The Differential Effects of Resveratrol and *trans*-ε-Viniferin on the GABA-Induced Current in $GABA_A$ Receptor Subtypes Expressed in *Xenopus Laevis* Oocytes

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ABSTRACT - Purpose: The natural products resveratrol and *trans*-ε-viniferin have been reported to have many beneficial effects, which include the enhancement of cognition and memory. There have been no studies which have reported the effects of these compounds on the different $GABA_A$ receptor subtypes and this study aimed to address this. **Methods:** The effects of both resveratrol, and its dimer, *trans*-ε-viniferin, have been investigated on different $GABA_A$ receptor subtypes expressed in *Xenopus laevis* oocytes, using the two-electrode voltage clamp technique. **Results:** Resveratrol induced a current of 22 ± 3.53 nA in the $\alpha_1\beta_2\gamma_{2L}$ subtype of the $GABA_A$ receptor (but not in the $\alpha_5\beta_3\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ subtypes) when applied alone. It also positively modulated the GABA-induced current (I_{GABA}) in $\alpha_1\beta_2\gamma_{2L}$ receptors, in a dose-dependent manner (EC_{50} 58.24 μM). The effects of resveratrol were not sensitive to the benzodiazepine antagonist flumazenil. *trans*-ε-Viniferin exhibited a different pattern of activity to resveratrol; it alone had no effect on any of the subtypes, but it did negatively modulate the GABA-induced current (I_{GABA}) in all three subtypes. The greatest inhibition was found in the $\alpha_1\beta_2\gamma_{2L}$ subtype (IC_{50} 5.79 μM), with the inhibition in the $\alpha_2\beta_2\gamma_{2L}$ (IC_{50} of 19.08 μM) and $\alpha_5\beta_3\gamma_{2L}$ (IC_{50} of 21.05 μM) subtypes being similar. The effects of *trans*-ε-viniferin in $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptors were also not sensitive to the benzodiazepine antagonist flumazenil while, in the $\alpha_5\beta_3\gamma_{2L}$ subtype the effect was not sensitive to the inverse agonist L-655,708, indicating different binding sites for this molecule. **Conclusions:** The results of the present study indicate that both resveratrol and *trans*-ε-viniferin modulate the GABA-induced current in different ways, and that *trans*-ε-viniferin may be a lead compound for the discovery of agents which selectively inhibit the GABA-induced current in α_1-containing subtypes.

INTRODUCTION

$GABA_A$ receptors are membrane bound pentameric chloride selective ion channel composed of α, β, and γ subunits. There are 19 genes for $GABA_A$ receptors, which include 16 subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ρ_{1-3}, θ, π, ε) that are assembled as the different subtypes of $GABA_A$ receptors (1). These differences in the combinations of receptor subunits result in variations in the biophysical and pharmacological properties of the receptors. The distribution of these receptors in the body also differs, with $GABA_A$ receptors being widely distributed in the CNS. Most importantly, agonist affinity, receptor kinetics, and sensitivity to a variety of clinically important drugs (including benzodiazepines and general anaesthetics) are determined by the composition of the subunits (2, 3). For example, receptors that are composed of α_{1-3}, α_5, γ_2, and β_2 or β_3 are sensitive to the benzodiazepines, whereas receptors composed of α_4 or α_6, or δ instead of γ_2, are not sensitive to this class of drugs (4). Simple changes in the receptor subunit combinations can lead to dramatically different activities; for example, receptors containing $\alpha_1\beta_2\gamma_2$ mediate the sedative and anticonvulsant effects of diazepam, $\alpha_2\beta\gamma_2$- and $\alpha_3\beta\gamma_2$-containing receptors are responsible for the anxiolytic and muscle relaxing effects of this drug, and $\alpha_5\beta\gamma_2$-containing receptors may mediate learning and memory processes (5). The involvement of α_5-containing $GABA_A$ receptors in cognition and memory is supported by both mutational and pharmacological studies on rats (6, 7), so these receptors have become attractive targets for the development of memory enhancing drugs (8).

Vitis vinifera (common grape vine) belongs to the family Vitaceae and the extracts and pure compounds from this plant exhibit a variety of

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biological activities, including effects on different neurological disorders. Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a phytoalexin from this plant, has been reported to improve scopolamine- but not mecamylamine-induced memory impairment in rats, in both passive avoidance and Morris water maze tests. The interaction of resveratrol with muscarinic cholinergic receptors has also been suggested by the same authors (9). The neuroprotective effects of resveratrol have been reported by a number of studies which include the protection of dopaminergic neurons from MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced toxicity in mice. It has also been reported to reduce the effect of acetylcholine esterase (AChE), with subsequent improvement of memory impairment in diabetic rats (10). The effect of resveratrol on the level of different neurotransmitters during ischemia/reperfusion in rats has been reported by Li *et al.,* who found that it significantly increased the basal extracellular level of GABA (11).

