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Research Article

Involvement of Mouse Cerebellar Neuronal Nitric Oxide Synthase (nNOS) System in the Functional Interaction and Cross-Tolerance between Nicotine and Ethanol

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Abstract Individuals who smoke tobacco overwhelmingly drink alcohol and vice versa. We previously observed functional interaction and cross-tolerance between alcohol and intracerebellar (ICB) nicotine. We now report their mediation by cerebellar nNOS in male CD-1 mice. Nicotine and nNOS inhibitor (nNOSI); N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidine tris(trifluoroacetate) salt, were directly infused into the cerebellum while ethanol was injected IP. Ethanol (2 g/kg)-induced ataxia (EIA) was potentiated by nNOSI (0.1, 0.5, 1.0 ng) suggesting nNOS-mediation. Pretreatment with nNOSI (0.5, 1.0, 2.0 ng) dose-dependently antagonized nicotine (5 ng)-induced attenuation of EIA suggesting involvement of cerebellar nNOS. When nNOSI (0.5, 1.0, 2.0 ng) and nicotine (5 ng) were microinfused once daily for 5 days, followed 16 h later by ethanol, virtual absence of cross-tolerance was noted. However, when aCSF was infused instead of nNOSI, robust cross-tolerance was noted confirming mediation by cerebellar nNOS system. Overall, these results support role of the cerebellar nNOS in cross-tolerance between ethanol and nicotine.

Keywords ethanol; Rotorod; intracerebellar microinfusion; cerebellar ataxia; nNOS; nitric oxide

1. Introduction

Alcohol and nicotine are the most extensively used psychoactive substances worldwide. Whereas, there is a decline in the extent of cigarette smoking in nonalcoholic population, it has remained consistently high among alcoholics providing strong circumstantial evidence for a functional interaction between alcohol and nicotine [29]. Nicotine markedly attenuates ethanol-induced ataxia via cerebellar nAChRs because it is antagonized by hexamethonium [2]. Ethanol, on the other hand, blocks nicotine-induced memory improvement as well as antagonizes many nicotinic functions within the CNS [24]. Nicotine antagonizes several physiological functions which are impaired by ethanol [3, 21, 24, 36]. It is plausible that a similar mechanism may be responsible for antagonism by nicotine of ethanol-induced ataxia and sedation in the cerebellum.

We targeted cerebellum in our studies because of its critical role in motor and movement control and the location of nAChRs on Purkinje cells and interneurons of the cerebellar cortex. Existence of a high degree of correlation between the sensitivity of some of the cerebellar cortical neurons and ethanol has indeed been reported [23, 28]. It is plausible that individuals who exhibit low sensitivity to ataxic action of ethanol may be more likely to suffer from alcoholism [25]. Therefore, understanding the mechanism of ethanol-induced cerebellar ataxia is an area of importance for continuing investigation.

The nitric oxide and cyclic guanosine 3′,5′-monophosphate (cGMP) signaling system is involved in several neuronal functions such as long-term potentiation (LTP) in the hippocampus [18] and long-term depression (LTD) in the cerebellum [16, 19]. Several reports have highlighted the central role of nitric oxide/cGMP signaling pathway in learning and memory [5, 7]. Furthermore, stimulation of nAChRs improves memory performance in experimental animals [20] as well as in the humans [20, 34]. Nicotine also increases the hippocampal LTP, a cellular mechanism that modulates intersynaptic communication during learning and memory [15]. Fedele et al. [14] have shown that nicotine stimulates the cGMP possibly via activation of nAChR. Overall, these literature reports support a functional interaction between nicotine and nitric oxide with participation of cGMP [14]. Ethanol, on the other hand, blocks nicotine-induced memory improvement as well as antagonizes many nicotinic functions within the central nervous system [20]. Nicotine pretreatment dose-dependently prevents ethanol-induced impairment of both aerial righting reflex and performance [30]. Since the nitric oxide/cGMP pathway has been identified as a neuromediator targeted by nicotine, possibly via nAChRs, and because nicotine antagonizes some of the same

physiological functions impaired by ethanol, it is possible that a similar relationship could exist in the cerebellum [24].

