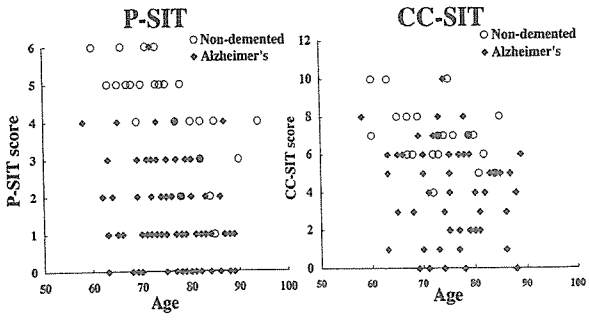

Figure 2. Correlations between age and Smell Identification Test (P-SIT, CC-SIT) scores in the non-demented elderly subjects (open circle) and in patients with Alzheimer's disease (closed square)

$\rho = -0.34, p = 0.13$; AD group: $\rho = -0.18, p = 0.18$) (Figure 2).

Correlations of the MMSE scores with the two olfactory test scores in the AD group are compared in Figure 3. The coefficient of correlation between the MMSE scores and the scores of the P-SIT was higher ($\rho = 0.57$, $p < 0.001$) than that between the MMSE scores and the scores of the CC-SIT ($\rho = 0.37$, $p < 0.01$) (Figure 3). Simple regression analyses indicated that the P-SIT score predicted MMSE score with a higher coefficient of determination (adjusted $R^2 = 0.33$) than did the CC-SIT (adjusted $R^2 = 0.11$).

ROC analyses for both olfactory tests indicated that the P-SIT discriminated AD group from elderly controls with higher sensitivity and specificity than the CC-SIT in varying cut-off scores. Az (area under the ROC curve) values for both tests were 0.993 (P-SIT) and 0.878 (CC-SIT) respectively (Figure 4). Based on the results of the ROC analyses, optimal cut-off scores for both tests (P-SIT: < or = 3, CC-SIT: < or = 5) were chosen for chi square analysis.

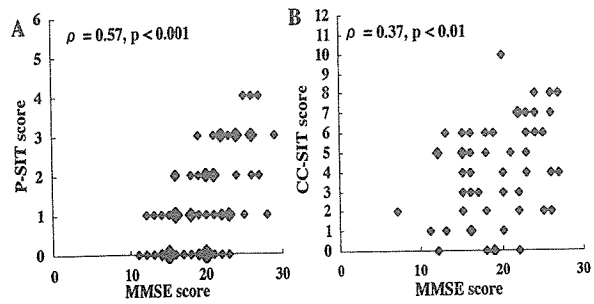

Figure 3. Scatterplots demonstrating relationship between MMSE score and (A) Picture-based Smell Identification Test (P-SIT) score and (B) Cross-Cultural Smell Identification Test (P-SIT) score and (B) Cross-Cultural Smell Identification Test (CC-SIT) score in the AD group. Range of scores: P-SIT: 0–4; CC-SIT: 0–10. Higher scores reflect better olfactory identification

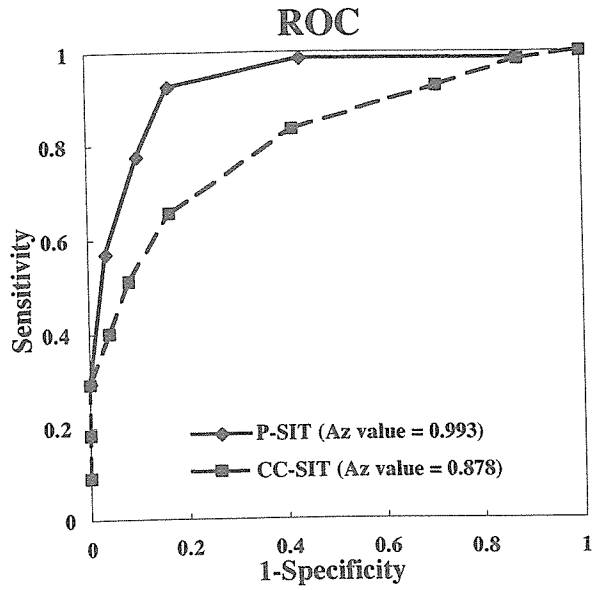

Figure 4. Receiver operating curve (ROC) analyses of the smell identification tests (P-SIT and CC-SIT) for discriminating AD patients from elderly controls

The result showed that the P-SIT discriminated AD group from elderly controls with high classification parameters (sensitivity; 0.94, specificity; 0.81, positive predictive value; 0.93, negative predictive value; 0.83, $p(\chi_0^2) < 0.0001$). Meanwhile, those parameters were lower in the CC-SIT (sensitivity; 0.90, specificity; 0.51, positive predictive value; 0.65, negative predictive value; 0.83, $p(\chi_0^2) < 0.001$).

To test whether ApoE genotype affects the observed correlation between cognitive performance and olfactory identification, we further examined correlations between the MMSE scores and the scores of the P-SIT depending on ApoE phenotypes. When dividing patients with AD according to the ApoE $\varepsilon 4$ allele, patients who were carrying the ApoE $\varepsilon 4$ allele had a higher coefficient of correlation between the MMSE scores and the scores of the P-SIT ($\rho = 0.67$) than that of patients without the ApoE $\varepsilon 4$ allele ($\rho = 0.46$). Simple regression analyses to calculate the predicted MMSE score from the P-SIT score indicated that in patients with the ApoE $\varepsilon 4$ allele, coefficient of determination was higher (adjusted $R^2 = 0.42$) than that of patients without the ApoE $\varepsilon 4$ allele (adjusted $R^2 = 0.18$).

DISCUSSION

Unlike other sensory modalities such as vision or hearing, dysfunction in olfaction may not be well

recognized as an objective symptom in clinical settings despite patient's subjective complaints or substantial impact on mood and social behavior (Kirk-Smith and Booth, 1987). Age-related decline in olfaction has been reported elsewhere (Doty *et al.*, 1984b; Schiffman, 1997). Phylogenetically, sense of smell developed early in mammalian animals and olfactory pathway within the brain has privileged access to major parts of the limbic areas associated with mnemonic function (Eslinger *et al.*, 1982; Eichenbaum *et al.*, 1996). Neuropathologic evidence suggests the involvement of regions related to olfactory processing in the pathogenesis typical of AD revealed by disproportionate numbers of neurofibrillary tangles and neuritic plaques relative to other sensory pathways (Reyes *et al.*, 1987; Reyes *et al.*, 1993). Therefore, a question arises about which level in olfactory processing explains the olfactory dysfunction observed in this study. The results of olfactory tests showing that disability in identifying smells precedes that in smell detection (Serby *et al.*, 1991; Morgan *et al.*, 1995) and neuropathologic findings suggesting the involvement of regions essential for olfactory processing (Hyman *et al.*, 1984; Pearson *et al.*, 1985; Hock *et al.*, 1998) may imply a central origin of olfactory dysfunction rather than peripheral alteration in AD. Although our study did not examine odor detection threshold, we confirmed throughout the two olfactory tests that all subjects detected the presence of odorants administered, which implies that the deficit in odor identification was not due to an inability to detect smells, but rather may have been caused by an alteration in higher olfactory processing, although the trigeminal effect cannot be excluded in interpreting the results.

There was a strong negative correlation ($\rho = -0.77$) between the age and the scores of the P-SIT in the elderly controls, which is in line with the results of a previous report showing that odor identification declines with age (Doty *et al.*, 1984b). However an age-related decline of odor identification scores in the non-demented elderly subjects was not evident when using CC-SIT ($\rho = -0.34$) as compared to using P-SIT, which raises a question about whether the test can be a relevant test battery for assessing olfaction in Japanese elderly subjects. Weak intensity of the smells, as many of the participants reported, may contribute to the poor correlation between the scores of the CC-SIT and the MMSE ($\rho = 0.37$) than that between the scores of the P-SIT and the MMSE scores ($\rho = 0.57$) in the AD group. However, it is unlikely that the lower intensity of the odorants used in the CC-SIT alone can account for the discrepancy of the results between the two tests. As observed from the responses of the subjects participated, some of the odorants such as turpentine or paint thinner included in the CC-SIT were not always familiar to Japanese elderly population, while all the odorants used in the P-SIT were equally familiar to all the subjects. Thus the difference in the intensity and the familiarity of the odorants chosen may have brought inconsistent results regarding correlation between the degree of cognitive impairment and the score of the tests.

