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## Evaluation of A1 Allele of the DRD2 Gene in Rat Model and Human

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### ABSTRACT

*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. In human study it has been shown that low levels of dopamine D2 receptors are associated with multi drug addiction. 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 no preferring (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. In human study TaqI RFLP was done for detection of one substitution change in 11q22-23 location, those with C(A2) allele have restriction site for TagI enzyme and will cuts while T(A1) allele remains intact. 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 [<sup>11</sup>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 [<sup>11</sup>C] raclopride specific binding in striatum than the NP rats. In human study there was no important difference in A1 allele frequency between two groups. 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.*

### INTRODUCTION

Addiction is a process in which the consumption of drugs in certain individuals lead to mandatory search drugs and inability to stop taking the drug(1, 2). Much research has been done to clarify the underlying neurobiological addiction. Most studies on the etiology have focused on diagnosis of genetic variants that predispose individuals to a range of addictive disorders. Some of the successes achieved in this area and polymorphisms in several genes related to neurotransmitter candidates with the potential for addiction(3). Genetic and environmental factors play a role in drug addiction. Advances in molecular genetics enable us to examine the entire human genome(4). In addition to genetic predisposition, individual temperament and personality traits as potential areas for the development of addiction, contribute to the risk of addiction medicine and clinical features related to treatment response and

prognosis is affected(5). Low DRD2 density in the brains of healthy people associated with high-risk behaviors(6). Dopamine is a chemical in the brain that creates a feeling of satisfaction and released in achievement, satisfaction and stimulation(7). Abuse drugs release dopamine, particularly in areas such as nucleus accumbens and frontal cortex stimulate Perry. Transient overexpression of the dopamine D2 receptor (DRD2) gene in the nucleus accumbency (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(8). In recent decades, a number of studies have done on the relationship between the DRD2 gene polymorphism and mood disorders. In a meta-analysis of the three polymorphisms of this gene, the -141 C ins/del and Ser317/Cys311 and TaqIA in the race Asian and Caucasian, was found no relationship between the -141 C ins/del and Ser317/Cys311 polymorphism and mood anomalies in both populations, but TaqIA DRD2 polymorphism associated with mood disorders, and those with A1A1 genotype there are more susceptible to mood disorders compared with those with the A2A2 and A1A2 genotype. This gene encodes D2 sub type of dopamine receptor. This receptor pairs to G protein which is located in post synaptic neurons and mainly involved in providing a reward in the mesocorticolimbic pathway and prevents the adenylyl cyclase activity. Alternative splicing the mRNA of this gene leads to different transcripts that encodes different isoforms, the long form (D2L) is a standard form and acts as a post synaptic receptor and short form (D2S) is presynaptic and acts as an autoreceptor and sets dopamine levels in the synaptic space(9). Dopaminergic neurons of human are optional in hormone controlling, movement and emotions, which is mainly mediated by D1 and D2 receptors. These macromolecules are belonging to a large family of receptors which binding to G-protein macromolecules belong. Dopamine is a neurotransmitter in the mammalian central nervous system(10). Signals via dopamine D2 receptor control physiological functions related to mobility, hormone production and drug abuse. D2 receptors mostly differentiated to the postsynaptic and somatodendritic auto receptors(9). D2 dopamine receptors are important for several reasons: Many mental illnesses drugs have a major impact on these receptors(11, 12) and their density increased in many psychiatric disorders(13), prolactin secretion from lactotrophs inhibited by D2 receptor stimulation(14). These receptors involve in movement abnormalities(15), drug abuse(16) and pituitary gland tumors(17). Dopamine D2 receptor located on the outer membrane of brain cells therefore forms motivation, emotion and movement. Receptor Protein enables dopamine neurotransmitter to connect these cells to influence the activity. Normally, the molecules of dopamine occupied some receptors of dopamine, while the rest remain available until a stimulus, such as exposure to a drug increase dopamine levels. One hypothesis says that the proportion of D2 receptors affect how severe that a person will respond to stimuli. Imaging studies of the human brain had shown low levels of D2 receptors among users of heroin, nicotine, amphetamines and alcohol(18).