Nitric oxide synthase (NOS) is highly expressed in the cerebellum [31]. Nicotine has also been reported to increase soluble cGMP production through activation of NOS, thereby, leading to an increase in the synthesis of nitric oxide [13]. We have provided strong pharmacological evidence that supports a functional relationship between cerebellar nitric oxide and ethanol-induced cerebellar ataxia [1,2]. Ethanol decreases the levels of cerebellar nitrite which were correlated with ethanol-induced cerebellar ataxia [1,2]. The evidence of a strong functional correlation between cerebellar nitric oxide and ethanol-induced cerebellar ataxia is not surprising because, as stated above, NOS is highly expressed in the cerebellum [31]. It has also been suggested that nitric oxide production plays a role in the development of tolerance to ethanol [35]. In view of these reports, it was considered relevant to study the possible involvement of cerebellar nitric oxide through neuronal nitric oxide synthase (nNOS) system in the behavioral interaction and cross-tolerance between nicotine and ethanol.

Therefore, we hypothesize that ethanol-induced cerebellar ataxia is likely modulated by nitric oxide involving nNOS system. Consequently, the hypothesis was tested to know if nNOS system participates in the (i) mediation of acute ethanol-induced cerebellar ataxia, (ii) motor behavioral interaction between nicotine and ethanol, and (iii) development of cross-tolerance between chronic nicotine and acute ethanol-induced cerebellar ataxia in CD-1 male mouse model.

2. Materials and methods

2.1. Animals

Male CD-1 mice (Charles River, Raleigh, NC, USA) weighing 22–24 g at the time of surgery and 5 to 6 weeks of age were used in the present work. For the mandatory 5-day rest and recovery following their arrival, the animals 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. The animals were fed commercial pellet food and water ad libitum except during the actual Rotorod test. The animals were brought to the laboratory for the stereotaxic survival surgery for guide cannula implantation and transferred back to animal housing in individual plastic cages with stainless steel wire lid. The East Carolina University Institutional Animal Care and Use Committee approved the use of animals for the procedures in the study under animal use protocol number AUP W125e. Laboratory facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and principles of laboratory animal care were consistent with the Declaration of Helsinki and Guide for the Care and Use of Laboratory Animals as adopted by National Institutes of Health.

2.2. Drugs

The drug solutions were either prepared on the day of behavioral experiment or one day earlier and were kept in the deep freezer at −70 °C. Chloral hydrate was prepared in sterile normal saline and injected in a volume of 0.01 mL/g body weight. Unless otherwise stated, the drugs were dissolved in the artificial cerebrospinal fluid (aCSF) containing (in 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. Nicotine as (-)-nicotine-di-l-tartrate and nNOS inhibitor, N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidine tris(trifluoroacetate), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (-)-Nicotine-di-l-tartrate was dissolved in aCSF. The ethanol solution (10% w/v) was prepared in normal saline and was always injected IP in a volume of 20 mL/kg to give a dose of 2 g/kg. For consistency in the ataxia-producing effect of ethanol, the latter was always purchased from the same vendor (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA). The chloral hydrate solution (pH 6.59) was sterilized and made pyrogen-free by filtration through 0.45 μm Millipore filtration system. The stability of solution was ensured by using the chloral hydrate solution for not beyond 24 h after its preparation.

2.3. Stereotaxic survival surgery

The animals were allowed to recover from the surgical trauma and effect of anesthetic for five days. In other words, the surgical implantation of guide cannulas was performed 6 days before the Rotorod test. Mice, anesthetized with chloral hydrate, were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The cannulas (12 mm long; 22-gauge) were targeted for direct drug infusion at site within the superficial layers of cerebellar cortex. They 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 were based on the atlas of Slotnick and Leonard [26], for guide cannula implantation 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). A previously conducted functional mapping study of the cerebellar cortex [27] led to the selection of the stereotaxic coordinates. Other details have been reported previously [10,11]. The surgery was conducted under strict aseptic conditions. In addition, each animal received a prophylactic dose of 3,000 units of Durelon (Vedco Inc., St. Joseph, MO, USA), a combination of benzathine and procaine penicillin G suspension, immediately after surgery. Each animal also received two injections of ketorolac tromethamine (Toradol 2 mg/kg,