We confirmed that non-lexical based test of odor identification could reliably discriminate well-defined AD patients from non-demented elderly subjects with high sensitivity and specificity. These results suggest a possible utility of assessing olfactory identification in differentiating AD patients from subjects with other medical conditions, although olfactory dysfunction is not specific to AD. Pathological evidences and observed correlation between the degree of impairment in olfactory identification and that of cognitive impairment in AD patients lead us to believe that the olfactory dysfunction may occur before cognitive symptoms manifest (Nordin and Murphy, 1996; Devanand *et al.*, 2000), which may give olfactory assessment a potential role as a predictor of the onset of the disease. A community-based cohort study showed that impaired olfaction on CC-SIT increased the odds for cognitive decline, which was verified by a global cognitive test (Graves *et al.*, 1999). Also, a study of patients with mild cognitive impairment demonstrated that olfactory deficits, accompanied by lack of awareness of the problem, was a reliable predictor for development of Alzheimer's disease (Devanand *et al.*, 2000). The results of these studies suggest a potential significance of olfactory dysfunction as a preclinical marker of AD, although we have to bear in mind that rigorous control of medical history and conditions affecting olfaction in sampled population may be crucial in assessing olfactory impairment specifically related to neurodegenerative disorders in elderly patients. Considering cross-sectional aspects of this study, the results do not support advantages of smell identification test over standard psychometric or neuropsychological measures for early detection of AD. Longitudinal follow-up of subjects with impaired smell identification may help examine the utility of smell identification test as a useful screening method for AD.

The strong correlation between the MMSE scores and the P-SIT scores in AD patients who were carrying one or two $\varepsilon 4$ alleles relative to those without $\varepsilon 4$ alleles is intriguing. The finding may suggest a

region-specific biological effect of the e4 allele, provided that in AD patients there is an association of ApoE ε4+ with atrophy (Juottonen *et al.*, 1998; Geroldi *et al.*, 1999), and a reduction of cerebral glucose metabolism (Hirono *et al.*, 2002) in the medial temporal lobe, a region where olfactory and cognitive processing are considered to take place. The observed difference of correlation between cognitive performance and olfactory identification depending on ApoE genotype in AD patients may endorse phenotypic heterogeneity of the disease. Stronger correlation in patients with the ε4 allele suggests higher involvement of medial temporal lobe dysfunction in the phenotypic subgroup relative to patients without the ε4 allele. However, considering a significant age difference between the two phenotypic groups, patients without the ε4 allele, who were older than those with the ε4 allele, were more likely to be affected by accompanying cerebrovascular lesions, which may have confounded the results in assessing the correlation between the P-SIT scores and the MMSE scores (Gray *et al.*, 2001).

In conclusion, this study confirmed that the non-lexical test of olfactory identification discriminates AD patients from non-demented elderly subjects with high sensitivity and specificity. Also, the impairment of olfactory identification correlates well with the degree of cognitive decline in AD patients. The correlation is more pronounced in AD patients who carry ε4 allele. We therefore propose a short and simple olfactory test appropriate for clinical use in Japanese elderly population to improve diagnostic accuracy in patients with AD.

ACKNOWLEDGEMENTS

This research was supported by funding from the Mitsui-Sumitomo Marine Welfare Foundation.

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0145-6008/04/2805-0720$03.00/0
ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

Vol. 28, No. 5
May 2004

DRD2 Gene Transfer Into the Nucleus Accumbens Core of the Alcohol Preferring and Nonpreferring Rats Attenuates Alcohol Drinking

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Background: Transient overexpression of the dopamine D2 receptor (DRD2) gene in the nucleus accumbens (NAc) using an adenoviral vector has been associated with a significant decrease in alcohol intake in Sprague Dawley rats. This overexpression of DRD2 reduced alcohol consumption in a two-bottle-choice paradigm and supported the view that high levels of DRD2 may be protective against alcohol abuse.

Methods: Using a limited access (1 hr) two-bottle-choice (water versus 10% ethanol) drinking paradigm, we examined the effects of the DRD2 vector in alcohol intake in the genetically inbred alcohol-preferring (P) and -nonpreferring (NP) rats. In addition, micro–positron emission tomography imaging was used at the completion of the study to assess in vivo the chronic (7 weeks) effects of ethanol exposure on DRD2 levels between the two groups.

Results: P rats that were treated with the DRD2 vector (in the NAc) significantly attenuated their alcohol preference (37% decrease) and intake (48% decrease), and these measures returned to pretreatment levels by day 20. A similar pattern of behavior (attenuation of ethanol drinking) was observed in NP rats. Analysis of the [^{11}C]raclopride micro–positron emission tomography data after chronic (7 weeks) exposure to ethanol revealed clear DRD2 binding differences between the P and NP rats. P rats showed 16% lower [^{11}C]raclopride specific binding in striatum than the NP rats.

Conclusions: These findings further support our hypothesis that high levels of DRD2 are causally associated with a reduction in alcohol consumption and may serve as a protective factor against alcoholism. That this effect was seen in P rats, which are predisposed to alcohol intake, suggests that they are protective even in those who are genetically predisposed to high alcohol intake. It is noteworthy that increasing DRD2 significantly decreased alcohol intake but did not abolish it, suggesting that high DRD2 levels may specifically interfere with the administration of large quantities of alcohol. The significantly higher DRD2 concentration in NP than P rats after 7 weeks of ethanol therefore could account for low alcohol intake.

Key Words: Alcoholism Adenovirus Addiction Positron Emission Tomography (PET) Gene Therapy.

ETHANOL STIMULATES BOTH dopamine (DA) neurons and DA release in the rat nucleus accumbens (NAc) (Blanchard et al., 1993; Imperato and Chiara, 1986; Weiss et al., 1993; Wozniak et al., 1990; Yoshimoto et al., 1991). In addition, it has been established that the various DA receptors play a significant role in alcohol and substance abuse (Caine et al., 1999; Di Chiara, 1995; Gonzales, 1996; Munzar and Goldberg, 2000; Volkow et al., 1993). Among the various DA receptor subtypes, the DRD2 receptor has been most frequently associated with the reinforcing effects of alcohol (Hitzemann et al., 2003; McBride et al., 1993; Nowak et al., 2000; Stefanini et al., 1992).

Similarly, chronic alcoholism produced significant changes in DRD2 levels (Guardia et al., 2000; Tajuddin and Druse, 1996; Volkow et al., 1996, 2002). These data have led to the hypothesis that DRD2 deficiency or down-regulation may predispose subjects to drug use as a means of compensating for the decrease in activation of reward circuits activated by these receptors (Blum et al., 1996; Volkow et al., 1996, 2002). Multiple genes and environmental conditions have been implicated in producing low levels of DRD2 receptors in the brain. Low levels of DRD2 in brain have been postulated to lead to a reward deficiency syndrome that predisposes an individual to multiple addictive, impulsive, and compulsive behavioral propensities,

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Received for publication March 18, 2003; accepted February 2, 2004.

This work was supported by the National Institute of Alcohol Abuse and Alcoholism (AA 11034, AA07574, and AA07611) and by the U.S. Department of Energy under contract DE-AC02-98CH10886.

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DOI: 10.1097/01.ALC.0000125270.30501.08

such as alcohol and drug abuse, glucose binging, pathologic gambling, sex addiction, attention-deficit/hyperactivity disorder, Tourette's syndrome, autism, chronic violence, posttraumatic stress disorder, schizoid/avoidant personality, conduct disorder, and antisocial behavior (Blum et al., 2000; Comings and Blum, 2000; Volkow et al., 1993, 1996, 2001, 2002; Wang et al., 1997).

Imaging studies (Volkow et al., 1999b, 2002) have shown significant correlation between DRD2 density and the reinforcing response to psychostimulants, suggesting that low DRD2 levels may be involved in the predisposition to drug abuse. Supporting this view, several animal studies have reported that DRD2 antagonists enhanced ethanol self-administration in selectively bred alcohol-preferring (P) rats (Dyr et al., 1993; Levy et al., 1991). In addition, it has been demonstrated that there was 20 to 25% lower DRD2 binding in the NAc of P rats when compared with alcohol nonpreferring (NP) rats (McBride et al., 1993), as well as 20% lower DRD2 binding between the Sardinian ethanol-preferring and the Sardinian ethanol nonpreferring rats (Stefanini et al., 1992).

