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# **Thymoquinone inhibition of acquisition and expression of alcohol-induced behavioral sensitization.**

Muhammad Sona Khan<sup>1</sup>, Aneela Gohar<sup>1</sup>, Ghulam Abbas<sup>1</sup>, Wajahat Mahmood<sup>1</sup>,  
Khalid Rauf<sup>1</sup>, Robert D. E. Sewell<sup>2\*</sup>

1. Basic and Applied Pharmacology Research Group, Department of Pharmacy, COMSATS Institute of Information Technology Abbottabad 22060, Pakistan.
2. Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, CF10 3NB, UK.

\*Correspondence to Professor Robert Sewell. Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff CF10 3NB., UK.  
[sewell@cardiff.ac.uk](mailto:sewell@cardiff.ac.uk)

**Short title:** Thymoquinone inhibition of alcohol behavioral sensitization

**Key words:** Alcohol, behavioral sensitization, thymoquinone, GABA, motive circuit

## **Abstract**

Repeated low doses of alcohol have been shown to progressively enhance locomotor activity in mice and this phenomenon is designated as behavioral sensitization. Thymoquinone, a major active component of *Nigella sativa* oil has been investigated in a number of studies for its neuroprotective effects against a variety of ailments. This study was conducted to explore the therapeutic potential of thymoquinone on the acquisition and expression of alcohol-induced behavioral sensitization. Mice treated with alcohol (2.2g/kg/day) or saline for 13 days and subsequently challenged with an acute alcohol dose (2.2g/kg) 5 days later were orally administered acute doses of thymoquinone (10, 20 and 30 mg/kg). Thymoquinone subacute treatment with all doses throughout alcohol exposure significantly inhibited both the development and expression phases of alcohol behavioral sensitization in a dose dependent manner. However, acute treatment with thymoquinone (30mg/kg) only reversed the expression phase of sensitization. These findings are explained in terms of the known GABA promoting action of thymoquinone in relation to the motive circuit within the limbic component of the basal ganglia. It is concluded that thymoquinone may be a potential therapeutic option for the treatment and prevention of alcohol induced behavioral sensitization.

## **INTRODUCTION**

Behavioral sensitization is a process whereby repeated administration of a stimulus or drug results in the progressive amplification/augmentation of a response (Shettleworth, 2010). The neural mediators which are thought to actuate long-term expression of behavioral sensitization are centered in a group of interconnected limbic nuclei, termed the 'motive' circuit (Pierce and Kalivas, 1997).

Ethanol (alcohol) induced behavioral sensitization has been postulated to play a key role in alcohol addiction (Robinson and Becker, 1986). This hypothesis has been supported by a considerable body of evidence. In this context, sensitization occurs to the enhanced locomotor activity induced by drugs of abuse such as alcohol, nicotine, amphetamine, and opioids (Vanderschuren and Kalivas, 2000). Moreover, this enhanced response is considered to underlie the motivational effects of drugs and the cues associated with them (Lett, 1989; Shippenberg and Heidbreder, 1995; Harmer and Phillips, 1999; Taylor and Horger, 1999). Consequently, those drugs which produce incentive sensitization are capable of restoring drug seeking behavior (De Vries et al., 1998, Steketee and Kalivas, 2011).

The phenomenon of alcohol-induced behavioral sensitization occurs because of neuronal adaptations in dopaminergic, glutamatergic and GABAergic circuitry linked through the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex, and amygdala (Stephans and Yamamoto, 1995; Pierce and Kalivas 1997; Cador et al., 1999, Vanderschuren and Kalivas, 2000; Nestler, 2001; Zhang et al., 2001; Carlezon and Nestler, 2002; Nordahl et al., 2003; Steketee and Kalivas, 2011; Miyazaki et al., 2013).

Behavioral sensitization has been characterized by two temporally distinguishable components, namely the initiation and expression phases. In this respect, the initiation domain is believed to be mediated anatomically through the ventral tegmental area (VTA), and the expression phase is considered to be associated with the NAc (Kalivas and Duffy, 1993, Vanderschuren and Kalivas, 2000).

Alcohol-induced behavioral sensitization is associated with repeated alcohol administration (Bahi and Dreyer, 2012) and the progressive persistent nature of addiction suggests that the

neuroadaptive responses that develop as a result of repeated exposure play an important role in this condition. Moreover, like addiction, sensitization persists for prolonged periods after cessation of drug use (Fish et al., 2002) and these characteristics support the idea that sensitization may contribute significantly to the addiction process (Robinson and Berridge, 1993).

