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Ethanol-induced hyperactivity is associated with hypodopaminergia in the 22-TNJ ENU-mutated mouse

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Abstract

Characterization of neurochemical and behavioral responses to ethanol in phenotypically distinct mouse strains can provide insight into the mechanisms of ethanol stimulant actions. Increases in striatal dopamine (DA) levels have often been linked to ethanol-induced hyperactivity. We examined the functional status of the DA system and behavioral responsiveness to ethanol, cocaine and a DA receptor agonist in an *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized mouse strain, 22-TNJ, generated by the Integrative Neuroscience Initiative on Alcoholism Consortium. The 22-TNJ mouse strain exhibited greater locomotor responses to 2.25 g/kg ethanol and 10 mg/kg cocaine, compared to control mice. *In vivo* microdialysis showed low baseline DA levels and a larger DA increase with both 2.25 g/kg ethanol and 10 mg/kg cocaine. In *in vitro* voltammetry studies, the 22-TNJ mice displayed increased V_{max} rates for DA uptake, possibly contributing to the low baseline DA levels found with microdialysis. Finally, 22-TNJ mice showed enhanced *in vitro* autoreceptor sensitivity to the D2/D3 agonist, quinpirole, and greater locomotor responses to both autoreceptor-selective and postsynaptic receptor-selective doses of apomorphine, compared to controls. Taken together, these results indicate that the dopaminergic system of the 22-TNJ mouse is low-functioning compared to control, with consequent receptor supersensitivity, such that mutant animals exhibit enhanced behavioral responses to DA-activating drugs such as ethanol. Thus, the 22-TNJ mouse represents a model for a relatively hypodopaminergic system, and could provide important insights into the mechanisms of hyperresponsiveness to ethanol's stimulant actions.

Keywords

Dopamine D2 receptor; *N*-ethyl-*N*-nitrosourea; ethanol; cyclic voltammetry and microdialysis

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Introduction

In the field of alcohol research, it is apparent that complex genetic and environmental factors contribute to the effects of ethanol, and a variety of animal models have been created, using targeted and random genetic alterations and selective breeding, to examine and understand these effects (e.g., Crabbe and Belknap, 1993; Phillips, 1993; Gorwood et al., 2002; Bergstrom et al., 2003; Hoplight et al., 2007; Bell et al., 2008). Recently, the Integrative Neuroscience Initiative on Alcoholism and Tennessee Mouse Genome Consortia used ENU-mutagenesis to induce random single base-pair mutations in mice and then characterized ethanol-related phenotypes in mutated mouse lines using high-throughput behavioral screens (Kermay et al., 2006; Hamre et al., 2007). In behavioral assays described by Hamre et al (2007), the 22-TNJ mutant mouse strain was noted because it exhibited a hyperactive locomotor response to ethanol compared to 1-TNH controls. Since stimulated locomotor behavior is often associated with activity of the dopamine (DA) system (Imperato and Di Chiara, 1986; Kalivas and Stewart, 1991), the goal of the present studies was to determine if there were alterations in the dopamine system of 22-TNJ mice.

The locomotor stimulant effects of ethanol and other abused drugs are often associated with activation of the ventral tegmental area - nucleus accumbens (NAc) pathway (Amalric and Koob, 1993; Imperato and Di Chiara, 1986) and increased DA release in the NAc (Imperato and Di Chiara, 1986; Kalivas and Stewart, 1991). Many studies show that both systemic injections of alcohol (Imperato and Di Chiara, 1986; Tang et al., 2003; Yim and Gonzales, 2000; Yoshimoto et al., 1992) as well as its voluntary consumption (Doyon et al., 2003; Doyon et al., 2005; Weiss et al., 1993) cause an increase in DA cell activity in the mesolimbic areas of the brain. Additionally, manipulating DA receptors, transporters or other targets within the DA system leads to alterations in ethanol-induced activity (Cohen et al., 1997; Le et al., 1997; Pastor et al., 2005; Jerlhag, 2008).

