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Involvement of the $\alpha_4\beta_2$ nicotinic receptor subtype in nicotine-induced attenuation of Δ^9-THC cerebellar ataxia: Role of cerebellar nitric oxide

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Abstract

We have recently reported that mediation of intracerebellar nicotine-induced attenuation of cerebellar Δ^9-THC ataxia was via the $\alpha_4\beta_2$ nAChR. The present study was meant to investigate the role of cerebellar nitric oxide (NO)-guanylyl cyclase (GC) signaling in the $\alpha_4\beta_2$-mediated attenuation in CD-1 male mice. Drugs were given via intracerebellar microinfusion using stereotaxically implanted guide cannulas, with ataxia evaluated by Rotorod. Intracerebellar microinfusion of SNP (sodium nitroprusside, NO donor; 15, 30, 60 pg) and SMT (*S*-methylisothiourea, inhibitor of inducible NO synthase; 70, 140, 280 fg) significantly enhanced and reduced, respectively, intracerebellar RJR-2403 (selective $\alpha_4\beta_2$ agonist)-induced attenuation of Δ^9-THC ataxia dose-dependently. Intracerebellar isoliquiritigenin (GC-activator; 1, 2, 4 pg) and ODQ (1H[1,2,4]oxadiazolo-[4,3-a] quinoxalin-1-one, GC inhibitor; 200, 400, 800 fg), significantly enhanced and reduced, respectively, intracerebellar RJR-2403-induced attenuation of Δ^9-THC ataxia dose-dependently. Further support for the role of NO was evidenced via increases in cerebellar NO_x (nitrate + nitrite) levels following microinfusion of nicotine or RJR-2403 as compared to control, whereas Δ^9-THC significantly decreased NO_x levels. "Nicotine/RJR-2403 + Δ^9-THC" treated mice had cerebellar NO_x levels significantly increased as compared to mice infused with Δ^9-THC alone. Results of the present investigation support the role of cerebellar NO–GC signaling in $\alpha_4\beta_2$ nAChR subtype-mediated attenuation of Δ^9-THC ataxia.

Keywords: Δ^9-THC; Ataxia; Nicotine; RJR-2403; $\alpha_4\beta_2$ nAChR subtype; Nitric oxide; Cerebellum

1. Introduction

Cannabis, more commonly known as marijuana, is the most widely abused illicit drug in the United States (Agosti and Levin, 2004) and most countries of the western world (Degenhardt et al., 2001). Cannabis' major psychoactive constituent is known to be Δ^9-tetrahydrocannabinol (Δ^9-THC) and is the primary reason for the "high" associated with its use (Adams and Martin, 1996). The effects of Δ^9-THC administration are quite varied, but elicit a range of effects, including altered perceptions to light and sound, euphoria, anxiolysis, as well as the loss of motor coordination and timing which suggests a strong cerebellar influence (Takahashi and Linden, 2000). Localization studies have revealed a density of CB_1 receptors located within the cerebellum (Matsuda et al., 1993), which may help explain the motor deficits, such as ataxia, catalepsy, and hypokinesia associated with Δ^9-THC/cannabinoid intoxication (Herkenham et al., 1991; Fonseca et al., 1998).

Nicotine is considered to be the primary component of tobacco smoke that establishes and maintains tobacco dependence despite the harmful consequences of cigarette smoking (Di Chiara, 2000). In addition, over five million individuals die annually from tobacco related diseases worldwide (Ezzati and Lopez, 2000). Actions of nicotine and its related agonists are mediated through binding to, and activation of, nicotinic acetylcholine receptors (nAChRs) (Picciotto et al., 2000) which elicit a profound impact on cognitive performance, locomotor activity, body temperature and pain perception (Lloyd and Williams, 2000). Electrophysiological studies have revealed the presence of a significant $\alpha_4\beta_2$ nAChR population in the cerebellum, located on the soma and dendrites of granule neurons (De Filippi et al., 2001). Behavioral actions, such as

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hyperlocomotion in rodents have been suggested to be mediated through the $\alpha_4\beta_2$ subtype (Picciotto et al., 2000).

A high frequency of association in the abuse of nicotine, in the form of tobacco, and marijuana together has been reported (Simmons and Tashkin, 1995; Watson et al., 2000). However, the specific behavioral and biochemical consequences of co-administration of Δ^9-THC and nicotine are poorly documented (Balerio et al., 2004). A single study (Valjent et al., 2002) showed that nicotine potentiates the Δ^9-THC-induced hypolocomotive effects in mice. However, recently Le Foll et al. (2006) have reported a failure to observe potentiation of the locomotor depressant effects of Δ^9-THC due to nicotine administration. This indirectly may support our recent findings of nicotine-induced attenuation of cerebellar Δ^9-THC ataxia (Smith and Dar, 2006). Additionally, in the same report we have shown the primary role of the cerebellar $\alpha_4\beta_2$ nAChR subtype, and not α_7, because attenuation of Δ^9-THC ataxia was noted following RJR-2403 (a selective $\alpha_4\beta_2$ nAChR agonist) administration. We chose these two nAChR subtypes for the study due to their significant distribution within the cerebellar cortex (Nakayama et al., 1997; De Filippi et al., 2001) and literature reports implicating the $\alpha_4\beta_2$ subtype in nicotine addiction (Tapper et al., 2004).

Nicotine via nAChRs activation is known to play a role in long-term potentiation (LTP) with involvement of nitric oxide-cGMP signaling system in both LTP (Kamisaki et al., 1994) and long-term depression (LTD) (Hartell, 2001). Nitric oxide and nitric oxide synthase are expressed in high levels in the cerebellar cortical granule cells, their axons, the climbing fibers (Vincent et al., 1998; Hartell et al., 2000), as well as stellate and basket cells (Rodrigo et al., 1994). However, the cerebellar Purkinje cells express nitric oxide-stimulated soluble guanylyl cyclase which allows for the generation, utilization, and degradation of cerebellar cGMP (Hartell et al., 2000). Finally, nicotine-induced increases in locomotor activity have been shown to be blocked by inhibitors of nitric oxide synthase (Shim et al., 2002).

