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Central α- and β-thujone: Similar anxiogenic-like effects and differential modulation on GABAA receptors in neonatal chicks

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ABSTRACT

The convulsant effects of α-thujone are attributed to inhibitory actions on the $GABA_A$ receptor. We investigated, for the first time, the effects of α-thujone or β-thujone administrated centrally on the fear/anxiety behaviour of 3-day-old chicks in an Open Field and their modulation on the $GABA_A$ receptor. Higher doses were convulsant by eliciting a toxic and excitatory action, with the results showing that a dose of 78 nmol of either of the two diastereoisomers had an anxiogenic-like effect observed as an increased latency to ambulate and a reduced locomotor activity in an Open Field. Nevertheless, only the central administration of α-thujone reversed the increase induced by acute stress in the flunitrazepam-sensitive $GABA_A$ receptor recruitment. These findings demonstrated that α-thujone, when intracerebroventricularly administered, suppressed the $GABA_A$ receptor recruitment induced by acute stress, maybe due to α-thujone blocking the benzodiazepine binding site or another site of the $GABA_A$ complex. However, it should not be discarded that acute stress associated with novelty may have induced the recruitment of a subpopulation of $GABA_A$ receptors more sensitive to α-thujone than to the constitutive receptors, or that this monoterpene could have inhibited any protein or enzyme trafficking that modulated the phosphorylation of the receptor involved in the turnover of $GABA_A$ receptor. β-Thujone showed behavioural effects similar to its diastereoisomer α-thujone. However, its action mechanism may have been mediated by other neurotransmitter systems, such as the serotonergic one or by a different biological effectiveness due to a distinct stereochemistry at the specific site of the $GABA_A$ receptor.

1. Introduction

GABA is the most important inhibitory neurotransmitter in the CNS. $GABA_A$ receptors ($GABA_AR$) being heteropentamers that are constituted from a combination of 19 known subunits ($\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, θ, π, and $\rho_{1–3}$) with an integral channel that is permeable to Cl^- ions (Lüscher et al., 2011). Many $GABA_AR$ contain two α subunits, two β subunits and one γ

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subunit, with two GABA binding sites being formed by the α and β subunits. The GABAergic synapses are critical for the development and coordination of the neuronal activity underlying the majority of physiological and behavioral processes in the brain (Lüscher and Keller, 2004; Jacob et al., 2008).

$GABA_AR$ are localized in the postsynaptic membrane of neurones, with experimental evidence indicating that synaptically released neurotransmitters saturate their receptors (Clements, 1996), and hence, the functional strength of the GABAergic synapses changes in proportion with the postsynaptic $GABA_AR$ density (Otis et al., 1994; Nusser et al., 1998). Central flunitrazepam (FNZ) binding expresses $GABA_AR$, and when the density was measured *ex vivo* in synaptosomes from chick forebrain, there is evidence that acute stress conditions induced a transient increase in the flunitrazepam sensitive-$GABA_AR$ density due to the stress accompanying a food discrimination task (Salvatierra et al., 1997), an Open Field (OF) test (Marin et al., 1997) or a novelty (Salvatierra et al., 2009).

The OF test is one of the most popular procedures in animal psychology (Belzung, 1999), with its use having been extended to a great number of species, including rodents, calves, pigs, rabbits, chicks, primates and also invertebrates. It is a convenient procedure for measuring not only anxiety-like behavior, but also sedation or locomotor activity, as well as the reaction of the subjects to a stressful event (Prut and Belzung, 2003).