Research in rodent models has provided many insights into behavioral and neurochemical phenotypes associated with alcoholism, and their underlying genetic correlates. There are several rodent lines which have been studied with regard to ethanol-induced locomotor activity. The FAST and SLOW mouse lines, selected for locomotor responsiveness to ethanol (Shen et al., 1995), have been postulated to have changes in their dopaminergic system which correlate with changes in locomotor responses to ethanol, as well as responses to psychostimulants acting on the DA system, such as cocaine and methamphetamine (Bergstrom et al., 2003). Additionally, the well-studied alcohol Preferring (P) and Non-Preferring (NP) rats have been found to exhibit enhanced locomotor responses to ethanol which correlate with their enhanced dopaminergic response to the drug (Engleman et al., 2006; McKinzie et al., 2002). Thus, we hypothesized that changes seen in the 22-TNJ mouse with regard to locomotor activity could be the result of changes in the dopaminergic system of these animals. While the FAST and SLOW mouse lines and the P and NP rat lines are selectively bred for ethanol-related phenotypes and may have differences at multiple gene loci, the single-base-pair, random mutagenesis method used to create the 22-TNJ mice may provide a more limited genetic alteration correlating with an ethanol-related phenotype.

In order to better understand the ethanol-induced hyperactivity phenotype of the 22-TNJ mouse strain, we embarked on a neurochemical characterization of their dopaminergic system, specifically focusing on the striatum (including the caudate-putamen (CPu) and NAc), a region linked to the rewarding and stimulating properties of ethanol and known for its abundance of DA. Electrically stimulated DA release, uptake and responses to the D2-type receptor agonist, quinpirole, were characterized using *in vitro* fast-scan cyclic voltammetry. Microdialysis was used to evaluate extracellular DA levels and DA responses to ethanol or cocaine in 22-TNJ

and control mice. The locomotor stimulating effects of cocaine and two doses of ethanol were also measured to characterize the behavioral phenotype. In addition, apomorphine, a non-selective DA receptor agonist, was used to examine vertical activity in 22-TNJ and control mice. D2-type autoreceptors are known to regulate DA synthesis through inhibition of tyrosine hydroxylase, therefore we measured synthesis rates of L-3,4-dihydroxyphenylalanine (L-DOPA) in the presence of an L-aromatic acid decarboxylase inhibitor and the effects of quinpirole on synthesis. These studies provide a comprehensive picture of DA system function in the 22-TNJ mice and their controls, elucidating neurochemical correlates of ethanol-related behaviors.

Materials and Methods

Animals

Male C57BL/6J mice were mutagenized with one 200mg/kg dose of ENU and bred, when fertility returned, to hybrid B6XC3H/RI females that carried the *hairy ear* (Eh) inversion. The F1 males of this mating were the progenitors strains of each pedigree (or line) of the TNJ experiment that consisted of a total of 30 lines. Thus 22-TNJ was one line of mice generated from this experiment and 1-TNH was the control that received no ENU but underwent the same breeding scheme. The resulting mutations were maintained on a segregating C3H:B6 background, and a mutant with hypersensitivity to the locomotor properties of ethanol was designated as the 22-TNJ strain. Non-mutated background C3H:B6 mice served as controls (1-TNH). Homozygous breeding pairs of 22-TNJ and 1-TNH mice were shipped from the University of Tennessee at Memphis to Wake Forest University Health Sciences, and a breeding colony was established. Locomotor phenotypes were maintained across all generations used. Animals were housed in groups of three or four per cage with food and water *ad libitum* on a 12-hr light-dark cycle with lights on at 7 am. All experiments used both male and female mice that were between 4 and 16 months old. Experimental protocols adhered to National Institutes of Health Animal Care Guidelines and were approved by the Wake Forest University Institutional Animal Care and Use Committee.

Locomotor Activity Monitoring

Initial activity measures were obtained using open field activity monitors (Med Associates, St. Albans, VT) located in a dimly lit room. The monitors consisted of small, square Plexiglas containers (27 × 27 × 20.3 cm) equipped with three 16-beam infrared arrays. All experiments were conducted between 0900 and 1700 hours during the light phase, and locomotor activity following cocaine or ethanol administration was measured as a total distance traveled (cm). Following a two-hour habituation period, mice were injected with either 10 mg/kg cocaine, 1.0 or 2.25 g/kg ethanol, or sterile buffered 0.9% NaCl (saline), given in a volume of 0.1 mL intraperitoneally (i.p.), immediately returned to the activity monitors, and data was collected for 120 minutes in 5-min non-cumulative bins. Locomotor activity in response to apomorphine (0.15, 0.3, 0.6, 1.2, 2.0 or 3.0 mg/kg) administration was measured by number of vertical rears. The effect of cocaine and ethanol on locomotor activity was assessed by twoway analysis of variance (ANOVA). The apomorphine dose-response curve was analyzed using a repeated measures ANOVA with a Bonferroni post-hoc analysis. Values of $P < 0.05$ were considered statistically significant.