It is well understood that DA projections from the ventral tegmental area (VTA) to the NAc are the most studied system implicated in the control of ethanol self-administration behavior (Kalivas et al., 1993; Koob et al., 1987). Previous studies have examined the subterritories of the NAc into core and shell with regard to alcohol preference (Samson and Hodge, 1996; Zocchi et al., 2003). It has been hypothesized that both are critical in alcohol consumption but that the NAc shell is involved in mediating the excitatory effects of stimuli, the anticipation of reward, and goal-directed behaviors (Corbit et al., 2001; Johnson et al., 1995; Sokolowski et al., 1998; Sokolowski and Salamone, 1998), whereas the NAc core is involved in mediating and encoding the incentive value of the instrumental outcome on the performance of goal-directed actions (Corbit et al., 2001; Samson and Chappell, 2003) and implicated in mechanisms of learning such as the expression of conditioned stimulus and unconditioned stimulus associations (Parkinson et al., 1999).

An adenoviral gene transfer technique was used to deliver the DRD2 gene into the NAc core of adult Sprague Dawley rats that were trained to self-administer alcohol (Thanos et al., 2000, 2001). Results indicated that DRD2 up-regulation in P rats produced marked reductions in alcohol preference and consumption, which returned to baseline as the DRD2 levels returned to their initial values. This was the first evidence that overexpression of DRD2 in the NAc core attenuated alcohol intake and suggested that high levels of DRD2 may be protective against alcohol abuse. More recently, it was reported that DRD2 gene transfer into the NAc core of rats that were trained to self-administer intravenous cocaine significantly attenuated the number of infusions and the number of lever presses associated with cocaine (Thanos et al., 2002a). This effect was significant for several days and was positively correlated with the time frame of peak DRD2 up-regulation. The present study used a similar approach of NAc core DRD2 up-regulation and examined the effects on alcohol consumption in the P and NP rats.

The biochemical and behavioral profile of the P and NP rats (developed by Li and colleagues at the Indiana University Alcohol Research Center) has been described extensively in the literature (Lumeng et al., 1982; Zhou et al., 1995). These rats were used in the current study and received a microinfusion of an adenoviral vector containing the rat D2cDNA insert into the NAc. Using a two-bottle-choice paradigm, ethanol preference and intake were assessed for each rat. We hypothesized that DRD2 gene transfer into the NAc of P rats would produce a significant attenuation in ethanol preference and ethanol consumption.

At the completion of this experiment, rats were imaged in vivo with micro–positron emission tomography (μPET) to assess the chronic effects (7 weeks) of ethanol exposure on DRD2 binding. Previous studies have indicated that quantitative assessment of DRD2 binding with μPET is a reliable, noninvasive technique (Alexoff et al., 2003; Ogawa et al., 2000; Thanos et al., 2002a,b). These data will help us better understand the effects of chronic (7 weeks) acute ethanol self-administration on DRD2 levels. Finally, these results were compared with previous binding data on naïve P and NP rats (McBride et al., 1993).

MATERIALS AND METHODS

All studies were conducted in accordance with the guidelines established by the National Institutes of Health in the *Guide for Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Brookhaven National Laboratory.

Subjects

Thirty male adult rats (350–450 g) were used in this study [15 P and 15 NP]. Rats were housed individually in a room controlled for temperature and humidity as well as a 12-hr light/dark (lights off at 7:00 AM) cycle. Food was provided ad libitum, whereas water and ethanol access were limited and available only during daily 1-hr testing sessions.

Procedures

Behavioral Testing and Microinfusions. The effect of the DRD2 vector was examined using a two-bottle-choice limited access paradigm, which captures aspects of voluntary alcohol consumption in humans. Limiting access to alcohol to a short period daily causes rats to drink alcohol immediately when first made available each day and at a constant amount during each access period. Moreover, ethanol drinking increased by limiting the opportunity to obtain alcohol (Files et al., 1994; Wilson et al., 1997). Briefly, the cage of each rat was fitted with two 150-ml (Kimax) drinking bottles. One bottle contained tap water, and the other contained a 10% (v/v) alcohol solution. The position of the bottles were reversed daily to prevent a position habit. Each morning (9:00 AM), the fluid intake and body weight of each animal were recorded. Each animal was given a 1-hr session daily (to a choice between the two drinking bottles). Behavioral assessment consisted of percentage of ethanol preference and ethanol intake. Ethanol preference was calculated from the ratio volume of ethanol consumed divided by the total fluid consumed (water bottle + ethanol bottle) each day × 100. Ethanol intake was calculated in grams

per kilogram using the mass of ethanol consumed divided by the rat's body weight.

After a 1-week adaptation period to the home cage environment [containing two bottles (water and 10% v/v ethanol)], rats that ranged in weight between 386 and 477 g (mean, 426 g) were given a second week of daily access and tested for their preference (water versus ethanol). This second week of drinking was considered the preoperative baseline. Next, each animal had a cannula surgically implanted into the NAc and was allowed 1 week of recovery time before being returned to the same two-bottle preference test for 1 week (postoperative baseline).

All animals next were treated with a microinfusion into the NAc of the control, replication-deficient adenovirus null vector (AdCMV.Null), as previously described (Thanos et al., 2001), and then returned to the home cage for two-bottle ethanol preference assessment for another week. Subsequently on day 0, all animals similarly received a microinfusion once into the NAc with the AdCMV.DRD2 vector (Thanos et al., 2001) and then were returned to the home cage for ethanol preference assessment. This final assessment continued for 24 days.

Microinfusion was carried out using an automated syringe pump (Razel, Stamford, CT) and a 26-gauge 5-μl Hamilton microsyringe connected to a 28-gauge internal cannula. Each microinfusion administered 2 μl of vector [adenoviral vector containing the cDNA for the DRD2 receptor (AdCMV.DopD2R; 10^{10} pfu/ml)] over 10 min so as to reduce the risk of procedure-induced lesions.

Surgery. Rats were anesthetized with ketamine and xylazine (100 mg/kg, 10 mg/kg) and placed in a Kopf stereotaxic apparatus. A 22-gauge guide cannula was then implanted unilaterally (Plastics One, Roanoke, VA) into the NAc core [+1.2 mm AP, ±1.4 mm ML, −6.6 mm DV (Paxinos and Watson, 1986)]. Laterality of cannula placement was randomly assigned so that half of the rats received left NAc implants and the other half received implants into the right NAc. The guide cannula was then secured to the skull with four small stainless steel screws and dental cement. The animals were then allowed 1 week to recover.

μPET. Upon completion of the behavioral experiment, 6 P and 6 NP rats were anesthetized intraperitoneally with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic head holder in a prone position on the bed of the μPET R4 scanner (Concorde Microsystems, Knoxville, TN). Animals then received an injection via the tail vein of a mean dose of 3.34 nmol/kg [^{11}C]raclopride (245.5 ± 26.2 μCi for P; 244.8 ± 27.8 μCi for NP; specific activity 1.4–2.2 mCi/nmol and injected volumes of 200 μl). [^{11}C]raclopride binding in the μPET R4 has been previously demonstrated as a reproducible and suitable method in studying in vivo the DRD2 availability in the rodent brain (Alexoff et al., 2003; Thanos et al., 2002b). The μPET R4 scanner has a 12-cm animal port with an image field of view of ~11.5 cm. Total acquisition time was 70 min [(22 frames: 1 (5 sec), 5 (10 sec), 1 (15 sec), 2 (20 sec), 1 (40 sec), 2 (60 sec), 1 (180 sec), 5 (300 sec), 1 (450 sec), 3 (600 sec)] and data were acquired in full three-dimensional mode with maximum axial acceptance angle (±28 degrees). Images were reconstructed using FORE rebinning (Matej et al., 1998) followed by two-dimensional filtered backprojection with a ramp filter cutoff at the Nyquist frequency. Using the rat stereotaxic atlas (Paxinos and Watson, 1986) and the Harderian glands as reference points, the coronal planes of striatum (ST) and cerebellum (CB) were identified in the same manner. Specifically, for each animal, the ST and CB were identified as 6 and 16 slices, respectively, caudal to the Harderian glands (slice thickness was 1.2 mm), which are routinely used as markers in rodent PET studies (Hume et al., 1996; Matej et al., 1998; Paxinos and Watson, 1986; Thanos et al., 2002b).