Various studies have demonstrated neuroactivity associated to essential oil due to terpene occurrence (Gomes Silva et al., 2007). The monoterpenes are composed of two units of isoprene, with different pharmacological properties of monoterpenes having been discovered, such as anesthetics, analgesics and gastric sedative agents, which produce strong effects in animal behavior when administered in an intravenous way. Monoterpene neuroactivity is due to an effect of neuronal intracellular signaling or through modulating ionotropic receptor ionic currents (Hall et al., 2004). The monoterpene, thujone is found in nature as a mixture of α-(−) y β-(+), in the essential oil of *Artemisia, Salvia and Thuja* (Nikolic, et al., 2011). Both these diastereomers are bicyclic monoterpenes that differ in stereochemistry in the C_4 methyl group, with the α- and β-thujone ratio in essential oil depending on the plant species. Essential oil has been used in alcoholic drink preparation, and also as flavoring in food and folk medicine (Arnold, 1988). α-Thujone is an active essential oil component of *Artemisia absinthium* L., which is used in "absinthe" liqueur preparation and is responsible for toxic effects such as convulsions, blindness, hallucinations and mental decline (Sirisoma et al., 2001; Lachenmeier and Uebelacker, 2010). However, its action mechanisms have not yet been elucidated, although Höld et al. (2000) y Olsen (2000) provided evidence that α-thujone acts as a $GABA_AR$ receptor chloride channel blocker, much like the plant convulsant picrotoxin and related synthetic analogs. Similarly, β-thujone acts as a $GABA_AR$ non-competitive blocker, but produces less toxic and powerful effects (Höld et al., 2001). Furthermore, Deiml et al. (2004) reported that α-thujone reduces 5-HT_3 receptor activity, indicating that its behavioral effects might not only be due to GABA-mediated current inhibition, but also to an enhancing effect on agonist-induced receptor desensitization. More recently, it has been reported that different $GABA_AR$ subtypes show distinct sensitivities to α-thujone, suggesting that this compound may differentially affect the tonic and phasic components of GABAergic inhibition (Czyzewska and Mozrzymas, 2013).

Taken into account the above findings, it is possible that α-thujone and β-thujone, when intracerebroventricularly administered, modulate the anxiety profile of neonatal chicks submitted to acute stress, perhaps through the activation of $GABA_AR$ by modulating the inhibitory synaptic transmission.

2. Results

2.1. *Effects of different i.c.v. doses of α-thujone or β-thujone on Open Field behavior*

First, we performed a dose–response curve for α-thujone to determine the optimum dose to use. Because α-thujone was being i.c.v. injected for the first time, we started by using doses of 624, 312 and 156 nmol obtained from calculations of the concentration in the brain according to biological activities described in the literature (Höld et al., 2000). However, these tested doses of α-thujone induced tonic–clonic convulsions in 100% of cases. Then, as a lower injection of 78 nmol of α-thujone induced no seizures, this dose was chosen for testing behavioral and the radioligand assay, with this same dose also being assayed for β-thujone in order to simplify the comparison between the effects of both terpenes. A β-thujone dose of 156 nmol was used but it also induced a convulsant action.

A one-way ANOVA revealed a significant effect of α-thujone (78 nmol) and of β-thujone (78 nmol) ($F(2,42)=56.25$, $p<0.001$) on latency to ambulate in 3-day-old chicks. The LSD Fisher test showed a significant latency increase for chicks injected with α-thujone ($p<0.05$) compared with those injected with vehicle. Similar results were also observed for the administration of β-thujone ($p<0.05$) compared to saline. In addition, the LSD test also revealed significant differences between the α-thujone group and of the β-thujone one ($p<0.05$) (Fig. 1).

A one-way ANOVA of the central administration effects of α-thujone and β- thujone showed a significant decrease in locomotor activity ($F(2,42)=5.26$, $p<0.010$), with the LSD Fisher test also revealing significant differences between α-thujone ($p<0.050$) and β-thujone ($p<0.050$) treatments compared to saline controls (Table 2). However, no significant effects were observed for the other behavior parameters studied: latency to defecate $F(2,42)=2.14$, $p=0.162$, number of defecations $F(2,42)=0.73$, $p=0.401$ and number of escapes $F(2,42)=1.36$, $p=0.263$.

2.2. *Effect of central 78 nmol of α-thujone or 78 nmol of β-thujone on the flunitrazepam sensitive-GABAAR density in synaptosomes from 3 day-old chick forebrain*

Fig. 2 shows that a two-way ANOVA on Bmax revealed a significant interaction between the stress accompanying the OF test and the i.c.v. terpenes administration $F(2,39)=8.17$,

$p<0.001$. The LSD Fisher test showed that Bmax was significantly greater in stressed chicks than in non-stressed ones ($p<0.020$) after being injected with vehicle. Subsequent to β-thujone administration, a rise in Bmax was also observed in stressed compared to non-stressed chicks ($p<0.010$). In contrast, i.c.v. α-thujone induced a significant decrease in the maximum density in stressed chicks compared to non-stressed ones ($p<0.001$). In the stressed group, there were significant differences between the α-thujone-injected chicks compared with β-thujone-injected ones ($p<0.001$) or with saline-injected chicks ($p<0.001$).

