

Dopamine D₂ Receptor Binding, *Drd2* Expression and the Number of Dopamine Neurons in the BXD Recombinant Inbred Series: Genetic Relationships to Alcohol and Other Drug Associated Phenotypes

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Background: It has not been established to what extent the natural variation in dopamine systems contribute to the variation in ethanol response. The current study addresses this issue by measuring D₂ dopamine (DA) receptor binding, the expression of *Drd2*, the number of midbrain DA neurons in the BXD recombinant inbred (RI) series and then compares these strain means with those previously reported for a variety of ethanol and other drug-related phenotypes.

Methods: Data were collected for 21 to 23 of the BXD RI strains and the parental strains. D₂ DA receptor autoradiography was performed using ¹²⁵I-epidepride as the ligand [Kanes S, Dains K, Cipp L, Gatley J, Hitzemann B, Rasmussen E, Sanderson S, Silverman S, Hitzemann R (1996) Mapping the genes for haloperidol-induced catalepsy. *J Pharmacol Exp Ther* 277:1016–1025]. *Drd2* expression was measured using the Affymetrix oligoarray system. Immunocytochemical techniques were used to determine the number of midbrain DA neurons [Hitzemann B, Dains K, Hitzemann R (1994) Further studies on the relationship between dopamine cell density and haloperidol response. *J Pharmacol Exp Ther* 271:969–976].

Results and Conclusions: The range of difference in receptor binding for the RI strains was approximately 2-fold in all regions examined, the core, the shell of the nucleus accumbens (NAc) and the dorso-medial caudate-putamen (CPu); heritability in all regions was moderate—($h^2 \sim 0.35$). *Drd2* expression in forebrain samples from the RI and parental strains ranged 1.5- to 2-fold and h^2 was moderate—0.47. Variation in the number of tyrosine hydroxylase (TH) positive neurons was moderate, 41% and 26% and h^2 was low—0.19 and 0.15 for the ventral tegmental area (VTA) and substantia nigra compacta (SNc), respectively. Significant correlations were found between D₂ DA receptor binding and the low dose (1.33 g/kg) ethanol stimulant response. ($p < 0.002$) and between *Drd2* expression and conditioned place preference (CPP) ($p < 0.0005$). No significant correlations were detected between ethanol preference and either receptor binding or *Drd2* expression; however, a significant correlation was found between preference and *Ncam* expression. *Ncam* is approximately 0.2 Mb from *Drd2*. Overall, the data suggest ethanol preference and CPP are associated with the expression of *Drd2* or closely linked genetic loci.

Key Words: Genetic, Recombinant Inbred, QTL, Dopamine Receptor, *Drd2* Expression, Microarray, Tyrosine Hydroxylase, Conditioned Place Preference.

THERE IS AMPLE evidence that dopamine (DA) neurotransmission, particularly the D₂ DA receptor mediated component, is involved in the acute reinforcing effects

of ethanol, ethanol-seeking behavior, ethanol sensitization and ethanol relapse (reviewed in Weiss, 2000). For example, it has been repeatedly demonstrated (see Belknap and Atkins, 2001) that a QTL for ethanol consumption/preference shows a peak on mouse chromosome 9 approximately at the *Drd2* locus; a QTL for ethanol place preference is also found near the *Drd2* locus (Cunningham, 1995). Other data (Hitzemann, 1998; Kanes et al. 1996) suggest that D₂ DA receptor density is, in part, regulated by either *Drd2* or a closely linked locus. Overall, these data plausibly suggest that there are likely to be relationships (perhaps genetic) between ethanol phenotypes and the D₂ DA receptor. However, the data supporting shared genetic mechanism(s) remain controversial, especially so from the clinical perspective. Clinical interest in this area was stimulated by the observation of Blum, Noble and colleagues (Blum et al. 1990; Noble et al. 1991) that an association existed between

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a *TaqI* polymorphism in DRD2 and alcoholism and that the *TaqI* polymorphism was associated with differences in receptor density. Although some subsequent studies have supported these associations (Blum et al. 1991, 1993; Comings et al. 1991; Noble et al. 1993; O'Hara et al. 1993; Parisan et al. 1991; Persico et al. 1996; Smith et al. 1992), the opposing evidence is also quite strong (Barr and Kidd, 1993; Chen et al. 1997; Cook et al. 1992; Goldman et al. 1998; Heinz et al. 1996; Turner et al. 1992;). What is not in doubt is that both among humans and animals, there is a remarkable variation in D₂ receptor binding. For example, both PET and SPECT measures of human receptor availability detect differences of more than 2-fold among "normal" controls (Laruelle et al. 1998; Pohjalainen et al. 1998; Volkow et al. 1999) and these differences may significantly affect central stimulant responsiveness (Volkow et al. 1999). Kanes et al. (1993, 1996) found large and reliable differences in receptor binding among both inbred mouse strains and a genetically segregating mouse population of 1.5 and 3-fold, respectively.

The question that has not been answered is how these apparent differences in D₂ DA receptor density affect acute and chronic responses to alcohol. One approach to this problem would be to determine both *Drd2* expression and D₂ receptor density in the BXD recombinant inbred (RI) series and compare these strain means with the reported strain means for those ethanol phenotypes which have been directly or indirectly related to the D₂ dopamine receptor system, especially including ethanol preference and consumption and conditioned place preference (CPP) (Cunningham et al. 2000; Risinger et al. 2000). In this way it is possible to determine (for the BXD RI series) the genetic relationship(s) between receptor regulation and ethanol phenotypes. Complimentary behavioral data are also available for a number of drugs which are known to increase or decrease dopamine receptor activity including, methamphetamine, cocaine and haloperidol (e.g., Kanes et al. 1996). This provides a broader context for assessing the specificity of the ethanol relationships.

Jones et al. (1999) have recently quantified the distribution of D₂/D₃ receptor density in 16 strains of the BXD RI series and used these strain means to map putative quantitative trait loci (QTLs) that modulate receptor density. No QTL(s) for receptor binding were detected in the region of the *Drd2* locus on chromosome 9. However, quite importantly, these authors found that the strain means varied as much as 3-fold in the nucleus accumbens (NAc) and by a remarkable 7-fold in the caudate-putamen (CPU). This range of variation far exceeds the rather modest differences between the parental C57BL/6J (B6) and DBA/2J (D2) inbred strains (Kanes et al. 1993) but is somewhat consistent with the marked transgressive segregation for D₂ receptor binding, previously noted in a B6×D2 F₂ intercross (Kanes et al. 1996). For the reasons noted above, we placed a high priority on independently replicating the Jones et al. (1999) data.