Brain Slices for Cyclic Voltammetry

Mice were sacrificed by decapitation and the brains rapidly removed and cooled in ice-cold, pre-oxygenated (95% O₂/5% CO₂), modified artificial cerebral spinal fluid (aCSF, Mateo et al., 2004a). The aCSF consisted of (in mM): NaCl (126), KCl (2.5), NaH₂PO₄ (1.2), CaCl₂ (2.4), MgCl₂ (1.2), NaHCO₃ (25), glucose (11), HEPES (20), L-ascorbic acid (0.4) and pH was adjusted to 7.4. The tissue was then sectioned into 400 µm-thick coronal slices containing

the NAc and CPu with a vibrating tissue slicer (Leica VT1000S, Vashaw Scientific, Norcross, GA). Slices were kept in a reservoir of oxygenated aCSF at room temperature until required. Thirty minutes before each experiment, a brain slice was transferred to a submersion recording chamber, perfused at 1 ml/min with oxygenated aCSF at ~ 30°C, and allowed to equilibrate.

Cyclic Voltammetry

Carbon-fiber microelectrodes were prepared as previously described (Cahill et al., 1996) and reference electrodes were Ag/AgCl wires. For DA recording, the electrodes was scanned linearly from -400 mV to 1200 mV and back to -400 mV vs. Ag/AgCl, at 400 V/s, repeated every 100 ms (Mateo et al., 2004a). Stimulation parameters used to evoke DA release produced consistent, measurable signals and these parameters were chosen based on previous work in mice (John et al., 2006; Mateo et al., 2004b). DA release was evoked by a single, rectangular, electrical pulse (monophasic, 350 μ A, 4 ms pulse width) every 5 min from an adjacent bipolar stimulating electrode (Plastics One, Roanoke, VA) placed on the surface of the slice, approximately 150 μ m away from the microelectrode. The carbon-fiber microelectrode was placed approximately ~ 150 μ m below the surface of the slice. Each slice served as its own pre-condition control. Quinpirole was applied to the slice by superfusion for 30 min per concentration, in a cumulative concentration paradigm (Jones et al., 1995b). DA was identified by its characteristic cyclic voltammogram, where the oxidation current for DA is ~ 600 mV and reductive current is ~ -200 mV. Electrodes were calibrated with 1 μ M DA to convert the signal from current to concentration.

Data Analysis for Cyclic Voltammetry

DA release and uptake kinetics were analyzed with a Michaelis-Menten based set of kinetic equations (Wightman and Zimmerman, 1990). Electrically stimulated DA release during the rising phase is a balance between the processes of release and uptake, and uptake is the primary process occurring during the decay phase. These parameters were characterized by the following formula:

$$\frac{d[DA]}{dt} = f[DA]p - \frac{V_{max}}{(K_m/[DA]) + 1}$$

In this equation, [DA] represents the instantaneous extracellular concentration of DA released, f is the stimulation frequency, [DA] p is the amount of DA released per stimulus pulse and V_{max} and K_m are Michaelis-Menten uptake rate constants. The following assumptions are made with the above equation: (1) the amount of DA ([DA] p) released into the extracellular space with each stimulus pulse is consistent, (2) uptake is a saturable process and (3) uptake occurs through the DAT, which is the primary mechanism for clearing DA. Furthermore, a control K_m value of 0.16 μ M for DA was used, and V_{max} , which is proportional to the number of monoamine transporters present, was determined. These assumptions are best suited for evaluating release and uptake in striatal regions where one-pulse stimulations are used, and where uptake rates are greater than or equal to 1 μ M/s (John and Jones, 2007). The curve-fitting algorithm, based on simplex minimization and goodness of fit, was described by a non-linear regression coefficient (r^2) (Jones et al., 1995a).