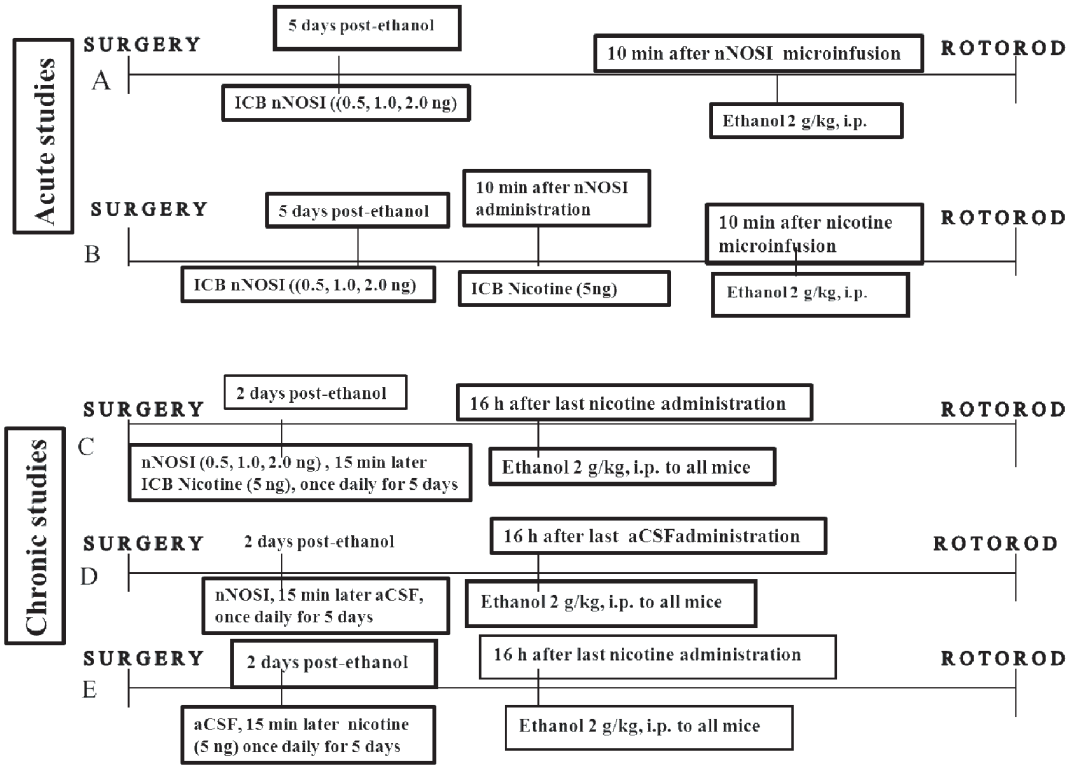

Figure 1: Protocol of acute and chronic nNOS inhibitor treatment studies.

subcutaneously; Abbott Laboratories, North Chicago, IL, USA) analgesic, first injection was made 60 min before surgery and the second 5 h post-surgically. Pre-surgically, after the administration of chloral hydrate anesthetic, bupivacaine (5 mg/kg subcutaneously) was injected at the nose clamp and at the skin site where incision was made. Currently, nearly 96 percent of animals undergo successful surgery and guide cannula implantation.

2.4. Direct intracerebellar microinfusions

The drugs used in the present study (nicotine, nNOS inhibitor) were microinfused directly into the anterior lobe of culmen via a stainless steel injector cannula (length 13 mm; 30 gauge) connected through PE-10 polyethylene tube (Clay Adams, Parsippany, NJ, USA) to a 25 μL Hamilton microsyringe (Hamilton Company USA, Reno, NV, USA). The Hamilton syringe, driven by a Model 22 Micro Injection Pump (Harvard Apparatus, South Natick, MA, USA), was used to make infusions at a regulated constant rate of 0.1 μL (100 nL)/min. The volume of infusions was kept constant throughout the present study at 100 nL. The guide cannulas were routinely cleaned with a fine tipped dental broach followed by injector cannula insertion in order to allow it to protrude 1 mm beyond the lower tip of the guide cannula. The proper delivery of the drug solution was ensured by monitoring the movement of an air bubble that was introduced in the PE-10 tubing between the drug solution and water. Other details of microinfusion have been previously reported [10].

2.5. Protocol for acute and chronic nNOS inhibitor and/or nicotine treatment

2.5.1. Acute studies

The effect of intracerebellar pretreatment with various doses of nNOS inhibitor on ethanol-induced cerebellar ataxia is shown in panel A of Figure 1. Panel B presents the protocol for the effect of intracerebellar pretreatment with various doses of nNOS inhibitor on nicotine-induced attenuation of cerebellar ataxia by acute ethanol.