Within the cerebellum, the majority of CB_1 receptors are localized on either parallel and climbing fibers which provide glutamatergic input to Purkinje cells (Szabo et al., 2004), or basket and stellate cells, which provide GABAergic input to Purkinje cells (Takahashi and Linden, 2000). Past research has elucidated that glutamatergic and GABAergic signaling are correlated with the nitric oxide pathway (Herman et al., 1995). This has been demonstrated by Levenes et al. (1998) which showed that a decrease in cerebellar LTD induction by WIN 55212–2, a CB_1 agonist, is due to the inhibition of glutamatergic neurotransmission at the parallel fiber-Purkinje cell synapse, with this inhibition mediated by the inhibition of nitric oxide synthesis. Additionally, CB_1-selective agonists, such as of CP 55940, have been shown to inhibit activation of nitric oxide synthase from primary cerebellar cultures (Hillard et al., 1999), and furthermore, the nitric oxide system has been shown to play a significant role in changes in Δ^9-THC-induced locomotor activity (Azad et al., 2001). Taken collectively, the current literature appears to suggest a functional interaction between both nicotine/RJR-2403 and Δ^9-THC involving the nitric oxide-guanylyl cyclase-cGMP signaling system. Therefore, the objective of the present study was to investigate if RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia was associated with an increase in activation of the cerebellar nitric oxide-guanylyl cyclase-cGMP pathway. Our hypothesis was that RJR-2403 would increase activation of the cerebellar nitric oxide-guanylyl cyclase-cGMP system and that administration of agents that increase cerebellar nitric oxide and guanylyl cyclase would elicit an enhancement of RJR-2403-induced attenuation of Δ^9-THC ataxia. Additionally, we believed drugs that decrease cerebellar nitric oxide and guanylyl cyclase would elicit decreases in RJR-2403-induced attenuation of Δ^9-THC ataxia. Finally, we wanted to look for a functional correlation between Rotorod behavioral studies in which Δ^9-THC produces cerebellar ataxia, and whereby RJR-2403 attenuates this ataxia, with changes in cerebellar NO_x levels following nicotine/RJR-2403 and/or Δ^9-THC microinfusion.

2. Methods

2.1. Animals

Male CD-1 mice were purchased from Charles River Labs (Raleigh, NC). The mice were 5 to 6 weeks old and weighed between 25–29 g at the time of behavioral experiments. The mice were maintained in a housing facility under controlled humidity (60–80%) and temperature (23 to 25 °C) and kept on a 12-hour light/dark cycle (lights on at 08:00 h). Mice were allowed to acclimate to their housing conditions for two days prior to stereotaxic surgery. Following the implantation of a stainless steel guide cannula for direct microinfusion into the cerebellum, each animal was housed in its own individual plastic cage. Mice had free access to water and commercial mouse chow. Each animal was used only once in the Rotorod experiment. All experiments were conducted under an Animal Use Protocol which was approved by the Animal Use and Care Committee of East Carolina University.

2.2. Stereotaxic surgery

The surgical procedure was performed under aseptic conditions and all surgical tools were sterilized via autoclaving. Under chloral hydrate (450 mg/kg, ip) anesthesia, the mouse with the skull in flat position was placed in a small stereotaxic frame (Model 900; David Kopf Instruments, Tujunga, CA). A 2-cm long mid-sagittal incision was made by sterile scalpel in order to expose the skull. Cannulation of the cerebellum was performed aseptically according to the following coordinates of Slotnick and Leonard (Slotnick and Leonard, 1995): AP −6.4 mm (from bregma); ML ±0.8 mm; DV −1.0 mm from the skull surface. The stainless steel guide cannula (22 gauge, 10 mm length; Small Parts Inc., Miami, FL) was lowered through a drilled craniotomy hole using a Masterlight® Hand Piece (Henry Schein, Port Washington, NY) into the anterior lobe region of the cerebellum. Durelon® cement (Premier Dental Products Co., Norristown, PA) was used to anchor the cannula to the skull surface. A removable stainless steel wire

plug was placed inside the guide cannula to prevent occlusion. Following surgery, each animal received 3000 units, subcutaneously, of procaine and benzathine penicillin G (Durapen®, VEDCO Inc., St. Joseph, MO) to prevent possible infection during post-surgical recovery. Each animal also received an injection of ketorolac tromethamine (Toradol®, Abbott Labs, N. Chicago, IL), subcutaneously at 2 mg/kg, for analgesia shortly after surgery and again four to six hours later. Animals were allowed to recover in their own individual cages at the Brody School of Medicine animal care facility for a minimum of five days before behavioral testing.

2.3. Drugs

The CB_1 receptor agonist Δ^9-THC was supplied free by DHHS from the NIDA Research Triangle Institute (Research Triangle Park, NC). Sodium nitroprusside (SNP); *S*-methylisothiourea (SMT); isoliquiritigenin; and ODQ (1H [1, 2, 4] oxadiazolo-[4, 3-a]quinoxalin-1-one), were purchased from Sigma Chemical Co. (St. Louis, MO). CNS selective $\alpha_4\beta_2$ nicotinic receptor agonist RJR-2403 fumarate was purchased from Tocris (Ellisville, MO). Δ^9-THC was dissolved in 100% dimethyl sulfoxide (DMSO) and all other drugs used in the present study were dissolved in artificial cerebrospinal fluid (aCSF) with the aid of a minimal volume of 100% DMSO (1%, v/v final concentration in aCSF). The composition of aCSF was the following (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).

The reagents used in the nitrite assay were as follows: 2, 3-diaminonaphthalene (DAN), D-glucose-6-phosphate disodium salt dihydrate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH; type IX), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), nitrate reductase [(NAD [P] H) from *Aspergillus niger*] and sodium nitrite. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Enzymes and cofactors were stored individually at −70 °C. The key reagents, DAN, NADPH and the standard sodium nitrite solutions were prepared fresh on the day of the NO_x (total sum of nitrite+nitrate) assay and were kept protected from light. Solutions of all other drugs were prepared before the day of the experiment and kept frozen.