The concentration of DA released and V_{max} values for uptake were determined from current vs. time curves before and after drug application. When quinpirole was used, the change in the current vs. time profile was evaluated as a change in [DA] p ; inhibition of DA release via D2-type autoreceptors. This change in electrically stimulated DA release was compared to pre-drug values (each animal served as its own control) resulting in a percent change in stimulated DA release. The dose response curve was then plotted as log concentration (M) of quinpirole

vs. percent of control DA response, and the data were fit using a non-linear regression curve fit (specifically, sigmoidal dose response curve) to determine EC₅₀ concentrations.

All voltammetry statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc. San Diego, CA). In all cases, data are mean $\pm$ S.E.M. of at least four brain slices, which were obtained from at least four animals. *In vitro* voltammetry data provided DA release and uptake data which were compared across mouse lines using Student's t-tests. Data obtained after administration of quinpirole were subjected to a two-way ANOVA with mouse line and dose of quinpirole as the factors. When significant interactions or main effects were obtained, differences between groups were tested using Bonferroni corrected post hoc tests. In all cases, statistical significance was set at $P < 0.05$.

In vivo Microdialysis

Briefly, mice were anesthetized with Avertin administered in a volume of 16 mL/kg, i.p. (Papaioannou and Fox, 1993). The skin over the skull was shaved, cleaned with alcohol, incised, and the exposed skull was cleaned and dehydrated with 10% H₂O₂. Mice were placed in a stereotaxic frame equipped with a mouse palate adapter and a burr hole (~1 mm diameter) was drilled in the skull. A guide cannula for a CMA/7 mouse microdialysis probe (CMA/Microdialysis, Chelmsford, MA) was implanted into the CPu using coordinates determined from a mouse atlas (Franklin & Paxinos, 2008) and refined by the Integrative Neuroscience Initiative on Alcoholism Consortium Neurohistology Core's (Principle Investigator: Andrea Elberger) histological mouse atlas (CPu: anterior, +0.6; lateral, -1.8; ventral, -2.5 from bregma, NAc: anterior, +1.2; lateral -0.6; ventral -3.3 from bregma). The guide cannula was anchored and the exposed skull sealed with a fast drying two-part epoxy from Loctite (Tak Pak 444, Accelerator 7452 Winona, MN). The microdialysis procedure was as described previously (Bungay et al., 2003, Mateo et al., 2004b), with the following modifications. As mice were recovering from anesthesia, microdialysis probes (1 or 2 mm membrane length (NAc or CPu), 0.24 mm o.d.; Cuprophane, 6 kDa cut-off; CMA-7, CMA/Microdialysis, Chelmsford, MA) were connected to a syringe pump and perfused with aCSF (in mM: 148 NaCl, 2.7 KCl, 1.2 CaCl₂ and 0.85 MgCl₂; pH= 7.4 with NaH₂PO₄) at a flow rate of 0.2 μ L/min overnight. Approximately twelve hours later, the flow rate was increased to 0.8 μ L/min and allowed to equilibrate for 2 hours before at least four baseline samples were collected at 20 minute intervals and analyzed immediately by high performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytical Instruments, West Lafayette, IN). Following determination of a stable baseline, ethanol (2.25 g/kg) or cocaine (10 mg/kg) was injected i.p. and samples were collected for 2 hours. Immediately after dialysis, mice were sacrificed by inhalation of halothane and cervical dislocation and the brains were removed for histological confirmation of probe placement.

Microdialysis data were calculated as the percentage change from baseline concentration, with 100% being defined as the average of the last three samples prior to drug treatment. 22-TNJ mutant mice were compared to their controls (1-TNH) using Student's t-tests. The effects of ethanol (2.25 g/kg, i.p.) or cocaine (10 mg/kg, i.p.) on extracellular concentrations of DA in the NAc were assessed by two-way ANOVA for repeated measures. Values of $P < 0.05$ were considered statistically significant.

Neurotransmitter Analysis

HPLC coupled to electrochemical detection was performed using two different methods depending on the sample type. Dialysate samples (10 μ L) were injected onto a Luna 50 $\times$ 2.0 mm C₁₈ 3 μ m HPLC column (Phenomenex, Torrance, CA) for separation followed by detection at a glassy carbon electrode (+0.65 V vs Ag/AgCl reference electrode, Bioanalytical Systems, West Lafayette, IN). The mobile phase consisted of (in mM): 19 Na₂HPO₄, 29 citric acid, 25

sodium acetate, 4.6 1-octane octanesulfonic acid, 0.5 EDTA and 20% acetonitrile (pH ~4). Neurotransmitter peak areas were integrated using Chromgraph software (Bioanalytical Systems, West Lafayette, IN) and quantified against known standards. Concentrations were expressed in nM $\pm$ SEM. The limit of detection for DA was 0.5 nM (3 times signal to noise).