[^{11}C]raclopride was synthesized according to previously described methods (Farde et al., 1986). [^{11}C]raclopride doses were small fractions of routine syntheses prepared for human subject studies. Specific activity determination was made using mass measurements acquired during radiotracer purification by high-performance liquid chromatography (Novapak C18, Milford, MA), and radioactivity measurements were obtained with a calibrated ion chamber (Capintec, Ramsey, NJ).

μPET Analysis. Regions of interest (ROI) in ST and CB were selected using a rat stereotaxic atlas (Paxinos and Watson, 1986). Specific binding was estimated using the ST/CB ratio, which was calculated for each animal as previously described (Thanos et al., 2002b). The ST is an area rich in DRD2 and can reliably be imaged and analyzed using the μPET R4 (Alexoff et al., 2003; Thanos et al., 2002b). Similarly, the CB is a structure that lacks DRD2 and is commonly used as a reference structure in PET studies by using a ratio of ST to CB DRD2 binding (Alexoff et al., 2003; Thanos et al., 2002b). Briefly, the left and right ST ROI were averaged and divided by CB ROI over the 70-min duration of each scan. Time activity data were then used to calculate distribution volume ratios (DVRs), using the Pixel-wise Modeling graphic analysis software (Mikolajczyk et al., 1998). This PET quantitation application is widely used and provides a linear function of receptor availability of PET data that does not require blood sampling (Logan et al., 1996; Mikolajczyk et al., 1998).

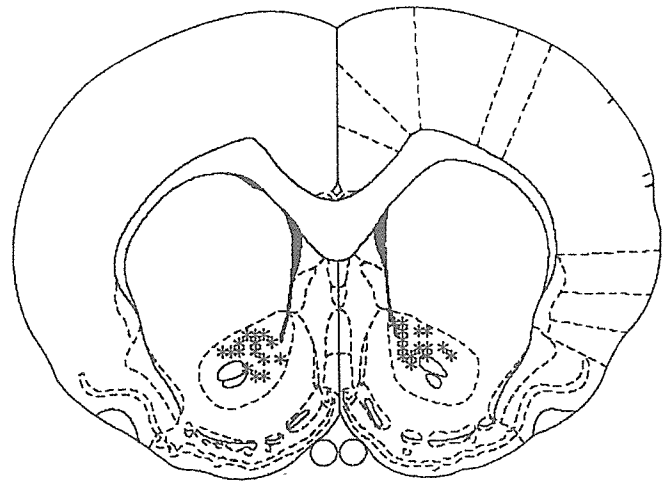

Fig. 1. Coronal section of the rat brain (AP +1.2 mm): the NAc and the location of microinfusion sites (adapted from Paxinos and Watson, 1986).

Histology. After completion of the behavioral and μPET experiments of the study, the brains were harvested from all rats for histological confirmation of the cannula placements. Briefly, each animal was deeply anesthetized with ketamine/xylazine, and the brain was rapidly removed and frozen in an isopentane/dry ice bath and stored in a freezer at −80°C. Next, 20-μm-thick coronal sections were cut on a cryostat (Leica CM3050S; Leica Microsystems, Nussloch, Germany).

Sections were stained with cresyl violet by serial immersion in the following solutions (time given in parentheses): dH_2O (1 min), 1% cresyl violet/0.3% acetic acid (15 min), dH_2O (2 min), dH_2O (2 min), dH_2O (2 min), 0.4% formalin/0.2% acetic acid (15 min), dH_2O (2 min), dH_2O (2 min), 95% ethanol (30 sec), 100% ethanol (30 sec), and xylene (2 min). Slides were then coverslipped with Permount and allowed to air dry before verification of cannula placement in the NAc under light microscopy (Fig. 1).

RESULTS

Rats did not show any signs of malaise or weight loss after treatment with the vector, and this was consistent with previous studies (Ikari et al., 1999, 1995; Ingram et al., 1998; Thanos et al., 2001; Umegaki et al., 1997). No alterations in general behavior or locomotor activity were noted (during observation of the animals).

Ethanol Preference

Baseline drinking behavior in both groups of rats (P and NP) was consistent with previous reports (Zhou et al.,

1995). A one-way, repeated measures ANOVA comparing drinking preference (preoperative baseline and postoperative drinking phases) revealed no statistical difference ($p > 0.05$). Subsequently, animals were treated with the AdCM-V.Null (vehicle) vector, and drinking preference was assessed for 7 days. Similarly, a one-way repeated measures ANOVA comparing baseline ethanol drinking and ethanol drinking after treatment with the control vector revealed no significant difference ($p > 0.05$).

All animals were then treated with the DRD2 vector (day 0), and ethanol drinking was monitored for 24 days. A one-way, repeated measures ANOVA, comparing baseline ethanol preference (day 0) and ethanol preference after DRD2 vector treatment revealed a significant difference in P rats ($F = 9.337$; $df = 24$; $p < 0.001$; Fig. 2A). Subsequently, a Tukey test was used to examine all pairwise multiple comparisons. Comparisons between baseline (day 0) ethanol preference and post-DRD2 vector treatment revealed several significant differences (illustrated in Fig. 2A by an asterisk; $p < 0.05$). Specifically, at day 2 (after DRD2 vector treatment), ethanol preference was decreased in the P rats from 81 to 44% and returned to pretreatment levels by day 20 (Fig. 2A).

Similarly, a one-way repeated measures ANOVA, comparing baseline ethanol preference (day 0) and ethanol preference after DRD2 vector treatment, revealed a significant difference in NP rats ($F = 1.88, df = 24, p < 0.01$; Fig. 2A). Pairwise multiple comparisons (Tukey test), however, did not yield any significant differences between baseline ethanol preference and post-DRD2 vector drinking.

Ethanol Intake

A one-way repeated measures ANOVA comparing baseline ethanol intake (day 0) in P rats with drinking after treatment with the active vector was significantly decreased ($F = 15.06$, $df = 24$, $p < 0.001$; Fig. 2B). At day 2 (after DRD2 vector treatment), ethanol intake was decreased in the P rats from 2.7 g/kg to ~1.3 g/kg, and intake returned to pretreatment levels by day 20 (Fig. 2B). Pairwise multiple comparisons (Tukey test) between baseline ethanol intake and post-DRD2 vector intake revealed several significant differences illustrated by an asterisk ($p < 0.05$; Fig. 2B).

A similar one-way repeated measures analysis of the data in NP rats baseline ethanol intake (day 0) and intake after treatment with the active vector revealed a statistical difference ($F = 1.687, df = 24, p < 0.05$; Fig. 2B). Subsequent, pairwise multiple comparisons (Tukey test), however, did not yield any significant differences between baseline ethanol intake and post-RD2 vector intake in NP rats.

Overall, it should be noted that there was no significant decrease in total fluid intake after treatment with the vector but rather a decrease in ethanol preference (drinking from the ethanol bottle versus the water bottle) and ethanol intake. Baseline total fluid (water + 10% ethanol) intake in the 1-hr two-bottle-choice procedure was similar in both the P (27.3 ml) and NP (25.2 ml) rats. Specifically, P rats showed a mean fluid intake of 21.1 ± 1.6 ml of ethanol and 6.2 ± 2.7 ml of water, whereas NP rats showed a mean fluid intake of 5.7 ± 1.8 ml of ethanol and 19.5 ± 5.2 ml of water.