It has been reported that the GABA<sub>B</sub> agonist baclofen but not THIP (GABA<sub>A</sub> agonist) obviates ethanol-induced locomotor behavioural sensitization without affecting its pharmacokinetics. Hence, GABA<sub>B</sub> receptors in particular play an important role in the development of behavioral sensitization to ethanol (Broadbent and Harless, 1999). Likewise, the benzodiazepine antagonist/inverse agonist flumazenil inhibits stress sensitization of the ethanol withdrawal-induced reduction in social interaction. Hence, adaptive changes in either neurotransmitter release or receptor function associated with GABA-containing pathways are likely to contribute to stress-induced sensitization of alcohol withdrawal reduction in social interaction (Breese et al., 2004)

Thymoquinone is an active principle of *Nigella sativa* that was first extracted by El-Dakhakhany (1963) and later isolated from the essential oils of this plant (Gali-Muhtasib et al., 2006). This compound has also been isolated from various other plant species including *Eupupatorium ayapana*, the leaves of *Origanum* species, the heartwood essential oils of *Calocedrus decurrens*, the oil of different *Saturaga* species, *Thymus vulgaris* and also from *Nepeta distans* (Trang et al., 1993; Lukas et al., 2009; Manter et al., 2007; Grosso et al., 2010; Hussain et al., 2010; Gohari et al., 2012).

Thymoquinone is not only an antioxidant, but also a potent neuroprotective agent against alcohol-induced apoptotic neurodegeneration (Ullah et al., 2012). Intracerebroventricular injection of the compound suppresses epileptic seizures in rats which are reversed by both flumazenil and naloxone and it has been proposed that these effects are elicited through an opioid receptor-mediated increase in GABAergic tone (Hosseinzadeh et al., 2005). More recently, in stressed mice, thymoquinone exhibited anxiolytic activity, accompanied by a decrease in plasma nitrite and a reversal of a decrement in brain GABA levels suggesting an involvement of nitric oxide-cGMP and GABAergic pathways in the anxiolytic-like activity (Gilhotra and Dhingra, 2011).

To date, there are no reports in the literature on thymoquinone regarding any activity on alcohol-induced behavioral sensitization. In view of its GABAergic-benzodiazepine activity, this study was designed to establish whether thymoquinone modifies the acquisition and expression of ethanol-induced behavioral sensitization in mice.

## **MATERIALS AND METHODS**

### **Drugs**

Alcohol (ethanol 99.9% v/v; Merck, Brazil) was diluted with 0.9% (w/v) NaCl solution to produce 15% (v/v) ethanol solution and it was administered via the intraperitoneal (i.p) route at a dose of 2.2g/kg/day. Control (Saline treated) animals were given an equivalent volume of saline via the i.p route. Thymoquinone (Santa Cruz USA), was also dissolved in 0.9% saline.

### **Subjects**

Five groups of adult female mice (24-34g body weight) were supplied by the animal house facility at COMSATS Institute of Information Technology Abbottabad. They were divided into the following groups and administered:

Group 1: Normal saline (control group, n=8).

Group 2: ethanol alone (n=8).

Group 3: thymoquinone (10mg/kg), as a single dose during the expression phase (n=8) or daily from day 1-13 (n=8).

Group 4: thymoquinone (20mg/kg), as a single dose during the expression phase (n=6) or daily from day 1-13 (n=8).

Group 5: thymoquinone (10, 20 and 30 mg/kg), as a single doses during the expression phase (n=8) or daily from day 1-13 (n=8).

Animals were housed in standard Plexiglas cages with access to food and water ad libitum. They were allowed to habituate to the colony room for a minimum of 12 days before the

start of experiments and maintained on a 12h/12h light/dark cycle with lights on at 0800 hours. All the experiments were conducted between 10.00 and 13.00 hrs and all procedures were carried out following approval by the “Ethical Care and Research committee” of CIIT Abbottabad.

### **Apparatus**

Animal locomotor activity was measured in one of a bank of six activity boxes measuring 45.6 x 45.6 cm divided internally into quadrants (22.8 x 22.8cm) by lines on the floor. Activity boxes were cleaned and swabbed between trials and activity was monitored using a VideoLAN client (VLC) media player on a PC coupled to a digital camera installed 300 cm above the boxes.