2.5.2. Chronic studies

The chronic studies involved repeated intracerebellar infusion of various doses of nNOS inhibitor, followed within 15 min by nicotine administration, both drugs given once daily for five days. Sixteen hours after the last nicotine treatment, acute ethanol was injected (Panel C). Panels D and E are similar to Panel C except that in Panel D, aCSF was infused instead of nicotine and in Panel E, aCSF was administered instead of nNOS inhibitor. It was considered logical to also conduct experiments in the present study in which chronic nicotine treatment was reversed with that of ethanol. However, experiments involving chronic ethanol posed two technical/interpretational problems. First, chronic

ethanol treatment leads to the development of tolerance due to which little or no ethanol-induced ataxia is observed. Second, behavioral outcomes are similar (i.e., no ethanol-induced ataxia following acute intracerebellar infusion of nicotine or chronic ethanol treatment). Consequently, it would be impossible to interpret the absence of ethanol-induced ataxia in animals treated with chronic ethanol followed by acute nicotine infusion.

2.6. Evaluation of motor coordination by Rotorod

The experimental procedure for the Rotorod Treadmill for mice (UGO Basile, Verese, Italy) has been previously explained [11,27]. Briefly, the procedure involves pre-screening of the mice on the Rotorod, where the animals act as their own control. Immediately prior to the actual experiment, mice are placed on Rotorod 2–3 times in order to acclimatize them to the treadmill. A successful prescreening of animals requires each animal to walk on Rotorod for an arbitrarily selected time of 180 s without a fall. Motor coordination of five successfully prescreened mice was simultaneously evaluated by Rotorod treadmill that was calibrated for a fixed speed of 24 rpm [9,27]. The animals received one of the drugs (nicotine, nNOS inhibitor) or aCSF by direct intracerebellar microinfusion which was followed within 5 min by the test dose of ethanol (2 g/kg IP) to all animals. The Rotorod evaluation of animals 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 to 11:30 AM to maximally minimize the influence of diurnal variation on ethanol-induced ataxia.

2.7. Histological confirmation of microinfusions

At the conclusion of each Rotorod experiment, each animal was microinfused 100 nL Fast Green dye via the same guide cannula in order to confirm the correctness of the cannula placement and accuracy of drug microinfusion. The animals under light isoflurane anesthesia (ISOFPo; Abbott Laboratories, North Chicago, IL, USA) were euthanized by cervical dislocation followed by decapitation. The brains were exposed and cut at the mark of cannulations. The confirmation of accuracy of cannula implantation was verified by visual observation of the presence of dye in the cerebellar anterior lobe region. Consequently, the Rotorod data from those animals in which the cannulas were found to have been accurately implanted was used for analysis. In addition, the integrity of the cerebellar tissue during the postsurgical period of guide cannula implantation was also checked. Damage to the integrity of the cerebellar tissue due to implanted cannulas could possibly impact the Rotorod performance of cannulated animals. Therefore, brains from randomly selected animals were removed, frozen at $-20\,^{\circ}$C and cut into 20-μm thick coronal sections using Tissue-Tek II cryostat (Mills Laboratories, Naperville, IL, USA). After mounting, the tissue sections were air dried, stained with cresyl violet and assessed for nonspecific tissue damage due to indwelling cannulas as described previously [11, 27]. 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 were observed. The histological data also confirmed that the drug dispersion following intracerebellar microinfusion was confined to the cerebellar tissue in agreement with our previous report [22].

2.8. Statistical analyses of Rotorod data

A commercial software program (SPSS for Windows, V19.0; SPSS Inc, Chicago, IL, USA) was used for Rotorod data analysis. Data collected from Rotorod experiments was subjected to two-way analysis of variance (ANOVA) with repeated measures in order to test for any significance of interaction between treatment groups and time periods. The multivariate criterion of Wilk's lambda (λ) was used to test the effect of the drug dosage x time interaction. A Dunnett's C post hoc test was performed whenever significance was found on treatment and/or time using one-way ANOVA. A P value of $< .05$ was considered significant. Group data are expressed as means $\pm$SEM throughout the investigation.

3. Results

3.1. Effect of test dose of ethanol on ataxia

Based on a dose-response study [8], a test dose of ethanol (2 g/kg; IP) was selected and used in the present investigation. The test dose of ethanol produced significant ataxia, starting at 15 min postethanol injection until animals regained their normal motor coordination, in agreement with our previous reports [9,11]. Typically, the animals were able to walk on Rotorod for an average of 40 s at 15 min postethanol evaluation. The walking time gradually increased to an average of 65 s and 125 s at 30 min and 45 min postethanol administration, respectively. The animals generally regained their normal motor coordination by 60 min postethanol injection. A remarkable consistency in the ataxic response in aCSF + ethanol-treated (control) animals was observed as in previous studies [9,11]. Therefore, to repeat ethanol control experiment in each treatment group was unnecessary and unethical. Consequently, the same ethanol control data was used to construct aCSF + ethanol curves shown in Figures 2, 3, and 4, without compromising the overall outcome of the investigation. This was also consistent with the approved "Animal Care and Use Protocol." As stated in Section 2, all drugs used in the present investigation, except ethanol, chloral hydrate, and ketorolac (Toradol), were administered by direct intracerebellar microinfusion.