No significant differences in Kd values were observed, as a two-way ANOVA of Kd data did not reveal any significant effects of acute stress $F(2,42)=0.07$ ($p=0.795$), monoterpenes administration $F(2,42)=0.97$ ($p=0.388$), or for the interaction between two $F(2,42)=0.05$ ($p=0.950$), suggesting that these terpenes did not induce any modification of the ligand affinity with its receptor (Table 3).

3. Discussion

The results show for the first time that the central administration of α-thujone and β-thujone, two isomeric forms of a bicyclic monoterpene from *A. absinthium* L. essential oil, is able to produce an anxiogenic-like behavior in neonatal chicks in an OF test. However, only i.c.v. administrated α-thujone eliminated the flunitrazepam sensitive-$GABA_AR$ recruitment induced by acute stress.

In chicks, an OF response is primarily a fear of novelty or isolation, in addition to a tendency to reinstate contact with conspecifics (Faure et al., 1983) that represent a compromise between opposing tendencies to reinstate contact and to avoid detection by potential predators (Gallup and Suarez, 1980). Thus, changes in the latency to begin ambulation, the number of squares crossed and escape attempts can be interpreted as a socially motivated behavior pattern in order

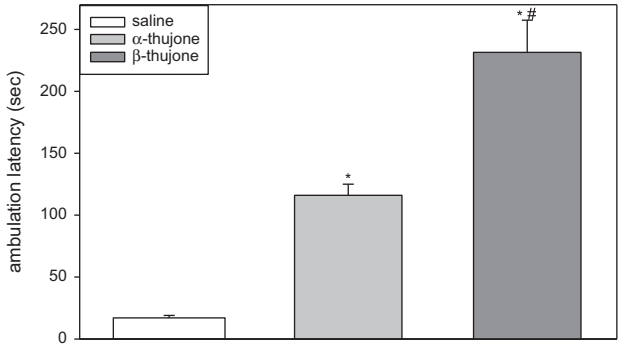

Fig. 1 – Effect of i.c.v. administration of 78 nmol α-thujone and of 78 nmol de β-thujone on the latency to ambulate in an Open Field in 3-day-old chicks. Bars represent mean ± S.E.M ($n=10$–19). *$p<0.05$, Significantly different from saline and \#$p<0.05$, significantly different from α-thujone (LSD Fisher).

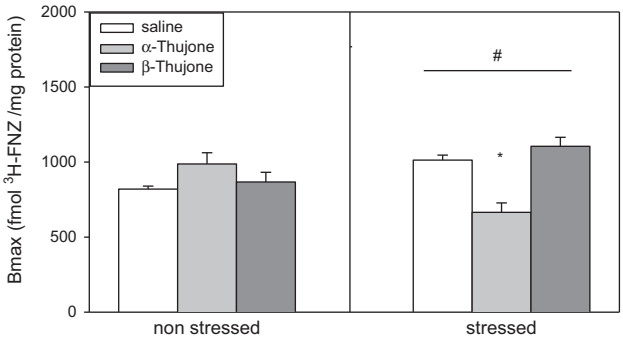

Fig. 2 – Binding maximum of [^{3}H]-FNZ in forebrain synaptosomes from non-stressed and stressed chicks following insulin i.c.v. administration of α-thujone and β-thujone. Bars represent the means ± SEM ($n=14$–19 chicks per group). \#$p<0.01$ Compared to respective groups of non-stressed chicks. *$p<0.001$ Compared to other groups of stressed chicks (LSD Fisher).

Table 1 – Concentration of terpenoid constituents of *Artemisia absinthium* L. essential oil, according to their elution order in the GC analysis.

