: a pathophysiological role. Arch Dermatol Res. 2010; 302:507-15.
70. Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, Steinman L. Inhibitory role for GABA in autoimmune inflammation. Proc Natl Acad Sci U S A. 2010; 107:2580-5.
71. Dionisio L, José De Rosa M, Bouzat C, Esandi Mdel C. An intrinsic GABAergic system in human lymphocytes. Neuropharmacology. 2011; 60:513-9.
72. Lee M, Schwab C, McGeer PL. Astrocytes are GABAergic cells that modulate microglial activity. Glia. 2011; 59:152-65.
73. Alam S, Laughton DL, Walding A, Wolstenholme AJ. Human peripheral blood mononuclear cells express GABAA receptor subunits. Mol Immunol. 2006; 43:1432-42.
74. Plummer PN, Colson NJ, Lewohl JM, MacKay RK, Fernandez F, Haupt LM, Griffiths LR. Significant differences in gene expression of GABA receptors in peripheral blood leukocytes of migraineurs. Gene. 2011; 490:32-6.
75. Tian J, Lu Y, Zhang H, Chau CH, Dang HN, Kaufman DL. Gamma-aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. J Immunol. 2004; 173:5298-304.
76. Reyes-García MG, Hernández-Hernández F, Hernández-Téllez B, García-Tamayo F. GABA (A) receptor subunits RNA expression in mice peritoneal macrophages modulate their IL-6/IL-12 production. J Neuroimmunol. 2007; 188:64-8.

77. Wheeler DW, Thompson AJ, Corletto F, Reckless J, Loke JC, Lapaque N, Grant AJ, Mastroeni P, Grainger DJ, Padgett CL, O'Brien JA, Miller NG, Trowsdale J, Lummis SC, Menon DK, Beech JS. Anaesthetic impairment of immune function is mediated via GABA(A) receptors. PLoS One. 2011; 6:e17152.
78. Tian J, Chau C, Hales TG, Kaufman DL. $GABA_A$ receptors mediate inhibition of T cell responses. J Immunol. 1999; 96:21-8.
79. Steidl U, Bork S, Schaub S, Selbach O, Seres J, Aivado M, Schroeder T, Rohr UP, Fenk R, Kliszewski S, Maercker C, Neubert P, Bornstein SR, Haas HL, Kobbe G, Tenen DG, Haas R, Kronenwett R. Primary human CD34+ hematopoietic stem and progenitor cells express functionally active receptors of neuromediators. Blood. 2004; 104:81-8.
80. Seidel J, Niggemann B, Punzel M, Fischer J, Zänker KS, Dittmar T. The neurotransmitter GABA is a potent inhibitor of the stromal cell-derived factor-1alpha induced migration of adult CD133+ hematopoietic stem and progenitor cells. Stem Cells Dev. 2007; 16:827-36.
81. Kuhn SA, van Landeghem FK, Zacharias R, Färber K, Rappert A, Pavlovic S, Hoffmann A, Nolte C, Kettenmann H. Microglia express GABA(B) receptors to modulate interleukin release. Mol Cell Neurosci. 2004; 25:312-22.
82. Duthey B, Hübner A, Diehl S, Boehncke S, Pfeffer J, Boehncke WH. Anti-inflammatory effects of the GABA(B) receptor agonist baclofen in allergic contact dermatitis. Exp Dermatol. 2010; 19:661-6.
83. Tian J, Yong J, Dang H, Kaufman DL. Oral GABA treatment downregulates inflammatory responses in a mouse model of rheumatoid arthritis. Autoimmunity. 2011; 44(6):465-70.
84. Samotrueva MA, Tiurenkov IN, Teplyĭ DL, Kuleshevskaia NR, Khlebtsova EV. [Immune-regulating effect of phenibut under lipopolysaccharide-induced immune stress conditions]. Eksp Klin Farmakol. 2010; 73:30-2.
85. Tyurenkov IN, Samotrueva MA. Comparative Study of Immunocorrective Activity of Phenibut and Its Organic Salts in Experimental Immunodeficiency. Bulletin of Experimental Biology and Medicine. 2009; 147:606-608
86. Brändén L, Fredriksson A, Harring E, Jensen J, Lehmann A. The novel, peripherally restricted GABAB receptor agonist lesogaberan (AZD3355) inhibits acid reflux and reduces esophageal acid exposure as measured with 24-h pHmetry in dogs. Eur J Pharmacol. 2010; 634:138-41.
87. Brennan FM, Maini RN, Feldmann M. Role of pro-inflammatory cytokines in rheumatoid arthritis. Springer Semin Immunopathol. 1998; 20:133-47.
88. Cho JY. Recent advances in mechanisms and treatments of airway remodeling in asthma: a message from the bench side to the clinic. Korean J Intern Med. 2011; 26:367-83.
89. Malik T, Mannon P. Inflammatory bowel diseases: emerging therapies and promising molecular targets.Front Biosci (Schol Ed). 2012; 4:1172-89.
90. de Kivit S, van Hoffen E, Korthagen N, Garssen J, Willemsen LE. Apical TLR ligation of intestinal epithelial cells drives a Th1-polarized regulatory or inflammatory type effector response in vitro. Immunobiology. 2011; 216:518-27.