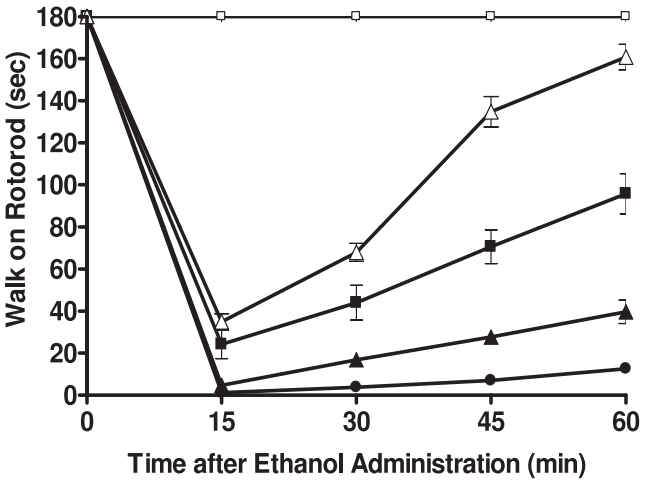

Figure 2: The accentuating effect of intracerebellar microinfusion of nNOS inhibitor (nNOSI, 0.1, 0.5, and 1.0 ng) on ethanol (2 g/kg; IP)-induced ataxia. Each point represents mean ±SEM of 10 mice. △: aCSF + ethanol; ■: nNOSI 0.1 ng + ethanol; ▲: nNOSI 0.5 ng + ethanol; ●: nNOSI 1.0 ng + ethanol; □: nNOSI 1.0 ng + saline.

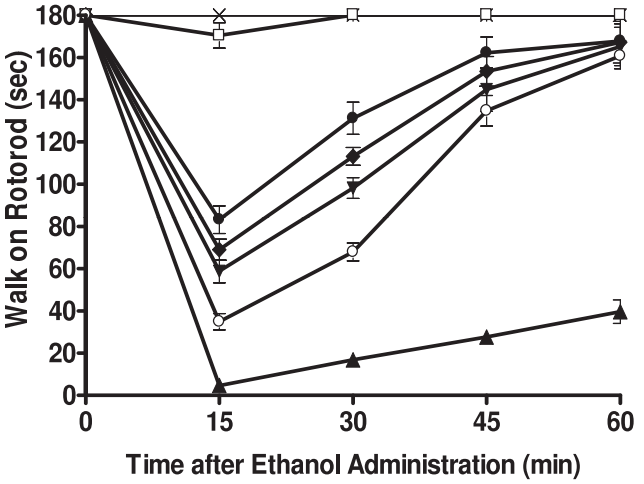

Figure 3: The antagonistic effect of intracerebellar (ICB) microinfusion of various doses of nNOS inhibitor (nNOSI, 0.5, 1.0, and 2.0 ng) on nicotine (5 ng; ICB)-induced attenuation of cerebellar ataxia following ethanol (2 g/kg; IP) administration. Each point represents mean ±SEM of 8 mice. ○: aCSF + ethanol; ▲: nNOSI 0.5 ng + ethanol; □: nicotine + ethanol; ●: nNOSI 0.5 ng + ethanol; ♦: nNOSI 1.0 ng + ethanol; ▼: nNOSI 2.0 ng + ethanol; x: nNOSI 1.0 ng + saline.

3.2. Dose-dependent accentuation of ethanol-induced ataxia by nNOS inhibitor

The potentiation of ethanol-induced cerebellar ataxia following microinfusion of various doses (0.1, 0.5, and 1.0 ng) of nNOS inhibitor in a dose-related manner is shown in Figure 2. A significant drug treatment and time interaction was observed [$F(9,60) = 21.121$, $P < .001$]. The accentuation following 0.5 ng and 1.0 ng doses of nNOS inhibitor was significant ($P < .001$) at all four postethanol evaluation periods. The animals were significantly ataxic and virtually unable to walk on Rotorod throughout the 60 min experimental period. A significant [$P < .001$] potentiation of ethanol-induced cerebellar ataxia was noted at 30, 45, and 60 min postethanol evaluation periods following 0.1 ng dose of nNOS inhibitor. When the microinfusion of the highest dose of nNOS inhibitor was followed by saline instead of ethanol, no change in the normal motor coordination was observed (Figure 2; □--□).