In the current study, we addressed the dopamine receptor hypothesis of differential ethanol sensitivity and response. Our receptor analysis was similar to the work of Jones and colleagues in that we used the same D₂/D₃ receptor specific ligand was used—¹²⁵I-epidepride. Our studies differed in that we used quantitative autoradiography (vs membrane binding) and we report separately on the core and shell of the nucleus accumbens; the shell is a widely agreed key to our understanding of drug reward (Weiss, 2000). The binding data are complimented by data on the forebrain expression of *Drd2* and these data have been used to examine the relationships among gene expression, receptor binding and various alcohol and drug related phenotypes.

In addition to viewing gene expression as a cause of the variation in D₂ receptor binding, we also now report on the relationships between receptor binding and the number of DA neurons in the substantia nigra compacta (SNc) and the ventral tegmental area (VTA). Previous work (Hitzemann et al. 1994) demonstrated that, among a series of inbred mouse strains, neuron number and the number of presynaptic receptors within the SNc were significantly correlated; however, no significant association was detected between neuron number and the number of receptors in the caudate-putamen or nucleus accumbens. These data suggest the independent genetic regulation of neuron number and receptor density; alternatively, failure to find association may have simply reflected the limited statistical power of the previous study. The current study partially addresses this issue as we were able to compare receptor binding and neuron number in 23 of the RI strains. Finally, Casu et al. (2002) have recently reported that there is no association between the number of DA neurons in the VTA and SNc and ethanol preference in the Sardinian Preferring and Non-Preferring selected rat lines. The BXD data set provided an additional opportunity to examine this issue.

METHODS

Animals

Male C57BL/6 (B6), DBA/2 (D2) and BXD RI mice were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were housed 2–4/cage in a constant temperature colony room with a 12 hr light/dark cycle. Food and water were provided ad libitum throughout the study. All mice were allowed a minimum of 10 days to acclimatize to the colony conditions prior to testing. All animal care protocols were approved by the Laboratory Animal Users Committee at the State University of New York at Stony Brook and conformed to the NIH Guidelines for Using Animals in Intramural Research.

Quantitative Receptor Autoradiography (QRA)

Binding to the D₂/D₃ receptor subtypes was determined using quantitative receptor autoradiography and [¹²⁵I]epidepride as the ligand (Clanton et al. 1991). Using the experimental conditions described below, raclopride (200 nM) completely inhibited the binding of epidepride. This concentration of raclopride is one that will block binding to D₂ and D₃ but not D₄ receptors (Seeman and Van Tol, 1994). Previously (Kanes et al.

1996) we examined the binding of [³H] 7-OH-DPAT (Levesque et al. 1992) to estimate the regional localization of D₃ receptor binding and the extent to which D₃ receptor binding contributes to the overall epidepride binding. Significant 7-OH-DPAT binding was detected in the nucleus accumbens; however, at 5 times the K_D concentration, the binding of 7-OH-DPAT was never more than 2.5% of the epidepride binding at a comparable concentration. In the caudate putamen (CPu), the binding of 7-OH-DPAT was < 1% of the epidepride binding.

Brains were sliced in 20 μ sections (six sets) and thaw mounted onto gelatin subbed microscope slides. Sections were collected across the rostral-caudal gradient of the accumbens and approximately matched plates 18 to 23 of Paxinos and Franklin (2001). In initial experiments, adjacent sections were used for nonspecific binding. For a second set of experiments, nonspecific binding was defined by that binding detected in the cerebellum, a region essentially devoid of D₂ and D₃ receptors—the advantage of this technique was that the nonspecific binding could be determined from the same slide as that used for specific binding, which reduced the average within strain variability by > 60%. This approach is widely used in clinical imaging studies (PET, SPECT) but is generally not used in quantitative autoradiography. However, since the goal of the current studies was an accurate determination of the rank order of the strain means and not an absolute value, the cerebellar subtraction design was utilized.

Slides were stored at -80°C until needed. Frozen slides were first warmed to room temperature under a gentle stream of air, then preincubated for 30 min at 4°C in incubation buffer (50 mM tris pH = 7, 120 mM NaCl) without ligand. These slices were transferred to fresh buffer at 24°C for 30 min and then incubated with [¹²⁵I]epidepride (300 pM; K_d = 50 pM) in standard incubation buffer at 30°C for 2 hr. After washing 4× in ice-cold buffer, slides were air dried, desiccated overnight at 4°C and then exposed to high performance autoradiography film (Hyperfilm ³H, Amersham, Inc.) for 4–6 hr. Since [¹²⁵I] epidepride also binds appreciably to α₂ adrenergic receptors, 100 nM idazoxan was included in all incubation mixtures.

All films were calibrated to ¹²⁵I radioactive standards (Amersham, Inc.) and read with a computer based image analysis system (MCID, Image Research Inc.) using landmarks and regional definitions identical to those previously described (see Kanets et al. 1993; Qian et al. 1992). Further details, including drawings of the regions analyzed are found elsewhere (Hitzemann et al. 1993, 1994; Kanets et al. 1993; Qian et al. 1992, 1993). All experiments were performed in duplicate; the data were averaged prior to analysis. The data obtained were analyzed by a general ANOVA program (CSS, Statsoft, Inc., Tulsa, OK) for the effects of strain, region and section and for the interactions among these effects.

Immunocytochemistry of the TH positive cells in the SNc and VTA

Animals (N = 10–15 strain) were anesthetized with a mixture of xylazine (8 mg/kg) and ketamine (60 mg/kg) and then were perfused *via* cardiac cannula in serial fashion with approximately 1) 20 ml of 0.1 M phosphate buffer (pH = 7.4) + 0.9% NaCl (PBS), 2) 50 ml of 4% paraformaldehyde + 0.1% glutaraldehyde in PBS, and 3) 20 ml of 10% sucrose in PBS. After perfusion, the brain was removed and stored in 30% sucrose in PBS. Thirty micrometer frozen coronal sections were collected. Data were collected here for sections beginning at the point the medial lemniscus intrudes into the TH cell mass and cleanly separates the SNc from the VTA. Sections were incubated with gentle rotation for 3 days with TH antibody (1:2000 dilution) (Eastacres Biologicals, Southbridge, MA) at 4°C. The manufacturer reported that there was no cross-reactivity with dopamine-β-hydroxylase or DOPA decarboxylase. Rabbit anti-sheep γ-globulin and Vectastain ABC kits were used in subsequent steps to complete the immunoperoxidase bridge. For the quantitative morphometric analyses, a Nikon Optiphot microscope and a camera lucida drawing tube was used to obtain a permanent record of cell distribution. Data were analyzed using standard ANOVA techniques.