## 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.

### *In animal study:*

Thirty male adult rats (350 – 450 g) were used in this study (15 P and 15NP). 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 (19, 20). 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 bottle was 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(8), 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, 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 5-1 Hamilton microsyringe connected to a 28-gauge internal cannula. Each microinfusion administered 2 $\mu$ l of vector [adenoviral vector containing the cDNA for the DRD2 receptor (AdCMV.DopD2R; 1010 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 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.

*In human study:*

Statistical analysis was conducted with 50 patients with 50 drug and 50 controls were studied.

After the initial consultation and consent, 2 cc blood samples collected into CBC vial containing Anticoagulants 0.05M EDTA, and gently inverted and mixed and stored in the freezer -20. Salting out method was used for DNA extraction.

PCR was done for the DRD2 polymorphisms via designed primers:

Forward primer: 5'-TCTTGGAGCTGTGAACTGGA -3'

And revers primer: 5'TGGCTTAGAACCACCCAGAG-3'

DNA amplified by PCR, using the Taq polymerase enzyme and these primers. These primers creates product with 415 bp. taqI restriction enzyme used for cutting PCR product .PCR product and taqI enzyme incubated in 65 ° C for 30 minutes. taqI enzyme cuts the C polymorphism (A2 allele) and creates DNA segments with 140 and 275 bp while T polymorphism (A1 allele) remains intact.this products electrophoresis in 3% agarose gel.

**DRD2 taqI A polymorphism:**

In taqI polymorphism low performance of DRD2 associated with different functions involved in character stability and susceptible to nerve disorders(21).A number of previous studies had shown the low performance of DRD2 taqI can be genetic background for alcoholism, dependence on opioids, cocaine and gambling. This polymorphism creates three genotypes: CC, CT and TT.T allele is associated with reduction in the binding sites of dopamine in brain that involve in addiction. This can be partially relieved by smoking or drug use.

Neville & etal in 2004 showed that TaqI RFLP is located on ankyrin domain kinase1 (ANKK1) in downstream of DRD2 gene on chromosome11q.23.1(22) DRD2 taqI A in fact is located on exon8 of ANKK1 gene that encodes X-kinase and changes Glu731 Lys amino acid.

## RESULTS

Rats did not show any signs of malaise or weight loss after treatment with the vector, and this was consistent with previous studies (8, 23-26). No alterations in general behavior or locomotors 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 (27). 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 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-DRD2 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.

#### **The outcome of the purity and quality of DNA:**

To consider the quality of extracted DNA electrophoresis pattern performed. If extracted DNA is good and not damaged, it should be form clear band in the gel. The results of DNA electrophoresis on 1% agarose gel is shown in Figure 4-1.

Using agarose gel electrophoresis, quality of DNA tested and confirmed. How quantity of extracted DNA achieved by optical density ratio at 260/280 wavelength, According to the optical density at 260 / 280 DNAs had good quality. Representative examples of coronal PET images of the ST after intravenous injection of [11C] raclopride are shown in Fig. 3

**In the human study:** results showed the percentage of A1 allele was no significantly different in the addicted group compared to the control.

## **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 post treatment. 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 post-treatment. 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 (8, 25, 26). In particular, ethanol preference and intake returned to baseline levels 20 days post treatment with the DRD2 vector, and this was similar to previous results observed in Sprague Dawley rats (8).

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 (8, 25, 26). Therefore, DRD2 vector treatment could not have influenced PET [11C] raclopride binding at 7 weeks. The difference in DRD2 binding (as observed with PET) between P and NP rats thus was negatively correlated with ethanol drinking. Specifically, although P rats exhibited greater alcohol consumption, they displayed lower [11C] 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. PET 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 PET comparison of specific (ST) DRD2 binding relative to non-specific (CB) binding. This popular method of PET analysis of DRD2 binding is unaffected by dose of the injected radiotracer and revealed that NP rats displayed 15% greater [11C] 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 (28, 29).

