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Sustained antagonism of acute ethanol-induced ataxia following microinfusion of cyclic AMP and cpt-cAMP in the mouse cerebellum

M. Saeed Dar *

Department of Pharmacology & Toxicology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA

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ABSTRACT

Ataxia is a conspicuous physical manifestation of alcohol consumption in humans and laboratory animals. Previously we reported possible involvement of cAMP in ethanol-induced ataxia. We now report a sustained antagonism of ataxia due to multiple ethanol injections following intracerebellar (ICB) cAMP or cpt-cAMP microinfusion. Adenylyl cyclase drugs cAMP, cpt-cAMP, Sp-cAMP, Rp-cAMP, adenosine A_1 agonist, N^6-cyclohexyladenosine (CHA) and $GABA_A$ agonist muscimol were directly microinfused into the cerebellum of CD-1 male mice to evaluate their effect on ethanol (2 g/kg; i.p.) ataxia. Drug microinfusions were made via stereotaxically implanted stainless steel guide cannulas. Rotorod was used to evaluate the ethanol's ataxic response. Intracerebellar cAMP (0.1, 1, 10 fmol) or cpt-cAMP (0.5, 1, 2 fmol) 60 min before ethanol treatment, dose-dependently attenuated ethanol-induced ataxia in general agreement with previous observations. Intracerebellar microinfusion of cAMP (100 fmol) or cpt-cAMP (2 fmol) produced a sustained attenuation of ataxia following ethanol administration at 1, 4, 7 and 25 h or 31 h post-cAMP/cpt-cAMP microinfusion. At 31 h post-cAMP, the ataxic response of ethanol reappeared. Additionally, marked antagonism to the accentuation of ethanol-induced ataxia by adenosine A_1 and $GABA_A$ agonists, CHA (34 pmol) and muscimol (88 pmol), respectively, was noted 24 h after cAMP and cpt-cAMP treatment. This indicated possible participation of AC/cAMP/PKA signaling in the co-modulation of ethanol-induced ataxia by A_1 adenosinergic and GABAergic systems. No change in normal motor coordination was noted when cAMP or cpt-cAMP microinfusion was followed by saline. Finally, Rp-cAMP (PKA inhibitor, 22 pmol) accentuated ethanol-induced ataxia and antagonized its attenuation by cAMP whereas Sp-cAMP (PKA activator, 22 pmol) produced just the opposite effects, further indicating participation of cAMP-dependent PKA downstream. Overall, the results support a role of AC/cAMP/PKA signaling in the expression of ethanol-induced ataxia and its co-modulation by adenosine A_1 and $GABA_A$ receptors.

1. Introduction

Activation of adenylate cyclase with subsequent intracellular formation of cyclic adenosine mono phosphate (cAMP) constitutes an important step in the mediation of cellular actions of several neurotransmitters. Ultimately the cAMP leads to activation of cAMP-dependent protein kinase (PKA) (Gilman, 1989). The activation of PKA follows the release of catalytic subunits of PKA within the nucleus which are responsible for phosphorylation of cAMP-responsive element binding protein (CREB) gene transcription factor. The latter regulates the expression of cAMP-inducible genes (Hunter and Michael, 1992; Meyer and Habener, 1993). The involvement of cAMP and cAMP-dependent PKA signaling pathway in the expression of CNS effects of ethanol has been reported by many investigators (Hoffman and Tabakoff, 1990; Meng et al., 1998; Mochly-Rosen et al., 1988). The cAMP signaling pathway has long been recognized to be important in the development of ethanol dependence and tolerance (Pandey, 1998).

Clinically, ethanol-induced motor impairment is an important and a consistent consequence of alcohol consumption and represents an excellent measure of ethanol intoxication. Ethanol-induced ataxia has also been used to study the differences in the initial sensitivity and acute tolerance in several inbred strains of mice (Browman and Crabbe, 2000; Deitrich et al., 2000; Kirstein and Tabakoff, 2001). Modulation of cAMP signaling pathway can result in an alteration in the sensitivity to ethanol-induced ataxia (Durcan et al., 1991; Yoshimura et al., 1998). Other investigators have also reported a role of cAMP signaling in neural sensitivity to ethanol and that ethanol-induced inhibition of cerebellar purkinje neurons involves cAMP signaling (Freund and Palmer, 1997). Furthermore, mutational

Abbreviations: ICB, intracerebellar; ANOVA, analysis of variance; mM, millimolar; CHA, N^6-cyclohexyladenosine; cAMP, cyclic adenosine mono phosphate; 8-(4-chlorophenylthio)-cAMP, cpt-cAMP; aCSF, artificial cerebrospinal fluid; AP, anterior–posterior; ML, medio-lateral; DV, dorso-ventral; MRI, magnetic resonance imaging; PET, positron emission tomography; ERK, extracellular signal-regulated kinases; PKA, protein kinase A.

* Tel.: +1 252 744 2885; fax: +1 252 744 3203.
E-mail address: darm@ecu.edu.

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studies in Drosophila have also demonstrated that cAMP signaling plays a role in the behavioral sensitivity to ethanol (Moore et al., 1998). For example, mutants exhibit greater sensitivity to ethanol on a measure of postural control due to a deficit in cAMP signaling.

We were first to report a functional link between ethanol and adenosine in mice (Dar et al., 1983). Subsequently, we demonstrated an adenosinergic modulation of ethanol-induced ataxia in mice (Dar, 1990, 2001) and rats (Barwick and Dar, 1998; Meng and Dar, 1995). Direct microinfusion of agonists and antagonists of adenosine (Dar, 1990) dose dependently accentuated and attenuated, respectively, ethanol-induced ataxia, supporting existence of a central mechanism of behavioral interaction between ethanol and adenosine systems. The adenosinergic studies were subsequently confirmed by several investigators (Carmichael et al., 1991; Nagy et al., 1989; Proctor and Dunwiddie, 1984) as well as extended by others (Arolfo et al., 2004; Thorsell et al., 2007). We have also shown (Dar, 2006) that ICB microinfusion of agonists and antagonists of $GABA_A$ accentuated and attenuated, respectively, ethanol-induced ataxia in a dose related manner indicating presence of a functional interaction between central GABAergic mechanisms and ethanol.