Microarray Procedures

Drd2 and *Ncam* expression were measured as part of a larger study to develop a transcriptome database for the BXD RI series. Gene expression was measured in 21 BXD RI strains, in both parental strains and the B6D2F1 hybrid using the Affymetrix U74Av2 oligonucleotide microarray. An average of three arrays were hybridized with cRNA samples from a pooled brain preparation that included the forebrain and midbrain from three individuals of the same sex and age (primarily females between 60 and 160 days). The olfactory bulbs were excluded from all dissections. Most estimates of D2 receptor transcript abundance are therefore averages of the hybridization signal from three arrays and a total of nine animals (3 × 3 design). The raw hybridization signal was processed using the Affymetrix MAS 5.0 statistical protocol. MAS 5.0 text output files were log transformed to produce normal or near normal distributions. Log values were then converted to z scores, thereby normalizing the mean and variance across the entire set of arrays. The z scores were rescaled to more closely match typical log base 2 expression data. This was done by increasing the variance from 1 to 4 units and shifting the mean from 0 to 8 units. The range of expression on this scale ranges from a noise floor at 4 units to a high of approximately 15 units (expression range of 2¹¹). Each unit is roughly equal to a 2-fold difference in hybridization signal. Analysis of 9 transcripts using these procedures and RT-quantitative PCR indicated good agreement. However, a 1 unit difference will typically correspond to a 2.2–2.5 fold difference in cycle number.

Focused QTL Mapping—Chromosome 9

A QTL analysis (limited to chromosome 9) was performed by correlating the strain means for D₂/D₃ receptor binding and *Drd2* expression with the strain distribution patterns of chromosome 9 marker gene loci showing allelic differences between the B6 and D2 strains. Data are presented as the *r* value obtained for each marker; a positive sign is used to denote D2 alleles that increase receptor binding and a negative sign is used to denote B6 alleles that increase receptor binding. The threshold for acceptance of a candidate QTL was set at *p* < 0.01 (Belknap et al. 1996); assuming 25 strains are entered into the analysis, this threshold is sufficient to detect an effect size of 0.25 of the genetic variance.

Correlations of DA Phenotypes with Behavioral Phenotypes

The published behavioral phenotypes and original sources are as follows: open-field activity (Koyner et al. 2000); haloperidol-induced catalepsy (Kanets et al. 1996); ethanol-induced locomotor activity (Demarest et al. 2001); sensitivity and sensitization to ethanol-induced locomotor activity (Phillips et al. 1995); acute and sensitized locomotor response to methamphetamine (Grisel et al. 1997); acute and sensitized response to cocaine (Miner and Marley, 1995; Phillips et al. 1998); phencyclidine induced locomotor activity (Alexander et al. 1996); ethanol-induced hypothermia (Crabbe et al. 1994, 1996); ethanol conditioned place preference (Cunningham, 1995); alcohol acceptance and preference (Rodriguez et al. 1994, 1995); ethanol preference and consumption (Phillips et al. 1994); ethanol induced locomotion (Crabbe et al. 1983). Given the multiple comparisons being made, the threshold of significance for the genetic correlations was set at *p* < 0.005; this increase in threshold was deemed appropriate as there were only four distinct ethanol phenotypes in the primary analysis. The other behavioral phenotypes were included only for comparative purposes.

RESULTS

Strain Means for D₂/D₃ Receptor Binding in the BXD RI Series

Data were collected for 23 of the BXD RI strains, in three brain regions (core and shell of the NAc and the dorsomedial CPu) and across five rostral-caudal sections

(see Methods for details). N was 9–15 for each strain and all experiments were performed in duplicate. Duplicates were averaged prior to analysis. The ANOVA revealed a significant effect for strain ($F_{22,4235} = 98, p < 10^{-6}$), region ($F_{2,4235} = 98, p < 10^{-6}$) and section ($F_{4,4235} = 3.4, p < 9 \times 10^{-3}$). The interaction effect was significant for strain \times region ($F_{44,4235} = 6.3, p < 10^{-5}$) and region \times section ($F_{8,4235} = 6.0, p < 10^{-5}$) but not for strain \times region or strain \times region \times section. The split halves reliability for the strain \times region data varied from 0.72 to 0.84. Heritability (h^2) (see Falconer and Mackay, 1996) estimates for the core, shell and CPU were, 0.34, 0.39, and 0.37, respectively.

The strain \times region data are presented graphically in Fig. 1. The actual strain means are available by e-mail request to the lead author. The percent difference of receptor binding from lowest to highest was 126%, 153% and 97% for the core, shell and CPU, respectively. Regardless of region, both the B6 and D2 strains were representative of the high binding phenotype. The inset of Fig. 1 shows the distribution of average *Drd2* expression among the RI strains—data are presented in terms of the “signal” measure from the Affymetrix MAS 5.0 analysis. The average signal was approximately 4 times higher than that found in whole brain but only 1/3 of the signal found in the dorsomedial CPU (R. Hitzemann, unpublished observations). The ratio of the B6/D2 signals is 1.27. The h^2 was estimated at 0.47; split-halves reliability could not be estimated.

Sixteen of the RI strains were common both to this study and Jones et al. (1999). The correlation for the binding data between either the core or shell (this study) and the total accumbens (Jones et al. 1999) was poor, 0.25 and 0.18, respectively (see Table 1). However, there was a moderate correlation ($r = 0.57, p < 0.026$) between the two data sets for the CPU; however, given the multiple correlations performed, this correlation was not viewed as significant.

For eighteen of the RI strains and the two parental strains data were available for both *Drd2* expression and D₂/D₃ DA receptor binding. There were no significant correlations between receptor binding and *Drd2* expression (Table 1).

Table 2 provides a QTL analysis for the expression and binding data (chromosome 9 only); the marker set used was edited from the full set available at www.jax.org to eliminate the redundant markers (identical strain distribution patterns). For comparison, the Phillips et al. (1994) data set for ethanol preference and consumption, the Cunningham (1995) data set for ethanol conditioned place preference and the Crabbe et al. (1983) data set for low dose ethanol activation have been included in the analysis. A strong QTL was detected for *Drd2* expression, with an apparent peak at *Xmv15* ($p < 0.001$). Note that it is the B6 allele that increases expression. No significant QTLs were detected for receptor binding. The QTL data for the four ethanol phenotypes reiterate the candidate QTLs previously reported (Cunningham 1995; Gora-Maslak et al. 1991; Phillips et al. 1994) for these phenotypes on chromosome 9.