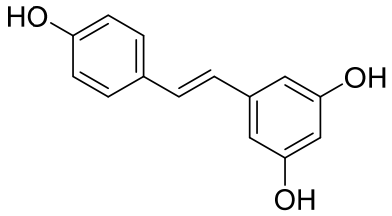
The suppression of β-amyloid (Aβ) fibril formation is considered to be an important target for the treatment of Alzheimer's Disease (AD) and ε-viniferin, the dimer of resveratrol, and resveratrol glucoside (at concentrations of 5 - 10 μM) have been reported to inhibit both fragment Aβ (25-35) and full length (Aβ (1-40) and Aβ (1-42)) peptide aggregation *in vitro* (12, 13). *trans*- ε-viniferin isolated from *Vitis amurensis,* at a concentration of 5 μM, also protects cultured cortical neuronal cells from glutamate-induced neurotoxicity (14).

The effect of resveratrol on different ligand-gated ion channels has been studied by Lee *et al.* (15, 16) and it has been found that it potentiates the 5-hydroxytryptamine (5-HT) induced current in the 5-HT_3 receptor, with an EC_{50} value of 28.0 ± 2.4 μM. At the same time, it inhibits the GABA-induced current in the $GABA_C$ ρ receptor expressed in *Xenopus laevis* oocytes, with an IC_{50} value of 28.9 ± 2.8 μM. Resveratrol also reported to inhibit 1 μM GABA-induced current at human ρ1 $GABA_C$ receptors with an IC_{50} value of 72 μM (17).To date, however, no studies have been reported on the effects of resveratrol on the different subtypes of $GABA_A$ receptor. In the present study, the effects of both resveratrol and *trans*-ε-viniferin (**Figure 1**) have been examined in three different $GABA_A$ receptor subtypes.

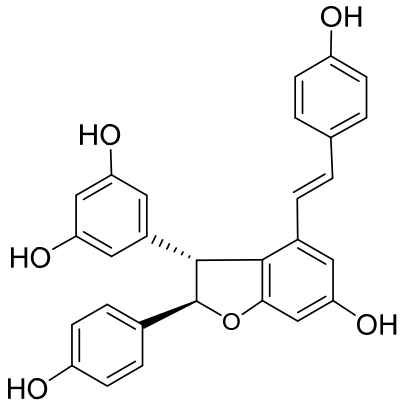
METHODS

Materials

Human α_1, α_5, β_2, β_3 and γ_{2L} DNA in pcDM8 (Invitrogen, CA, USA) were a kind donation from Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK). *Xenopus laevis* were obtained from NASCO, Fort Atkinson, Wisconsin, USA and housed in the Department of Veterinary Science, University of Sydney. DMSO, GABA, and zinc sulphate were purchased from Sigma Aldrich Chemical Co. Ltd. (St Louis, MO, USA). *trans*-ε-Viniferin was purchased from Cfm Oskar Tropitzsch GmbH, Germany and resveratrol was purchased from Sigma Aldrich, Australia. Flumazenil and L655,708 were purchased from Tocris Bioscience, Minneapolis, USA. The compounds used were dissolved in DMSO and any further dilution was made with ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg/mL gentamycin, pH 7.5) buffer before use (all drug solutions were standardised to contain 0.8% DMSO).



Resveratrol



***trans*-ε-Viniferin**

Figure 1. Structures of resveratrol and *trans*-ε-viniferin.

Oocyte preparation

After surgical removal, the ovarian lobes of female *Xenopus laevis* were rinsed with oocyte releasing buffer 2 (OR2; 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_2O$, 5 mM HEPES, pH 7.5), then suspended for 2 hours in collagenase (2 mg/ml in OR2, Bohringer Manheim, Germany) to allow the separation of oocytes from connective tissues and follicular cells. The separated oocytes were then washed several times with ND96 buffer solution. The oocytes were then sorted under a microscope in order to obtain mature and healthy cells with clear animal / vegetal pole divisions and without any spots or markings on the surface. Before injection, the oocytes were stored in a refrigerator at 2-8 ^{0}C.