### **Procedures**

Over a total of 5 days, all the animals were habituated to the experimental room for one hour. On habituation day-1, all animals were administered an i.p injection of normal saline and five minutes after injection, they were placed in the activity boxes. The incidence of line crossings by the animals signified their locomotor activity. The same procedure was repeated on habituation day-2. On day-3 (Experimental Day-1), all the alcohol treated groups were given i.p. injections of 15% alcohol (v/v) at a dose of 2.2g/kg/day. Five minutes after injection, they were placed in the activity boxes and locomotor activity was recorded for a period of 30 minutes. The control group received an equivalent volume of normal saline via i.p. injection. On Day-2, alcohol treated groups were given i.p. alcohol injections (2.2g/kg/day), and an equivalent volume of saline was administered intraperitoneally to control groups, but the locomotor activity test was not performed i.e. animals after injections were placed into their home cages. Locomotor activity tests were performed on days 1, 5, 9, and 13. Following 13 days of treatment, all the animals were abstained from their respective treatments for a period of 5 days (i.e. day14 - 18). On the treatment day (i.e. day19), alcohol treated animals were administered thymoquinone doses (10, 20 and 30mg/kg) orally (p.o.) one hour before alcohol according to their respective groups and the saline and alcohol only groups received corresponding equivalent volumes followed by locomotor activity recording.

The experimental paradigm for the subacute experiment was identical with the exception of subacute administration of thymoquinone doses orally one hour before i.p alcohol administration to the respective treatment groups.

## **Statistical analysis**

Graphpad prism-5 was used for statistical analysis. Data was expressed as mean  $\pm$  sem for different groups and Student t-tests, one way ANOVA followed by Bonferroni's test were applied. Data were considered significant at  $*(P<0.05)$ ,  $** (P<0.01)$ , and  $*** (P<0.0001)$ .

## **RESULTS**

### **Ethanol challenge during the expression of ethanol-induced behavioral sensitization.**

As shown in the Fig. 1, there was no variation in the expression of locomotor activity in the alcohol-treated group from day 1 – 9 ( $P=0.0818$ ). Locomotor activity testing was performed on day 9 and day13. On day 13 there was significant hyperlocomotor activity ( $P <0.01$ ).

After five days of abstinence, a subsequent challenge injection of alcohol (2.2g/kg) on the treatment day significantly increased ( $P=0.0091$ ) the locomotor activity of the alcohol treated group as compared to saline controls indicating the presence of behavioral sensitization with respect to locomotor activity.

### **Acute action of thymoquinone on the expression of alcohol-induced behavioral sensitization.**

Acute administration of thymoquinone at the two lower doses (10 and 20 mg/kg) on day 19 of the protocol did not significantly modify the expression of behavioral sensitization and there was no significant difference in the locomotor activity in either case from the synchronized controls on day 19 ( $P>0.05$ ) (data not shown). However, thymoquinone (30 mg/kg) on its own as a control induced less locomotor activity than the group treated with saline plus alcohol on



treatment day 19. Additionally, it was clearly evident that this acute dose of thymoquinone reversed the expression of alcohol-induced behavioral sensitization, there being a highly significant difference in locomotor activity expression ( $P<0.01$ ) between the alcohol control saline group (alcohol sensitization) and the alcohol plus thymoquinone acutely treated group (Fig 2).

### **Activity of subacute thymoquinone treatment on the acquisition of ethanol induced behavioral sensitization.**

As shown in Fig 3, thymoquinone administered daily on a subacute basis orally by itself, induced some reduction in locomotor activity by protocol day 19 at the highest dose (30 mg/kg). However, when given subacutely one hour before alcohol, it also produced a dose graded inhibition of ethanol induced sensitization as signified by a progressive decrement in hyperlocomotion. In the case of the 10 mg/kg dose, the onset of activity occurred by protocol day 13 ( $P<0.05$ ) (Fig 3A) whilst at the two higher doses (20 and 30 mg/kg) expression of this declination occurred earlier on day 9 (20 mg/kg,  $P<0.05$ ; 30 mg/kg,  $P<0.01$ ) and by day 19 the following decreases were observed: 10mg/kg = - 50.8%,  $P<0.01$ ; 20 mg/kg = - 56.3%,  $P<0.001$  ; 30 mg/kg = - 65.6%,  $P<0.001$  (Fig 3B and C).