The adenosine receptors are members of G-protein family and are coupled to adenylate cyclase (AC)-cAMP signaling pathway. The cerebellar adenosinergic modulation of ethanol-induced ataxia mainly involved the adenosine A_1 receptors subtype which is negatively coupled to AC-cAMP system (Dar, 1990, 2001). This led to further studies in which a functional relationship between cAMP and ethanol-induced ataxia was shown (Dar, 1997; Meng et al., 1998).

We have previously reported the involvement of cAMP in ethanol-induced ataxia because intracerebellar (ICB) microinfusion of forskolin and miconazole markedly attenuated and accentuated, respectively, ethanol-induced ataxia (Dar, 1997). These observations suggested a role of AC-cAMP system in motor impairing effect of ethanol with a further possible involvement downstream of cAMP-dependent PKA signaling. Ethanol-induced stimulation of cAMP-PKA signaling in a specific brain region may reflect participation of that brain region in the expression of CNS responses of ethanol (Asyyed et al., 2006).

Cyclic AMP-dependent PKA signaling transduction pathway has been shown to play an important role in the modulation of ethanol-induced behavioral actions such as sedation (Wand et al., 2001). The purpose of the present study was to have further insight into the functional role of cAMP signaling on ethanol-induced ataxia. Three parameters were evaluated. First, the duration of antagonism by a single ICB cAMP or 8-(4-chlorophenylthio)-cAMP (cpt-cAMP) microinfusion on ataxia involving multiple ethanol injections was determined. Second, the antagonising effect of cAMP or cpt-cAMP also involved the adenosinergic A_1 and GABAergic co-modulation of the ethanol-induced ataxia. Last, evidence for the hypothesis that a cAMP-dependent protein kinase (PKA) signaling transduction pathway may regulate the sustained antagonism of ethanol-induced ataxia by cAMP was obtained. We examined the effects of ICB microinfusion of specific membrane-permeable inhibitor and stimulator of cAMP-dependent PKA on ethanol-induced ataxia and its antagonism by cAMP or cpt-AMP treatment. Using Rp-cAMP and Sp-cAMP, the inhibitor and activator of cAMP-dependent PKA as drug tools, the study also identified downstream participation of PKA in the modulation of ethanol-induced ataxia by cAMP .

2. Materials and methods

2.1. Subjects

Subjects were male CD-1 mice (Charles River, Raleigh, NC) weighing 22–25 g at the time of surgery and were 5 to 6 weeks of age. Immediately after arrival, mice were housed in groups of eight in Plexiglas cages in temperature- and humidity-controlled animal housing, lights from 8:00 AM to 8:00 PM. Commercial pellet food and water were available *ad libitum* except during rotorod behavioral testing. However, after surgical implantation of guide cannulas, the animals were housed individually in plastic cages with stainless steel wire lid. All experiments including survival stereotaxic surgery were approved by East Carolina University Animal Care and Use Committee in accordance with the Declaration of Helsinki and with Guide for the Care and Use of Laboratory Animals as adopted by National Institutes of Health.

2.2. Drugs

Drug sources were as follows: cAMP sodium salt, cpt-cAMP sodium salt, Rp-8-cpt-cAMP sodium salt, Sp-cAMP sodium salt, chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA); N^6-cyclohexyladenosine (CHA), muscimol (Research Biochemicals, Natick, MA, USA). Unless otherwise stated, all these drugs were dissolved in artificial cerebrospinal fluid (aCSF) containing (mM): NaCl, 127.65; KCl, 2.55; $CaCl_2$, 0.05; $MgCl_2$, 0.94; $Na_2S_2O_5$, 0.05; at pH 7.4, a day before the rotorod experiment and the drug solutions were stored at −74 °C. The CHA and muscimol were dissolved in aCSF with the aid of dimethylsulfoxide (DMSO). For consistency in the ataxia-producing effect of ethanol, the latter was always purchased from the same vendor (AAPER Alcohol and Chemical Co., Shelbyville, Kentucky, USA) and the source of animals has always been the Raleigh (NC) facility of Charles River. The solution of ethanol (10% w/v) was prepared in sterile 0.9% saline and injected at 20 ml/kg (i.p.). Similarly, chloral hydrate was dissolved in sterile 0.9% saline and injected at 10 ml/kg (i.p.).

2.3. Stereotaxic surgery

Surgery was performed following two days (current protocol requires 6 days) rest after delivery of animals from the vendor. Five days were allowed for the animals to recover from the surgical trauma and effect of anesthetic. Therefore, surgical implantation of permanent stainless steel guide cannulas was performed 7 (currently 11) days before the start of rotorod test. Mice were anesthetized with chloral hydrate (450 mg/kg; i.p.) before placement in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and implanted the cannulas (length 12 mm; 22-gauge) targeted for direct drug infusion at site within the superficial layers of cerebellar cortex. The cannulas were anchored to skull surface, scraped clean of periostium in the horizontal plane (skull flat), with quick setting dental cement (Durelon®, Premier Dental Products, Morristown, PA, USA). Coordinates for the cannula implantation were as follows (in mm): anterior–posterior plane – 6.4 (A–P) from bregma, lateral plane from midline ± 0.8 (L–M), dorso-ventral plane from the skull surface −1.0 (D–V). All coordinates are based on the Atlas of Slotnick and Leonard (1975). Other details have been reported previously (Dar, 1997, 2006). The surgery was conducted under strict aseptic conditions. In addition, each animal received 3000 units of Durapen® (Vedco Inc., MO, USA), a combination of benzathine and procaine penicillin G suspension, immediately after surgery prophylactically. Each animal also received an injection of ketorolac tromethamine (2 mg/kg; s.c., Abbott Laboratories, North Chicago, IL.) analgesic immediately before and 4 h after surgery, respectively.