Retention index[a]	Compounds	Essential oil (μl/ml)	Methods of identification
937	α-Pinene	1,6	GCMS-Co
978	Sabinene	24	GCMS-Co
984	β-Pinene	0.7	GCMS-Co
992	β-Myrcene	0.7	GCMS
1031	p-Cymene	4	GCMS-Co
1035	Limonene	5	GCMS-Co
1039	1,8-Cineole	6	GCMS-Co
1065	γ-Terpinene	3	GCMS-Co
1077	Terpinolene	4	GCMS
1115	α-Thujone	22	GCMS-Co
1131	β-Thujone	924	GCMS-Co
1149	Trans-sabinol	2	GCMS
1191	Terpinen-4-ol	1	GCMS-Co
1256	Carvone	2	GCMS-Co

GCMS: peak identifications are based on an MS comparison with file spectra.
Co: peak identification is based on a standard comparison with relative retention time.
[a] The retention indexes were determined on the basis of homologous *n*-alkane hydrocarbons under the same conditions.

to reinstall contact for an isolated chick, while the freezing and silence function are behaviours that preclude a potential predatory encounter as a consequence of contact and restraint by a human being during removal from the home cage and placement in the Open Field (Suarez and Gallup, 1983). In the present study, a 78 nmol dose of α-thujone (Fig. 1) induced a significant increase in latency to begin to ambulate compared to the vehicle-group, indicating an anxiogenic-like effect. Related to this, Gallup and Suarez (1980) reported that an increase in latency in an OF correlated with a rise in the level of fear/anxiety in chicks. In addition, Marin et al. (1997) found that the systemic administration of a beta-carboline (anxiogenic) or diazepam (anxiolytic) in chicks induced an increase or decrease, respectively, in the locomotor activity in and OF test. Similar results were previously described by Salvatierra and Arce (2001) and Marin et al. (1997) using diazepam anxiolytic doses, which induced a decrease in latency to ambulate in a T-maze and to peck in a new environment in subpopulations with a higher level of anxiety. Table 2 shows that α-thujone (78 nmol) reduced locomotor activity, indicating that the increase in the latency to ambulate corresponded with a decrease in the total ambulatory activity, suggesting an anxiogenic action.

In the present report, no significant differences in other behaviour parameters studied in the OF were found, perhaps because the interpretation of defecation is difficult in birds, whereas in rodents it is a primary pattern of fear (Gallup and Suarez, 1980). β-Thujone, at dose of 78 nmol, also induced a significantly greater increase than α-thujone in the latency to start ambulating in this test. In this way, both terpenes induced an anxiogenic-like effect, showing that the latency to ambulate increased as locomotor activity decreased. The doses used in this study were selected from previous results of α-thujone systemic injections in mice (Höld et al., 2000), whose demonstrated that the i.p. LD_{50} of α-thujone in mice was about 45 mg/kg, with generally 100% mortality at 60 mg/kg. Therefore, the final adjustment of the dose was performed by taking into account the values reported by these groups. However, when we tested α-thujone doses of 156, 312 and 624 nmol/per chick and β-thujone dose of 156 nmol/per one, they all caused tonic–clonic seizures in 100% of individuals, but which did not lead to the death of the animals. Due to this toxic effect and the strong excitatory action on CNS, a final dose of 78 nmol was chosen in both terpenes.

It has been previously reported that exposure to various types of acute stressors induces modifications in the maximum density of $GABA_AR$ expressed on synaptosomes (Medina et al., 1983; Trullas et al., 1987; Salvatierra et al., 1997, 2009; Martijena et al., 1992; Marin et al., 1997; Salvatierra and Arce, 2001; Cid et al., 2008, 2013). Moreover, Martijena et al. (1992) reported that, in synaptosomes from chick forebrain, the Bmax increase induced by acute stress was due to a higher recruitment of flunitrazepam-sensitive-$GABA_AR$. In fact, the traffic velocity and the anchorage of $GABA_AR$ are related to several proteins associated with the receptor, which may be phosphorylated. It is then possible that acute stress stimulates the phosphorylation of these proteins, and consequently increases the velocity of the vesicular transport, as well as their fusion with membranes and final receptor insertion at the membrane surface (Cid et al., 2007). Thus, a density increase of receptors *in vivo* was quantified *ex vivo* at 4 °C, because changes in receptor density by acute stress under these cold conditions are maintained after decapitation when the synaptosomes are then obtained. Our results (Fig. 2) showed that Bmax increased after acute stress, independent of the doses used of α-thujone and β-thujone, which suggests that OF test may have acted as a stressor by inducing an increase in the recruitment of receptors after the acute exposure. Similarly, Salvatierra and Arce (2001) observed significant differences in the maximum number of $GABA_AR$ in the subpopulations categorized by an anxiety profile in an OF test. Therefore, the chicks suffered a stress due to isolation and novelty that was explained by a rapid recruitment of already synthesized receptors.