Although animals were not repeatedly examined with PET over time, these results provided evidence that PET 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 (30). That is, P rats displayed lower [11C] raclopride binding in comparison with NP rats. These PET 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 (31-33). However, our PET 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 (34-37), including most recently in P rats (38). In addition, our findings were consistent with the clinical report that alcoholics showed a DRD2 down-regulation compared with controls (39).

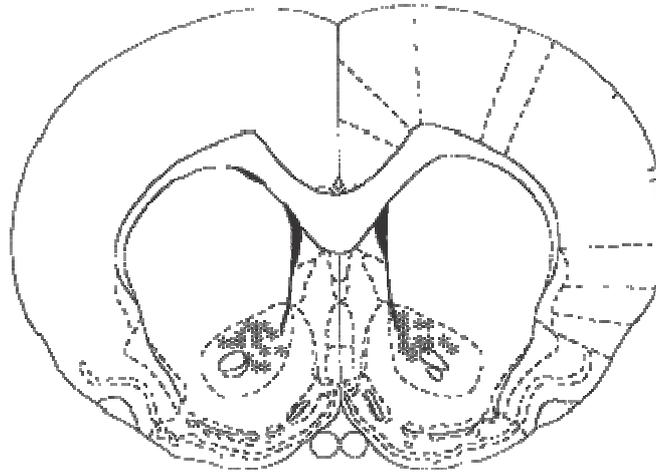
The PET data helped assess the effects of chronic acute ethanol exposure (albeit different doses/group) on DRD2 levels in P and NP rats. These results were comparable to the DRD2 data reported previously in naïve P and NP rats (30). Recently, evidence was provided that alcohol drinking by P rats attenuated D2 autoreceptor.

Function in the NAc (38). In the present study, rats were scanned for PET 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 PET data requires caution because these results were compared with DRD2 binding data from naïve P and NP rats in the literature (30).

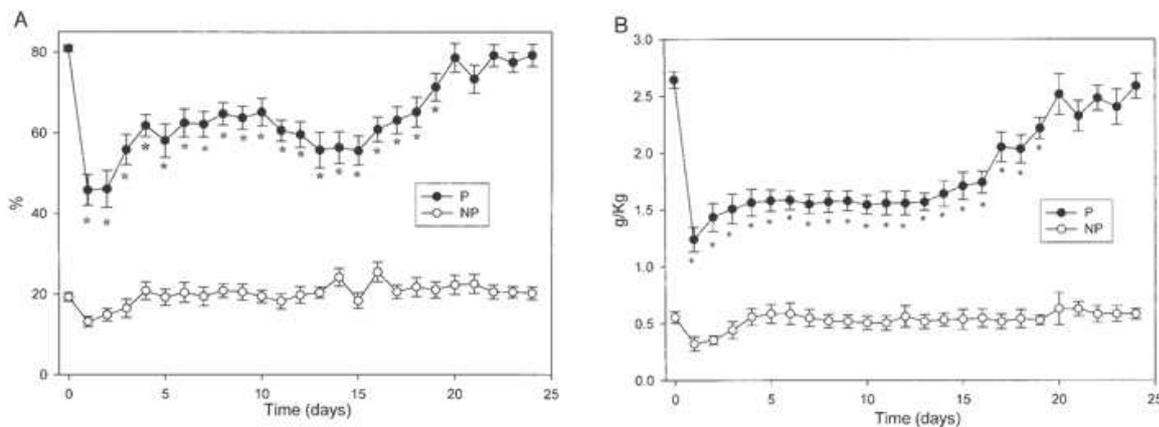
The present results emphasized the important role that DRD2 levels in the NAc may play in the unique ethanol self-administration behavior profile of the P rats. This was in agreement with previous studies that have reported that DRD2 levels were significantly different between naïve P and NP rats. Specifically, DRD2 density was reported to be 20% lower in the olfactory tubercle and NAc of the Sardinian P rats compared with Sardinian NP rats (40). In a comparable study in P and NP rats, P rats showed 20 to 25% lower [3H] sulpiride binding in the caudate putamen, medial and lateral NAc, and VTA compared with NP rats (30). 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