2.4. Intracerebellar microinfusion

In the present study all drugs (cAMP, cpt-cAMP, Rp-cAMP, Sp-cAMP, CHA and muscimol) were microinfused directly into the anterior lobe of culmen via an injector cannula (length 13mm; 30-gauge). Infusions were made via a stainless steel injector cannula attached by PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ, USA) to a 25-µl Hamilton microsyringe driven by a Model 22 Micro Injection Pump (Harvard Apparatus, South Natick, MA, USA).The

microsyringe was fitted onto a mini-pump which was set to infuse the drug solution at a regulated constant rate of 0.1 μl (100 nL)/min. The infusion of drugs via guide cannula was targeted to bathe the superficial layers of the cerebellar cortex, mainly the molecular, Purkinje and in part granular cell layers. The volume of infusions was kept constant at 100 nL. Prior to microinfusion, stylets were removed and the guide cannulas were cleaned with a fine tipped dental broach. This was followed by inserting the injector cannula into the guide cannula and allowing it to protrude 1 mm beyond the lower tip of the guide cannula. The proper delivery of the drug solution was adjusted by monitoring the movement of an air bubble that was introduced in the PE-10 tubing between the drug solution and the water. Other details of microinfusion have been previously reported (Dar, 1997).

2.5. Rotorod treadmill

The rotorod apparatus (UGO Basile, Verese, Italy), and the general rotorod procedure have been described previously (Al-Rejaie and Dar, 2006; Smith and Dar, 2007). In brief, the procedure consisted of pre-screening the mice on the rotorod in which the animals act as their own control. Mice become acclimatized to the treadmill in the manner when they are placed on it 2–3 times 30 min prior to the actual experiment. A successful pre-screening of the animals requires each animal to walk on rotorod for an arbitrarily selected time of 180 s without a fall. Motor coordination was evaluated by rotorod treadmill for mice that was calibrated for a fixed speed of 24 rpm (Dar, 1990; Smith and Dar, 2007). At a time five successfully prescreened mice were evaluated on the rotorod. The animals received one of the drugs (cAMP, cpt-cAMP, Rp-cAMP, Sp-cAMP, CHA, muscimol) or aCSF by direct ICB microinfusion which was followed within 5 min by the test dose of ethanol (2 g/kg; i.p.) to all animals. The evaluation of animals by the rotorod was carried out every 15 min from the moment of ethanol administration during the 60 min experimental period. The rotorod experiments were conducted between 8:00 AM and 11:30 AM to avoid the influence of diurnal variation on the motor behavior of the animals.

2.6. Histology

At the conclusion of each rotorod experiment, each animal was microinfused 100 nL of Fast Green dye via the same guide cannula in order to confirm the correctness of the cannula placement and consequently the accuracy of the drug microinfusion. To minimize personal bias in the data collection and interpretation, the technician that performed the rotorod and the histological studies was not informed what drug was actually being microinfused. All animals had the correct cannula placement in the present study. The animals were euthanized by cervical dislocation followed by decapitation under Isoflurane anesthesia (ISOFPo; Abbott Laboratories, North Chicago, IL., USA). The brains were exposed and cut at the mark of cannulation and confirmation of accuracy of cannula implantation was verified by visual observation of the presence of dye in the cerebellar anterior lobe region. Only rotorod data from those animals in which cannulas were found to have been accurately implanted was used for statistical analysis. In order to assess the integrity of the cerebellar tissue during the period of implantation of the guide cannulas and the execution of the rotorod experiment, brains were randomly selected, frozen at −20 °C and cut into 20 μm thick coronal sections using Tissue-Tek II cryostat (Mills Laboratories, Naperville, IL, USA). The sections, following mounting and air drying, were stained with cresyl violet to assess non-specific damage due to indwelling cannulas and examined under a light microscope as described previously (Al-Rejaie and Dar, 2006; Smith and Dar, 2007). A minimal variation between and within groups and treatments in the drug dispersion sites of microinfusion and extent of tissue damage due to cannula implantation was observed. The histological data also supported that the drug dispersion following intracerebellar microinfusion was confined to the cerebellar tissue in agreement with our previous report (Meng and Dar, 1996), assuming no significant differences in the pharmacokinetics of dye and the drugs used in the study.

2.7. Statistical data analysis

A commercial software program (SPSS for Windows, V15.0; SPSS Inc, Chicago, IL, USA) was used for rotorod data analysis. Data collected from rotorod experiments was subjected to analysis of variance (ANOVA) with repeated measures in order to test for any significance of interaction between treatment groups and time periods. A two-way repeated measures ANOVA was used to evaluate the effect of various drug doses and time on motor coordination. The drug dosage × time interaction was tested using the multivariate criterion of Wilk's lambda (λ). A Dunnett's C post hoc test was performed whenever significance was found on treatment and/or time. A p value of <0.05 was considered significant.

3. Results

The test dose of ethanol, 2 g/kg; i.p, used in our ongoing work and throughout this investigation was selected based on a dose–response study conducted and published previously (Dar, 1988). Since that report, the ethanol-induced ataxic response evaluated by rotorod has been consistent. The onset of the ataxia was observed within 30–60 s with maximum effect noted at 15 min post-ethanol evaluation with a mean of 40 s walking time. The animals exhibited no loss of righting reflex and usually regained their normal coordination within 60-min post-ethanol injection. The aCSF + ethanol control groups were the same in each treatment group. The ethanol response was consistent and therefore it was not considered ethical to repeat the ethanol control experiment with each treatment group.