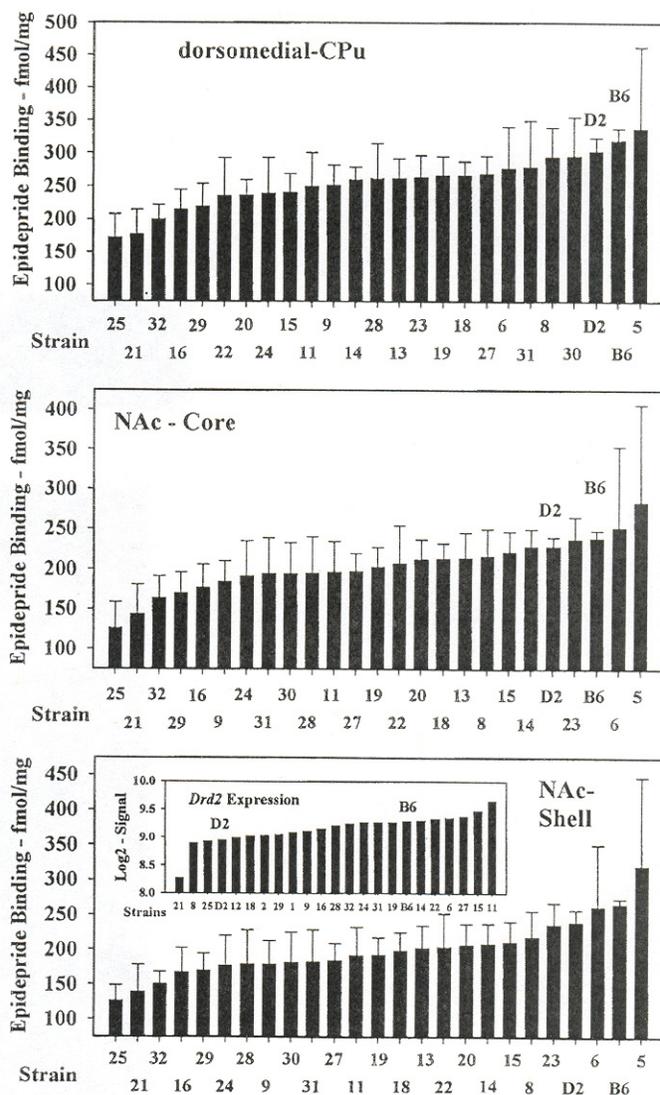


Fig. 1. Strain means for [¹²⁵I]-epidepride binding in the BXD recombinant inbred (RI) series. Data are presented for the dorsomedial caudate-putamen, the core and shell of the nucleus accumbens. Data were collected for six sections through each region; since there was no strain \times region \times section interaction, data were collapsed across section. Also shown are the strain means for the parental DBA/2J (D2) and C57BL/6J (B6) parental lines. Data are expressed as the means \pm SE. N = 10–15 animals per strain; each assay was run in duplicate. The concentration of [¹²⁵I]-epidepride was 4–5 times the estimated K_D . The inset shows the data for *Drd2* expression in the mouse forebrain; data are expressed as the log of the relative signal (base 2). Details are found in the methods. N averages three for each of the strains.

Strain Means for the Number of Tyrosine Hydroxylase (TH) Positive Neurons in the BXD RI Series

Data were collected for 25 of the BXD RI strains, in two brain regions (substantia nigra zona compacta [SNc] and ventral tegmental area [VTA]) and across six rostral-caudal sections (see Methods for details). N was 10–15 for each strain. The ANOVA revealed a significant effect for strain ($F_{25,3424} = 39, p < 10^{-6}$), region ($F_{1,3424} = 8000, p < 10^{-6}$) and section ($F_{5,3424} = 130, p < 10^{-6}$). The interaction effects were significant for strain \times re-

Table 1. Correlation Matrix in the BXD RI Series for the Dopamine Transporter (DAT), the Number of Tyrosine Hydroxylase (TH) Positive Cells in the Ventral Tegmental Area (VTA) and Substantia Nigra Compacta (SNc), D₂/D₃ Dopamine Receptor Binding in the Nucleus Accumbens (NAc) (Core and Shell) and Dorsomedial Caudate Putamen (CPu) and Forebrain *Drd2* Expression

	DAT ^a	TH VTA	TH SNc	D ₂ /D ₃ Core	D ₂ /D ₃ Shell	D ₂ /D ₃ CPu	D ₂ /D ₃ NAc ^b	D ₂ /D ₃ CPu ^b	<i>Drd2</i>
DAT ^a									
TH - VTA	0.28								
TH - SNc	-0.09	0.59^c							
D ₂ /D ₃ Core	-0.08	-0.16	0.07						
D ₂ /D ₃ Shell	0.09	0.17	-0.06	0.96					
D ₂ /D ₃ CPu	-0.26	-0.05	-0.23	0.83	0.82				
D ₂ /D ₃ NAc ²	-0.38	-0.14	-0.02	0.24	0.18	0.44			
D ₂ /D ₃ CPu ²	-0.23	-0.38	-0.05	0.34	0.26	0.57	0.71		
<i>Drd2</i> ^d	-0.12	-0.04	0.24	0.46	0.36	0.46	-0.12	-0.05	

^a Data from Janowsky et al. (2001).^b Data from Jones et al. (1999).^c Correlations in bold significant at $p < 0.01$.^d Data from mouse forebrain - Affymetrix oligomer array (U74aV2).**Table 2.** QTL Analysis on Mouse Chromosome 9 for Various Dopamine and Ethanol Phenotypes

Marker	cM	Phenotype							
		<i>Drd2</i> Expression	D ₂ Binding NAc-Core	D ₂ Binding NAc-Shell	D ₂ Binding Cpu	Ethanol Preference	Ethanol Consumption	Ethanol CPP	Ethanol Activation
<i>Ets 1</i>	15	-0.17	-0.33	-0.29	-0.46	-0.33	-0.37	0.16	-0.07
<i>Lap 1</i>	20	-0.28	-0.38	-0.34	-0.45	-0.47	-0.51	0.13	0.18
<i>D9Rik69</i>	25	-0.56	-0.34	-0.35	-0.27	-0.64	-0.63	-0.39	0.46
<i>Drd2</i>	28	-0.56	-0.29	-0.27	-0.36	-0.64	-0.63	-0.3	0.42
<i>Ncam</i>	28	-0.61	-0.4	-0.38	-0.4	-0.64	-0.64	-0.33	0.49
<i>Xmv15</i>	36	-0.68	-0.2	-0.14	-0.12	-0.58	-0.52	-0.55	0.69
<i>D9Rik59</i>	37	-0.63	-0.19	-0.13	-0.16	-0.58	-0.52	-0.49	0.69
<i>Myo5a</i>	42	-0.56	-0.12	-0.01	-0.03	-0.36	-0.36	-0.4	0.58
<i>D9Byu2</i>	44	-0.56	-0.31	-0.23	-0.19	-0.44	-0.43	-0.31	0.6
<i>D9Rik62</i>	48	-0.53	-0.19	-0.13	-0.14	-0.26	-0.25	-0.21	0.47
<i>D9Ncvs53</i>	50	-0.53	-0.19	-0.13	-0.14	-0.26	-0.25	-0.21	0.47
<i>D9Rik65</i>	54	-0.19	-0.03	0.01	-0.13	-0.34	-0.36	-0.05	0.12
<i>D9Byu3</i>	58	-0.58	-0.26	-0.21	-0.29	-0.33	-0.33	-0.21	0.47
<i>D9Rik66</i>	61	-0.19	-0.1	-0.1	-0.08	0.01	-0.02	0.03	0.12
<i>Bgl</i>	66	-0.53	-0.27	-0.22	-0.23	-0.08	-0.07	-0.28	0.12
<i>D9Byu5</i>	71	-0.23	-0.09	-0.09	-0.21	-0.1	-0.13	0.24	0.3