The results obtained from the binding assay of [^{3}H]-FNZ showed no significant differences in the maximum density or the affinity constant of $GABA_AR$ in the non-stressed groups, indicating that neither α-thujone nor β-thujone exerted a modulator effect on the recruitment of $GABA_AR$. However, under acute stress conditions, the Bmax revealed only a significant reduction in the group injected with α-thujone. Moreover, novelty and isolation induced an increase that was

Table 3 – Effects of injection of 78 nmol of α-thujone or 78 nmol of β-thujone on Kd values of GABAAR in forebrain synaptosomes from non-stressed and stressed chicks.

Treatments	Non-stressed	Stressed
Saline	2.63±0.11	2.75±0.30
α-Thujone	2.49±0.48	2.68±0.27
β-Thujone	3.14±0.47	3.09±0.22

Each Kd value represents the mean±SEM of values obtained by non-linear regression of experimental data from saturation curves (n=14–19 chicks/group).

Table 2 – Open Field test: behavioral changes induced by injection of 78 nmol of α-thujone or 78 nmol of β-thujone.

Treatment	Am	L_{def}	ND	NE
Saline	82.29±18.94	89.00±39.22	1.38±0.21	5.10±2.20
α-Thujone	21.50±6.18	207.00±78.70	1.20±0.29	2.80±2.03
β-Thujone	22.86±6.09	41.57±15.72	1.60±0.20	2.14±1.24

Behaviors were scored for 10 min after drug administration of α-thujone or β-thujone or of saline (control). Am: ambulation, as an index of exploratory activity. L_{def}: latency to defecate. ND: number of defecations. NE: number of escapes. Values are expressed as mean±SEM (n=10–11 chicks/group). $^*p<0.05$ Compared to saline (control) (LSD Fisher).

eliminated by the action of this monoterpene, suggesting that α-thujone may have modulated the receptor density in the final step of insertion into the postsynaptic membrane by decreasing the amount of $GABA_AR$ in the synaptosomal membrane. In another investigation, Szczot et al. (2012) obtained evidence that α-thujone affected the functionality of $GABA_AR$, by observing a decrease in the frequency of miniature inhibitory postsynaptic currents via an unknown mechanism that regulated the presynaptic release of GABA and thereby caused reduction in the phasic GABAergic transmission. Consequently, decreased receptor insertion in the postsynaptic membrane leads to a weak GABAergic inhibition, in contrast to that described for acute stress in chicks. In agreement, it has been shown that α-thujone induces a negative modulation of the inhibitory GABAergic current in recombinant receptors, thus indicating an inhibitory effect on the GABAergic function (Hall et al., 2004). Consequently, it is possible that isolation plus an associated novelty induced the recruitment of a subpopulation of $GABA_AR$ that was more sensitive to α-thujone than the receptors expressed constitutively in the synaptosomal membrane, or that this terpene inhibited the traffic of any protein or enzymes that modulated phosphorylation in the turnover of $GABA_AR$. Nevertheless, it cannot be excluded that α-thujone administered *in vivo* might have prevented the $GABA_AR$ recruitment induced by acute stress, perhaps due to α-thujone binding to the site of the benzodiazepines or to another site of the complex, thereby not permiting a proper interaction with FNZ (a ligand used for the radioligand assays *in vitro*) through a competitive inhibition mechanism (Benavidez and Arce, 2002; Lüscher and Keller, 2004).