Synthesis

Tissue samples were assayed for L-DOPA accumulation following inhibition of L-aromatic acid decarboxylase to measure the activity of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis. Mice were injected with the inhibitor 3-(hydrazineomethyl)phenol dihydrochloride (NSD-1015), 100 mg/kg, i.p., and because autoreceptor regulation of DA synthesis was measured, γ -butyrolactone (GBL), 750 mg/kg, was used to inhibit DA neuron firing and reduce extracellular DA levels to a minimum (Jones et al., 1999; Wang et al., 1997). This was done to eliminate autoreceptor activation by endogenous DA tone. In experiments measuring autoreceptor regulation, quinpirole (1 mg/kg) was administered 10 min before NSD-1015 and GBL was administered 5 min before NSD-1015. Mice were sacrificed 40 minutes after NSD-1015 injection and the striatum was dissected and immediately frozen. Tissue samples were stored at -80°C until time of analysis. Tissue samples were removed from the freezer and homogenized in 0.2 M HClO_4 and 1 mM EDTA containing 250 nM 3,4-dihydroxybenzylamine (DHBA) as an internal standard (Wang et al., 1997). Homogenates were centrifuged for 8 min at 10,000 r.p.m. Separation of L-DOPA was obtained using a Luna 50×2.0 mm C_{18} 3 μm HPLC column followed by detection with an ESA 5011 analytical cell ($E_1 = +220$ mV, ESA Coulochem III, ESA Inc., Chelmsford, MA). A guard cell (ESA 5020) was placed before the injection loop and set at a potential of +350 mV. The mobile phase consisted of (in mM): 80 citric acid, 10 Na_2HPO_4 , 7.8 chloroacetic acid, 1.0 EDTA, 2.4 sodium octanesulfonate and 3 % acetonitrile in a volume of 500 mL (pH =3.0) at a flow rate of 0.36 mL/min. L-DOPA was identified using PowerChrom Software (eDAQ Inc., Colorado Springs, CO) and quantified against known standards. Protein content of 20 μL aliquots was determined with the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Co., Rockford, IL). Analytes were quantified and normalized with respect to mean concentrations measured in control mice.

Chemicals and Drugs

Components of HPLC mobile phases, aCSF, Krebs' buffer and neurotransmitters were of HPLC grade or the highest quality obtainable from Sigma-Aldrich (St. Louis, MO). Sodium octanesulfonate was from Acros Organics (Morris Plains, NJ). The BCA Protein Assay Kit containing all chemicals necessary for the protein assays was purchased from Pierce Co. (Rockford, IL; part #23225).

Results

Locomotor activity responses to cocaine or ethanol challenge

Following two hours of habituation to the locomotor chambers, mice were given an i.p. dose of 10 mg/kg cocaine, 0.1 mL saline, or 1.0 or 2.25 g/kg ethanol. A 10 mg/kg dose of cocaine was chosen because this dose is known to stimulate locomotor activity in mice (Elliot et al., 2002). The 2.25 g/kg dose of ethanol was chosen to be consistent with the high throughput screening analysis used by the Integrative Neuroscience Initiative on Alcoholism and Tennessee Mouse Genome Consortium (Hamre et al., 2007). Interestingly, the 1-TNH control mouse strain showed an augmented response to saline injection relative to the 22-TNJ strain ($p < 0.05$, data not shown). Given this disparity, the subsequent 22-TNJ response to psychostimulants may be considered more pronounced. Cocaine significantly elevated levels of locomotor activity in both 22-TNJ and 1-TNH mice during the 2 hr test session ($F_{28,56} = 126.1$, $P < 0.0001$), with the activity of the 22-TNJ significantly increased (two-fold) over control ($F_{1,58} = 115.7$, $P < 0.0001$) (Fig. 1A). There was a significant mouse line $\times$ time