2.4. Intracerebellar microinjections

All drugs administered in the present investigation were given via intracerebellar microinfusion. A Harvard Model 22 (Harvard Apparatus, Holliston, MA) microinfusion syringe pump was used for drug infusions. Drugs were microinfused through PE-10 (Clay Adams; Parsippany, NJ) polyethylene tubing fitted with a 25 μl Hamilton syringe. The sterile stainless steel injection cannula (30 gauge; 0.31 mm diameter; Small Parts Inc., Miami, FL) was fitted to the PE-10 tubing so that the total length of exposed cannula was 11 mm. This allowed for protrusion of the injector cannula 1 mm beyond the lower tip of the guide cannula. Injection cannulas were left in guide cannulas for one additional minute with the infusion pump cut off to allow for adequate diffusion of the solution. An air bubble separating drug solution and water in the tubing was monitored for continuous movement to indicate that no blockage was occurring and that the desired drug dose was administered. The intracerebellar dose of Δ^9-THC chosen for the current experiments was based on our laboratory's previous dose response studies (Dar, 2000; Smith and Dar, 2006). All experiments in the present investigation used a median dose of Δ^9-THC at 20 μg/1 μl microinfused at a rate of 1.0 μl/min. This dose of Δ^9-THC was selected is because its ability to produce significant cerebellar ataxia. However, all drugs other than Δ^9-THC used in this investigation were administered into the cerebellum at a rate of 0.1 μl/min, for a total volume of 100 nl. Sodium nitroprusside, SMT, isoliquiritigenin, and ODQ were always administered 10 min prior to the administration of RJR-2403 and/or Δ^9-THC. For those drugs (SMT and ODQ) which were expected to functionally oppose RJR-2403-induced attenuation of Δ^9-THC ataxia, the highest dose of RJR-2403 (750 ng) was used; for those drugs (SNP and isoliquiritigenin) which were expected to enhance the effects of RJR-2403, a median dose of RJR-2403 (375 ng) was used. The targeted cerebellar area was in accordance with the mouse brain atlas of Slotnick and Leonard (Slotnick and Leonard, 1995) and the Atlas of the Mouse Brain and Spinal Cord (Sidman et al., 1971).

2.5. Rotorod evaluation

Mice were evaluated for motor coordination using a standard Rotorod treadmill (Ugo Basil, Verese, Italy) set at a fixed speed of 24 rpm. As previously described, normal motor coordination was arbitrarily defined as the ability of a mouse to walk continuously on the Rotorod, without falling off, for 180 s (Dar et al., 1983). All mice were screened prior to intracerebellar microinjection in order to establish normal motor coordination, and thus, the mice served as their own control. Screening was performed the morning of the experiment typically 20 min prior to microinjection and subsequent Rotorod evaluations. Any mouse unable to walk 180 s in three attempts during screening was considered to have abnormal coordination and was excluded from the experiment. In the present investigation, all animals passed the Rotorod screening test. Motor coordination experiments were performed between 8 and 11 AM.

The Rotorod experiments were conducted five days after surgery to allow the animals to recover from the effects of the anesthetic and surgical trauma. The Rotorod evaluation times used were 10, 20, 30 and 40 min starting from the moment of Δ^9-THC intracerebellar microinjection. After evaluation on the Rotorod at each time point, the animal was returned to its original cage until the next evaluation time. Each treatment group consisted of ten mice. Since only five mice can be evaluated on the Rotorod at one time, at least two Rotorod experiments were conducted for a single dose of a drug. Results are expressed in seconds the animal walked on the Rotorod so that the longer an animal walks on the Rotorod, the lesser the motor incoordination and *vice versa.* Accentuation or attenuation of cerebellar Δ^9-THC ataxia by other drugs is thus indicated by either a decrease or an increase, respectively, in the time period the animals walked on the Rotorod.

2.6. Histology

In order to confirm the accuracy of drug microinjections, mice were microinjected with 100 nl Fast Green dye at the end of each Rotorod experiment. Only mice verified to have correct cannula placement were included in data analysis. The cannulation success rate in the present study was 100%. Representative histologic photomicrographs (not shown) were examined which demonstrated minimal variation between and within groups and treatments in both the drug dispersion sites of the microinfusions and the extent of tissue damage due to cannula implantation. Drug dispersion following intracerebellar drug microinfusion remains confined to the cerebellar tissues because the dispersion of drugs microinfused directly into the brain areas was confined to tissues immediately surrounding the microinfusion site (Zhi-Hong and Dar, 1996).

2.7. Sample preparation

It has been previously reported that freezing brain tissue samples results in interference in the NO_x assay (Daiber et al., 2003). Therefore, all assays were performed on the same day when tissue samples were prepared following animal euthanasia. Mice were euthanized by cervical dislocation and decapitation 10 min post-Δ^9-THC or DMSO microinfusion. This time point for animal euthanasia was chosen because peak Δ^9-THC ataxia was observed within this post-Δ^9-THC microinfusion time interval. Brains were carefully removed and placed on a cold plastic dish. The assay was conducted via tissue homogenization, where the tissue was cut out around the drug microinfusion site in the cerebellum. After removal of the brain from the skull, the guide cannula mark was used as a landmark for dissecting out cerebellar tissue. Using the tip of a RIB-BACK® carbon-steel surgical blade #11 (Bard-Parker® Becton Dickinson AcuteCare, Franklin Lakes, New Jersey), tissue was dissected as close and as deep to the guide cannula mark as possible. Upon successive experiments and practice, the dissection of the cerebellar tissue was standardized by maintaining tissue weight between approximately 15–20 mg. The dissected tissue was immediately frozen in liquid nitrogen. The tissues from two animals were pooled to increase the sample volume and NO_x concentration. The frozen pooled tissue sample was added to a tarred plastic homogenizing tube and weighed to obtain net tissue weight. Seven volumes of 20 mM Tris/10 mM EDTA (pH 7.4) buffer was added and the tissue sample was homogenized twice at setting six for 10 s using a tissue homogenizer (Polytron Model PT 10/35, Brickman Instruments, Westbury, NY). All samples were subsequently centrifuged at 4000 ×*g* for 20 min. The supernatant was then filtered through a 10,000 MW cut off microcentrifuge filters (Microcon® YM-10, Amicon® Bioseparations, Millipore Co., Bedford, MA) by centrifugation at 16,000 ×*g* for 60 min at 4 °C to remove possible interference from other proteins (Fernandez-Cancio and Fernandez-Vitos, 2001). A colorless filtrate was obtained after filtration.

2.8. Measurement of cerebellar nitric oxide

Changes in nitric oxide concentrations were monitored by measurement of nitric oxide metabolic breakdown products, nitrite and nitrate (NO_x = total sum of nitrite + nitrate). The assay is based on the measurement of NO_x that permits indirect determination of the amount of actual nitric oxide present in tissue samples. NO_x was measured in the present investigation by a modification of the fluorometric assay in which the reaction is based around the 2, 3-diaminonaphthalene (DAN) reagent (Rao et al., 1998). The DAN reagent displays a 50-fold greater sensitivity (nM range) compared to the Griess and most other methods (Misko et al., 1993).