3.3. Nicotine-induced attenuation of cerebellar ataxia by ethanol: dose-dependent antagonism following pretreatment with nNOS inhibitor

Figure 3 shows the antagonism to the attenuating effect of nicotine on ethanol-induced cerebellar ataxia following pretreatment with various doses of nNOS inhibitor. Microinfusion of nNOS inhibitor (0.5, 1.0, and 2.0 ng) markedly ($P < .001$) decreased the ability of nicotine to attenuate ethanol-induced cerebellar ataxia in a dose-related manner at all four motor coordination evaluation time periods (Figure 3; □--□ vs. ▼--▼, ♦--♦, and ●--●). A significant drug treatment and time interaction was observed [$F(9,44) = 6.274$, $P < .001$]. However, the lowest dose (0.5 ng) of nNOS inhibitor drug produced the optimum decrease in the attenuation by nicotine of ethanol-induced cerebellar ataxia. When the highest dose (2 ng) of nNOS inhibitor was microinfused followed by saline, no change in normal motor coordination was noted.

3.4. Repeated intracerebellar microinfusion of nNOS inhibitor + nicotine: effect on acute ethanol-induced cerebellar ataxia

The effect of chronic pretreatment with nNOS inhibitor followed within 15 min by nicotine microinfusion was evaluated 16 h after the last nicotine administration, on acute ethanol-induced cerebellar ataxia. The consequence of chronic microinfusion of nicotine is the development of cross-tolerance between nicotine and ethanol because acute ethanol injected 16 h after the last nicotine microinfusion failed to produce cerebellar ataxia (Figure 4; □--□). However, when chronic nicotine microinfusion was preceded by pretreatment with various doses of nNOS inhibitor and followed by injection of acute ethanol 16 h after the last nicotine administration, significant cerebellar ataxia was observed (Figure 4; □--□ vs. ●--●, ▼--▼, and ■--■). The cross-tolerance between nicotine and ethanol was observed only partially. There was a significant drug treatment and time interaction [$F(12,61) = 35.382$,

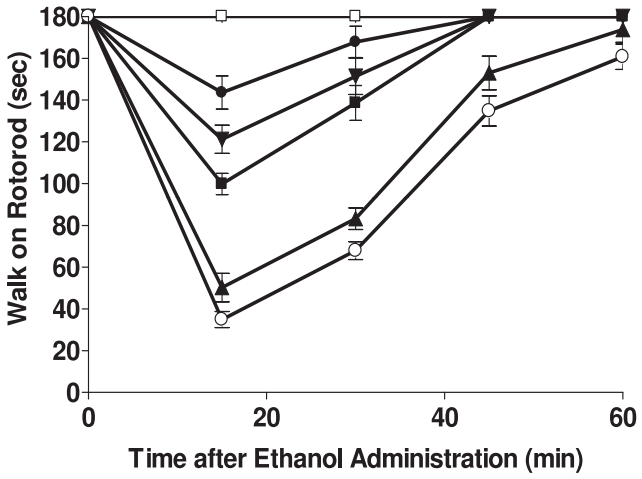

Figure 4: The effect of chronic (once daily for five days) intracerebellar microinfusion of various doses of nNOS inhibitor (nNOSI, 0.5, 1.0, and 2.0 ng) given 15 min before chronic (once daily for five days) intracerebellar nicotine (5 ng) microinfusion, on acute ethanol (2 g/kg, IP injected 16 h after the last nicotine treatment)-induced cerebellar ataxia. Each point represents mean ±SEM of 10 mice. ○: aCSF + ethanol; ■: nNOSI 0.5 ng + nicotine + ethanol; ▼: nNOSI 1.0 ng + nicotine + ethanol; ●: nNOSI 2.0 ng + nicotine + ethanol; ▲: nNOSI 0.5 ng + aCSF + ethanol; □: aCSF + nicotine + ethanol.