The QTL Analysis was performed as described elsewhere (Kanes et al. 1996). The strain means for *Drd2* expression and D₂ DA receptor binding are found in Fig. 1. The strain means for ethanol preference and consumption (Phillips et al. 1994), conditioned place preference (Cunningham, 1995) and ethanol-induced activation (Crabbe et al. 1983) are taken from the original publications. Values in bold are significant at $p < 0.01$. A negative sign indicates that the B6 allele increases the phenotypic value.

region ($F_{25,3424} = 8.6$, $p < 10^{-6}$), strain \times section ($F_{125,3424} = 3.8$, $p < 10^{-6}$), region \times section ($F_{5,3424} = 180$, $p < 10^{-6}$) and strain \times region \times section ($F_{125,3424} = 3.6$, $p < 10^{-6}$). The split halves reliability for the strain \times region data were 0.65 and 0.78 for the SNc and VTA, respectively. Heritability estimates for the VTA and SNc were, 0.15 and 0.19, respectively. The strain \times region data are presented graphically in Fig. 2. The data for strain, region, section and the relevant interactions are available by request. Figure 2 illustrates that the percent difference of TH positive neurons from lowest to highest was 41 and 26% for the VTA and SNc, respectively. In both regions the D2 strain was the extreme high TH neuron number phenotype.

Correlations among the DA Phenotypes

The complete correlation matrix for D₂/D₃ receptor binding, *Drd2* expression and TH neuron number is shown in Table 1; data for the dopamine transporter (DAT) bind-

ing and taken from Janowsky et al. (2001) are also included. The minimum number of common strains for any correlation was $N = 18$. No significant correlations across parameters (DAT, D₂/D₃, *Drd2*, TH) were detected.

Genetic Correlations Between Dopamine and Ethanol Phenotypes

The list of ethanol and drug-related phenotypes used in the analysis are found in Table 3; basal locomotor activity was also included since many of the drug phenotypes are a constructed as a difference score from basal activity. Correlations are provided for the three DA phenotypes where significant effects (defined here as $p < 0.005$) were detected. The most significant association was found between ethanol conditioned place preference and *Drd2* expression ($p < 0.0005$). The behavioral phenotype entered into the analysis was the percent time spent on the drug-associated grid during a 30 min trial (see Cunningham, 1995). The training dose of ethanol

DISCUSSION

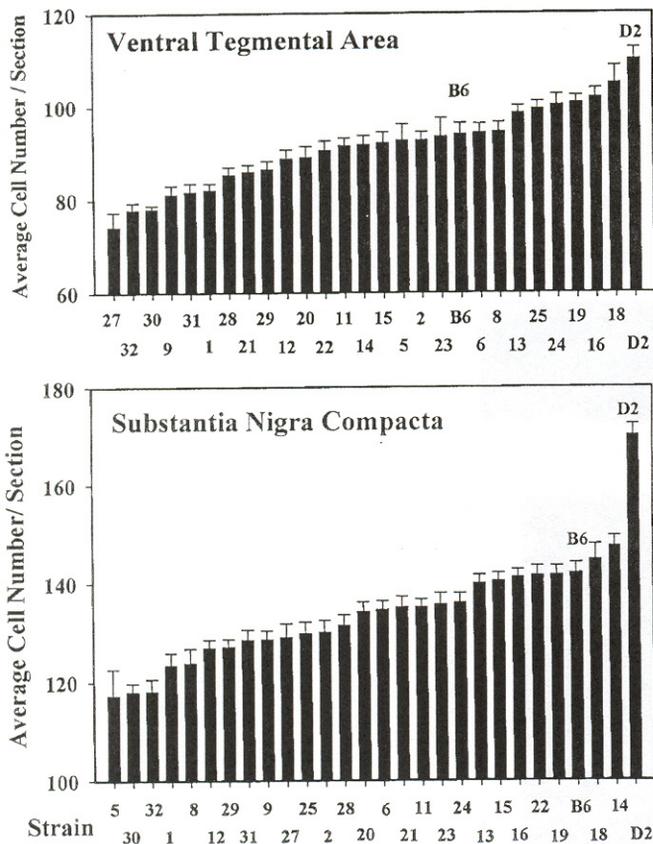


Fig. 2. Strain means for the number of tyrosine hydroxylase (TH) neurons in the ventral tegmental area and substantia nigra compakta of the BXD RI series. Data were collapsed across sections for each region (see Methods). Also shown are the strain means for the parental DBA/2J (D2) and C57BL/6J (B6) parental lines. Data are expressed as the means \pm SE. $N = 10$ –15 animals per strain.

was 2 g/kg. This association is also illustrated in Fig. 3; the correlation was positive, i.e., the animals showing the highest level of *Drd2* expression exhibited the highest place preference. Ethanol-induced locomotor activation has been measured for the RI series in several laboratories, with different doses of ethanol; the phenotype is a difference score obtained for each animal between a saline response and an ethanol response. A significant correlation ($p < 0.002$) was detected between receptor binding and the lowest dose ethanol response (1.33 g/kg) (Crabbe et al. 1983). Significant associations were not noted for cocaine and methamphetamine-induced activation. No significant associations were noted for sensitization to ethanol or cocaine, with the exception of a moderate relationship between the number of DA neurons in the VTA and low dose (5 mg/kg) cocaine sensitization. A significant association was detected between *Drd2* expression and cocaine activation (Miner and Marley, 1995) but this was not confirmed with a larger data set (Phillips et al. 1998). No significant associations were detected between any of the DA phenotypes and ethanol acceptance, preference or consumption.