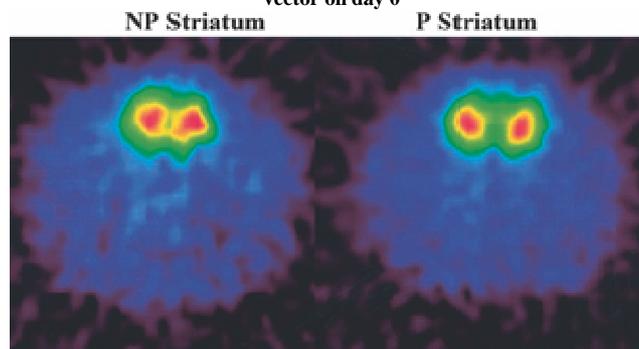
which further stimulation becomes aversive to explain why in individuals with high striatal D2R levels a relatively large dose of a stimulant drug (methylphenidate) was perceived as aversive, whereas in individuals with low D2R levels, it was perceived as pleasurable (41).



**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)

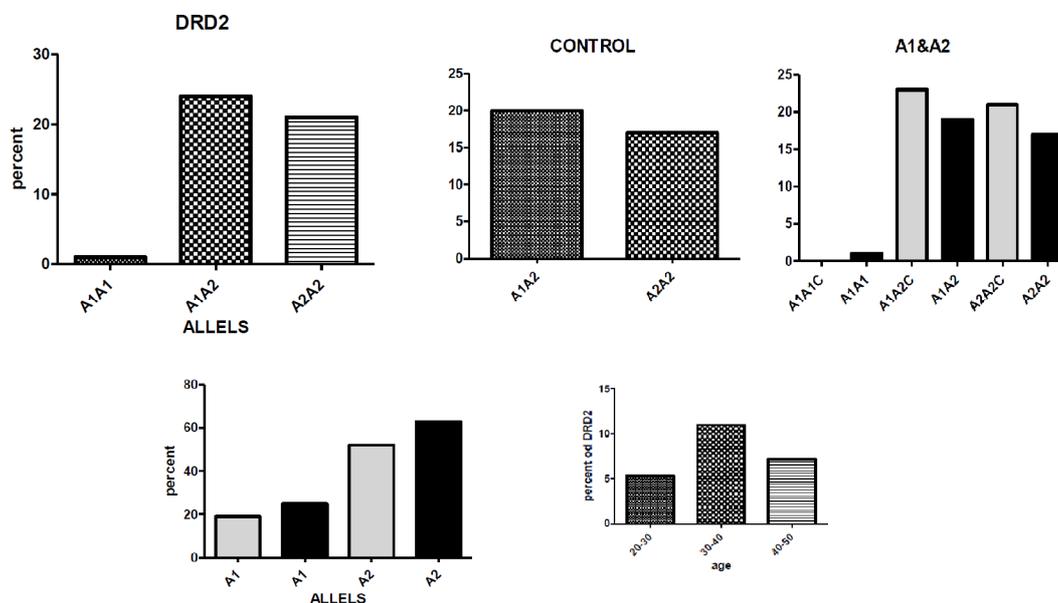


**Fig. 2.** (A) Mean percentage (SE) of ethanol preference over time in P and NP rats in a daily 1-hr limited-access session (\*p < 0.05). Day 0 represents baseline drinking. Animals were treated with the DRD2 vector on day 0. (B) Mean (SE) ethanol intake (g/kg) over time in P and NP rats in a daily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



**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 PET R4 scanner with [11C]raclopride

Figure4: The DRD2 gene expression half a per cent, and the results showed that the alleles A1A2 and A2A2 25 percent to 20 percent compared to the control group were significantly different( $p<0.005$ ).The results showed that in the control group, while in Mtadad A1A1 zero percent to 20 percent( $p<0.005$ ). The percentage of allele A1 allele were significantly different in the addicted group compared to the control. In terms of the age of genes between the ages of 30 and 40 were higher( $p<0.005$ )



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