interaction ($F_{28,56}=19.8$, $P < 0.0001$) indicating that overall, the different lines of mice responded differently to cocaine with respect to time. In addition, we examined two doses of ethanol, 1.0 and 2.25 g/kg. The lower dose of ethanol caused a significant increase in activity in both 22-TNJ and control mice ($F_{10,250} = 11.27$, $P < 0.0001$), with the 22-TNJ mice showing a significantly greater enhancement ($F_{1,250} = 12.33$, $P < 0.001$). A significant interaction between mouse line $\times$ time was found ($F_{16,204}=3.958$, $P < 0.0001$). The 2.25 g/kg ethanol dose (Fig. 1C) also caused a significant increase in activity in both 22-TNJ and 1-TNH mice during the 30 min test session ($F_{10,320}=14.51$, $P < 0.0001$), with the 22-TNJ exhibiting a significantly greater increase ($F_{1,320}=19.65$, $P < 0.0001$). A significant interaction between mouse line and time was also found at the higher dose ($F_{10,320}=7.132$, $P < 0.0001$). Thus, the 22-TNJ mutant mice are hyper-responsive to the locomotor stimulating effects of cocaine and ethanol.

Measures of DA release and reuptake using in vitro voltammetry

The 22-TNJ mutant was selected for study based on increased locomotor activation following ethanol injection. Since locomotor stimulant activity is often associated with the DA system, we first used voltammetry to characterize DA release and uptake in controls and mutant 22-TNJ mice. Exemplary traces for the 1-TNH mouse and the 22-TNJ mouse are shown in Fig. 2. $V_{\max}$ for DA uptake was significantly faster ($2.8 \pm 0.2 \mu\text{M/s}$) in the core of the NAc of 22-TNJ mice ($n=30$) compared to $2.1 \pm 0.1 \mu\text{M/s}$ in the controls ($n=31$) ($t = 2.64$, $P < 0.05$, data not shown); however, there was no difference in the amount of electrically-stimulated DA release between the control ($[\text{DA}_p]$, the concentration of DA released per stimulus pulse = $0.95 \pm 0.09 \mu\text{M}$) and 22-TNJ ($[\text{DA}_p] = 1.06 \pm 0.10 \mu\text{M}$; $t = 0.7852$; $P = 0.4355$, data not shown) mice. Therefore, the 22-TNJ mutant mice have increased maximal DA uptake rates in the core of the NAc.

Measurement of presynaptic D2 autoreceptors in the NAc

The activity of DA release-regulating autoreceptors was evaluated in the core of the NAc. Increasing concentrations of the D2-like receptor agonist, quinpirole ($0.001 - 1 \mu\text{M}$), were added to slices at 25-min intervals. After reaching a plateau effect at each quinpirole concentration, the peak DA efflux was evaluated and expressed as percent of control (Fig 3). The software Graphpad Prism was used to construct a sigmoidal dose-response curve to determine the best fit values for the EC_{50} . Quinpirole significantly reduced DA release in the NAc core ($F_{6,73}=47.08$; $P < 0.001$). The log-concentration of the half-maximal response ($\log \text{EC}_{50}$) was -6.9 ± 0.1 (140 nM) for controls ($n=6$) and -7.3 ± 0.2 (60 nM) for 22-TNJ mice ($n=8$). There was a significant effect of mouse line on EC_{50} ($F_{1,73}=29.27$; $P < 0.001$), illustrating supersensitivity of D2 autoreceptors in 22-TNJ mice. No interaction between groups was found ($F_{6,73}=0.5563$).

In vivo microdialysis DA measurements in the CPu and NAc

Using microdialysis, baseline DA levels were compared in the NAc and CPu of the 22-TNJ and 1-TNH mice. Extracellular DA levels were lower in the CPu of the 22-TNJ mice ($1.0 \pm 0.4 \text{ nM}$) compared with their controls ($3.7 \pm 0.8 \text{ nM}$; $t = 2.927$; $P < 0.05$, data now shown). Extracellular DA levels in the NAc were not significantly different between the 22-TNJ mice ($1.9 \pm 0.2 \text{ nM}$) and controls ($2.1 \pm 0.3 \text{ nM}$) (data not shown).