2.9. Nitrite assay

Measurement of NO_x concentration in the cerebellar tissue samples involved a two-step reaction procedure. First, the tissue nitrate was converted to nitrite by an enzymatic reaction with NADPH-dependent nitrate reductase. Second, the reaction with the DAN reagent was followed by the measurement of total nitrite present in the sample. The reaction between tissue nitrite and DAN yielded the fluorescent 2, 3-naphthotrazole product which indirectly was the basis for the quantification of total tissue nitrite. NADPH higher than a concentration of 3 μM reduces the sensitivity of the fluorometric assay (Rao et al., 1998). Therefore, the optimal NADPH concentration of 3 μM was maintained by utilizing the NADPH regenerating system comprised of glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PDH). The DAN reaction was carried out with the filtrate of cerebellar tissue homogenates. The actual assay, conducted in triplicate, involved pipeting 100 μl of sample mixture into a separate well in a white 96-well flat bottom plate (Costar, Corning Inc., Corning, NY). Each sample mixture consisted of 20 μl of G-6-P 750 μM, 20 μl of G-6-PDH 48 mU, 20 μl of NADPH 3 μM, 20 μl of nitrate reductase 30 mU, and 20 μl of ultra-filtrate sample in a 20 mM Tris/10 mM EDTA buffer pH 7.4. Samples were incubated for 90 min at 25 °C. 100 μl each of freshly prepared sodium nitrite standard solutions (50–1000 pmol) were transferred into separate wells. At the end of the incubation, 30 μl of DAN solution (0.05 mg/ml in 0.62 N HCl) was added to each well and incubated in the dark for an additional 10 min. The reaction was stopped by adding 30 μl of 1.4 N of NaOH. The intensity of the fluorescent signal was immediately measured by a fluorometer (FL600 Microplate Reader; Bio-Tek Instruments, Winooski, VT) at λ_{ex}=360, λ_{em}=460. Concentration of NO_x in each sample was calculated from a standard curve prepared by plotting the Fluorescent Units (FU) with sodium nitrite concentration in pmol using KC4 software (Bio-Tek Instruments, Winooski, VT) for linear regression and concentration analysis of the data. Results were expressed in pmol/mg fresh tissue consistent with others (Seo and Rivier, 2003). Total NO_x present in each sample was calculated as follows: total NO_x = [NO_x in sample (pmol) × total volume added (μl)/20 μl] / mg tissue.

2.10. Statistical analysis

Rotorod data was analyzed by a two-way repeated measure analysis of variance (ANOVA) in order to evaluate the effect of various drug doses and time on Rotorod motor coordination using the multivariate criterion of Wilk's lambda (Λ). Significance in drug versus time interaction was evaluated with a one-way ANOVA. A Dunnett's C post hoc test was performed whenever significance was found on treatment and/or time. A p-value <0.05 was taken as the level of significance in all statistical tests. Statistical analyses were performed using SPSS for Windows, version 13.0. Area under the curve (AUC) analysis was performed using GraphPad Prism 4.0©. Data comparing NO_x levels was analyzed by one-way analysis of variance (ANOVA) and Bonferroni post hoc analysis. A level of $p<0.05$ was considered statistically significant.

3. Results

Cerebellar ataxia due to Δ^9-THC was quick in onset; a maximum ataxic response was evident 10 min post-Δ^9-THC microinfusion. Ataxia continued to diminish over the next 30 min, and an almost complete return to normal motor coordination was seen by 40 min post Δ^9-THC administration. Fig. 1A shows the effect of intracerebellar SNP (sodium nitroprusside; 15, 30, 60 pg), a nitric oxide donor, on RJR-2403 (375 ng)-induced attenuation of cerebellar Δ^9-THC ataxia. A significant drug treatment and time interaction was observed ($F_{18,159}=9.68$; $p<0.0001$). The attenuation of cerebellar Δ^9-THC ataxia following microinfusion of RJR-2403 was further enhanced by pretreatment with SNP in a dose-dependent manner. Sodium nitroprusside pretreatment (30, 60 pg) significantly ($p<0.01$) enhanced RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia at 10, 20, and 30 min post Δ^9-THC microinfusion intervals as compared to the "RJR-2403 + Δ^9-THC" treatment. Sodium nitroprusside given alone ("SNP 60 pg + Δ^9-THC") produced significant ($p<0.01$) attenuation of cerebellar Δ^9-THC ataxia at 10, 20 and 30 min post Δ^9-THC microinfusion intervals as compared to "aCSF + Δ^9-THC" control.

Animals who received pretreatment of 60 pg SNP regained 100% of their normal motor coordination by 20 min post Δ^9-THC administration, and mice receiving 30 pg SNP regained full normal motor function by 30 min post Δ^9-THC administration as compared to 40 min for "RJR-2403 + Δ^9-THC" treated animals. Mice treated with SNP (15 pg) did not regain full normal motor coordination until 40 min post Δ^9-THC microinfusion, the same as "RJR-2403 + Δ^9-THC" treated animals. No change in motor coordination was seen when "SNP 60 pg + RJR-2403" treatment was followed by DMSO. Additionally, microinfusion of SNP alone at its highest dose, 60 pg, when followed by DMSO had no impact on normal motor coordination (data not shown). Even though SNP, when infused alone with Δ^9-THC significantly attenuated Δ^9-THC ataxia, the effect of SNP on RJR-2403-induced attenuation of Δ^9-THC ataxia was relatively much stronger and quicker in onset. Fig. 1B shows the Area Under the Curve (AUC) of the same treatment groups as in Fig. 1A. Area Under the Curve is an additional quantitative tool that can be used to look at the total level of ataxia present in different treatment groups. As the dose of SNP increased in the presence of RJR-2403 and Δ^9-THC, the total AUC decreased in a dose-related manner consistent with a decrease in cerebellar ataxia.