cRNA preparation of different $GABA_A$ receptors and microinjection

Human α_1, α_2, α_5, β_2, β_3 and γ_{2L} cDNAs subcloned in pcDM8 were linearised using the restriction enzyme NOTI, 3 µL buffer (50 µM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 100 mM NaCl, 0.1 mg/mL BSA). Linearised plasmids containing α_1, α_2, α_5, β_2, β_3 and *γ2L* cDNAs were transcribed using T7 RNA polymerase and capped with 5,7-methylguanosine using a ''mMESSAGE mMACHINE'' kit (Ambion, Austin, TX, USA). Reaction buffer (2 µL), NTP/CAP nucleotide bases (10 µL) and enzyme mixture (2 µL) were added to linearized DNA and incubated at 37 ^{0}C for 1.5 h. The synthesized RNA was then purified and quantified. The quantification of RNA was carried out by heating 2 µL RNA at 94 ^{0}C for 1 min, then running it on a 0.9 % agarose electrophoresis gel containing 1 µL ethidium bromide (5 mg/mL) to check the integrity of the RNA. This was further quantified using a Thermo Scientific NanoDrop 1000 Spectrophotometer and the samples were combined to achieve the desired combinations and ratio of subunits. Forty nanograms per 50 nl of a 1:1:2 mixture of (α_1, α_2, or α_5): (β_2 or β_3):γ_{2L} cRNAs were injected using a 15 to 20 µM diameter tip micropipette (micropipette puller, Sutter Instruments, USA) into the cytoplasm of individual defolliculated oocytes using a Nanoject injector (Drummond Scientific, Broomali, PA, USA). The oocytes were incubated in buffer solution at 18 ^{0}C in an orbital shaker with a once daily change of buffer.

Oocyte recording

Two-three days after injection, the two-electrode voltage clamp technique was performed to measure the receptor activity with Digidata 1200, Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA). Microelectrodes were made by pulling glass capillaries (0.94 mm I.D.x1.2 mm O.D.; Harvard Apparatus Ltd., Kent, UK) using an automated micropipette puller (PUL-100, World Precision Instruments, Inc.) filled with 3M potassium chloride solution.

Oocytes were placed in the oocyte bath chamber, impaled by electrodes with resistance of less than 10 MΩ (usually 0.5 to 2.0 MΩ). In the oocyte chamber, the cells were always perfused with ND96 buffer solution. The current traces elicited due to the application of drugs and / or GABA were recorded using a Mac Lab 2e recorder (ADInstruments, Sydney, NSW, Australia) and Chart Version 5.1 program. For all the electrophysiological experiments, the oocytes were clamped at a holding potential of -60 mV.

Data analysis

Data analysis was performed as described previously, with slight modifications (18). The analysis was performed on GraphPad Prism version 5; concentration–response curves were obtained from the currents recorded from the applied GABA concentrations (EC_{10} for potentiation and EC_{50} for inhibition) in the presence of range of resveratrol and *trans*-ε-viniferin concentrations. The data are expressed as a percentage of the averaged maximum current (I_{max}) and fitted by least squares non-linear regression with the empirical Hill equation.

$$I/I_{max} = [A]^{nH}/ (EC^{nH}_{50}+ [A]^{nH})$$

where [A] is the agonist concentration, n_H is the Hill coefficient and EC_{50} is the effective concentration that evoked a 50% of I_{max} response. Similarly, inhibition curves were assembled from the peak currents recorded from the range of ε-viniferin concentrations applied in the presence of a fixed concentration (EC_{50}) of GABA. The data were expressed as a percentage of the peak current (I_{max}) obtained from the application of the GABA concentration alone. The concentration that inhibited 50% of I_{max} (IC_{50}) was estimated from fitting the data with the Hill equation, where the concentration of the ε-viniferin is substituted for the agonist concentration. Unless otherwise stated, parameters were calculated from individual oocytes and then averaged.

RESULTS

Resveratrol

The addition of the maximal concentration of GABA (1 mM) induced a large inward current

(I_{GABA}) in all three subtypes of receptors, confirming the expression of the respective $GABA_A$ receptors by the oocytes. This current was not inhibited by either 10 or 100 µM solutions of zinc chloride, indicating the incorporation of the γ_{2L} subunit(19). Resveratrol (**Figure 1**), at a concentration of 100 µM induced a slight current (22 ± 3.53 nA) (**Figure 2B**) at $\alpha_1\beta_2\gamma_{2L}$, but not at the $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_5\beta_3\gamma_{2L}$ $GABA_A$ receptor subtypes. Resveratrol at 100 µM concentration did not modulate the GABA-induced current at the $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_5\beta_3\gamma_{2L}$ subtypes of $GABA_A$ receptor but potentiated the EC_{10} (3 µM) GABA-induced current at $\alpha_1\beta_2\gamma_{2L}$ by 126 ± 15 %. In a dose-response study, when applied with a fixed dose of GABA (EC_{10}, 3 µM), resveratrol positively modulated the GABA-induced current (62 ± 2.35 nA) in a concentration dependent manner, with an EC_{50} of 58.24 µM (**Figure 2A**). Moreover, the effect of resveratrol was not sensitive to the benzodiazepine antagonist, flumazenil (**Figure 3**), indicating that it does not interact with the high sensitivity benzodiazepine binding site, which is sensitive to flumazenil and is located at the interface of the α-γ subunits (20, 21).