The data presented here confirm earlier observations from this laboratory (Hitzemann, 1998; Kanes et al. 1993, 1996;) and elsewhere (e.g., Jones et al. 1999; Laruelle et al. 1998; Pohjalainen et al. 1998; Volkow et al. 1999) that there is a marked natural variation in dopamine D_2/D_3 receptor binding. In particular, these data extend our findings of marked differences in receptor binding among a panel of inbred mouse strains (Kanes et al. 1993) and both confirm and extend the previous observation (Jones et al. 1999) of marked differences in binding among the BXD RI series. For four reasons it appears that the differences are largely associated with D_2 and not D_3 receptor binding: a) direct measurement in mice (Kanes et al. 1996) revealed that for both the CPU and the NAc, D_3 receptor binding is only a few percent of D_2 receptor binding; b) the expression of *Drd3* in the CPU is 30 to 40 fold less than that of *Drd2* (unpublished observation); c) Using the conditions described in the Methods, we are unable to detect any specific binding of [125 I]epidepride in the CPU of D_2 receptor KO mice; d) the binding data between the CPU and the NAc are strongly correlated (Table 1).

An issue of some concern for interpreting the current results was the observation that our binding data only moderately correlated with that of Jones et al. (1999) in the CPU and poorly correlated with their NAc data. There are several potential reasons for these differences: a) the autoradiographic data were obtained using discrete targets (dorsomedial CPU, shell and core of the NAc); b) membrane binding will more readily access all receptor pools, including the receptors undergoing recycling; c) the strategies used for determining specific binding were different (see Methods); and d) there were only 16 common strains in both studies and thus, the effect(s) of one or two aberrant data points are amplified. This latter point may in fact be the most important; in our data set BXD-6 is reported as having relatively high binding (Fig. 1), whereas Jones et al. (1999) reported this a low binding strain. Eliminating the data from the analysis, improves the correlation between the CPU data to $r = 0.64$ ($p < 0.012$) and the NAc data now approaches significance ($r = 0.52$, $p < 0.06$).

Despite the less than very strong correlation between data sets, both the current study and Jones et al. (1999) do provide strong evidence that genetic factors contribute significantly to the variation in D_2 receptor binding. Further, these data confirm our earlier results in a B6 \times D2 *F2* intercross (Kanes et al. 1996) showing that D_2 receptor binding is both a quantitative and complex trait, likely to be under the control of multiple genes. In the current study we have again focused on whether or not the regulatory elements are associated with the *Drd2* locus. One of us (RH) has most recently argued that the *Drd2* locus may have only a minor role in the regulation of D_2 receptor binding (Hitzemann, 1998). The current study allowed us to revisit this issue with important new data.

Table 3. Correlations Between Various Ethanol and Other Drug Related Phenotypes and Three Measures of Brain DA Systems: D₂/D₃ Receptor Binding - NAC Shell; Forebrain *Drd2* Expression and the Number of DA Neurons - VTA

Phenotypes	Reference	D ₂ /D ₃ Binding-Shell			<i>Drd2</i> Expression			DA Neurons - VTA		
		N	r	p<	N	r	p<	N	r	p<
Ethanol-Induced Activation - 1.33 g/kg	Crabbe et al. (1983)	17	-0.71	0.002	18	-0.3	0.22	20	-0.32	0.17
Ethanol-Induced Activation - 1.50 g/kg	Demarest et al. (2001)	23	-0.27	0.21	21	-0.25	0.27	24	0.28	0.18
Ethanol-Induced Activation - 2.00 g/kg	Phillips et al. (1995)	18	-0.43	0.072	20	-0.4	0.08	21	0.15	0.15
Sensitization to Ethanol-Induced Activation - 2.00 g/kg	Phillips et al. (1995)	18	-0.21	0.21	20	0.15	0.54	21	0.19	0.4
Ethanol Acceptance	Rodriguez et al. (1994, 1995)	18	0.19	0.45	21	0.03	0.91	21	-0.05	0.82
Ethanol Preference	Rodriguez et al. (1994, 1995)	18	-0.13	0.61	21	0.19	0.42	21	-0.19	0.4
Ethanol Preference	Phillips et al. (1994)	17	0.2	0.44	18	0.4	0.1	19	-0.26	0.27
Ethanol Consumption	Phillips et al. (1994)	17	0.27	0.29	18	0.45	0.06	19	-0.29	0.22
Ethanol Conditioned Place Preference	Cunningham (1995)	17	0.11	0.67	20	0.72	0.0004	20	0	0.99
Ethanol-Induced Hypothermia - 2.0 g/kg	Crabbe et al. (1994)	20	-0.32	0.18	22	0.27	0.23	23	-0.04	0.86
Ethanol-Induced Hypothermia - 3.0 g/kg	Crabbe et al. (1994)	20	-0.34	0.14	22	-0.07	0.74	23	0.14	0.52
Ethanol-Induced Hypothermia - 3.0 g/kg	Crabbe et al. (1994)	20	-0.39	0.09	22	-0.14	0.53	23	0.27	0.22
Cocaine-Induced Activation - 5 mg/kg	Phillips et al. (1998)	22	-0.17	0.46	23	-0.52	0.011	25	0.12	0.57
Cocaine-Induced Activation - 10 mg/kg	Phillips et al. (1998)	22	-0.1	0.66	23	-0.34	0.11	25	-0.33	0.11
Cocaine-Induced Activation - 40 mg/kg	Phillips et al. (1998)	22	-0.08	0.72	23	-0.09	0.67	25	-0.41	0.04
Cocaine-Induced Activation - 32 mg/kg	Miner and Marley (1995)	16	-0.22	0.41	14	-0.7	0.005	16	0.15	0.58
Sensitization to Cocaine-Induced Activation - 5 mg/kg	Phillips et al. (1998)	22	-0.26	0.24	23	0.07	0.77	25	-0.55	0.004
Sensitization to Cocaine-Induced Activation - 10 mg/kg	Phillips et al. (1998)	22	0.08	0.71	23	-0.02	0.92	25	-0.03	0.91
Sensitization to Cocaine-Induced Activation - 40 mg/kg	Phillips et al. (1998)	22	0.14	0.54	23	-0.13	0.54	25	0.16	0.44
Sensitization to Cocaine-Induced Activation - 32 mg/kg	Miner and Marley (1995)	16	0.07	0.79	14	0.43	0.13	16	0.25	0.35
Cocaine-Induced Stereotyped Behavior - 32 mg/kg	Miner and Marley (1995)	16	0.29	0.28	14	0.43	0.13	16	-0.22	0.42
Methamphetamine-Induced Activation - 4 mg/kg	Grisel et al. (1997)	22	0.14	0.53	23	-0.28	0.19	25	-0.2	0.33
Methamphetamine-Induced Activation - 8 mg/kg	Grisel et al. (1997)	22	0.13	0.56	23	-0.18	0.42	25	-0.31	0.13
Methamphetamine-Induced Activation - 16 mg/kg	Grisel et al. (1997)	22	0.06	0.79	23	0.01	0.95	25	-0.12	0.57
Phencyclidine-Induced Activation	Alexander et al. (1993)	22	-0.06	0.78	21	0.16	0.5	25	0.18	0.4
Haloperidol-Induced Catalepsy	Kanes et al. (1996)	23	-0.09	0.65	23	-0.12	0.59	26	0.25	0.22
Basal Locomotor Activity	Koyner et al. (2000)	23	-0.15	0.48	21	-0.07	0.76	24	-0.02	0.92

Data entered into the correlations are found in Figs. 1 and 2 and the cited publications. Values in **bold** are significant at $p < 0.005$ or greater.

