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# Effects of bilobalide, ginkgolide B and picrotoxinin on $GABA_A$ receptor modulation by structurally diverse positive modulators

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ABSTRACT

Anxiolytics and anticonvulsants generally positively modulate the action of GABA, whereas many convulsants (including the chloride channel blocker picrotoxinin) negatively modulate the action of GABA on $GABA_A$ receptors. Like picrotoxinin, bilobalide and ginkgolide B, active constituents of *Ginkgo biloba*, have been shown to negatively modulate the action of GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. However, unlike picrotoxinin, bilobalide and ginkgolide B are not known to cause convulsions. We have assessed the action of bilobalide, ginkgolide B and picrotoxinin on a range of $GABA_A$ modulators (etomidate, loreclezole, propofol, thiopentone sodium, diazepam, and allopregnanolone), using two-electrode voltage clamp electrophysiology at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. The results indicate that bilobalide and ginkgolide B differ from picrotoxinin in their ability to inhibit the actions of a range of these structurally diverse $GABA_A$ positive modulators consistent with these modulators acting on a multiplicity of active sites associated with $GABA_A$ receptors. In the presence GABA, ginkgolide B was more potent than bilobalide in inhibiting the GABA-potentiating effect of propofol, equipotent against loreclezole and allopregnanolone, and less potent against etomidate, diazepam, and thiopentone sodium. This indicates that in comparison to picrotoxinin, bilobalide and ginkgolide B differ in their effects on the different modulators.

## 1. Introduction

γ-Aminobutyric acid (GABA) is a major neurotransmitter, regulating the overall balance between neuronal excitation and inhibition in the central nervous system (Chebib and Johnston, 2000). $GABA_A$ receptors are classified as members of the cys-loop ligand-gated ion channel (LGIC) superfamily. They mediate fast synaptic neurotransmission *via* the gating of chloride ion movement through the channel by GABA.

$GABA_A$ receptors are made up of five subunits arranged pseudo-symmetrically, around a central conducting chloride ion channel. Each subunit comprises four transmembrane domains, M1, M2, M3 and M4. The helical M2 domain surrounds the ion channel. The ion channel of these receptors is activated (opened) following the binding of GABA to its recognition site to permit chloride permeability. The channel helical structure is extended to the M2-M3 loop residues (Bera et al., 2002). The extracellular M2-M3 loop is required for ion channel activation through the rotation of M2 domains while the intracellular M3-M4 loop is responsible for the attachment to the cytoskeleton and modulating protein phosphorylation (Absalom et al., 2004). In addition, the extracellular N-terminal domain forms most, if not all, of the agonist and antagonist binding residues. The intracellular loop linking to both M1 and M2 domains is involved in ion selectivity (Jensen et al., 2005).

Many current therapeutic agents, including general anaesthetics, benzodiazepines, barbiturates, neurosteroids and loreclezole, act *via* positive modulation of $GABA_A$ receptors (Hales and Lambert, 1991; Krasowski and Harrison, 1999; Study and Barker, 1981; Tomlin et al., 1998; Wafford et al., 1994). These positive modulators enhance the action of GABA, by increasing the probability or duration of chloride channel opening, resulting in anti-anxiety and anticonvulsant effects. In contrast, negative modulators, including the chloride channel blocker picrotoxinin (Inoue and Akaike, 1988; Zhang et al., 1995), keep the channels in inactive/closed states, resulting in over-excitation of the neurons and convulsions.

*Ginkgo biloba* extract EGb 761 contains 24% flavonoids and 6% terpenoids (bilobalide, and ginkgolides A, B, C and J), and has been shown in clinical studies to reduce symptoms of anxiety without causing sedation with minimal unwanted side effects (Scripnikov et al., 2007; Van Beek, 2002; Woelk et al., 2007). This is in contrast to currently used benzodiazepine anxiolytics that also produce sedation and cognitive impairment. Bilobalide, ginkgolide B and picrotoxinin

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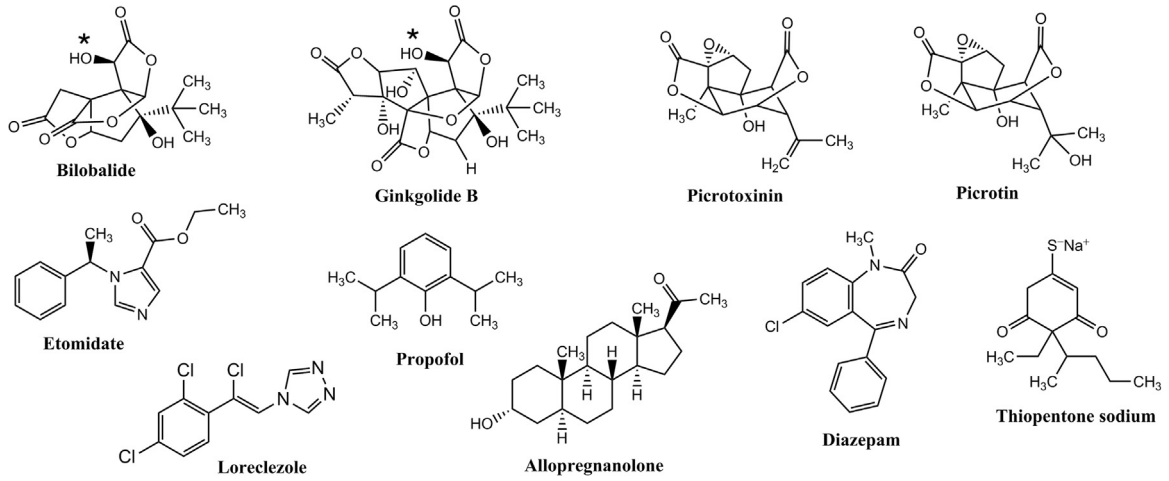


**Fig. 1.** Chemical structures of the negative modulators bilobalide, ginkgolide B, picrotoxinin and picrotin, and the positive modulators etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone. Key hydroxyl groups in biolobalide and ginkgolide B are marked with an asterisk.