3.1. Effect of ICB microinfusion of cAMP on ethanol-induced ataxia

Fig. 1 shows the dose-related effect of cAMP on ethanol-induced ataxia. The doses (0.1, 1, 10 fmol) of cAMP following their ICB microinfusion were followed 60 min later by the test dose of ethanol and rotorod evaluation. There was dose-dependent attenuation of ethanol-induced ataxia. The 1 and 10 fmol cAMP doses virtually abolished the ethanol-induced ataxia. A significant drug dosage and time interaction was observed [F (6, 40) = 26.702, $p<0.01$]. The attenuation following both cAMP doses was significant ($p<0.01$) at all four (15, 30, 45, 60 min) evaluation periods. The animals regained complete normal motor coordination within 30 min post-ethanol

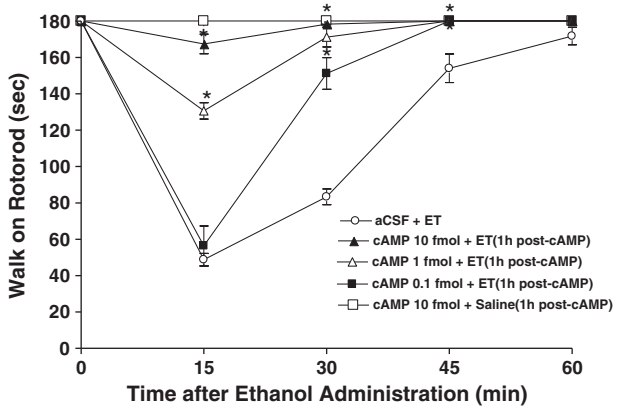

Fig. 1. The effect of acute intracerebellar microinfusion of various doses of cAMP given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia in mice. Each point represents the mean and S.E. of maximum of 10 mice. (*) Significantly different ($p<0.01$) from the ethanol control (aCSF + ethanol) group.

injection. Following 0.1 fmol cAMP pretreatment, the attenuation of ethanol-induced ataxia was significant ($p<0.05$) at 30, 45 and 60 min post-ethanol administration. When the ICB microinfusion of 10 fmol cAMP was followed by saline, no change in the normal motor coordination was noted (Fig. 1).

3.2. Effect of ICB microinfusion of cpt-cAMP on ethanol-induced ataxia

We also observed a similar dose-dependent attenuation of ethanol-induced ataxia following ICB microinfusion of various doses (0.5, 1.0, 2.0 fmol) of cpt-cAMP as shown in Fig. 2. There was a significant drug treatment and time interaction [$F(4, 42) = 8.874$, $p<0.01$]. The highest ICB dose of cpt-cAMP, 2 fmol, markedly attenuated ethanol-induced ataxia at 15, 30, and 45 min. No change in normal motor coordination was observed when 2 fmol dose of cpt-cAMP was infused followed by saline injection instead of ethanol.

3.3. Effect of multiple acute ethanol injections on ataxia following single ICB microinfusion of cAMP and cpt-cAMP

An interesting observation in the present study was that the prolonged attenuating effect of a single ICB microinfusion of cAMP (100 fmol) or cpt-cAMP (2 fmol) on ataxia produced by the test dose of ethanol administered at 60 min (first dose), and repeated at 4 h (second dose), 7 h (third dose), 25 h (fourth dose), and 31 h (fifth dose) post-cAMP/cpt-cAMP microinfusion (Figs. 3 and 4). A single dose of cAMP (100 fmol) or cpt-cAMP (2 fmol) administered by direct ICB microinfusion remained markedly effective in attenuating the ataxia produced following each of the multiple test doses of ethanol at 1, 4, 7, 25, or 31 h post-cAMP/cpt-cAMP microinfusion. A significant time and dosage interaction [$F(12, 48) = 19.180$, $p<0.01$ and $F(8, 48) = 7.929$, $p<0.01$] was observed with cAMP and cpt-cAMP treatment groups, respectively. Significant ($p<0.01$) attenuation of ethanol-induced ataxia at all four (15, 30, 45, 60 min) post-ethanol evaluation time periods was observed following first, second, third, and fourth ethanol dose at 1, 4, 7, 25 h post-cAMP (Fig. 3) and -cpt-cAMP (Fig. 4), respectively. A significant ($p<0.05$) attenuating effect was also observed on ethanol-induced ataxia at 31 h post-cpt-cAMP microinfusion (Fig. 4) but the effect was not significant ($p>0.05$) in cAMP-treated animals (Fig. 3). This suggested that: (i) cpt-cAMP has more potent attenuating effect on ethanol-induced ataxia compared to cAMP and (ii) the duration of the attenuating effect lasted between 25 and 31 h post-cAMP/cpt-cAMP microinfusions.

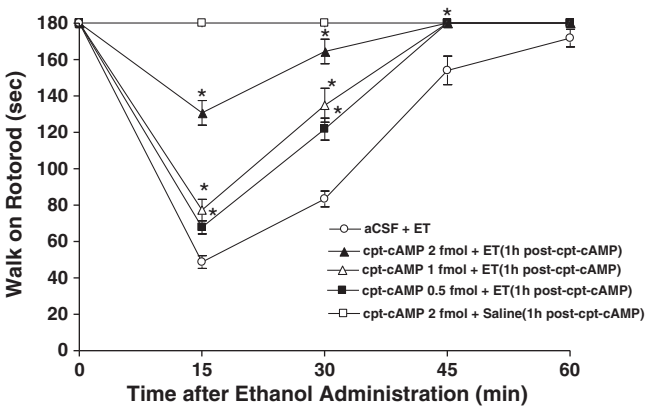

Fig. 2. The effect of acute intracerebellar microinfusion of various doses of cpt-cAMP given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia in mice. Each point represents the mean and S.E. of maximum of 10 mice. (*) Significantly different ($p<0.01$) from the ethanol control (aCSF + ethanol) group.

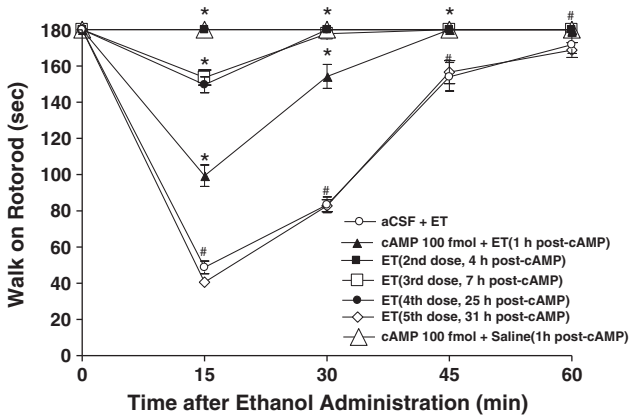

Fig. 3. The effect of acute intracerebellar microinfusion of cAMP (100 fmol) given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia in mice. Each point represents the mean and S.E. of maximum of 10 mice. (*) Significantly different ($p<0.01$) from the ethanol control (aCSF + ethanol) group.