Data for *Drd2* expression are reported for 21 RI strains and the two parental strains (Fig. 1). The variation in *Drd2* expression is substantial (~3-fold) and although the data set is small, the data appear to be continuously distributed, i.e., suggesting that *Drd2* expression is itself a complex trait that is suitable for a transcriptome-QTL analysis. The data in Table 2 confirm that a strong QTL for *Drd2* expression is found on chromosome 9 ($r^2 = 0.46$, $p < 0.0004$). Although the data in Table 2 suggest that the peak of the *Drd2* QTL is distal to the *Drd2* locus, this interpretation should be viewed cautiously. Clearly, the *Drd2* locus will lie within the 95% confidence interval for the QTL. Furthermore, the inclusion or exclusion of even a single strain in the QTL analysis can easily move the QTL peak substantially forward or back. This point also needs to be considered when comparing the *Drd2* analysis with the analyses for other phenotypes, given that strains included in all analyses are not identical. Overall, we would argue that the most parsimonious explanation of the *Drd2* expression QTL is the presence of polymorphisms in the *Drd2* regulatory elements that have the net effect of the B6 allele(s) increasing gene expression.

In contrast to the data for *Drd2* expression, no QTL(s) were detected for D₂ binding data in the shell or core of the NAC or the dorsomedial CPU (Table 2). A similar conclusion for their data set was reached by Jones et al. (1999). Complimenting these observations, no significant associations were detected between *Drd2* expression and receptor binding (Table 2). This observation that there is a substantial disconnect between *Drd2* expression and receptor binding has been noted elsewhere. For example, Qian et al. (1993) using a nuclease protection assay was able to demonstrate that, while the regional receptor gradients correlated generally well with *Drd2* expression, differences in receptor binding among animals selectively bred for differences in haloperidol response did not follow *Drd2* expression. Subsequent studies pointed to differences in post-translational processing as the likely candidate for individual differences in D₂ receptor binding (Qian et al. 1993). Both Qian et al. (1992) and Hitzemann et al. (1994) provided some evidence that individual differences in the number of DA neurons may be associated with differences in receptor density. The current study provided an opportunity to also re-examine this issue. These data summa-

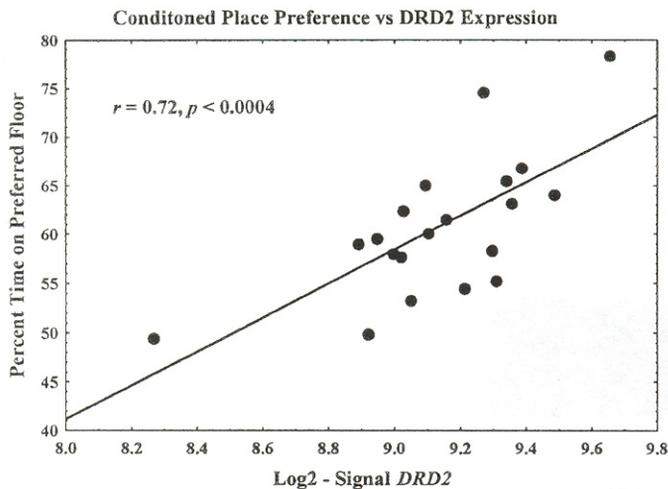


Fig. 3. The relationship between *Drd2* expression and alcohol conditioned place preference in the BXD RI series. The data for *Drd2* expression are taken from Fig. 1. The data on conditioned place preference are taken from Cunningham (1995); these data are expressed as the percent time on the "alcohol" floor.

ized in Table 2, could not detect an association. However, it should be noted that the variation in the number of DA neurons within both the VTA and SNc was relatively modest and the heritability of these phenotypes was poor. Overall, the data in Table 2, which also include data for the striatal DA transporter (DAT) (Janowsky et al. 2001), illustrate that there does not appear to be a set of simple rules which organize the natural variation in brain DA systems. From the BXD transcriptome database, one could also add *Drd3* and *Drd4* expression but these data provide no greater clarity to the problem (unpublished observations).

Using an experimental design essentially identical to that of the current study, Janowsky et al. (2001) found in a sample of 20 BXD RI strains that DAT binding was correlated with cocaine and methamphetamine-induced locomotor activation and thermic responses (hypo- or hyperthermia), but was not correlated with behaviors related to sensitization, reward, voluntary consumption, stereotypy, or drug-induced seizures. These authors also found a major QTL for DAT binding on chromosome 19 near the *Pomc-*ps1** locus but were unable to detect a QTL on chromosome 16 near the *Dat* locus. *Dat* expression data are not available in the BXD transcriptome database, so at present it remains unknown if there is a disconnect between *Dat* expression and DAT binding similar to that found in the current study for *Drd2* expression and D₂ receptor binding; however, it is clear that neither DAT nor D₂ receptor binding map to their respective gene loci.

The analyses summarized in Table 3 illustrate several points of interest. First, no significant associations were found between the DA parameters and ethanol acceptance, preference and consumption. These observations can also be extended with similar negative results to the data for

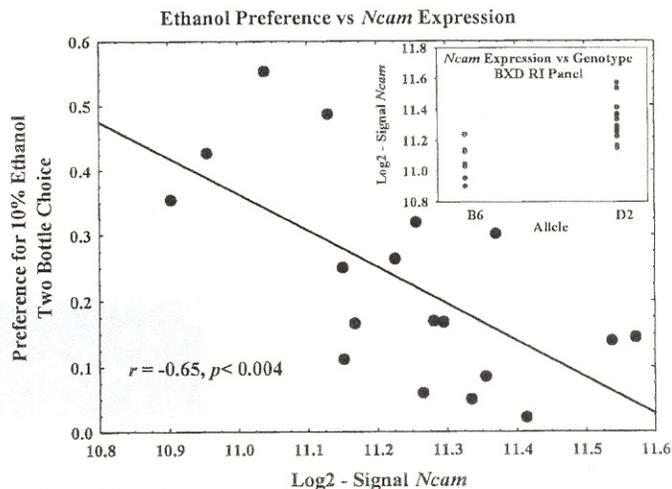


Fig. 4. The relationship between *Ncam* expression and preference for 10% ethanol (two-bottle choice) in the BXD RI series. The data for *Ncam* expression were extracted using the same procedures as for the extraction of *Drd2* expression. The ethanol preference data are taken from Phillips et al. (1994). The inset shows the relationship between *Ncam* expression and *Ncam* genotype (data taken from the BXD database at www.jax.org).