## DISCUSSION

There are two distinguishable components to alcohol-induced sensitization which comprise induction and expression phases. The former phase is indicative of transient neuronal maladaptive alterations occurring during the acquisition of sensitization and the latter phase reflects neuronal adaptive alterations evoked by persistent alcohol exposure (Camarini et al., 2000; Robinson and Berridge, 1993). In this study, alcohol treatment over 13 days followed by abstinence from day 13-19 instigated locomotor sensitization. Acute administration of thymoquinone at the highest dose examined (30 mg/kg) in the absence of alcohol, did yield some reduction in locomotor activity (Fig. 2). This accords with the findings of Hosseinzadeh and Parvardeh (2004) who reported that thymoquinone doses in excess of 20 mg/kg reduced locomotion in the open-field. Moreover, when given acutely one hour before alcohol challenge during the alcohol sensitization expression phase on the protocol treatment day (i.e day 19) (Fig. 2), thymoquinone was found to suppress the manifestation of alcohol-induced sensitization only at the highest dose (30 mg/kg) (Fig 2) but not at 10 or 20 mg/kg. This response may indicate a potential of thymoquinone to diminish the behavioral sensitization response which ultimately stems from synaptic plasticity at the level of the nucleus accumbens (Steketee and Kalivas, 2011). Interestingly, It has been reported in rodents that at higher doses, thymoquinone induces abnormal liver and kidney parameters and accordingly, dose levels of 30mg/kg or lower have been recommended to avoid this prospect in experimental studies (Kurt et al., 2015). In light of this, acutely administered thymoquinone (20 mg/kg) reverses a decline in brain GABA content, there being an added involvement of nitric oxide-cGMP associated with consequential anxiolytic-like activity (Gilhotra and Dhingra, 2011). Similarly, elevated GABAergic tone, probably arising from an opioid receptor mechanism in response to peripherally or centrally administered thymoquinone attenuates pentylenetetrazole-induced epileptic seizures (Hosseinzadeh and Parvardeh, 2004; Hosseinzadeh et al., 2005). In addition to the action on GABA, thymoquinone has been reported to raise both noradrenaline and dopamine brain concentrations whilst decreasing the content of serotonin (5-HT) (Hamdy and Taha, 2009). However, repeated dosing with *Nigella sativa* oil (containing thymoquinone) during a four week period brought about an increase in 5-HT but a decrease in its turnover (Perveen et al., 2009; 2014).

Subacutely administered thymoquinone treatment, in combination with daily alcohol, significantly inhibited the developmental stages of alcohol sensitization at all three doses tested (10, 20 and 30 mg/kg). There was a differential between the lowest dose and the two higher doses with respect to the chronology of onset since the appearance of suppressed sensitization occurred earlier within the protocol in the case of the two higher doses than at 10 mg/kg (Fig. 3).

This may reflect a therapeutic potential for thymoquinone to inhibit alcohol-enhanced behavioral sensitization. A possible mechanism underlying the sensitization phenomenon derives from alcohol induced adaptations that are believed to motivate misuse, develop addiction and consequently relapse because of alcohol induced positive reinforcement (Grahame et al., 2000, Hunt and Lands, 1992).

Among the neurotransmitters initiating neuronal changes and expressing alcohol-induced sensitization, GABA is notable in mediating alcohol reinforcement and reward (Koechling et al., 1991, Vlachou and Markou, 2010). Likewise, a GABAergic-benzodiazepine activity is thought to be implicated in sensitization of alcohol withdrawal-induced reduction in anxiety-like behavior. In this context, it has been concluded that agents which increase brain levels of GABA and GABA<sub>A</sub> receptor activity may diminish the stimulant effects of alcohol by accentuating its intoxicating and sedative properties (Holstein et al., 2009). On the other hand, selective activation of GABA<sub>B</sub> receptors specifically counteracts alcohol generated stimulation, implying that GABA<sub>B</sub> agonists may exhibit proclivity as pharmacotherapies for alcohol use disorders (Holstein et al., 2009).