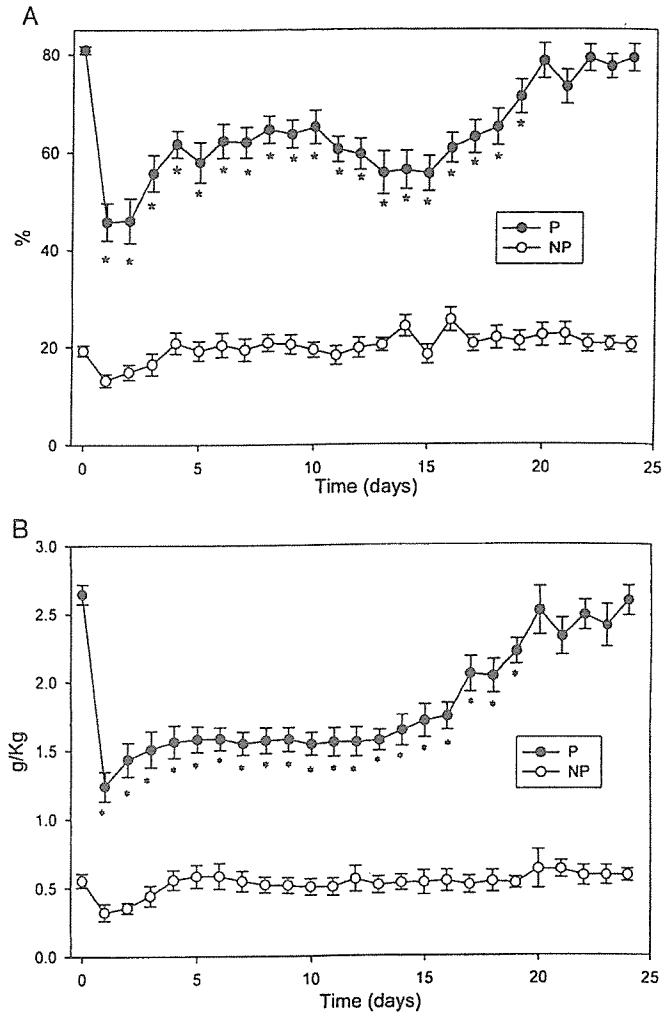

Fig. 2. (A) Mean percentage (± SE) of ethanol preference over time in P and NP rats in a daily 1-hr limited-access session (*$p < 0.05$). Day 0 represents baseline drinking. Animals were treated with the DRD2 vector on day 0. (B) Mean (± SE) ethanol intake (g/kg) over time in P and NP rats in a daily 1-hr limited-access session (*$p < 0.05$). Day 0 represents baseline drinking. Animals were treated with the DRD2 vector on day 0.

μPET

Representative examples of coronal μPET images of the ST after intravenous injection of [^{11}C]raclopride are shown in Fig. 3. A significant difference in [^{11}C]raclopride binding in the ST was observed between the P and NP rats.

Quantitative analysis of the [^{11}C]raclopride μPET consisted of (1) the ST/CB binding ratios over time, (2) [^{11}C]raclopride binding over time, and (3) the DVR. *T* test comparisons between the two strains revealed a statistically significant difference in the ST/CB ratios ($t_{obs} = 3.26$, $df =$

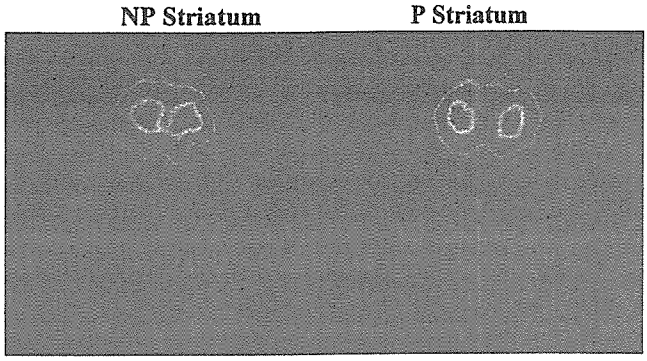

Fig. 3. Coronal images (plane thickness = 1.2 mm) of a P and NP rat brain at the level of the ST obtained using the μPET R4 scanner with [^{11}C]raclopride.

21, $p < 0.001$). Specifically, the mean ratio from time point 0 to 70 min for each strain was 1.84 ± 0.08 and 1.56 ± 0.05 for the NP and P rats, respectively (Fig. 4A). The [^{11}C]raclopride binding kinetics or time activity curve (TAC) is shown for each strain in Fig. 4B. A t test comparison of the TAC for the ST between the P and NP rats revealed a significant difference (t_{obs} = 3.31, df = 21, $p < 0.001$). Finally, the time activity data were then used to calculate DVRs, using the Pixel-wise Modeling graphic analysis software. The DVR was calculated for each strain (NP = 2.66 and P = 2.25) and provided a linear function of the DRD2 receptor availability between P and NP rats as observed with μPET.

Histology

Localization of the area of microinfusion was assessed using a stereotaxic atlas (Paxinos and Watson, 1986) (Fig. 1). Histological examination of the area of microinfusion did not reveal any unusual neuropathology or significant signs of inflammation associated with sites infected with AdCMV.DRD2 or control vector.

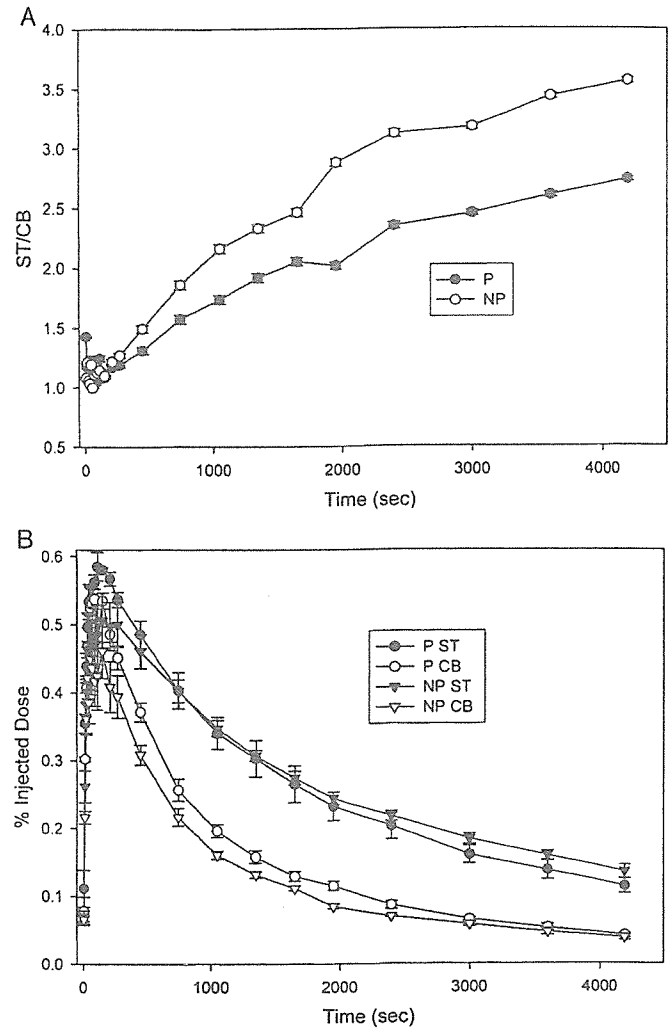

Fig. 4. (A) ST/CB, [^{11}C]raclopride binding ratio (mean ± SE) in P and NP rats. (B) [^{11}C]raclopride TAC in P and NP rats. Mean (± SE) percentage injected dose/ml binding in ST and CB.

DISCUSSION

The present study examined the role of DRD2 gene transfer and selective up-regulation in a rodent ethanol self-administration paradigm. Ethanol intake and preference were significantly reduced in P rats that were treated with the DRD2 vector. Specifically, P rats that were treated with the DRD2 vector showed that ethanol preference was attenuated for a period of 20 days before returning to baseline levels, with a maximum effect seen (37% decrease) within the first few days posttreatment. Similarly, ethanol intake was attenuated after treatment with the DRD2 vector before returning to baseline, in 20 days, with a maximum effect (48% decrease) within the first few days posttreatment. These data further supported our hypothesis that DRD2 levels in the NAc play an important role in ethanol drinking and may be associated with the significant differences in ethanol preference and consumption observed between P and NP rats. NP rats that were treated with the DRD2 vector also showed a significant main effect on ethanol preference and intake; however, these changes were not as large in magnitude or specific over time and could be associated with the already low baseline consumption of NP rats.

The duration of the effect of the DRD2 vector on behavior was consistent with previous studies (Ingram et al., 1998; Thanos et al., 2001; Umegaki et al., 1997). In particular, ethanol preference and intake returned to baseline levels 20 days posttreatment with the DRD2 vector, and this was similar to previous results observed in Sprague Dawley rats (Thanos et al., 2001). This effect on ethanol consumption by the DRD2 vector was observed beyond the 2 weeks reported in previous binding studies. Specifically, previous in vitro autoradiography studies reported the transient nature of DRD2 overexpression induced by the vector in the rat, with DRD2 levels returning to baseline within 2 weeks and peak expression at days 2 to 5 after infusion (Ingram et al., 1998; Thanos et al., 2001; Umegaki et al.,

1997). Therefore, DRD2 vector treatment could not have influenced μPET [^{11}C]raclopride binding at 7 weeks. The difference in DRD2 binding (as observed with μPET) between P and NP rats thus was negatively correlated with ethanol drinking. Specifically, although P rats exhibited greater alcohol consumption, they displayed lower [^{11}C]raclopride binding, and vice versa for the NP rats.