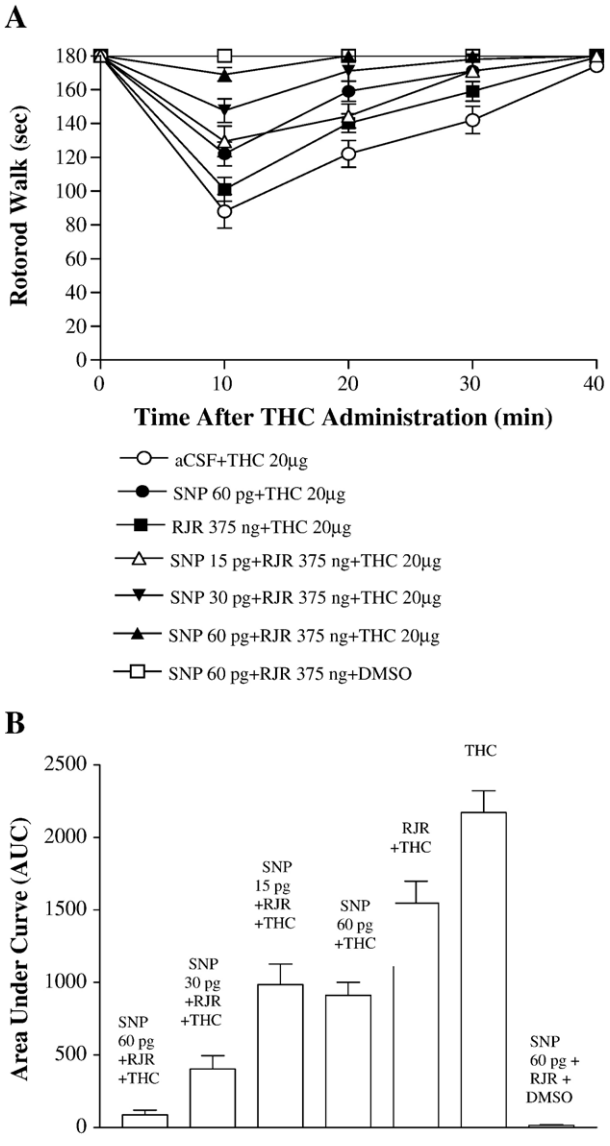

Fig. 1. A: effect of acute intracerebellar microinfusion of various doses (15, 30, 60 pg) of sodium nitroprusside (SNP) on the attenuation of Δ^9-THC (20 µg) cerebellar ataxia by intracerebellar RJR-2403 (375 ng) in mice. Each point represents the mean ± S.E. of 10 mice. B: area under the curve (AUC) from the same treatment groups presented in A which are mentioned above the corresponding bar.

Fig. 2A illustrates the effect of intracerebellar SMT (70, 140, 280 fg), on RJR-2403 (750 ng)-induced attenuation of cerebellar Δ^9-THC ataxia. A significant drug treatment and time interaction was observed ($F_{15,130}=33.94$; $p<0.0001$). *S*-methylisothiourea (70 fg) significantly ($p<0.01$) antagonized RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia at 10 and 20 min post Δ^9-THC microinfusion intervals as compared to "RJR + Δ^9-THC". *S*-methylisothiourea (140 fg) significantly inhibited RJR-2403-induced attenuation of cerebellar Δ^9-THC

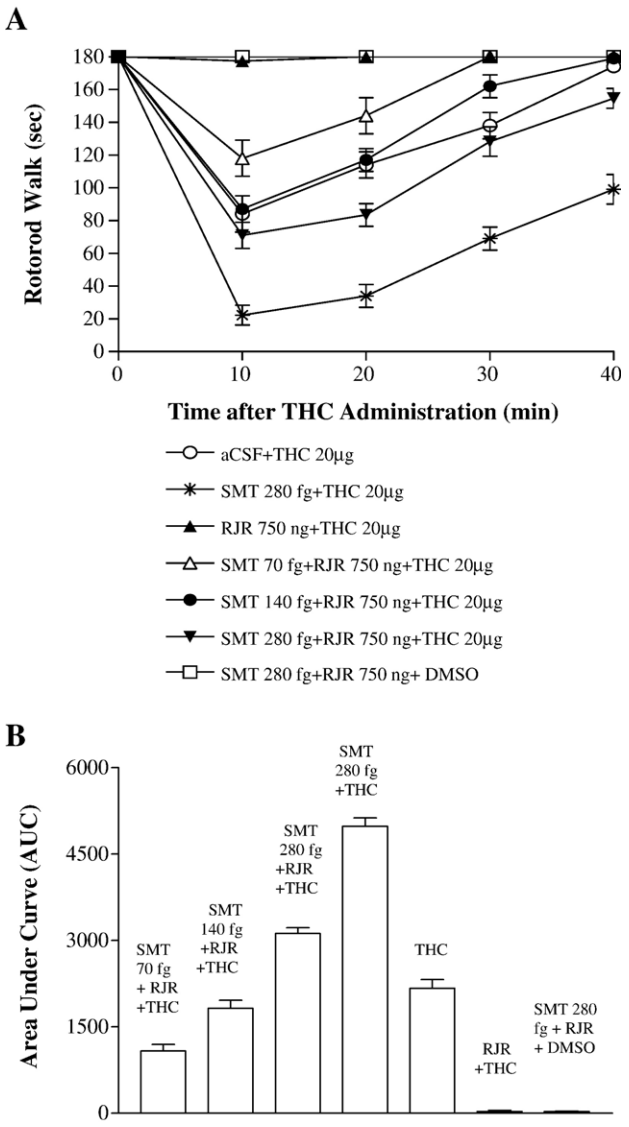

Fig. 2. A: effect of intracerebellar microinfusion of various doses (70, 140, 280 fg) of *S*-methylisothiourea (SMT; inducible nitric oxide synthase inhibitor) on the attenuation of intracerebellar Δ^9-THC (20 µg) ataxia by ICB RJR-2403 (750 ng) in mice. Each point represents the mean ± SE of 10 mice. B: area under the curve (AUC) from the same treatment groups presented in A which are mentioned above the corresponding bar.

ataxia at 10 ($p<0.01$), 20 ($p<0.01$) and 30 ($p<0.05$) min post Δ^9-THC administration times. The highest dose of SMT (280 fg) significantly ($p<0.01$) antagonized RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia at all time intervals. There was a markedly prolonged motor impairment in both treatment groups which received 280 fg of SMT, resulting in a lack of full recovery of motor coordination by the usual 40 min post Δ^9-THC infusion. Microinfusion of "SMT 280 fg + RJR-2403" when followed by DMSO did not alter normal motor coordination. Additionally, microinfusion of SMT or RJR-2403 alone at their highest dose, 280 fg or 750 ng, respectively, when followed by DMSO had no impact on normal motor coordination (data not shown). Fig. 2B shows that as the dose of SMT increased in the presence of RJR-2403 and Δ^9-THC, the AUC, and thus the level of cerebellar ataxia, also increased.