3.4. Effect of multiple acute ethanol injections on ataxia in the absence of ICB microinfusion of cAMP

Fig. 5 shows the ethanol control data where the animals instead of cAMP or cpt-cAMP received aCSF microinfusion prior to the administration of the test dose of ethanol. The animals exhibited the typical ethanol-induced ataxia following each ethanol administration at 1, 4, 7, 25, and 31 h post-aCSF microinfusion. There was no difference in the degree of ethanol-induced ataxia following each ethanol injection as the ethanol response curves essentially overlapped (Fig. 5). A statistical analysis in which rotorod data from treatment groups in Fig. 4 was compared with the corresponding ethanol control groups (Fig. 5), indicated significant time and dosage interaction [$F(21, 86) = 8.743$, $p<0.01$]. The difference between cAMP + ethanol treatment groups (Fig. 3) and aCSF + ethanol control groups (Fig. 5) at 1, 4, 7, 25, and 31 h ethanol administration was significant ($p<0.01$) at 15 and 30 min but not at 45 and 60 min evaluation periods.

3.5. Prolonged antagonism, following ICB microinfusion of cAMP, of the accentuation of ethanol-induced ataxia by ICB adenosine A_1 and $GABA_A$ receptor agonists

We also evaluated the effect of single cAMP microinfusion on the adenosinergic A_1 and GABAergic modulation of ethanol-induced

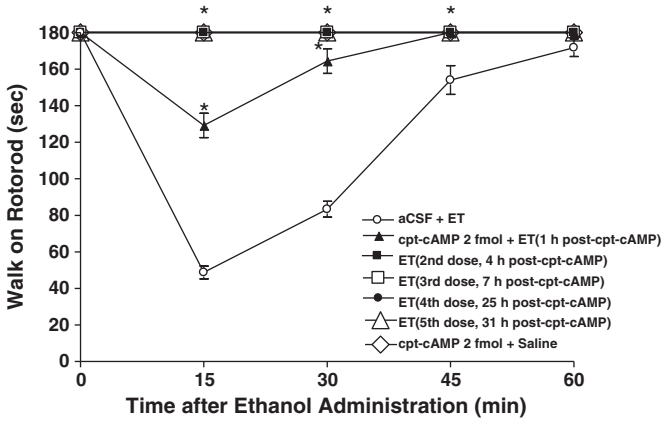

Fig. 4. The effect of acute intracerebellar microinfusion of cpt-cAMP given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia in mice. Each point represents the mean and S.E. of maximum of 10 mice. (*) Significantly different ($p<0.01$) from the ethanol control (aCSF + ethanol) group.

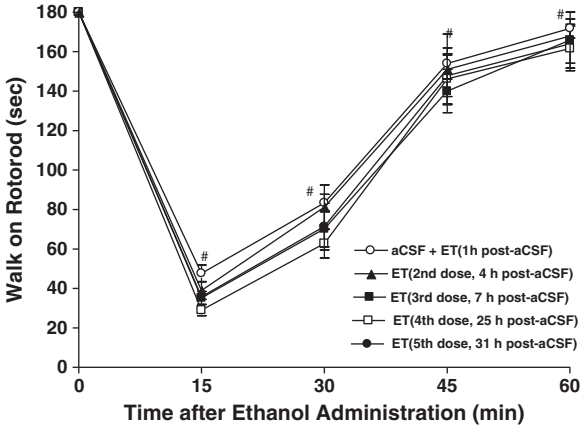

Fig. 5. The effect of acute intracerebellar microinfusion of aCSF (100 nl) given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia in mice. Each point represents the mean and S.E. of maximum of 10 mice. (#) Not significantly different ($p>0.05$) from the ethanol control (aCSF + ethanol) group.

ataxia. Intracerebellar pretreatment with cAMP produced a similarly significant antagonism of the adenosinergic A_1 modulation of ethanol-induced ataxia that lasted at least 24 h post-cAMP microinfusion (Fig. 6). Fig. 6 shows that even though cAMP was microinfused 24 h prior to the ICB microinfusion of CHA, it was still able to virtually block not only ethanol-induced ataxia but its accentuation by a relatively high dose [3-fold higher dose than that used in previous study (Dar, 1997)] of adenosine A_1 receptor agonist, CHA (11.4 pmol vs.34 pmol; ICB). Similarly, cAMP microinfused 24 h before the ICB infusion of $GABA_A$ agonist muscimol (88 pmol; ICB) virtually abolished its potentiating effect on ethanol-induced ataxia. Accentuation of ethanol-induced ataxia by CHA and muscimol was so marked that the animals practically were unable to walk on the rotorod during the entire 60-min experimental period (Fig. 6). In spite of this the attenuating effect of cAMP was marked and sustained. There was a significant time and dosage interaction [$F(15, 97) = 22.359$, $p<0.01$]. Ethanol-induced ataxia was virtually eliminated in cAMP + CHA + ethanol and cAMP + muscimol + ethanol groups compared to CHA + ethanol and muscimol + ethanol treatment groups at all four evaluation periods ($p<0.01$) (Fig. 6).

3.6. Effect of ICB pretreatment of Rp-cAMP and Sp-cAMP on the attenuating effect of ICB cAMP on ethanol-induced ataxia

Finally, the results presented in Fig. 7 demonstrated that the attenuating effect of cAMP on ethanol-induced ataxia was significantly mediated by a signaling pathway that involved PKA. Intracerebellar pretreatment with Rp-cAMP, an inhibitor of cAMP-dependent PKA: (i) significantly antagonized cAMP-induced attenuation of ethanol-induced ataxia and (ii) markedly accentuated ethanol-induced ataxia (Fig. 7). On the contrary, ICB pretreatment with stimulator of cAMP-dependent PKA, Sp-cAMP, virtually abolished the ethanol-induced ataxia (Fig. 7). The attenuation of ethanol-induced ataxia was significant ($p<0.01$) at all four evaluation periods. One-way ANOVA indicated a significant time and Rp-cAMP/Sp-cAMP treatment interaction [$F(12,153) = 18.738$, $p<0.01$].