β-Thujone did not induce a modulation on the recruitment of $GABA_AR$ for the unstressed condition, and there was also no additional effect on Bmax under conditions of acute stress, indicating that β-thujone did not directly modulate the inhibitory GABAergic transmission. This imply that the induced increase in latency to ambulate in the OF might have been mediated by another neurotransmitter system. Related to this, it is known that several subtypes of serotonin receptors including $5\text{-}HT_{1A}$, $5\text{-}HT_{2A–2C}$, $5\text{-}HT_3$ mediate behavioral consequences (Barnes and Sharp, 1999; Sonavane et al., 2002) such as anxiogenesis or antipsychotic effects (Jones and Piper, 1994; Costall and Naylor, 1994; Brunello et al., 1995). Therefore, it is possible that the anxiogenic-like effect of β-thujone was mediated by other systems rather than the GABAergic system. It has also been previously described that diastereomers can exert physiochemical differences in their biological functionality, as observed in behavioral tests, which depend on a structure-activity relationship with the receptor binding site (Carliss et al., 2009). Thus, β-thujone may also have a different biological effectiveness compared to that of α-thujone, which is associated with a complex stereochemistry.

4. Experimental procedure

4.1. Animals

Chicks (Cobb) of both sexes (*Gallus gallus domesticus*) were obtained immediately after hatching from the commercial hatchery INDACOR (Argentina). On arrival, these chicks were housed in a white wooden box that measured 90 × 40 × 60 cm (length × width × height) before performing the OF test. This box was illuminated with an incandescent lamp hanging just above it and was kept in a small room (3 × 3 m) at controlled temperature (30–32 °C) in a 12–12 h dark–light cycle (lights on at 7 a.m.). Tap water and food being freely available. The chicks were socially reared until they reached 3 days of life, with daily food replenishment (Cargill, broiler BB, and 20% minimum crude protein 12.34 MJ/kg) and maintenance chores being performed at 9 a.m.

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of the Universidad Nacional de Córdoba, and every effort was made to minimize animal suffering and to keep the number of animals used to a minimum.

4.2. Terpenes

α-Thujone ((1S,4R,5R)-4-methyl-1-(propan-2-yl) bicyclo[3.1.0] hexan-3-ona) was purchased from Merck and β-thujone ((1S,4S,5R)-4-methyl-1-propan-2-yl) bicyclo[3.1.0]hexan-3-ona) was obtained as described below. Dried leaves (100 g) of *A. absinthium* plant were hydrodistilled for 1 h using a Clevenger-like apparatus, with the oil obtained being dried over anhydrous sodium sulphate and stored at 5 °C until analysis. For the quantification of individual components, essential oil (EO) was analyzed using a Perkin-Elmer Clarus 500 gas chromatograph equipped with a flame ionization detector (GC-FID), and a capillary column DB-5 (30 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for the separation of individual components of the EO. Helium was employed as the carrier gas with a flow rate of 1 ml/min. The temperature program was 60 °C for 4 min, from 60 to 240 °C at 5 °C/min, and a final hold time of 10 min, with the injector and detector being maintained at 260 and 280 °C, respectively. The sample was diluted 1:100 in *n*-hexane and 0.2 μl was injected with a 1:100 split ratio (Zunino et al., 2000). For the determination of the composition, EO samples were diluted with hexane, using an injection volume of 1 μl. The identification of the components of the EO was realized by GC–MS, utilizing a Perkin-Elmer Q 700 GC–MS coupled with an ion trap mass detector. A capillary column DB-5 (60 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for the separation of the components, with Helium employed as the carrier gas at a flow rate of 0.9 ml/min. The temperature program for the oven and injector was the same as that for the GC-FID, and ionization was carried out by electron impact at 70 eV. The mass spectral data were acquired in the scan mode in the *m/z* range 35–450; with retention indices (RI) of the sample components being determined on the basis of homologous *n*-alkane hydrocarbons under the same conditions. The compounds were identified by comparing their retention indices and mass spectra with published data (Adams, 2007) and NIST and Adams libraries. The principal components were also identified by co-injection of authentic standards (SIGMA, USA), and the quantitative composition was obtained by peak area normalization, with the response factor for each component being considered to equal 1 (Table 1).

4.3. Intracerebroventricular injection

The terpenes were dissolved in 0.85% saline containing 0.1% Evans Blue solution, and then α-thujone was administered at doses of 78, 156, 312 and 624 nmol or β-thujone administered at doses of 78 and 156 nmol. Intracerebroventricular (i.c.v.) injections were given freehand at a volume of 10 μl using a Hamilton syringe (Carvajal et al., 2009), with the depth of the injection of 3 mm being controlled by using a plastic sleeve on a 27-gauge needle. As the chicks had soft unossified skulls, this procedure did not require an anesthetic and could be routinely performed without any administration of analgesics (Andrew, 1991). The control group was given saline containing 0.1% Evans Blue solution.