Fig. 3A shows the effects of intracerebellar isoliquiritigenin (1, 2, 4 pg), an activator of guanylyl cyclase, on RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia. A significant drug treatment and time interaction was observed ($F_{18,173}=15.26$; $p<0.0001$). The attenuation of cerebellar Δ^9-THC ataxia following microinfusion of RJR-2403 was further enhanced by pretreatment with isoliquiritigenin in a dose-dependent manner. Isoliquiritigenin pretreatment (2, 4 pg) significantly ($p<0.01$) enhanced RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia at 10, 20, and 30 min post Δ^9-THC microinfusion intervals as compared to the "RJR-2403 + Δ^9-THC" treatment. Isoliquiritigenin given alone ("ISO 4 pg + Δ^9-THC") produced

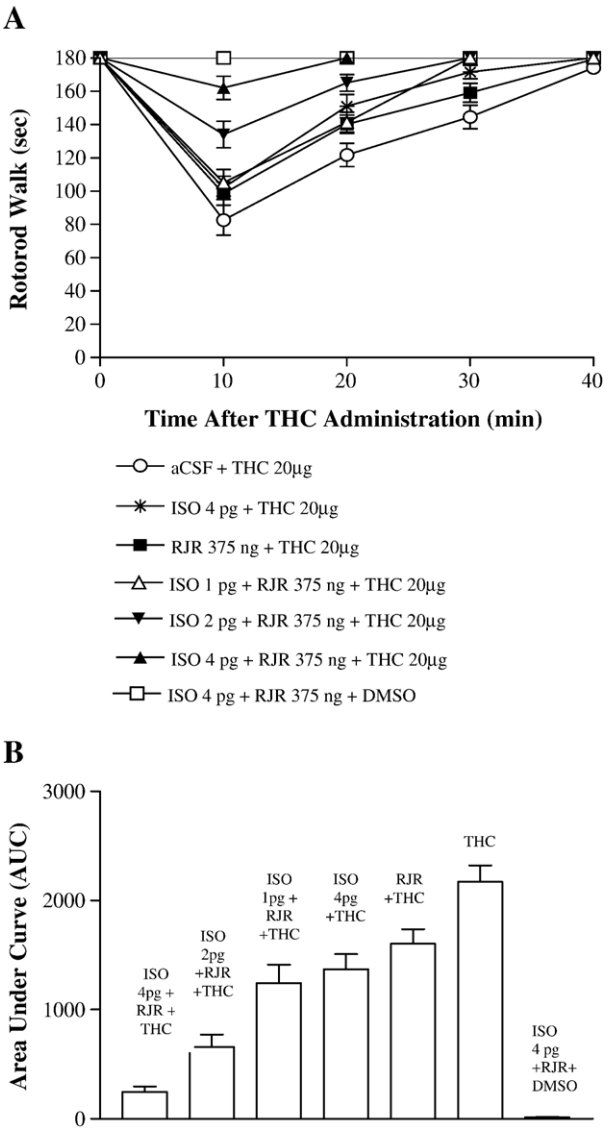

Fig. 3. A: effect of intracerebellar microinfusion of various doses (1, 2, 4 pg) of isoliquirtigenin (ISO, activator of soluble guanylyl cyclase) on the attenuation of acute Δ^9-THC (20 µg) ataxia by intracerebellar RJR-2403 (375 ng) in mice. Each point represents the mean ± SE of 10 mice. B: area under the curve (AUC) from the same treatment groups presented in A which are mentioned above the corresponding bar.

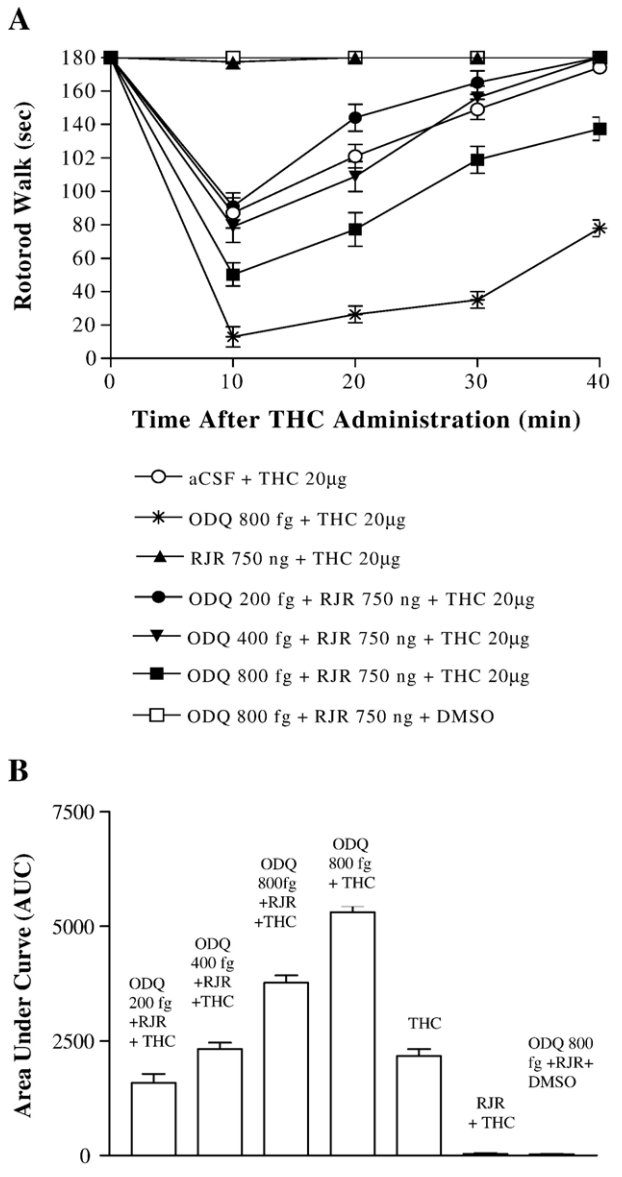

Fig. 4. A: effect of intracerebellar microinfusion of various doses (200, 400, 800 fg) of ODQ (soluble guanylyl cyclase inhibitor) on the attenuation of intracerebellar Δ^9-THC (20 µg) ataxia by intracerebellar RJR-2403 (750 ng) in mice. Each point represents the mean±SE of 10 mice. B: area under the curve (AUC) from the same treatment groups presented in A which are mentioned above the corresponding bar.

significant ($p<0.05$) attenuation of cerebellar Δ^9-THC ataxia at 10, 20 and 30 min post-Δ^9-THC microinfusion intervals as compared to "aCSF+Δ^9-THC" control. All animals receiving isoliquiritigenin pretreatment prior to RJR-2403 and Δ^9-THC regained their normal motor coordination by 30 min post Δ^9-THC administration, as compared to 40 min for the "RJR-2403+Δ^9-THC" treatment. No change in normal motor coordination was noted when "ISO 4 pg+RJR-2403" treatment was followed by DMSO microinfusion. Additionally, microinfusion of isoliquiritigenin at its highest dose, 4 pg, followed by DMSO had no impact on normal motor coordination (data not shown). Even though isoliquiritigenin, when microinfused alone with Δ^9-THC significantly attenuated cerebellar Δ^9-THC ataxia, the effect of isoliquiritigenin on RJR-2403-induced attenuation of Δ^9-THC ataxia was relatively much stronger and quicker in onset. Fig. 3B shows that as the dose of isoliquiritigenin increases in the presence of RJR-2403, the AUC conversely decreases, with the highest dose of isoliquiritigenin, 4 pg, yielding the smallest AUC and the biggest decrease in cerebellar ataxia.