4. Discussion

We have previously demonstrated that some of the central effects of ethanol are modulated by brain adenosine (Dar et al., 1983; Dar, 1990, 2001). An underlying important mechanism responsible for the expression of behavioral effects of ethanol involves inhibition of adenosine uptake by ethanol (Clark and Dar, 1989). The adenosine uptake inhibition has been elegantly confirmed to be the result of selective inhibition of alcohol-sensitive equilibrative nucleoside transporter 1 (ENT1) by ethanol (Nagy et al., 1990). Adenosine uptake is one of the many mechanisms that regulate adenosine levels. The resultant increase in the extracellular adenosine concentration mediates acute and chronic effects of ethanol through activation of the adenosine receptors (Diamond and Gordon, 1994; Dunwiddie and Masino, 2001). The subtype of adenosine receptors that modulates the motor impairing effect of ethanol has been demonstrated as A_1 (Barwick and Dar, 1998; Dar, 1990, 2001; Meng and Dar, 1995). Within the mouse cerebellum, the adenosine A_1 receptors are negatively coupled through pertussis toxin sensitive G_i/G_o protein to adenylate cyclase (AC)-cAMP system (Dar, 1997). Adenosine A_1 receptors upon activation, therefore, would decrease the intracellular cAMP levels. The results of the present study (Fig. 7) demonstrated that the adenosine A_1 receptors are further linked through AC-cAMP to cAMP-dependent PKA thereby implicating AC/cAMP/PKA signaling system in the expression of ethanol-induced ataxia. Pretreatment with potent and specific membrane permeable inhibitor Rp-cAMP and activator Sp-cAMP resulted in marked accentuation and attenuation,

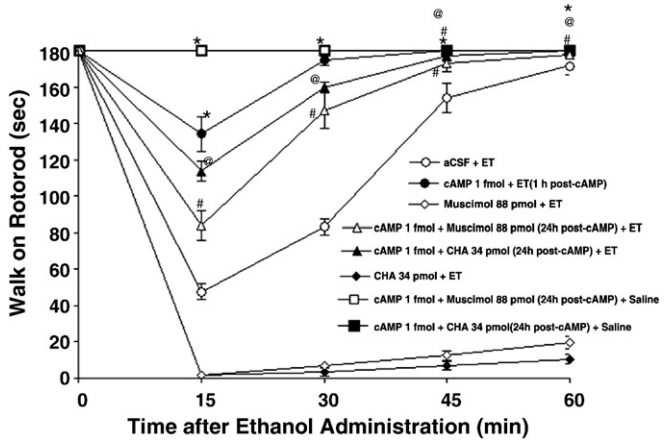

Fig. 6. The effect of acute intracerebellar (ICB) microinfusion of cAMP (1 fmol) given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia and its accentuation by ICB agonists of adenosine A_1 receptor subtype cyclohexyl-adenosine (CHA) and $GABA_A$ muscimol in mice. Each point represents the mean and S.E. of maximum of 10 mice. (*) Significantly different ($p<0.01$) from the ethanol control (aCSF + ethanol) group. (#) Significantly different ($p<0.01$) from the muscimol + ethanol treatment group. (@) Significantly different ($p<0.01$) from the CHA + ethanol treatment group.

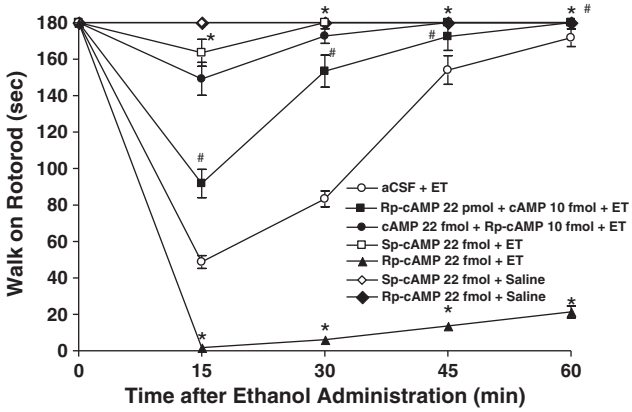

Fig. 7. The effect of acute intracerebellar microinfusion of specific membrane-permeable inhibitor (Rp-cAMP) and activator (Sp-cAMP) of cAMP dependent protein kinase on the antagonistic effect of cAMP given 60 min prior to ethanol (2 g/kg; i.p.) administration on ethanol-induced ataxia in mice. Each point represents the mean and S.E. of maximum of 10 mice. (*) Significantly different ($p<0.01$) from the ethanol control (aCSF + ethanol) group; (#) Significantly different ($p<0.01$) from the Rp-cAMP + ethanol treatment group.

respectively, of ethanol-induced ataxia (Fig. 7). The Rp-cAMP treatment not only blocked the powerful attenuation of ethanol-induced ataxia by cAMP (Fig. 1) but resulted in marked ataxia (Fig. 7), indicating that the tonic adenosinergic A_1 receptor modulation of ethanol-induced ataxia would be via AC/cAMP/PKA signaling pathway.

Cerebellum remained the focus of our investigation due to its significance in alcoholism and the relatively recent finding of its role in cognition (Schmahmann, 2004). It is especially susceptible to ethanol toxicity during neurodevelopment (Maier et al., 1999). Functional MRI (Schneider et al., 2001) and PET studies have implicated it in drug addiction (Olbrich et al., 2006; Volkow et al., 1999). Finally, the CREB transcription activity has been linked to ethanol sensitivity to cerebellum (Acquaah-Mensah et al., 2006).