***trans*-ε-Vinferin**

trans-ε-Viniferin (**Figure 1**), at a concentration of 100 µM, did not induce any current at all three subtypes of $GABA_A$ receptors when applied alone, but there was a small outward current for *trans*-ε-viniferin on the $\alpha_5\beta_3\gamma_{2L}$ subtype (**Figure 4F**) of the $GABA_A$ receptor. However, it did negatively modulate the GABA-induced current (I_{GABA}) at all three subtypes. In dose-response experiments, involving co-application with the EC_{50} GABA concentration, *trans*-ε-viniferin inhibited the GABA-induced current in a concentration dependent manner. The highest inhibitory potency was observed at the $\alpha_1\beta_2\gamma_{2L}$ subtype, with an IC_{50} value of 5.79 µM **Figure 4 (A-B),**, followed by the $\alpha_2\beta_2\gamma_{2L}$ (IC_{50} 19.08 µM) **Figure 4 (C-D),** and then the $\alpha_5\beta_3\gamma_{2L}$ (IC_{50} 21.05 µM) (**Figure 4 (E-F)**).

Further studies showed that the effect of *trans*-ε-viniferin on both the $\alpha_1\beta_1\gamma_{2L}$ (**Figure 5A**), and $\alpha_2\beta_2\gamma_{2L}$ subtypes is not affected by the benzodiazepine antagonist flumazenil (**Figure 5B**), indicating that it does not interact with the high affinity benzodiazepine binding site (which is sensitive to flumazenil). In addition, the effect of *trans*-ε-viniferin on the $\alpha_5\beta_3\gamma_{2L}$ subtype is not sensitive to L-655,708, a preferential inverse agonist of this subtype of $GABA_A$ receptor (**Figure 5C**) (22).

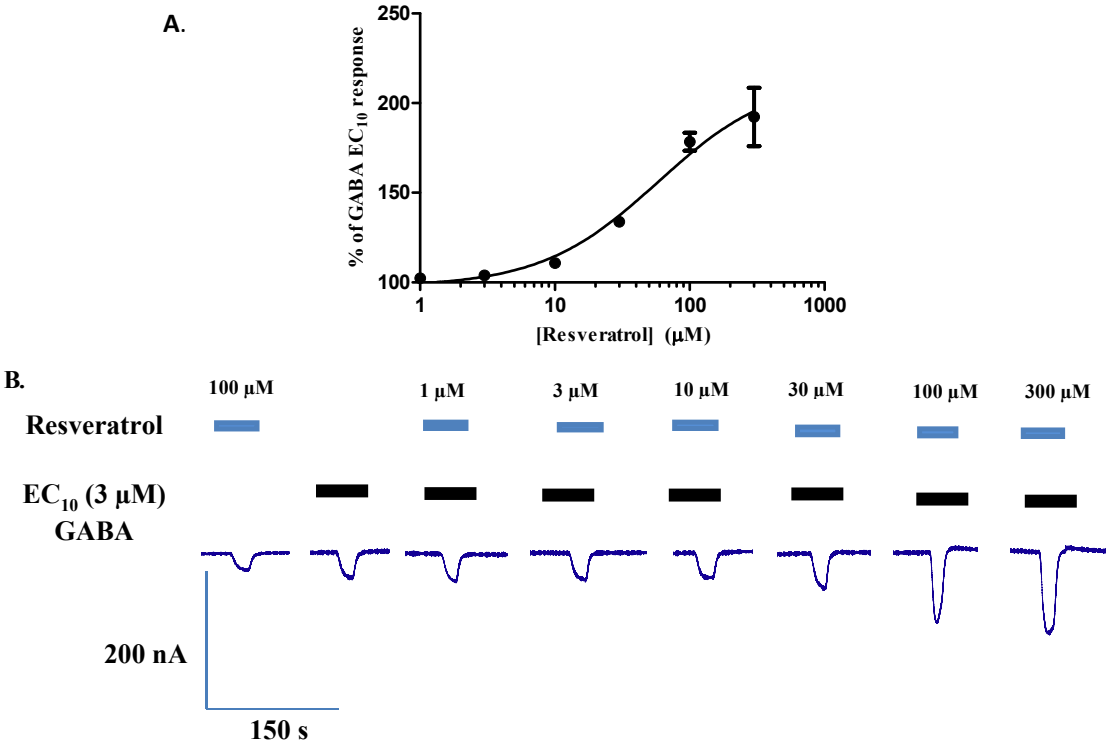


Figure 2. A. Dose-response curve for the effect of resveratrol on the GABA EC_{10} (3 µM) response at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. **B.** Typical traces for the positive modulation of the GABA EC_{10} (3 µM) induced current by different concentrations of resveratrol.