Corresponding to this tenet, GABA<sub>B</sub> receptors localized in the reward pathway have been associated with acute and chronic alcohol induced psychomotor effects (Boehm et al., 2002; Vlachou and Markou, 2010). Hence, the GABA<sub>B</sub> receptor agonist baclofen, when coadministered with alcohol, has been reported to attenuate alcohol-induced hyperlocomotion (Holstein et al., 2009). Additionally, baclofen blocks both the induction and expression phases of alcohol psychomotor sensitization (Broadbent and Harless, 1999) and this concurs with our findings on thymoquinone. Moreover, a study conducted recently by Gilhotra and Dhingra (2011), evaluated anxiolytic-like effects of thymoquinone in mice which coincided with an enhancement of brain GABA levels. In a similar vein, an anticonvulsant activity of thymoquinone has been proposed via an opioid receptor-mediated increase in GABAergic tone (Hosseinzadeh et al., 2005).

A brain circuitry model within the limbic component of the basal ganglia has been proposed for agents causing behavioural sensitization (Pierce and Kalivas, 1997). In the model, there are reciprocal GABAergic projections between the core of the nucleus accumbens (NAc) and the ventral pallidum (VP). The NAc core and VP are further sources of GABAergic connections to the ventral tegmental area (VTA) and there is also a GABAergic projection from the VP to the mediodorsal thalamus (M-D Thal). In addition, a dynorphin (Dyn) input to the VTA from the NAc core has been described in this motive system (Fig. 4) (Pierce and Kalivas, 1997) and studies have shown that activation of this opioid system through its  $\kappa$ -receptors decreases DA transmission (Shippenberg, 2009). The circuitry is completed by glutamatergic pathways from the M-D Thal to the prefrontal cortex and then back to the VTA (Pierce and Kalivas, 1997) and NAc core (Pierce and Kumaresan, 2006).

In this circuitry, alcohol may facilitate DA release in the NAc by increasing the firing rate of DA neurons in the VTA (Bunney et al., 2001) and increased DA transmission is consistently associated with sensitization (Pierce and Kalivas, 1997). The expression of sensitization also involves a reduction in GABA transmission in the VTA which can stimulate the frequency of firing or even the bursting activity of dopamine neurons (Lacey et al., 1988; Grace et al., 1984; Johnson et al., 1992) promoting DA release in the NAc (Garris et al., 1994).

Contrary to the sensitizing mechanisms outlined above, stimulation of GABA<sub>B</sub> receptors in the VTA inhibits spontaneous motor activity (Kalivas et al., 1990) and increased GABA transmission in the VP may also act to limit sensitized motor responses (Pierce and Kalivas, 1997). Thus, thymoquinone is likely to inhibit sensitization through an opioid receptor-mediated increase in GABAergic tone (Hosseinzadeh et al., 2005) in the NAc core pathway to the VTA and also by promoting GABA transmission from the VP to the VTA both of which are conducive to reduced DA activity and inhibition of alcohol sensitization (Fig. 4). It is noteworthy, in relation to this concept, that an earlier study indicated that supraspinal  $\mu$ - and  $\kappa$ - but not  $\delta$ -opioid receptor subtypes were implicated in thymoquinone mediated antinociception (Abdel-Fattah et al., 2000). Indeed more recently, concurrent administration of thymoquinone (10mg/kg) with morphine in a 7-day paradigm offset the development not only of tolerance to morphine antinociception but also dependence. This was thought to be the outcome from inhibition of morphine-augmented brain glutamate, oxidative stress and nitric oxide overproduction (Abdel-Zaher et al., 2013).

There is a hypothesis that positive GABA modulators such as thymoquinone (Gilhotra and Dhingra, 2011) possess advantages in the management of alcohol/drug dependence due to a better side-effect propensity attributable to a lack of intrinsic agonist activity in the absence of GABA itself. Such agents only exert their modulatory actions in tandem with endogenous GABAergic function. Therefore, GABA positive modulators, embracing elevated GABA<sub>B</sub> receptor function, represent promising therapeutic prospects for aspects of dependence including initiation, maintenance and relapse to substances of abuse such as alcohol (Vlachou and Markou, 2010). Thymoquinone appears to show promise since it resides in this category.