In the present investigation, the ICB pretreatment with cAMP and cpt-cAMP markedly attenuated acute ethanol-induced ataxia dose dependently (Figs. 1 and 2). It is commonly accepted that the levels of cAMP in most cells are typically <1 to 10 μM (Bender and Beavo, 2006). Therefore, the doses of cAMP in the range of 1 to 100 fmol used in the present investigation, were well below the physiological range and physiologically relevant. The potency of cpt-cAMP compared to cAMP was greater because: (i) relatively smaller doses of cpt-cAMP produced comparable attenuating effect; and (ii) the attenuating effect of a single ICB microinfusion of cpt-cAMP lasted relatively longer (31 h) compared to cAMP treatment (25 h). The key observation was the sustained antagonism, following single ICB microinfusion of cAMP or cpt-cAMP, of ataxia due to ethanol injections at 1, 4, 7, 25 or 31 h post-cAMP/cpt-cAMP microinfusion in the same group of animals (Figs. 3 and 4). The attenuating effect of cpt-cAMP lasted relatively longer (31 h) because ethanol injection failed to produce its usual ataxic response at 31 h post-cpt-cAMP treatment. On the contrary, the attenuating effect of cAMP lasted 25 h because at 31 h post-cAMP treatment, the usual ethanol-induced ataxia was observed. The aCSF + ethanol and cAMP + ethanol treatment response curves were virtually overlapped (Fig. 3; ◊—◊).

The virtual loss of ethanol's ability to produce its ataxic response during 25 or 31 h of post-cAMP and cpt-cAMP treatment, respectively, could have been the result of the development of acute tolerance because of repeated acute ethanol administration to same animals (Figs. 3 and 4). This possibility was ruled out by the results of the control ethanol rotorod experiment (Fig. 5) in which the same animals were administered ethanol at 1, 4, 7, 25 h following ICB microinfusion of aCSF instead of cAMP or cpt-cAMP. Usual ataxic response was observed following each ethanol injection and the ethanol response curves at 1, 4, 7, 25 h post-aCSF administration overlapped (Fig. 5). Therefore, whereas the results presented in Figs. 3 and 4 supported that the absence of ethanol-induced ataxia clearly was due to the participation of AC-cAMP signaling pathway, the results shown in Fig. 5 ruled out the development of acute tolerance due to multiple ethanol injections as a participating factor in the elimination of ethanol-induced ataxia in cAMP- or cpt-cAMP-treated animals. Consequently, the results of ethanol control experiment (Fig. 5) provided indirect strong evidence that the AC/cAMP/PKA signaling pathway primarily was responsible for significant and sustained attenuation of ethanol-induced ataxia. The onset of antagonism to ethanol-induced ataxia was within 60 min of ICB microinfusion of cAMP or cpt-cAMP, the antagonism lasted for 25 to 31 h indicating significant modulation downstream of the events of AC/cAMP/cAMP-dependent PKA signal transduction cascade. It has been reported that cAMP signal transduction pathway may be the intracellular targets mediating the CNS actions of ethanol and ultimately lead to molecular events that constitute as the basis of development of ethanol tolerance and dependence (Pandey, 1998).

The AC-cAMP pathway appeared to be similarly effective in antagonizing the accentuation of ethanol-induced ataxia both by adenosine A_1 receptor agonist CHA and $GABA_A$ agonist muscimol. For example, the activation of adenylyl cyclase by forskolin not only attenuated the ethanol-induced ataxia but also the accentuating effect of CHA on ethanol-induced ataxia (Meng et al., 1998). The observed antagonizing effect of cAMP on CHA-induced accentuation of ethanol-induced ataxia also lasted 24 h (Fig. 6) similar to the animal group that received multiple ethanol injections (Fig. 3). The accentuation of ethanol-induced ataxia by CHA obviously occurred via the high affinity adenosine A_1 receptors. As stated above, these metabotropic receptors are coupled through G_i/G_o proteins to AC, with moderate density within the cerebellum. The $GABA_A$ receptors, however, are ionotropic, coupled to GABA-benzodiazepine-chloride ionophore complex, and modulate opening of the chloride ion channels. Within the cerebellar cortex, $GABA_A$ receptors are localized primarily on the granule cell (Simantov et al., 1976). Activation of $GABA_A$ receptors by muscimol markedly accentuated ethanol-induced ataxia which was abolished 24 h post-cAMP microinfusion (Fig. 6). Accentuation by muscimol was likely due to the combined effect of ethanol and muscimol to increase chloride influx. This observation was consistent with the report that ethanol-induced ataxia was reversed by Ro15-4513, a partial inverse agonist of benzodiazepine (Dar, 1995). Ro15-4513 is a potent inhibitor of the chloride channels and interestingly also antagonizes the accentuation of ethanol-induced ataxia by CHA and muscimol (Meng et al., 1997). Also, K-ATP channel antagonist, glipizide, and channel opener pinacidil, following their ICB pretreatment markedly antagonized and accentuated, respectively, ethanol-induced ataxia in an adenosine A_1 receptor sensitive manner (Dar, 2004). This would suggest that adenosine A_1 receptors also modulate ethanol-induced ataxia via the chloride and K-ATP channels through specific but most likely different G_i/G_o proteins, thereby implicating the same AC/cAMP/PKA signaling pathway.

A plausible explanation for the observed blockade of ethanol-induced ataxia by cAMP/cpt-cAMP treatment would be a subtractive functional interaction between ethanol and muscimol at the granule cell site and cAMP/cpt-cAMP at the axonal and axonal terminal sites of the granule cells. This functional interaction, therefore, most likely involved both ends of the granule cells i.e., ethanol and muscimol acting on the granule cell sites and cAMP/cpt-cAMP at the granule axonal (parallel fibers) terminal sites resulting in a net functional outcome of marked attenuation of ethanol-induced ataxia. This indicates the critical significance of the role of AC/cAMP/PKA signaling pathway in the expression of ethanol-induced ataxia via co-modulation by adenosine A_1, $GABA_A$, chloride and K-ATP channels. The antagonism by cAMP/cpt-cAMP appears to be physiological in nature and consistent with our hypothesis that ethanol-induced ataxia is caused by a decrease in the intracellular levels of cAMP and *vice versa*. Acute ethanol lowers the level of cAMP that is further reduced by CHA consistent with marked accentuation of ethanol-induced ataxia by CHA (Meng et al., 1998). Therefore, ICB microinfusion of cAMP would be expected to elevate the levels of cAMP that would oppose the reduction in cAMP levels by the combined CHA and ethanol. Thus, as long as the tissue cAMP remained at or above the basal levels, the ethanol administration would not produce ataxia. The cAMP-ataxia hypothesis in no way has overlooked the elegant literature reports in which ethanol has been reported to increase the cAMP levels through adenosine A_{2A} receptor activation via G_s protein (Newton and Messing, 2006). Much of the experimental data was generated using cultured NG108-15 neuroblastoma cells that express adenosine A_{2A} but not A_1 subtype (Nagy et al., 1990). The adenosine A_1 receptors selectively mediate the negative central effects of ethanol such as ataxia (Barwick and Dar, 1998; Dar, 1990, 2001; Meng and Dar, 1995) and sleep (Dar et al., 1983; Thakkar et al., 2003a,b). Consequently, one would expect to observe a decrease in cAMP levels through activation of pertussis toxin sensitive G_i/G_o protein (Meng et al., 1997).