cocaine and methamphetamine preference and consumption found in Janowsky et al. (2001). Thus, despite the overwhelming evidence that DA systems and, in particular, the D₂ receptor mediated component, have important roles in the regulation of these phenotypes and despite the QTL evidence which points to the *Drd2* locus (Table 2), we were unable to find evidence for a genetic link between the drug and DA phenotypes. One cannot ignore the possibility that in some discrete brain region or regions, D₂ dopamine receptors do play an important role; however, we would argue that our attention should turn elsewhere. Given this perspective we returned to the BXD transcriptome database and queried for significant correlations between gene expression and ethanol preference/consumption (Phillips et al. 1994). The query was limited to ± 10 cM from the *Drd2* locus; the region included 140 unique genes or transcripts. Three significant correlations were detected for ethanol preference: *2310045A07Rik* ($r = 0.60, p < 0.008$), *Ncam* ($r = -0.65, p < 0.004$) and *D9Wsu138e* ($r = 0.68, p < 0.002$). *2310045A07Rik* is predicted to be a gene but the function is unknown and there are no known homologies; the *Wsu* transcript has homology with a putative ionotropic glutamate receptor *Grin11a* (fragment). Given the position of the preference QTL (Table 2) and the position of the *Wsu* transcript as approximately 20 Mb from either of the other genes (Ensembl Database), both *Ncam* and *2310045A07Rik* appear to be better candidates. The data for *Ncam* are presented in Fig. 4, illustrating that increased preference is associated with decreased *Ncam* expression. The inset of Fig. 4 shows that *Ncam* like *Drd2* appears to have strong cis-regulatory elements. Further, given the focus of the current study, it is of interest to note that *Ncam* has been shown to be important for the development and maintenance of brain DA systems (e.g., Shults and Kimber 1992).

Thus, *Ncam* could be viewed as an attractive quantitative trait gene for ethanol preference. However, we argue that it should be viewed as such only most cautiously and that an exhaustive proof is necessary (see, e.g., Belknap et al. 2001). More importantly we would argue that the data found in Fig. 4 are a nascent example of the discovery process that is possible by integrating QTL analysis and functional genomics.

Differently than ethanol drinking preference, ethanol conditioned place preference (CPP) showed a strong association with *Drd2* expression. The data sets used here for drinking preference and CPP are not genetically correlated ($r = 0.34, p > 0.19$) suggesting that compared to drinking preference, CPP engages a different set of the variables associated with ethanol's rewarding or hedonistic properties. However, like drinking preference, CPP shows strong regulation by brain DA systems (see, e.g., Cunningham et al. 2000 and references therein). The question immediately arises as to how to interpret the relationship between CPP and *Drd2* expression given the lack of correlation between *Drd2* expression and D₂ receptor binding (see above). As a first step to investigating this issue, the expression data need to be independently replicated preferably in a target region of interest, such as the shell of the NAc and preferably using a different analytic platform, e.g., RT-PCR. Assuming the strain distribution pattern is replicated, our preference for the next step would be to determine whether or not there are brain regions where receptor density parallels gene expression. This analysis could begin with the phenotypic extreme RI strains (BXD-11 (high) and BXD-21 (low)). BXD-21 is a low binding strain (see Fig. 1) but BXD-11 is not a high binding strain and despite a 170% difference in gene expression, the difference in receptor binding between strains is only 39% (NAc shell). Assuming that 2- to 3-fold larger differences could be found in other D₂ receptor rich brain regions not investigated in the current study (e.g., the central nucleus of the amygdala) and/or larger differences could be detected in more discrete aspects of those regions already investigated, the relevance of these differences to both gene expression and CPP could be ascertained by adding additional RI strains to the analysis. Although it will be investigated, this type of analysis is probably not possible with the existing binding data set given the substantial loss of spatial resolution with iodinated ligands. Identification of these expression-dependent regions will naturally lead to new rounds of hypothesis testing and clarification of the relevant circuit(s).

The current study was initiated to determine the relationships between D₂ receptor binding and a variety of ethanol phenotypes. Only the low dose (1.33 g/kg) ethanol-induced locomotion (Crabbe et al. 1983) showed a significant ($p < 0.001$) association with D₂ receptor binding. Interestingly, higher locomotor activity is associated with lower receptor binding. However, given that the ethanol response to higher doses was not associated with receptor binding and that the locomotor responses to neither co-

caine nor methamphetamine were associated with receptor binding, the low dose data must be viewed cautiously and needs replication. Despite these concerns, the data remain of considerable interest. Previous studies have posited that the abuse potential of ethanol may in part be associated with the stimulant effects of the drug which appear to always be associated with the ascending limb of the blood alcohol curve (de Wit et al. 1987, 1989b; Holdstock and de Wit, 1998; Martin et al. 1993). The stimulant effects depend on dose (Holdstock and de Wit, 1998), expectations about the effects of alcohol (Earleywine and Martin, 1993), environmental factors (Doty and de Wit, 1995) and other factors (de Wit et al. 1987, 1989a; Sher, 1985). We now provisionally suggest that under certain conditions the stimulant response may also depend on D₂ receptor density.

The original BXD RI panel was developed to detect and map Mendelian (single gene) traits (Taylor, 1978). Subsequently, it was recognized that the panel could be used to characterize genetically complex traits, including behavioral traits (Plomin et al. 1991). From the strain means one could extract information about genetic correlation and candidate QTLs. From almost the outset, it was recognized that a QTL analysis based solely on the BXD RI panel was statistically underpowered, that the QTL analysis must be considered provisional and that alternative mechanisms of confirmation would be required (Belknap et al. 1996). With the discovery of microsatellite markers that could be rapidly and easily genotyped, murine QTL analysis turned to the use of large intercross populations (e.g., Demarest et al. 2001) and the use of the RI panel as a primary QTL strategy dramatically decreased. The results described here suggest that there may be a renaissance in the use of the RI panel, stimulated by the development of the transcriptome database. Although no more than 30% of the known and predicted genes in the mouse genome are represented on U74Av2 chip, the value of the information that can be obtained on the cis and trans regulation of gene expression and on the integration of QTL and expression data are quite clear. The certain likelihood of improvements in array technology, coupled with the accumulation of additional data in multiple brain regions and in a larger RI panel, seems certain to provide new insights into the genetic architecture of complex behavioral traits.

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