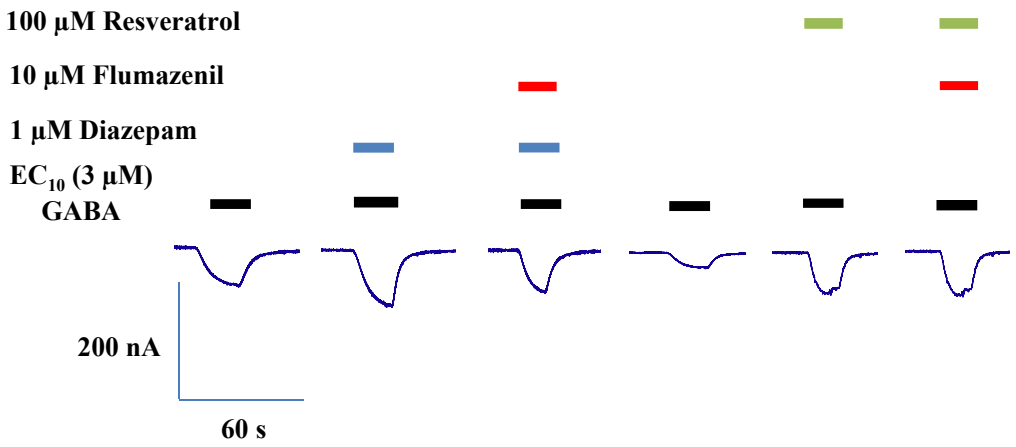
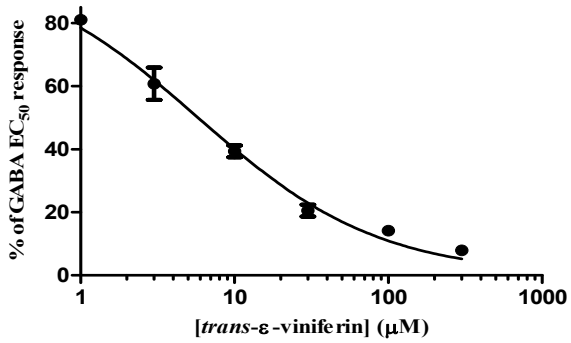
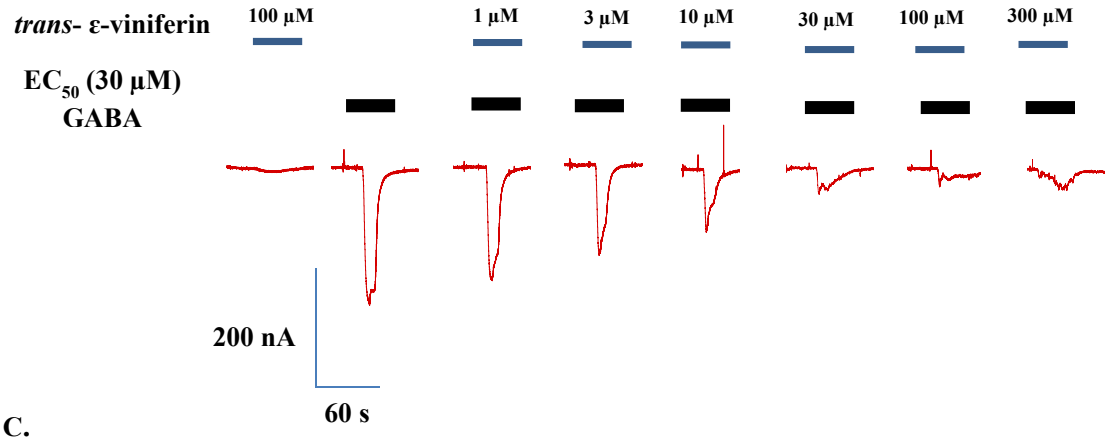


Figure 3. Traces showing that the positive modulation of the EC_{10} (3 µM) GABA-induced current by resveratrol is insensitive to the benzodiazepine antagonist flumazenil.

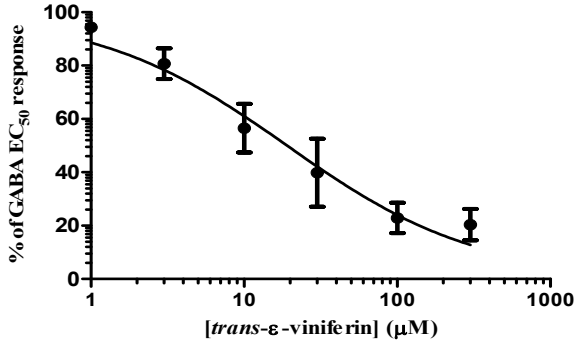
A.



B.



C.