Several investigators (Asyyed et al., 2006; Lai et al., 2007; Newton and Messing, 2006; Pandey, 1998; Wand et al., 2001) as well as we

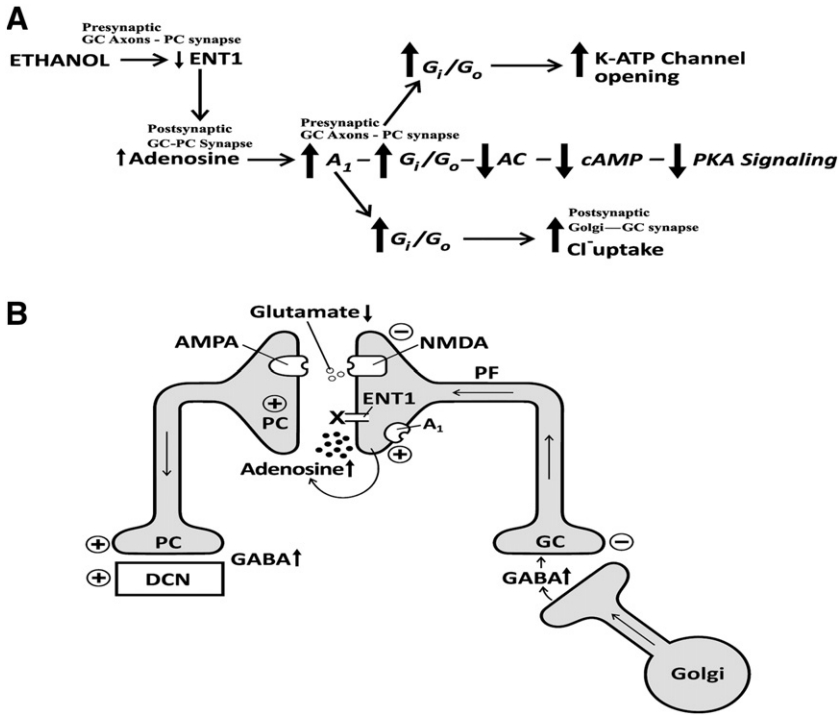

Fig. 8. A: Schematic diagram showing the various signaling pathways involved in the expression of ethanol-induced ataxia. B: Simplified illustration of the basic cerebellar cortical neural connections showing only the components of the circuit that are relevant to the expression of ethanol-induced ataxia. GC: Granule cell, PC: Purkinje cell, PF: parallel fibers, DCN: deep cerebellar nuclei, ENT1: ethanol nucleoside transporter 1.

(Dar, 1997; Meng et al., 1998) have strongly implicated cAMP-PKA signaling in the CNS responses of ethanol. The present study provided yet another evidence for the role of cAMP-PKA signaling in ethanol-induced ataxia. Fig. 8 summarizes schematically the signaling events based on the results of the present investigation and some of our previously published reports (Dar, 2004).

It must be stated that the rotorod method represents only one measure of ataxia and/or cerebellar dysfunction. As so, it seems that all ataxic effect of ethanol could be explained by cAMP pathways, since cAMP reverses completely the ataxic effect. However, ethanol has many direct antagonist and agonist effect in synaptic transmissions in cerebellum which are also clinically relevant. It is also important to discuss possible confounding effect on rotorod results due to sleep deprivation as mice are nocturnal animals. The sleep deprivation effect may gradually increase as the day advances and could appear significant in the later part of the day i.e., in the afternoon. While an effect of sleep deprivation cannot be ruled out, the authors have attempted to minimize this possible confound by performing rotorod experiments during the morning (8–11:30 AM) rather than later in the day. It should also be noted that each animal is only tested for a total of one hour, and thus, any effect of sleep deprivation on behavior is expected to be marginal.

The involvement of cAMP-dependent PKA signaling in ethanol-induced ataxia and in the extended protective response of this signaling pathway indicates possible participation of complex cascade downstream. Signaling pathways are known to be stimulated by ethanol and many drugs of abuse resulting in the activation of protein kinases including cAMP-dependent PKA which in turn leads to altered gene transcription and protein synthesis. As a consequence, long-term changes in synaptic functions may occur that ultimately may constitute the basis of addiction (Lee and Messing, 2008). Ethanol and other drugs of abuse also regulate the activity of transcription factors and increase gene expression through ERK-dependent mechanisms (Lee and Messing, 2008). It would be only speculative on the part of the author to suggest precise mechanism to explain the key observation in the present study of the protracted effectiveness of single ICB administration of cAMP/cpt-cAMP against ethanol-induced ataxia as well as its accentuation by agonists of adenosine A_1 receptor subtype CHA and $GABA_A$.

5. Conclusions

The results of the present investigation demonstrated the critical importance of cAMP in ethanol-induced ataxia and reemphasize the participation of AC/cAMP/PKA signaling in the central effects of ethanol. Ruling out the development of tolerance to ethanol, the results suggest that the observed sustained antagonism of ethanol-induced ataxia was through participation of downstream AC/cAMP/PKA signaling.

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