The in vivo effects of chronic ethanol exposure on DRD2 levels in P and NP rats were also examined. μPET analyses revealed significantly lower DRD2 binding in the P rats that were chronically exposed to ethanol compared with NP rats. The ST/CB ratio over time provided a relative μPET comparison of specific (ST) DRD2 binding relative to nonspecific (CB) binding. This popular method of μPET analysis of DRD2 binding is unaffected by dose of the injected radiotracer and revealed that NP rats displayed 15% greater [^{11}C]raclopride binding compared with P rats. Next, the DRD2 binding kinetics (TAC data) were consistent with the ratio data and revealed a consistently and significantly higher ST binding in the NP rats. Similarly, the DVR analysis described DRD2 receptor availability between P and NP rats and showed a highly significant difference between the groups (~16% greater in the NP versus P rats). Comparing the DVR of the P and NP rats with the ST/CB ratio data revealed a high degree of concurrence, and this was consistent with the literature (Logan et al., 1996; Thanos et al., 2002b). Although animals were not repeatedly examined with μPET over time, these results provided evidence that μPET could be used to effectively examine quantitatively and noninvasively DRD2 binding in P and NP rats. Furthermore, these data indicated that chronic alcohol consumption in these animals maintained a similar DRD2 profile as previously observed with autoradiography in naïve P and NP rats (McBride et al., 1993). That is, P rats displayed lower [^{11}C]raclopride binding in comparison with NP rats. These μPET findings were not in agreement with some previous studies that reported an up-regulation of DRD2 in several rodent strains in a variety of forced chronic ethanol administration procedures (Hruska, 1988; Lograno et al., 1993; Tajuddin and Druse, 1996). However, our μPET findings were in accordance with several other rodent studies that have described that chronic ethanol administration produced functional down-regulation or desensitization of DRD2 in several rat strains (Lucchi et al., 1988; Muller et al., 1980; Rommelspacher et al., 1992; Syvalahti et al., 1988), including most recently in P rats (Engleman et al., 2003). In addition, our findings were consistent with the clinical report that alcoholics showed a DRD2 down-regulation compared with controls (Volkow et al., 1996).

The μPET data helped assess the effects of chronic acute ethanol exposure (albeit different doses/group) on DRD2 levels in P and NP rats. These results were comparable to the DRD2 data reported previously in naïve P and NP rats (McBride et al., 1993). Recently, evidence was provided that alcohol drinking by P rats attenuated D2 autoreceptor function in the NAc (Engleman et al., 2003). In the present study, rats were scanned for μPET after ~7 weeks of ethanol exposure and ~4 weeks after treatment with the DRD2 vector. Finally, although the present study provided important insight into the chronic effects of ethanol exposure on DRD2, it is important to point out a limitation in interpreting these results, which is the disparate ethanol dose consumed between the two groups of rats, which is unavoidable in self-administration procedures. Furthermore, interpretation of the μPET data requires caution because these results were compared with DRD2 binding data from naïve P and NP rats in the literature (McBride et al., 1993).

The present results emphasized the important role that DRD2 levels in the NAc may play in the unique ethanol self-administration behavior profile of the P rats. This was in agreement with previous studies that have reported that DRD2 levels were significantly different between naïve P and NP rats. Specifically, DRD2 density was reported to be 20% lower in the olfactory tubercle and NAc of the Sardinian P rats compared with Sardinian NP rats (Stefanini et al., 1992). In a comparable study in P and NP rats, P rats showed 20 to 25% lower [^{3}H]sulpiride binding in the caudate putamen, medial and lateral NAc, and VTA compared with NP rats (McBride et al., 1993). This intrinsic lower DRD2 profile observed in P rats may suggest that some type of adaptive process to the chronic effects of ethanol is present before ethanol exposure.

In evaluating the mechanisms underlying the protective effects of high D2R level in alcohol intake, it is worth noting that increasing D2R produced a marked decrease in alcohol intake but did not abolish it. This suggests that high D2R levels may be specifically interfering with the administration of high concentrations of alcohol. This could explain why NP rats, which have high D2R levels, still consume alcohol, albeit at much lower concentrations. Indeed, we had postulated that there is an optimal level for the activation of reward circuits after which further stimulation becomes aversive to explain why in individuals with high striatal D2R levels a relatively large dose of a stimulant drug (methylphenidate) was perceived as aversive, whereas in individuals with low D2R levels, it was perceived as pleasurable (Volkow et al., 1999a, 2002).

Although the complex interaction within the mesocortical/mesolimbic system that processes the various stimuli that regulate ethanol self-administration is beyond this discussion, the data suggest that DRD2 levels in the NAc core play an important role in ethanol drinking. One possible mechanism influencing self-administration alcohol consumption is a complex network of feedforward and feedback loops within the mesolimbic system between the NAc core, the medial prefrontal cortex (mPFC), and the VTA (Kalivas et al., 1993; Samson and Chappell, 2003; Samson and Hodge, 1996). According to this system organization, alcohol consumption is influenced by environmental stimuli that function as discriminative and conditioned reinforcing

stimuli (processed by the mPFC). The mPFC then sends excitatory input to the NAc core that influences the release of DA (You et al., 1998), as well as having direct actions on the medium spiny output neurons in the NAc core (Kiyatkin and Rebec, 1996). In addition to the environmental stimuli, internal stimuli that reach the mPFC from the hypothalamus, amygdala, and hippocampus interact to determine the degree of glutamate output to the NAc core (Kalivas et al., 1993; Pennartz et al., 1994). This part of the system, therefore, is testing the salience of the external environmental stimuli in conjunction with internal generated stimuli (e.g., degree of deprivation, anxiety). Alcohol is known to increase the extracellular levels of DA (Fadda et al., 1989; Imperato and Chiara, 1986) and will significantly increase DA output to both the NAc and the mPFC (Kalivas et al., 1993). Furthermore, excess DA in the NAc core can extend an ethanol self-administration session well beyond the "normal" limits (Samson et al., 1991). DA release in the NAc provides a feedforward process to maintain the current behavior in relation to the salience of the current multiple-stimulus inputs to the NAc core. However, the release of DA in the mPFC would tend to decrease mPFC output, thus reducing the excitatory facilitation of NAc core and VTA cells in a negative feedback function. The termination of conditioned responding (i.e., to ethanol-related stimuli) is most likely controlled by a shift in the NAc core output to the ventral pallidum and its projections to the thalamus, which send additional feedback projections to the mPFC (Pennartz et al., 1994). Therefore, DRD2 up-regulation in the NAc core (in the present study) potentiated the output signal of these GABAergic medium spiny neurons, which in turn feedback and inhibit the mPFC, which provides control over the onset and offset of a self-administration bout (Samson and Hodge, 1996). In contrast, DRD2 deficiency or down-regulation in the NAc may predispose a subject to drug use as a means of compensating for the decrease in activation of reward circuits activated by these receptors.

The expression of DRD2 in the brain, which has been shown to be modulated by both genetic and environmental factors such as stress (Morgan et al., 2002; Papp et al., 1994), provides a molecular mechanism that can account for the involvement of both genetic and environmental factors in the predisposition to drug and alcohol abuse. Thus, possibilities exist for developing strategies to modulate the expression of DRD2 as a means of attenuating alcohol preference and dependency.

Understanding alcohol preference in rodent models requires the elucidation of the genotypes and phenotypes involved. Previous studies have reported several differences (besides in the mesolimbic dopamine reward system) in biochemistry between the P and NP rats. Specifically, with these selected lines, the data include differences in serotonin, GABA, endogenous opioid, and neuropeptide Y systems (McBride et al., 1990; Thielen et al., 1997). In addition, phenotypic and genotypic mapping studies have identified quantitative trait loci influencing alcohol consumption on chromosomes 3, 4, and 8 in the inbred P/NP rats (Murphy et al., 2002). Therefore, it is imperative to study further the interaction and regulation/modulation of the above neurotransmitters and genes to understand better the mechanism(s) of alcohol abuse.