### **Conflict of interest**

The authors declare no competing interests

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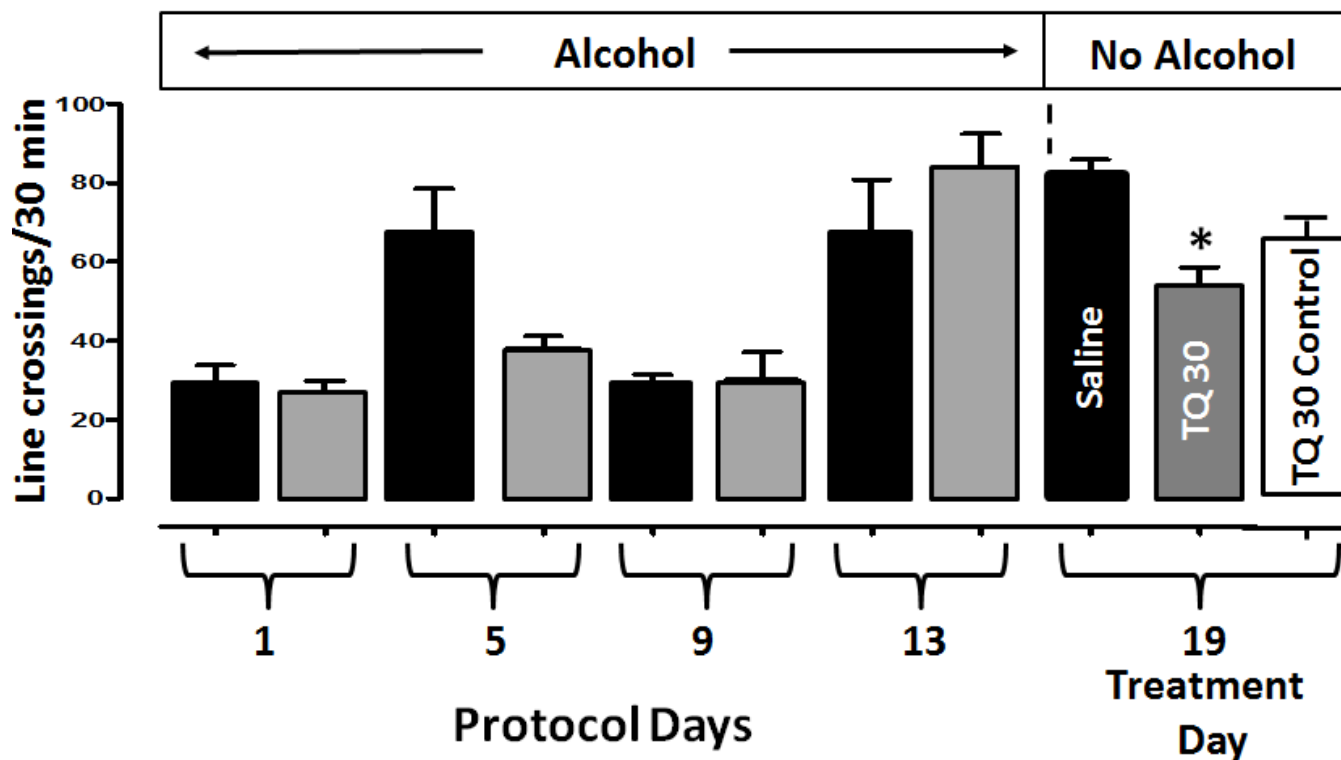
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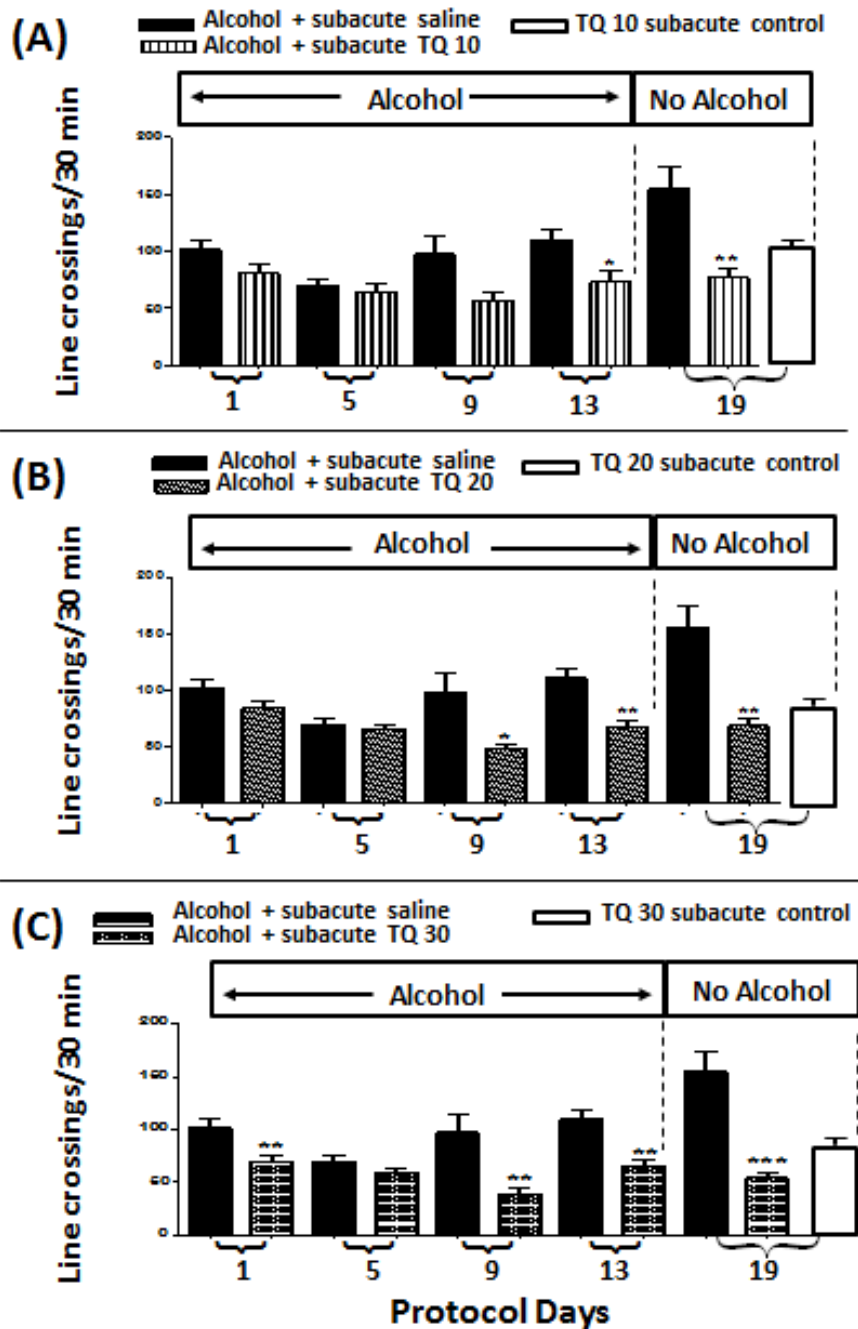
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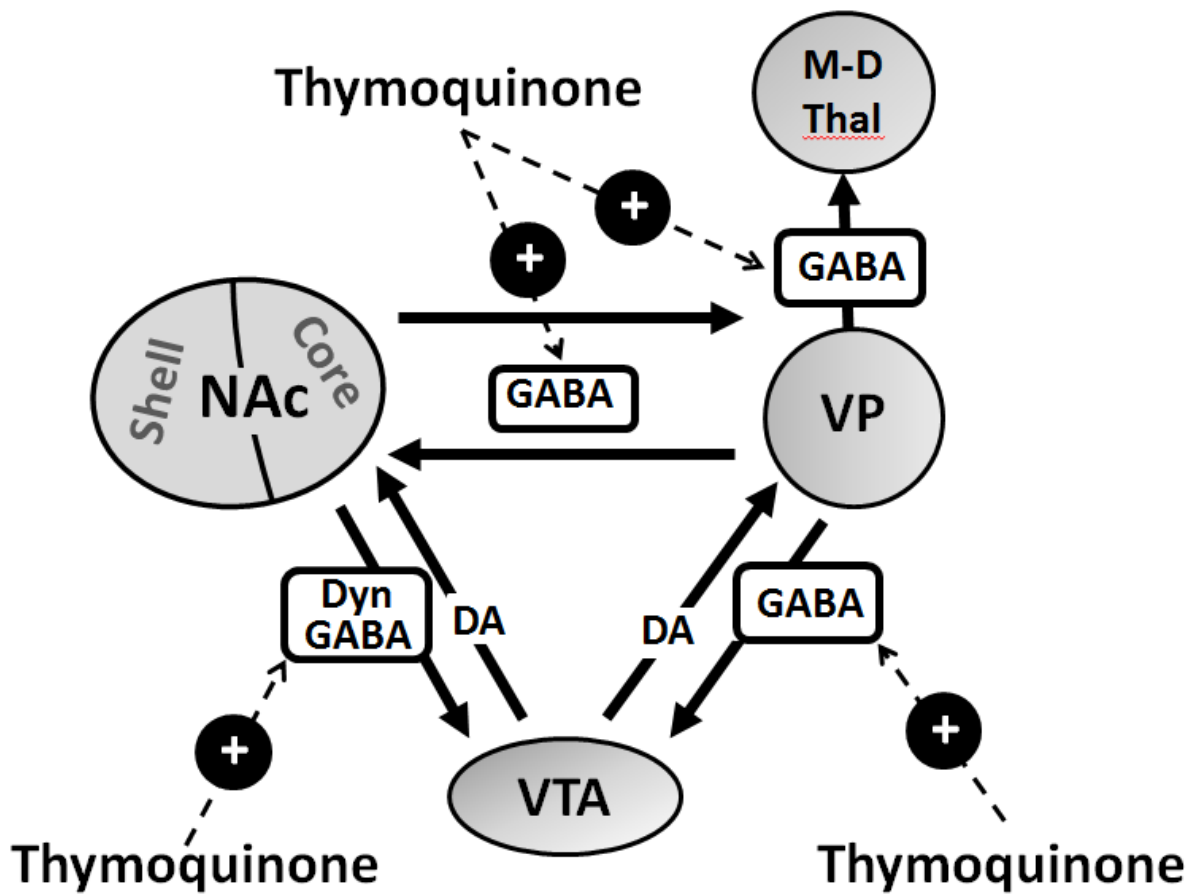
**Figure 1.** Behavioural locomotor sensitization response to alcohol treatment. Groups of mice (n=8) were treated with alcohol or saline vehicle i.p. daily for 13 days and locomotor activity was tested on days 1, 5, 9 and 13. This was followed by 5 days of abstinence then locomotor activity was retested on day 19 (treatment day). Each column represents the mean  $\pm$  sem. \*\* $P < 0.01$  compared with the saline vehicle control.