4.4. Open Field test

Chicks (3 days old) were individually captured, injected with the different doses of α-thujone or of β-thujone as indicated above, or just with saline, and immediately placed in a cardboard box before being carried to a separate room where the chick was then placed in the centre of a 60 × 60 cm OF apparatus with sides 30 cm high. This OF was made of white wood, and the floor was marked off to give 25 squares of 12 × 12 cm and illuminated by a 100 W overhead bulb (Gallup and Suarez, 1980). The following types of behaviors were analyzed for 10 min: latency to ambulate, locomotor activity (number of squares crossed), latency for defecation, number of defecations and number of escapes. After testing, the floor of the OF apparatus was cleaned with towels moistened with 70% ethanol. Spontaneous activity was recorded by a digital camera suspended 1.5 m above the center of the apparatus (Day 1 of the experiment), with the monitoring system being set up in a separate room to avoid disturbing the birds. After the experiment, the birds were immediately decapitated and their brains were removed and inspected in order to control the accuracy of the placement of the injection.

4.5. Preparation of crude synaptosomal fraction

The crude synaptosomal fraction was obtained essentially as described previously (De Robertis et al., 1961), with all procedures being carried out at 4 °C. Briefly, the forebrain was first homogenized in 20 volumes of ice-cold 0.32 M sucrose/g original forebrain tissue, using a Potter glass–Teflon homogenizer, before being centrifuged at 1000*g* for 10 min. The supernatant was then centrifuged at 10,000 × *g* for 20 min, and the pellets were resuspended in a solution containing 50 mM Tris–HCl buffer, pH 7.4, thereby obtaining a final concentration of 0.3 mg protein/ml (Lowry et al., 1951). Finally, these pellets were used immediately for the binding assay.

4.6. [^{3}H]-flunitrazepam binding assay

The specific binding of [^{3}H]-FNZ (85 Ci/mmol) was measured by a filtration technique, with binding being carried out in the presence of a radioligand at final concentrations of 0.5, 1, 2, 4, 6, 8, 10 and 12 nM at 4 °C (Cid et al., 2008). Each assay was performed in triplicate using 1 ml aliquots containing 0.3 mg of protein from the synaptosomal fraction, and non-specific binding was measured in the presence of 10 mM diazepam. After a 60 min incubation, samples were filtered under vacuum through Whatman GF/B filters using a Brandel M-24 filtering manifold, before being washed three times with 4 ml of ice-cold Tris–HCl buffer (50 mM, pH 7.4) and the radioactivity counted in an LKB-1214-RackBeta counter at 60% efficiency. The Bmax and Kd values were obtained by nonlinear regression using the equation for a hyperbola (one binding site): $Y = Bmax/(Kd+X)$, where Bmax is the maximal binding, and Kd is the concentration of ligand required to reach half maximal binding. The Bmax of the [^{3}H]-FNZ binding is representative of the $GABA_AR$ density.

4.7. Statistical analysis

The experimental data were expressed as the mean ± S.E.M. Behavioral data from the OF were subjected to a one-way analysis of variance (ANOVA). The $GABA_AR$ density data for groups injected with terpenes were compared with those of the stress accompanying the OF test using a two-way ANOVA. Whenever, the one-way and two-way ANOVA indicated significant effects ($p<0.05$), a pairwise comparison of means was carried out by the LSD Fisher test. A p value <0.05 was considered to represent a significant difference.

5. Conclusion

α-Thujone administered centrally induced anxiogenic-like behavior and suppressed the increase in recruitment of $GABA_AR$ induced by acute stress by decreasing the postsynaptic membrane receptors, in neonatal chicks. In addition, β-thujone showed similar behavioral effects to that of its diastereoisomer α-thujone. However, the mechanism of action of this terpene may have been mediated by other neurotransmitter receptors, including the serotonergic system or have resulted from a different biological effectiveness due to a distinct stereochemistry.

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