Finally, it was demonstrated that longitudinal in vivo assessment of vector-mediated DRD2 expression in the rat brain can be accomplished with μPET. This technique also provides future opportunities in studying rodent models of alcoholism as well as evaluating possible new-generation viral vectors that could offer longer duration of action and, hopefully, longer functional effects. Further studies will seek to identify the types and number of cells transduced by the DRD2 vector.

Future studies will examine this DRD2 hypothesis and alcoholism in transgenic mice, as well as develop and evaluate a second-generation DRD2 vector that is capable of providing DRD2 up-regulation beyond the 2 weeks observed with the present vector. Similarly, this approach should be used to examine the role of other receptors (within the D1 and D2 family) on alcoholism.

ACKNOWLEDGMENTS

The authors thank Drs. T.K. Li, William J. McBride, and Larry Lumeng and the Indiana University Alcohol Research Center (PHS AA 07611) for providing the alcohol-preferring and non-preferring rats. The authors also thank Drs. Yu-Shin Ding and Paul Vaska for PET operations; and Dr. David Schlyer, Michael Schueller, Richard Ferrier, Colleen Shea, Yonweu Xu, and Victor Garza for cyclotron operations and radiotracer preparation; and Maryann Kershaw and staff for veterinary care.

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The entorhinal cortex regulates blood glucose level in response to microinjection of neostigmine into the hippocampus.

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Key words: **entorhinal cortex; ibotenic acid; neostigmine; hippocampus; glucose**

Abstract

OBJECTIVE: Microinjectin of neostigmine, an inhibitor of acetylcholine esterase, into the rat hippocampus elicited stress-like responses reflected by the release of adrenocorticotropic hormone (ACTH) and blood glucose elevations. The entorhinal cortex (EC) is regarded as an interfrace between the hippocampus and neocortex. The current study was designed to examine the role of the entorhinal cortex in regulation of blood glucose elevation induced by hippocampal neostigmine injection. MARERIAL AND METHODS: We produced the entorhinal cortex lesions in 9 week-old male Wistar rats by the bilateral injections of the cell-selective neurotoxin, ibotenic acid (15 μg / μl). Two weeks after the injections, neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected into the rat hippocampus in a volume of 1 μl for 1 min using a CMA/100 microinjection pump. Plasma ACTH levels were measured by radioimmunoassay. Plasma glucose concentrations were determined by the immobilized enzyme membrane/H_2O_2 method with a compact glucose analyzer Antsense II (Bayer Medical Co.Ltd,Tokyo, Japan). RESULTS: Compared with sham-operated control rats, the entorhinal lesions produced by ibotenic acid significantly attenuated the elevations of blood glucose evoked by the microinjection of neostigmine into the hippocampus. However, no significant difference of plasma ACTH in response to the injection was observed between the entorhinal-lesioned rats and controls. CONCLUSION: The results of the present study indicate that the entorhinal cortex plays a role in the central nervous systems (CNS) regulation of blood glucose and may be involved in a stress response presumably via an alternative pathway.

Abbreviations:

ACTH	adrenocorticotropic hormone
EC	entorhinal cortex
CNS	central nervous systems
CRH	corticotropin releasing hormon
PVN	paraventricular nucleus
HPA	hypothalamic-pituitary-adrenal
BNST	bed nucleus of the stria terminalis

Introduction

Stress is common to all living creatures regardless of differences in its quality or intensity. The imposition or perception of environmental or physical change, negative or positive, elicits a spectrum of physiologic changes that can be construed as adaptive to the organism. Prominent among these is the release of glucocorticoids by the adrenal glands, which serves to alert the organism to environmental or physiologic changes and to preserve homeostasis. Levine and Ursin [10] provided a definition of stress that consists of three elements: stimulus input, central processing system, and response output; with biological and psychological processes viewed as integral parts of the general homeostatic principle. The brain perceives inputs of various stressors and

responds via the nervous, endocrine and immune systems, which are called stress responses [17]. In this sense, the brain plays a role in governing the stress responses. Elevations of corticotropin-releasing factor, ACTH and glucocorticoids are the main features of reactions to diverse and acute stressful stimuli 2, 24]. During stress, neurons of the hypothalamic paraventricular nucleus (PVN) release corticotropin-releasing hormone into the pituitary portal circulation, and ACTH secreted from the anterior pituitary gland in response to corticotropin-releasing hormone, stimulates the secretion of glucocorticoids from the adrenal gland. This constitutes the hypothalamic-pituitary-adrenal (HPA) axis, which is the major regulator of neuroendocrine stress responses [1,5,7,11,13,15]. Involvement of the limbic system in neuro-endocrine responses to some stressors has been documented. A wealth of evidence suggests that the hippocampal cholinergic system is involved in some stress responses [9,12,21]. In particular, the cholinergic system in the hippocampus plays a role in regulating the peripheral metabolism of glucose and catecholamines [7,21]. Under stress, the release of acetylcholine in the hippocampus increases, which coincides with the elevation of plasma glucose and catecholamines [19]. In our previous experiments, we observed that the administration of neostigmine, an acetylcholine esterase inhibitor, into the hippocampus elevates the levels of blood glucose and ACTH. Thus, we concluded that the microinjection of neostigmine into the hippocampus is a potential experimental model for acute stress responses [7,8]. The entorhinal cortex is a gateway to the hippocampus. Many sensory inputs and other information reach the hippocampus via the entorhinal cortex. It receives inputs from the neocortex, including the temporal and frontal lobes, amygdala and olfactory bulbs [3]. Information enters the hippocampal formation via the entorhinal cortex and exits via the fornix. Also, the entorhinal cortex is the primary supplier of converging neocortical sensory input to the ipsilateral dentate gyrus of the hippocampal formation [20]. We previously reported on the involvement of the entorhinal cortex in the stress response to immobilization [22]. Lesions in this area produced by ibotenic acid attenuate ACTH elevation during immobilization stress but not during insulin-induced hypoglycemia. The aim of this study was to investigate the role of the entorhinal cortex in stress responses. We produced bilateral entorhinal lesions using ibotenic acid in rats, and observed the peripheral responses of stress markers induced by microinjections of neostigmne into the hippocampus.

Material and Methods

Subjects:

We used 9 week-old male Wistar rats (200-300 g) for the experiment. The animals were individually housed under standard laboratory conditions in temperature-controlled rooms (25 °C), and were maintained under a 12 h light/dark cycle (light on at 06.00) with food pellets and water available *ad libitum*. The rats were cared for in accordance with the ethical guidelines approved by the Animal care and Use Committee of Nagoya University.

Experimental protocol:
Rats were randomly assigned to one of two major groups: unlesioned or lesioned.
The rats in each group were then divided into two subgroups: Group 1: unlesioned neostigmine-injected, Group 2: unlesioned saline-injected, Group 3: lesioned neostigmine-injected, and Group 4: sham-operated rats, neostigmine-injected.
Surgery:
The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and mounted in a stereotaxic frame (Narishige Scientific Instruments Laboratory, Tokyo, Japan). For insertion of the stainless steel needle, the skull was exposed and a burr hole was drilled overlying the injection coordinates. Ibotenic acid was injected through a stainless steel needle (outside tip diameter of 28 μm), which was connected to a 1.0 μl syringe via a 30 cm tube filled with the injection solution. Coordinates for the entorhinal cortex were calculated relative to Bregma with the incisor bar set at –3.30 mm. The coordinates used were anterior-posterior –6.04 mm, medial-lateral ±6.50 mm and dorsal-ventral 7.00 mm from the skull surface in accordance with the atlas of Paxinos and Watson [14]. Entorhinal cortex lesions were produced by pressure-injection of 0.1 μl of ibotenic acid (15 μg / μl in 0.9% NaCl, Sigma Chemical Co., St Louis, MO, USA) bilaterally over 5 min. The tip was allowed to remain in the brain for 5 min after injection to minimize dorsal diffusion of the drug along the needle tract. Sham-operated rats were treated in an identical manner to the ibotenic acid-lesioned rats but were injected with the same volume of saline without ibotenic acid.