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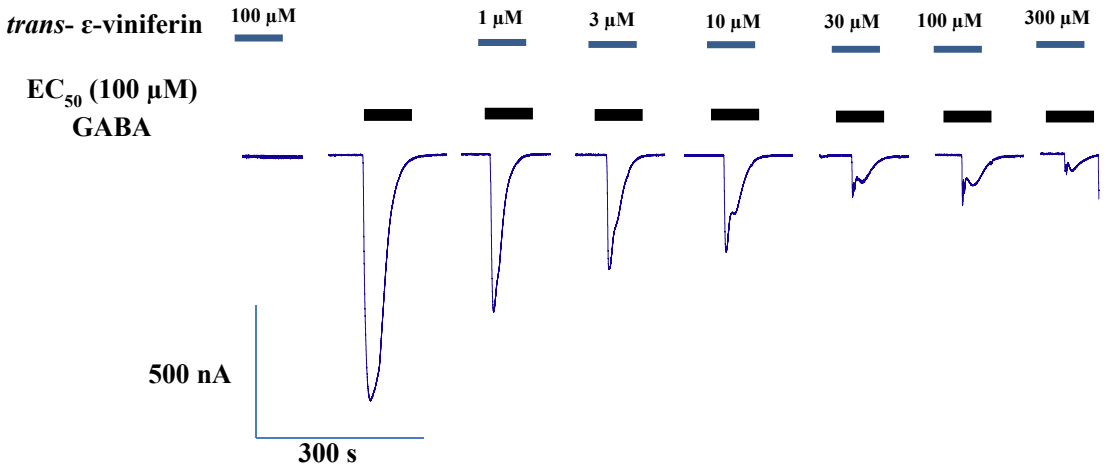
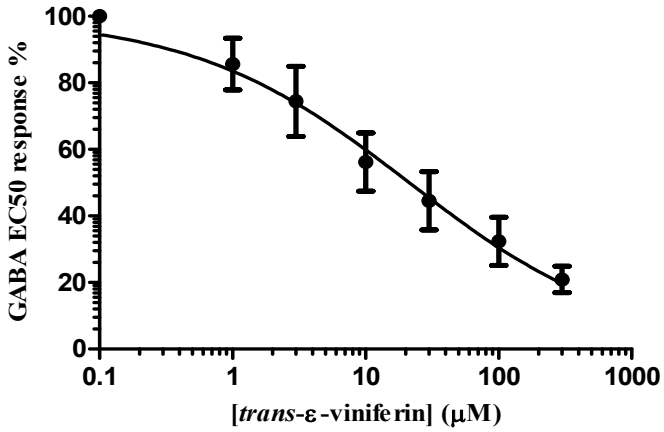


E.



F.

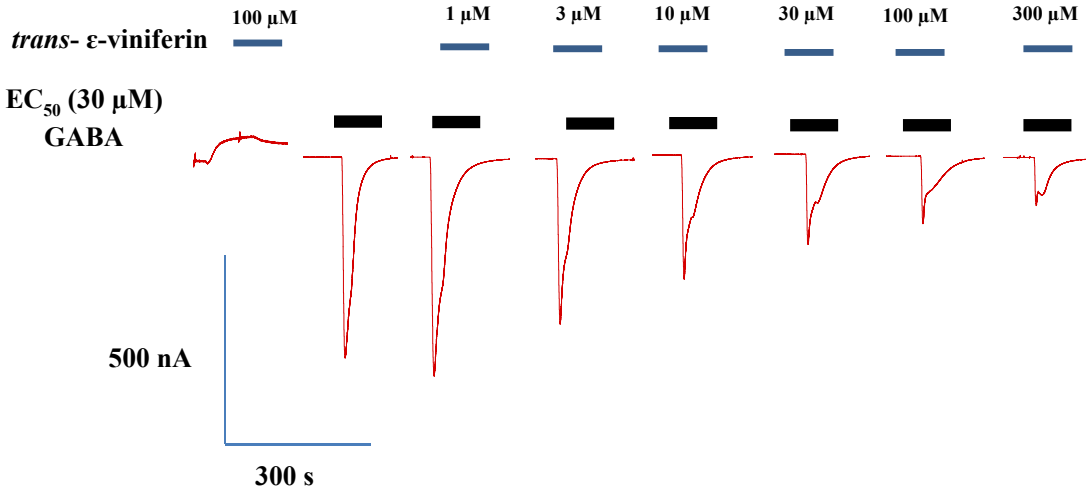
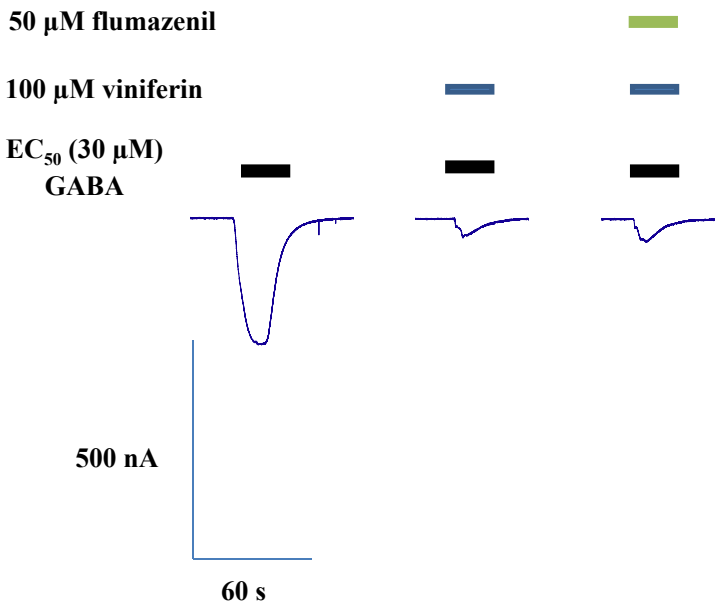
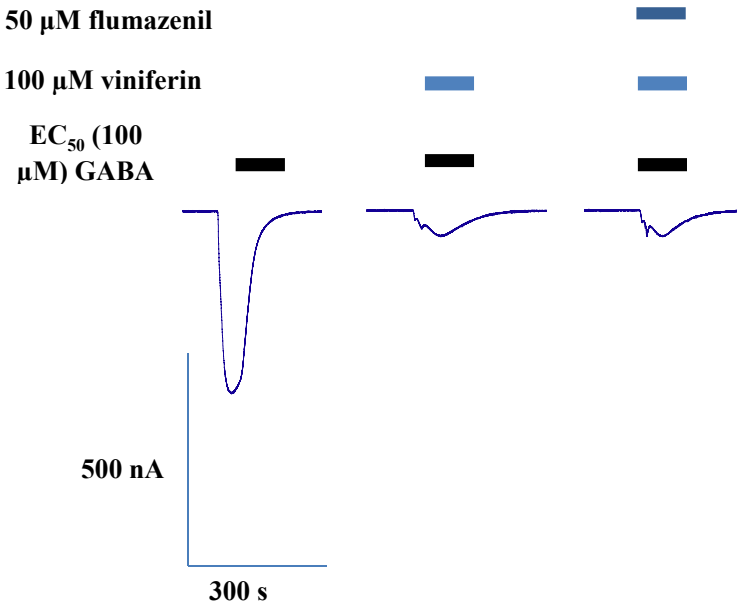