$P < .001$]. The chronic microinfusion of nNOS inhibitor, when followed by aCSF instead of nicotine, did not alter the ataxic effect of acute ethanol (Figure 4; ○–○ vs. ▲--▲). The dose of nNOS inhibitor (0.5 ng) produced the optimal antagonism to the development of cross-tolerance between nicotine and ethanol (Figure 4; ■--■). The antagonism was lesser with the higher nNOS inhibitor pretreatment doses.

4. Discussion

In the present investigation, the behavioral consequence of direct intracerebellar microinfusion of nNOS inhibitor was a marked dose-dependent accentuation of ethanol-induced cerebellar ataxia (Figure 2). The inhibition of cerebellar nNOS also antagonized the attenuation by nicotine of ethanol-induced cerebellar ataxia in a dose-related manner (Figure 3), which indicated an involvement of nitric oxide in the nicotine-ethanol behavioral interaction. Finally, following repeated intracerebellar nicotine treatment which preceded infusion of nNOS inhibitor, the degree of cross-tolerance between nicotine and ethanol-induced cerebellar ataxia was markedly reduced (Figure 4). This key observation of an absence of total cross-tolerance provided an interesting evidence that the cerebellar nNOS may be a participating factor in the development of functional cross-tolerance between nicotine and ethanol.

The marked accentuation of ethanol-induced cerebellar ataxia following intracerebellar pretreatment with nNOS inhibitor suggested that nNOS-mediated production of nitric oxide exerted a robust tonic mediating role (Figure 2). It also confirmed our previous hypothesis that so long the basal production of cerebellar nitric oxide remained normal or above normal, ethanol would not cause ataxia [2,35]. It is important to note that the treatment with nNOS inhibitor alone, however, produced no change in the normal motor coordination. The nNOS also appeared to be important in the functional interaction between ethanol and nicotine. We have repeatedly demonstrated that intracerebellar nicotine virtually abolishes acute ethanol-induced cerebellar ataxia [2,35]. Pretreatment with nNOS inhibitor markedly prevented the established attenuating effect of nicotine on ethanol-induced cerebellar ataxia in a dose-related manner (Figure 3). The observed reversal of attenuation by nicotine of ethanol-induced cerebellar ataxia following microinfusion of the highest dose of nNOS inhibitor suggested an involvement of nitric oxide. These results clearly implicate cerebellar nNOS in the expression of (i) ethanol-induced cerebellar ataxia and (ii) functional interaction between ethanol and nicotine.

The production of cerebellar nitric oxide has been attributed to the stimulated release of endogenous glutamate by nicotine via activation of nAChRs and subsequent activation of glutamate receptors [6]. There is a direct interaction between ethanol and glutamate-NMDA receptor in agreement with others [12] as well as non-NMDA receptor mechanisms. The ethanol-induced ataxia is markedly attenuated directly by L-glutamate and NMDA via their specific receptors because this attenuation is virtually abolished by NMDA and L-glutamate antagonists [17]. Furthermore, the antagonists of NMDA and L-glutamate markedly accentuate ethanol-induced ataxia indicating a tonic role of glutamate and NMDA [17]. Ethanol and glutamate-NMDA may also be interacting indirectly through adenosine A_1 receptor mechanisms. Activation of adenosine A_1 receptors on the glutamatergic neuronal membrane would lead to hyperpolarization and decreased release of glutamate [4]. Simultaneously, ethanol could antagonize the glutamatergic NMDA receptor and, in effect, synergize with adenosine to decrease glutamatergic neurotransmission. Under physiological conditions, the release of glutamate from mossy fibers would activate NOS-containing granule cells and parallel fibers [1]. These signaling events lead to nitric oxide-induced stimulation of GC-cGMP that suppresses firing of Purkinje neurons and GABA release. Since GABA is the primary transmitter within the Purkinje cells, its decrease results in disinhibition of deep cerebellar nuclei (main cerebellar output) explaining marked attenuation of ethanol-induced ataxia.

The NOS localized in the granule and Basket cells generates nitric oxide that diffuses to target soluble GC

in Purkinje cells [13]. Arginine is the crucial regulator of nitric oxide (NO) bioavailability and is the substrate for NO synthase (NOS). The NO has < 10 s half-life. Changes in NO concentration are indirectly related to the concentrations of its stable and nonvolatile metabolites, nitrites, and nitrates. Intracerebellar nicotine most likely activates C_6-type nAChRs located on the Purkinje dendrites and C_{10}-type α-bungarotoxin/curare sensitive receptors located on the Basket and Stellate cells. Activation of C_6-nAChRs is inhibitory to the Purkinje cells while activation of C_{10}-nAChRs is excitatory to these interneurons. However, Basket and Stellate interneurons are inhibitory to the Purkinje cells. Therefore, the intracerebellar nicotine inhibits the Purkinje cells directly as well as indirectly via C_{10}-nAChRs of the Basket and Stellate interneurons. The overall consequence of intracerebellar microinfusion of nicotine will be inhibition of Purkinje cells with decreased GABA release. Decreased GABA release, as stated above, means disinhibition of deep cerebellar nuclei and attenuation of ataxia.