Fig. 4A illustrates the effect of intracerebellar ODQ (200, 400, 800 fg), a nitric oxide-sensitive inhibitor of guanylyl cyclase, on RJR-2403 (750 ng)-induced attenuation of cerebellar Δ^9-THC ataxia. A significant drug treatment and time interaction was observed ($F_{18,158}=26.14$; $p<0.0001$). ODQ (200, 400 fg) significantly antagonized RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia at 10 ($p<0.01$), 20 ($p<0.01$), and 30 ($p<0.05$) min post Δ^9-THC microinfusion intervals as compared to the "RJR-2403+Δ^9-THC" treatment. The highest dose of ODQ (800 fg) significantly ($p<0.01$) antagonized RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia at all time intervals. Pretreatment with ODQ at all three doses resulted in a failure of the mice to regain normal motor function by 40 min post Δ^9-THC infusion. Microinfusion of "ODQ 800 fg+RJR-2403" when followed by DMSO did not alter normal motor coordination. Additionally, microinfusion of ODQ at its highest dose, 800 fg, followed by DMSO had no impact on normal motor coordination (data not shown). Fig. 4B shows that as the dose of ODQ was increased in the presence of RJR-2403 and Δ^9-THC, there was a resultant increase in the AUC corresponding with an increase in cerebellar ataxia.

Fig. 5 shows the effect of intracerebellar microinfusion of nicotine (5 ng) and RJR-2403 (750 ng) on the Δ^9-THC-induced decrease in cerebellar NO_x levels in mouse cerebellar tissue using the fluorometric DAN method. One-way ANOVA revealed a significant difference between drug treated and control groups ($F_{5,59}=16.20$, $p<0.0001$). Contralateral cerebellar brain tissue served as control. Δ^9-THC treatment significantly ($p<0.03$) decreased NO_x levels by 24% as compared to contralateral control, while both nicotine and RJR-2403 significantly ($p<0.01$) increased cerebellar NO_x levels 30% and 25%,

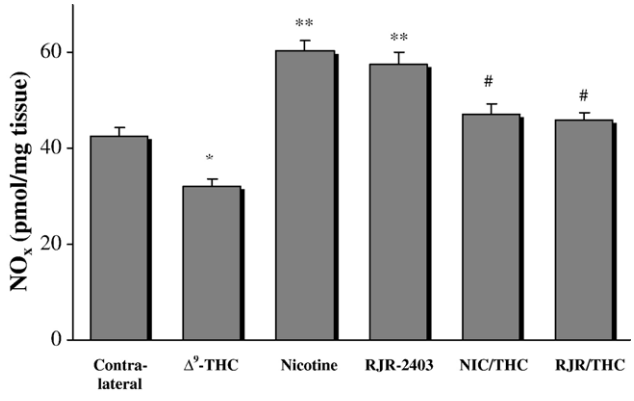

Fig. 5. Effect of acute intracerebellar Δ^9-THC (20 µg), nicotine (5 ng), RJR-2403 (750 ng), "nicotine+Δ^9-THC", or "RJR-2403+Δ^9-THC" on cerebellar NO_x (total sum of nitrite+nitrate) levels, using the fluorometric DAN (2, 3, diaminonapthalene) method. Contralateral cerebellar tissue served as control. Each bar represents the mean±S.E. of at least 10 mice. *$p<0.03$ from control, **$p<0.01$ from control, $^{\#}p<0.01$ from Δ^9-THC treatment.

respectively, as compared to contralateral control. Although Δ^9-THC significantly decreased cerebellar NO_x levels, pretreatment with either nicotine or RJR-2403 significantly ($p<0.01$) antagonized the Δ^9-THC-induced decrease in cerebellar NO_x levels by 32% and 28%, respectively.

4. Discussion

Results of the present investigation suggest a functional involvement of the nitric oxide-guanylyl cyclase system in the behavioral interaction between the cerebellar $\alpha_4\beta_2$ nAChR and Δ^9-THC because drugs that enhanced or reduced nitric oxide availability further increased or decreased, respectively, RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia. Similarly, drugs that activated or inhibited guanylyl cyclase resulted in a further increase or decrease, respectively, of cerebellar Δ^9-THC ataxia. It should be noted that administration of the highest dose of RJR-2403 (750 ng), when followed by DMSO, and not Δ^9-THC, exerted no influence on animal motor coordination. This lack of effect of RJR-2403 alone on motor function may suggest relative specificity of the RJR-2403-Δ^9-THC interaction.

We hypothesized that the behavioral interaction between RJR-2403 and Δ^9-THC was primarily mediated by the $\alpha_4\beta_2$ nAChR subtype and involved participation of the cerebellar nitric oxide-guanylyl cyclase-cGMP signaling system. Results of the Rotorod data presented here provide strong evidence that supports our hypothesis. Sodium nitroprusside, a nitric oxide donor, and SMT, an inhibitor of inducible nitric oxide synthase, enhanced and decreased, respectively, RJR-2403-induced attenuation of Δ^9-THC ataxia which suggested a functional role of nitric oxide in the interactions between RJR-2403 and Δ^9-THC. Additionally, the results also indicated a tonic participation of nitric oxide in the attenuation of cerebellar Δ^9-THC ataxia because both SNP and SMT, when microinfused with just Δ^9-THC, significantly attenuated and accentuated, respectively, cerebellar Δ^9-THC ataxia. These results strengthened the hypothesis that cerebellar nitric oxide mediates RJR-2403-induced attenuation of Δ^9-THC ataxia.

Once the role of nitric oxide in the functional interaction between RJR-2403 and Δ^9-THC was evident, the observation that isoliquiritigenin, a guanylyl cyclase activator, and ODQ, a GC inhibitor, enhances and attenuates, respectively, RJR-2403-induced attenuation of Δ^9-THC ataxia suggested a functional role of GC in the interactions between RJR-2403 and Δ^9-THC. The results also indicate a tonic participation of guanylyl cyclase in the attenuation of cerebellar Δ^9-THC ataxia because both isoliquiritigenin and ODQ, when microinfused with just Δ^9-THC, significantly attenuated and accentuated, respectively, cerebellar Δ^9-THC ataxia. Taken collectively, the isoliquiritigenin and ODQ data together with the SNP and SMT data supports our hypothesis that the functional interactions between RJR-2403 and Δ^9-THC involved participation of the cerebellar nitric oxide-guanylyl cyclase-cGMP system.