Figure 4. Dose-response curve and typical traces showing the effect of trans-ε-viniferin on the GABA-induced current in different subtypes of $GABA_A$ receptors; **A-B** $\alpha_1\beta_2\gamma_{2L}$, **C-D** $\alpha_2\beta_2\gamma_{2L}$, **E-F** $\alpha_5\beta_3\gamma_{2L}$. Data for all dose-response curves are the Mean ± SEM (n=3-4 oocytes).

A.


50 µM flumazenil
100 µM viniferin
EC50 (30 µM) GABA
500 nA
60 s

B.


50 µM flumazenil
100 µM viniferin
EC50 (100 µM) GABA
500 nA
300 s

C.

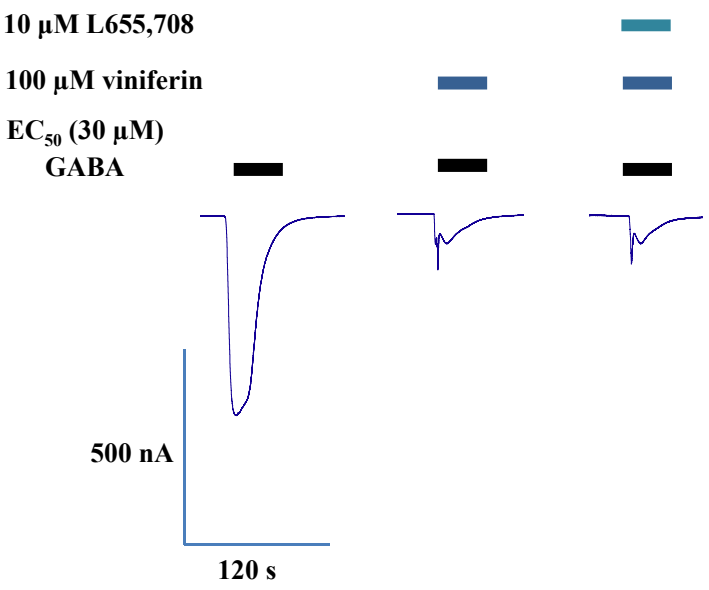


Figure 5. Effect of flumazenil on *trans*-ε-viniferin inhibition of EC_{50} GABA-induced current in the $\alpha_1\beta_2\gamma_{2L}$ (**A**) and $\alpha_2\beta_2\gamma_{2L}$ subtype (**B**). **C.** Effect of L-655,708 on *trans*-ε-viniferin-induced current on $\alpha_5\beta_3\gamma_{2L}$.