The mechanism of alcohol-nicotine functional interaction has also been explained electro-physiologically [33]. Ethanol increases Golgi cell firing leading to an inhibition of granule cells due to increased GABA release at Golgi-granule cell synapse [33]. The increase in GABAergic tone by ethanol may decrease glutamatergic neurotransmission in granule cells and parallel fibers. Thus, activation of granule cells by nicotine, as explained above, most likely functionally opposes ethanol-induced increase in GABAergic neurotransmission. The participation of NO/cGMP pathway in the functional interaction between nicotine and ethanol has been reported [35]. Nicotine enhances nitric oxide production and cGMP levels via activation of nAChRs and NMDA receptors, respectively [14]. Thus, nicotine via nAChRs activation stimulates glutamate (NMDA)/NO/cGMP pathway causing decreased Purkinje cell firing [13, 14]. Overall, nicotine via nitric oxide suppresses Purkinje cell firing thereby decreasing GABA release as explained above in detail.

Finally, the results of chronic experiments (Figure 4) indicated a role of cerebellar nNOS in the functional interaction and the development of cross-tolerance between these psychoactive substances. Repeated intracerebellar nicotine infusion, when followed by acute dose of ethanol, virtually abolishes cerebellar ataxia due to the development of tolerance to acute ethanol-induced cerebellar ataxia (Figure 4). However, when the treatment protocol was modified such that the repeated microinfusion of nicotine preceded the intracerebellar infusion of nNOS inhibitor, a significant reduction in the degree of cross-tolerance was observed. In other words, the acute ethanol-induced cerebellar ataxia was not totally abolished and only a partial cross-tolerance was noted. Administration of nNOS inhibitor would be expected to result in a reduction in the production of cerebellar nitric oxide which would functionally produce an opposite effect (i.e., an increase in ethanol-induced cerebellar ataxia based on our hypothesis) [2]. Consequently, chronic intracerebellar nicotine treatment leads to the development of cross-tolerance to acute ethanol ataxia, most likely, via an increase in glutamatergic neurotransmission within the granule cells of the cerebellar cortex. Again as explained above, the increase in glutamatergic neurotransmission leads to an increase in the production of nitric oxide via nNOS. The nitric oxide thus generated diffuses to Purkinje cells where it activates GC-cGMP system. Activation of GC-cGMP is associated both with a decrease in Purkinje cells firing and GABA release, ultimately causing disinhibition of deep cerebellar nuclei with attenuation of ethanol-induced cerebellar ataxia. A considerable body of literature supports that the neuronal mechanism of acute ethanol involves blockade and potentiation of glutamatergic and GABAergic transmission, respectively [32]. The motor coordination activities are regulated by equilibrium balance between GABAergic inhibitory and glutamatergic excitatory signals operating within the cerebellar cortical Purkinje and granule cells, respectively. Any disruption within this balance can lead to loss of motor control. To help explain the underlying mechanism for the functional nicotine and ethanol interaction, nicotine and ethanol modulate excitatory and inhibitory signals in the cerebellum, respectively. The observed ethanol-induced ataxia and its attenuation by nicotine microinfusion is the net outcome of whether inhibitory or excitatory signals are dominant. Ethanol is known to potentiate GABAergic transmission by stimulating Golgi cell firing within the cerebellum [33]. The GABA release at Golgi-granule cell synapse depresses the firing of granule cells which represent the only excitatory neuronal cells within the cerebellum [32]. In the absence of adequate excitatory signals, this balance is shifted in favor of inhibitory signals producing inhibition of Purkinje cells. Therefore, deep cerebellar nuclei undergo profound inhibition resulting in ataxia. On the other hand, ethanol enhances GABAergic transmission via Golgi cells which provide negative feedback to granule cells [32]. Since granule cells are the sole excitatory cells in cerebellum, their inhibition by Golgi cells will allow inhibitory signals to take over and dominate the cerebellar cortical activity resulting in the ataxia.

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