**Figure 2.** Activity of acute thymoquinone administration on the expression of alcohol-induced behavioural locomotor sensitization. Groups of mice (n=8) were treated i.p with alcohol (grey bars) or saline vehicle and no alcohol (black bars) daily for 13 days and locomotor activity was tested on days 1, 5, 9 and 13. This was followed by 5 days of abstinence then thymoquinone 30 mg/kg was administered p.o (TQ 30) and one hour later, locomotor activity was retested on day 19 (treatment day). A further group of animals was treated with saline vehicle for 13 days and locomotor activity was tested on days 1, 5, 9 and 13. This was followed by 5 days of no treatment and then thymoquinone 30 mg/kg was acutely administered p.o (TQ 30 Control, open bar) and one hour later, locomotor activity was retested on day 19 (treatment day). Each column represents the mean  $\pm$  sem. \* $P < 0.05$  compared with the saline vehicle control.



**Figure 3.** Activity of subacute administration of thymoquinone on the expression of alcohol-induced behavioural locomotor sensitization. Groups of mice ( $n=8$ ) were treated subacutely with either saline vehicle p.o (black bars) or (A) thymoquinone 10 mg/kg p.o (TQ 10, striped bars), (B) thymoquinone 20 mg/kg p.o (TQ20, grey bars) or (C) thymoquinone 30 mg/kg p.o (TQ 30, patterned bars) plus alcohol one hour later (black bars) daily for 13 days and locomotor activity was tested on days 1, 5, 9 and 13. This was followed by 5 days of abstinence then thymoquinone (A) 10, (B) 20 or (C) 30 mg/kg was administered respectively p.o and one hour later, locomotor activity was retested on day 19 (treatment day). In each case, groups ( $n=8$ ) subacutely treated with corresponding control doses of thymoquinone alone ( $n=8$ , open bars) were run. Each column represents the mean  $\pm$  sem. \* $P<0.05$ , \*\*  $P<0.01$ . \*\*\* $P<0.001$  compared with the corresponding alcohol plus saline treated control.



**Figure 4.** Schematic illustration of prospective sites of action for thymoquinone on alcohol locomotor sensitization within the motive circuitry of the limbic component of the basal ganglia. Thymoquinone promotion of neurotransmitter function is indicated by the positive signs. GABA,  $\gamma$ -aminobutyric acid; DA, dopamine; Dyn, dynorphin; NAc, nucleus accumbens; VTA, ventral tegmental area; VP, ventral pallidum; M-D Thal, mediodorsal thalamus.