DISCUSSION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS), and GABAergic neurons constitute 17-20% of all neurons in the brain (23). There are three different types of GABA receptors, which are classified as $GABA_A$, $GABA_B$, and $GABA_C$ ($GABA_\rho$) based upon their subunit composition, gating properties, and pharmacological profiles. $GABA_A$ and $GABA_C$ are ligand-gated ion channel receptors (LGICs), whereas $GABA_B$ are G-protein coupled receptors (24, 25). $GABA_A$ receptors are an important target for anxiolytics, sedative, hypnotics, anticonvulsant and muscle relaxants (26) and, in spite of having a range of drugs for the treatment of anxiety, there is an increased demand for herbal preparations for the treatment of anxiety, depression, insomnia *etc*. (27). In the present study, we report the differential effects of resveratrol, and its dehydrodimer *trans*-ε-viniferin, which was originally obtained from plant, on different $GABA_A$ receptor subtypes expressed in *Xenopus laevis* oocytes. The effects of resveratrol on ligand gated ion channels have been investigated by many researchers and it has been reported that the neuro-protection by resveratrol in a cerebral ischaemia model is a result of its interaction with NMDA receptors (28). Resveratrol has been found to inhibit the acetylcholine-induced current in rat $\alpha_3\beta_4$ nicotinic acetylcholine receptors (IC_{50} 25.9 µM), inhibit the GABA-induced current in $GABA_C$ receptors, and to potentiate the 5-HT induced current in $5\text{-}HT_{3A}$ receptors (15, 16, 29). It also inhibits the effect of GABA (1 µM) at the human ρ_1 $GABA_C$ receptor as a non-competitive inhibitor with an IC_{50} of 72 µM (30). In the current study, resveratrol had no direct effect on the different subtypes of $GABA_A$ receptors, except $\alpha_1\beta_2\gamma_{2L}$, when applied alone. It did, however, positively modulate the GABA-induced current at the $\alpha_1\beta_2\gamma_{2L}$ subtype (but not the $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_5\beta_3\gamma_{2L}$ subtypes) in a dose-dependent manner. It appears, therefore, that the α_1 subunit is essential for the modulatory effects of this compound on $GABA_A$ receptors. Moreover, the effect of resveratrol is not sensitive to the benzodiazepine antagonist flumazenil, indicating that its binding site is distinct from that of high affinity benzodiazepine binding site. In the previous studies, similar data has been obtained where resveratrol had no influence on the positive modulation of diazepam. At the same time, there was no significant effect on the effect of higher (40 µM) concentration of GABA at $\alpha_1\beta_2\gamma_{2L}$ receptors (31). In the present study, resveratrol positively modulated the current induced by a lower (3 µM) GABA concentration.

Although both resveratrol and *trans*-ε-viniferin are present in comparable amount in grapes (32), the effects of *trans*-ε-viniferin have not been well studied (33), despite it having been found to be more active than resveratrol in a range

of biological assays. For example, it is more active than resveratrol in inducing the relaxation of rat thoracic aorta preparations, has greater *in vitro* antioxidant activity, is a more potent inhibitor of platelet-derived growth factor-induced cell proliferation, and induces nitric oxide generation in vascular smooth muscle cells (VSMCs) (34-36). A number of reports on the modulatory effect of resveratrol on ion channel receptors have been published (15, 16), however, to date, no reports on the modulatory effects of *trans*-ε-viniferin have been published. In the present study, *trans*-ε-viniferin, the dehydrodimer of resveratrol, has been shown to negatively modulate the GABA-induced current (I_{GABA}) in all three subtypes of $GABA_A$ receptor in a dose-dependent manner. The effect of *trans*-ε-viniferin on the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ subtypes is also not sensitive to benzodiazepine antagonist flumazenil, while the effects on the $\alpha_5\beta_3\gamma_{2L}$ subtype are not sensitive to the inverse agonist L-655,708, indicating that this compound does not interact with the high affinity benzodiazepine binding site.

The α_5 subunit containing receptors are mainly located in the hippocampus, where they mediate a tonic chloride leak current and contribute a slow component to GABAergic inhibitory synaptic currents. The inhibitory effect of these receptors on the excitation of hippocampal neurons is thus partly responsible for their association with cognition, learning and memory. These receptors have thus become an important target for different pathological conditions including age related dementia, schizophrenia, and Down syndrome (37). Moreover, it has also been reported that the chronic treatment of TS mice (mouse model of Down syndrome) with an α_5 negative allosteric modulator (NAM) reversed their deficit in spatial learning and memory (38). In the present study, *trans*-ε-viniferin negatively modulated the GABA-induced current at $\alpha_5\beta_3\gamma_{2L}$ $GABA_A$ receptor with an IC_{50} of 21.05 μM, which indicate the potential of this molecule for the development of drug for the treatment age related dementia, Down syndrome and schizophrenia.

In conclusion, despite the structural similarity between resveratrol and *trans*-ε-viniferin, these compounds modulate the GABA-induced current in $GABA_A$ receptors in different ways. The effects of *trans*-ε-viniferin are subtype selective but, in order to increase the selectivity, particularly selectivity towards $\alpha_5\beta_3\gamma_{2L}$ $GABA_A$ receptor, analogues of this compound should be designed and, in addition to being tested on $GABA_A$ receptors *in vitro*, should be tested in animal models.

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