Additional support to our hypothesis was obtained from the results of the cerebellar NO_x (total sum of nitrite and nitrate) assays. Results from the assays revealed that RJR-2403 and nicotine, when given alone, significantly increased cerebellar NO_x levels compared to contralateral cerebellar control. This was in agreement with others (Pogun et al., 2000; Smith et al., 1998) who have shown increases in CNS levels of nitric oxide production due to nicotine administration. Conversely, Δ^9-THC administration significantly decreased cerebellar NO_x levels as compared to contralateral cerebellar control, which was also in general agreement with others (Levenes et al., 1998; Hillard et al., 1999). When either RJR-2403 or nicotine was given as intracerebellar pretreatment followed by Δ^9-THC, significant antagonism to the Δ^9-THC-induced decrease in cerebellar NO_x levels was noted. Overall, these findings demonstrate a functional correlation between the behavioral experiments in which RJR-2403 attenuated Δ^9-THC-induced ataxia and the observed changes in cerebellar NO_x levels. Cerebellar ataxia due to Δ^9-THC was consistent with Δ^9-THC-induced decreases in cerebellar NO_x. Similarly, RJR-2403-induced attenuation of Δ^9-THC ataxia was consistent with RJR-2403-induced increases in cerebellar NO_x levels. The results of the NO_x assays when compared with the Rotorod data indicate that RJR-2403 overcomes the decrease in nitric oxide production following intracerebellar Δ^9-THC microinfusion by stimulating cerebellar nitric oxide. This demonstrates that the Δ^9-THC-induced decrease in cerebellar nitric oxide may be the basis for the expression of cerebellar Δ^9-THC ataxia.

The SNP and SMT behavioral data also functionally correlate with the biochemical NO_x assay data because the changes (decrease and increase) in cerebellar NO_x due to Δ^9-THC or RJR-2403 administration, respectively, were functionally associated with Δ^9-THC ataxia and its attenuation due to RJR-2403. SNP is a nitric oxide donor which would be expected to increase cerebellar nitric oxide levels, and was shown to enhance RJR-2403-induced attenuation of Δ^9-THC. We have shown that a decrease in NO_x levels due to Δ^9-THC is associated with Δ^9-THC ataxia; therefore, the observed attenuation of Δ^9-THC ataxia by SNP would logically be due to antagonism of the Δ^9-THC-induced decrease in cerebellar NO_x by an increase in nitric oxide production via SNP. Similarly, pretreatment with SNP may have accentuated RJR-2403-induced attenuation of Δ^9-THC ataxia by supplementing the RJR-2403-induced increase in nitric oxide levels which would compensate and/or overcome the reduction in cerebellar NO_x levels due to Δ^9-THC, even when a median dose of RJR-2403 was given. Conversely, SMT markedly accentuates Δ^9-THC ataxia most likely by producing an additional decrease in cerebellar nitric oxide production corresponding with a further increase in cerebellar ataxia as noted on the Rotorod. The synergism between SMT and Δ^9-THC to promote an additive decrease in cerebellar nitric oxide production correlates since Δ^9-THC has been shown to inhibit the induction of iNOS alone (Jeon et al., 1996). The antagonism of RJR-2403's effect on nitric oxide production due to SMT microinfusion most likely was the basis of the decreased ability of RJR-2403 to attenuate Δ^9-THC ataxia in the presence of SMT.

The overall results of the present investigation, therefore, show a functional role of cerebellar nitric oxide in the behavioral

interactions between RJR-2403 and Δ^9-THC. Since RJR-2403, acting through the $\alpha_4\beta_2$ nAChR, attenuates cerebellar Δ^9-THC ataxia (Smith and Dar, 2006), and antagonizes Δ^9-THC-induced decreases in cerebellar NO_x levels, it may indicate possible mediation by the cerebellar nAChR via participation of the nitric oxide-guanylyl cyclase-cGMP system. It has been shown previously (Fedele et al., 1998) that nAChR activation via nicotine accentuates central glutamate neurotransmission and is coupled with the production of nitric oxide and cGMP which may ultimately be the basis for many cellular events. Within the cerebellum, the production of nitric oxide and subsequent increase in cerebellar cGMP levels is due to the stimulation of glutamate receptors with a resultant increase in the release of endogenous glutamate (Reno et al., 2004; Fedele and Raiteri, 1999). In addition, nitric oxide synthase has been shown to be localized in significant quantities in cerebellar granule and basket cells (Hillard et al., 1999). When these cells are stimulated, the nitric oxide produced is then able to freely diffuse to target soluble guanylyl cyclase known to be located within Purkinje cells causing an increase in cGMP (Fedele et al., 1998; Hartell et al., 2000). These cerebellar signaling events producing increases in cerebellar glutamatergic signaling would increase GABAergic neurotransmission onto Purkinje cells, thereby opposing the Δ^9-THC-induced decrease of GABAergic neurotransmission onto Purkinje cells. This would ultimately result in a decrease of Purkinje cell GABAergic neurotransmission (De la Garza et al., 1987; Fedele et al., 1998). This decrease in GABAergic transmission from Purkinje cell terminals due to RJR-2403 administration would ultimately act to disinhibit the deep cerebellar nuclei (DCN). DCN disinhibition would be expressed in the form of antagonism of both a decreased cerebellar output, which has been associated with motor dysfunction, and ataxia due to Δ^9-THC (Patel and Hillard, 2001). In other words, RJR-2403 would act to disinhibit the deep cerebellar nuclei via decreasing Purkinje cell GABAergic transmission, and thus, antagonize cerebellar Δ^9-THC-induced ataxia.

The cerebellar ataxia due to Δ^9-THC administration could be due to an enhanced decrease in nitric oxide synthase activation with a consequent decrease in cerebellar nitric oxide concentration in granule and basket cells (Hillard et al., 1999), with a concurrent decrease in GABAergic transmission between basket cell axon terminals and Purkinje cells (Patel and Hillard, 2001). Thus, this decrease in GABAergic neurotransmission at the basket-Purkinje cell synapse would lower the threshold for Purkinje cell firing thereby causing an increase in Purkinje cell GABAergic neurotransmission resulting in inhibition of the deep cerebellar nuclei (Takahashi and Linden, 2000). The inhibition of the DCN would decrease the total cerebellar output with a resultant development of cerebellar dysfunction and ataxia (Patel and Hillard, 2001). Our overall findings support our hypothesis that RJR-2403-induced attenuation of cerebellar Δ^9-THC ataxia via the cerebellar $\alpha_4\beta_2$ nAChR involves participation of the nitric oxide-guanylyl cyclase-cGMP pathway, with a functional correlation between behavioral results and cerebellar NO_x levels.

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