(Fig. 1) have been shown to negatively modulate the action of GABA at $GABA_A$ receptors (Huang et al., 2003, 2004). However, unlike picrotoxinin, bilobalide and ginkgolide B have not been reported to cause convulsions. Moreover, animal studies have demonstrated that bilobalide has an anticonvulsant action (Sasaki et al., 1995) and ginkgolide A reduces anxiety (Kuribara et al., 2003).

Bilobalide ($IC_{50}$ 3.9 μM) and ginkgolide B ($IC_{50}$ 19 μM) have been shown to potently displace binding of the radioligand for picrotoxinin binding site [$^{35}$S]-*t*-butylbicylophosphorothionate ([$^{35}$S]TBPS) (Chatterjee et al., 2002, 2003). These findings suggest that bilobalide and ginkgolide B may act at overlapping and/or coupled binding sites to picrotoxinin at $GABA_A$ receptors. We have recently shown in a study of cysteinyl mutants of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors that bilobalide and ginkgolide B differ from picrotoxinin in their binding within the chloride channel (Ng et al., 2016). The present study aimed to investigate the action of bilobalide and ginkgolide B in comparison to picrotoxinin on the actions of a range of structurally diverse positive $GABA_A$ modulators (etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors using two-electrode voltage clamp electrophysiology. The $\alpha_1\beta_2\gamma_{2L}$ subunit combination was employed as it constitutes the major $GABA_A$ receptor subtype in the mammalian brain (McKernan and Whiting, 1996).

## 2. Material and methods

The materials and methods used were essentially as described by Ng et al. (2016). The procedures involving the use of *Xenopus laevis* were approved by the Animal Ethics Committee of the University of Sydney.

### 2.1. Materials

Human $\alpha_1$, $\beta_2$ and $\gamma_{2L}$ deoxyribonucleic acid (DNA) subcloned in pCDM8 were provided by Dr. Paul Whiting (then at Merck, Sharp and Dohme Research Labs, Harlow, UK). Picrotoxinin, bilobalide and ginkgolide B were kind gifts of Dr. Rujee Duke (The University of Sydney, NSW, Australia). GABA, dimethyl sulfoxide (DMSO), propofol and allopregnanolone were purchased from Sigma Aldrich (St Louise, MO, USA). Zinc sulphate ($ZnSO_4$), thiopentone sodium and diazepam were obtained from Ajax Finechem (Seven Hills, NSW, Australia), Jurox (Rutherford, NSW, Australia) and APIN Chemicals (Abingdon, Oxon, UK), respectively. Etomidate and loreclezole were purchased from Tocris Bioscience (Bristol, UK). GABA, $ZnSO_4$ and thiopentone sodium were prepared from 50 or 100 mM milli-Q water stock solutions. Picrotoxinin, bilobalide, ginkgolide B, etomidate, propofol, diazepam, loreclezole and allopregnanolone were prepared from 100 or 200 mM DMSO stock solutions. The highest DMSO concentration used was 0.6%, which was shown to have no significant effect on the oocytes.

### 2.2. Expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors in Xenopus laevis oocytes

Female *Xenopus laevis* were anaesthetised with 0.17% ethyl 3-aminobenzoate in saline for 10–15 min, and ovarian lobes were surgically removed. The lobes were rinsed with OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_20$, 5 mM HEPES, pH 7.5) and treated with 2 mg/ml collagenase A in OR-2 buffer for 2 h to separate oocytes from follicle cells and connective tissues. Released stage V to VI oocytes were collected and rinsed in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 0.5 mM theophylline, pH 7.5). The oocytes were then stored at 15–16 °C in ND96 storage solution (ND96 solution supplemented with 50 μg/ml gentamycin) in an orbital shaker (Ratex Instruments, Victoria, Australia).

Human DNA plasmids of $\alpha_1$, $\beta_2$ and $\gamma_{2L}$ subunits were linearised with Not 1 restriction enzyme and then transcribed using T7 mMessage mMachine kit from Ambion (Austin, TX, USA). A Nanoject injector (Drummand Scientific, Broomali, PA, USA) was used to inject 10 ng per 50 nl of a 1:1:2 mixture of $\alpha_1$, $\beta_2$ and $\gamma_{2L}$ RNAs into the cytoplasm of each oocyte. The purpose of injecting a higher concentration of $\gamma_{2L}$ subunit was to prevent the formation of $\alpha_1\beta_2$ subtype $GABA_A$ receptors. The oocytes were kept in ND96 storage solution at 15–16 °C in an orbital shaker with a twice-daily change of buffer.

### 2.3. Electrophysiological recording

Receptor activity was measured using two-electrode voltage clamp recording 2–7 days after RNA injection. Two glass microelectrodes (Harvard Apparatus, Edenbridge, Kent, UK) were made using a micropipette puller and filled with 3 M KCl solution. Both microelectrodes were inserted into the membrane of an oocyte placed in a cell bath continuously superfused with ND96 recording solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). The membrane potential was clamped at −60 mV using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, USA). The current traces (nA) were detected by a MacLab2e recorder (ADInstruments, Sydney, NSW, Australia) and recorded using LabChart 3.6.3 (ADInstruments, Sydney, NSW, Australia). The sampling frequency used was 10 kHz. The GABA-modulatory and GABA-mimetic effects of etomidate (0.01 μM to 1 mM), propofol (0.01 μM to