A recovery period of 7 days was given to the above operated rats (Group 3, 4), otherwise all rats were anesthetized one week before the experiment to stereotaxically implant a guide cannula (Bas, Tokyo, Japan) into the left dorsal hippocampus at the following coordinates: anterior-posterior –2.0 mm, medial-lateral 1.5 mm, dorsal-ventral 3.5 mm in accordance with the Paxinos and Watson atlas [14] one week before the experiments.

The day before the experiments, the rats were anesthetized with diethyl ether (Kanto Chemical Co. Inc, Tokyo, Japan), and a catheter was inserted into the jugular vein for repeated blood sampling. A 2 cm longitudinal incision was made in the neck directly over the trachea. The underlying muscles were separated using blunt dissection and the right jugular vein was catheterized with Silastic tubing (Shiniest Polymer, Nagoya, Japan) filled with heparinized saline. The catheter was threaded through the vein over a distance of 2.5 cm, which allowed the tip of the cannula to rest in or near the atrium. The free end of the catheter was plugged with a knot and the catheter exteriorized and secured at the back of the neck with a special cap. The rats were kept in individual cages with free access to water and food.
Procedures:
Two weeks after developing entorhinal cortex lesions, saline containing neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected in a volume of 1μl for 1 min using a CMA /100 microinjection pump (BSA, Tokyo, Japan) through the guide cannula into the

left dorsal hippocampus of free moving rats. To determine the plasma concentration of ACTH and glucose, blood was intermittently sampled (0.8 ml), starting at time 0, just before injection, and at 10, 30, 60 and 120 min after. To minimize the effect of volume loss, an equal volume of heparinized saline was retuned to the general circulation at each sampling. The blood samples were kept on ice, centrifuged, and the plasma was removed and stored at –20 °C in 400 µl aliquots for subsequent determination of ACTH by radioimmunoassay [16]. Plasma glucose concentrations were determined by the immobilized enzyme membraine/H_2O_2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) (21). All experiments were completed between 10.00 h and 13.00 h to minimize variability resulting from circadian rhythm. Two hours after neostigmine injection, the rats were deeply anesthetized with a lethal dose of sodium pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). They were postfixed over night and cryoprotected in phosphate-buffered saline containing 30% sucrose for 2 days.

Histological verification

For the verification of lesions and the effect of the vehicle, the postfixed brains were frozen with powdered dry ice and serial sections 20 µm in thickness were processed from the region of the entorhinal cortex and mounted on glass slides. Selected regions were stained with Cresyl Violet to assess the extent of the lesions in the entorhinal cortex.

Data and statistical analysis: All sections were assessed by means of microscopic examination using an Olympus BX50 microscope (Tokyo, Japan). Photographs were made of representative lesions and vehicle injection sites in the entorhinal cortex. Blood glucose concentrations were expressed as means ± S.E.M, and differences between the four experimental groups were assessed using repeated measures of one factor ANOVA. Plasma ACTH levels were measured by radioimmunoassay [16], and plasma glucose concentrations were determined by the immobilized enzyme membrane/H_2O_2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) [18].

Results

Figure 1 shows Nissle staining of representative sections including the entorhinal cortex. The significant loss of neurons accompanied by extensive glial proliferation was observed in the entorhinal cortex sections of animals that received ibotenic acid injections (Group 3) (Fig. 1D,E). Animals that received injections of vehicle in the entorhinal cortex did not show any histological signs of neuronal damage (Fig. 1B,C). Figure 2 shows the ACTH and blood glucose concentrations for the lesoined and unlesioned rats following microinjectin of neostigmine into the hippocampus. Figure 2A shows the blood glucose concentration after microinjection of neostigmine into the hippocampus. For Group 1, the plasma concentration of glucose increased after 10 min and reached a peak after 60 min. The saline injected group (Group 2) showed no effect. For the lesioned groups, blood glucose levels

for Group 3 were significantly lower than those of Group 4 (Fig. 2A). ANOVA showed that there was a statistically significant difference among the groups ($p<0.0001$), and Scheffe's post–hoc analysis indicated that ibotenic acid lesions significantly attenuated blood glucose release evoked by the microinjection of neostigmine into the hippocampus (Fig. 2A). No significant difference was observed in the plasma ACTH concentration between Group 1, 3 and 4 after the microinjection of neostigmine into the hippocampus (Fig. 2B).

Discussion

In the current study, we have discovered that the entorhinal cortex is involved in the regulation of stress-like responses induced by hippocampal neostigmine injection. The lesions in this area significantly attenuated the blood glucose elevation but did not affect ACTH secretion. No significant difference of weight was observed before and after lesion.

During stress, an adaptive or compensatory response by the organism is activated to sustain homeostasis. Stress induces adaptation through the production of various mediators such as adrenal steroids, catecholamines, cytokines, and tissue mediators [13]. Stress-related signals in the central nervous system initially act upon the hypothalamus. From there, signals, which respond to stress stimuli in the central nervous system, reach the peripheral nervous system through several pathways. Two main ones are; (1) autonomic neurons and the adrenal medulla system, which release catecholamines; and (2) the hypothalamic-pitutitary-adrenal system, known as the HPA axis, which releases glucocorticoids. The secretion of adrenocortical glucocorticoids is driven by the release of ACTH from corticotropes in the anterior pituitary gland. Neurons in the PVN are the most potent structures capable of inducing ACTH release in response to stress through the release of corticotropin-releasing factor [24].

Microinjections of neostigmine into the hippocampus produce hyperglycemia associated with the secretion of plasma cathecholamines, which showed similarities to stress responses. Regarding the mechanism responsible for the neostigmine-induced elevation of plasma glucose, at least four pathways had been hypothesized; (1) secreted ephinephrine may directly act on the hepatic release of glucose, (2) epinephrine may induce the release of glucagon, (3) direct neuronal control in the pancreas causes glucagon secretion, (4) direct innervation in the liver induces glucose release [6]. Our previous studies showed that ACTH release is accompanied by c-fos expression, a universal marker of neuronal activation, in the PVN of the hypothalamus [24]. We also found that the bed nucleus of the stria terminalis (BNST) is involved in the regulation of ACTH release in response to hippocampal neostigmine injection [4,23]. This structure receives inputs directly from the ventral hippocampus area and sends a heavy axonal projection into neuroendocrine cell regions of the PVN [5,23]. Although lesions of the BNST attenuated the elevation in ACTH, and c-fos expression in the PVN induced by hippocampal neostigmine injection, blood glucose elevation was not altered by the BNST lesions [23,24]. This suggests that

glucose and ACTH are regulated differently within the brain, and that BNST is not involved in the glucose regulation pathways.

The entorhinal cortex occupies a key position in the limbic system, functioning as a relay station between the hippocampus and neocortex. The entorhinal cortex is a major gateway for sensory information into the hippocampal formation. In the current study, the entorhinal cortex lesion attenuated blood glucose elevation but did not affect the ACTH response. These results suggest that the entorhinal cortex plays role in blood glucose regulation and not in ACTH regulation when the hippocampal cholinergic system is activated.

We previously found that the entorhinal cortex is involved in the stress response to immobilization but not to insulin-induced hypoglycaemia [22]. Lesions in this area attenuate ACTH release induced by immobilization but have no effect on the blood glucose response. These findings are not compatible with what we observed in this study using hippocampal neostigmine injections. Although the hippocampal neostigmine–injection model shows similarities to the stress responses in terms of ACTH and glucose profiles in the plasma, the model may activate a pathway in the brain different from what is activated in response to immobilization.

Recent evidence suggests that various stressors activate different regulatory pathways. According to Herman and Cullinan [5], stressors can be divided into two categories. One category is processive (emotional/ psychological) stressors, which activate cortical and limbic areas before the PVN is activated. Signals from multiple sensory modalities are processed in these structures prior to final elaboration of the stress response. The other group is systemic (physical) stressors, which directly threaten the survival and activate the PVN through the ascending catecholaminergic pathway from the brainstem. According to Herman and Cullinan's definition immobilization stress is processive stress. Since the brain pathways activated in the hippocampal neostigmine-injection model were different from the responses to immobilization, this model may show similarities to other types of stress responses. Further investigation should be performed to elucidate the mechanism responsible for this. In conclusion, the present study showed the role of entorhinal cortex in regulating stress response induced by microinjection of neostigmine and the relationship between the entorhinal cortex and hippocampus in stress responses.

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