Microdialysis measurements of ethanol effects on DA levels

Ethanol (2.25 g/kg) was administered i.p. at the end of the last baseline microdialysis sample, and subsequently samples were collected in 20-min intervals for two hours. DA levels in the three samples prior to ethanol administration were averaged to obtain mean baseline values, to determine the effect of ethanol on extracellular DA levels in striatum by calculating the percent change from baseline. After administration of 2.25 g/kg ethanol, DA dialysate levels

were measured in the NAc (Fig. 4). A significant increase in DA levels was observed ($F_{8,101}=2.00$; $P < 0.05$) in response to ethanol and there was a significant difference between the 22-TNJ and 1-TNH mouse lines ($F_{1,96}=25.66$; $P < 0.0001$). Finally, there was a significant interaction between strains over time ($F_{8,101} = 2.539$; $P < 0.05$), indicating that the 22-TNJ strain exhibited a significant increase in DA levels over time, above and beyond that of the 1-TNH.

Cocaine-stimulated DA increases in 22-TNJ and 1-TNH mice

Cocaine (10 mg/kg, i.p.) was administered and extracellular DA levels in the CPu and NAc were monitored using *in vivo* microdialysis. Cocaine elevated extracellular DA concentrations ($F_{8,90}=6.746$; $P < 0.0001$) in the CPu (Fig 5A); and there was a significant difference between mouse lines ($F_{1,90}=7.358$; $P < 0.01$), indicating a greater increase in the 22-TNJ mice. There was no significant interaction between groups over time ($F_{8,90}=1.247$; $P = 0.2813$).

In addition to monitoring extracellular DA levels in CPu, we also evaluated DA in the NAc following cocaine administration (Fig 5B). In these animals, as observed in the CPu, systemic administration of cocaine (10 mg/kg, i.p) elevated extracellular DA concentrations in the NAc in both the 22-TNJ and 1-TNH mouse lines ($F_{8,144}=5.322$; $P < 0.0001$). The DA response to cocaine between the lines of mice was also significantly different, with the 22-TNJ mice showing an enhanced DA response to cocaine compared to the 1-TNH ($F_{1,144}=5.360$; $P < 0.05$) and a significant interaction between mouse lines over time was found ($F_{8,144}=2.587$; $P < 0.05$).

Behavioral responses to the non-selective DA receptor agonist apomorphine

Following a two-hour habituation period to the locomotor activity monitoring apparatus, mice were injected with the non-specific DA receptor agonist, apomorphine (0.15, 0.3, 0.6, 1.2, 2.0, or 3.0 mg/kg) or saline in a volume of 0.1 mL, i.p. At low doses, apomorphine is known to suppress vertical rearing in mice, through activation of high-affinity inhibitory presynaptic D2-type autoreceptors. Conversely, at doses over 0.6 mg/kg, apomorphine is known to stimulate vertical activity, through post-synaptic DA receptor actions (Protais et al., 1976). A significant interaction between genotype and dose was found ($F_{6,154}= 3.662$, $p<0.01$) indicating that, overall, the two genotypes responded differently to both low and high doses of apomorphine. In response to 0.3 mg/kg apomorphine (Fig.6), 22-TNJ mice exhibited a suppression in vertical activity exceeding that of controls ($F_{11,11}= 19.04$, $p<0.05$). Further, in response to the highest dose of apomorphine given (3.0 mg/kg, Fig. 5), 22-TNJ mice exhibited a significant increase in vertical activity compared to control ($F_{11,11}= 10.89$, $p<0.05$). Therefore, 22-TNJ mice are supersensitive to both pre- and post-synaptic effects of apomorphine on vertical activity.

Measurement of D2 autoreceptors controlling synthesis

DA synthesis rates were measured by accumulation of L-DOPA following NSD-1015, an aromatic amino acid decarboxylase inhibitor, and accumulation of L-DOPA was similar in the striatum (containing both the NAc and CPu) of both lines of mice (Fig 7A). The GBL and NSD-1015 treatment combination (Fig 7A) was used to assess the effects of autoreceptors on synthesis. Administration of GBL following NSD-1015 will block cell firing, providing a rate of synthesis in the absence of autoreceptor tone (Walters and Roth, 1976). Mice treated with both GBL and NSD-1015 had significantly elevated L-DOPA accumulation in the CPu compared to NSD treatment alone ($F_{1,25}= 14.02$; $P < 0.001$). L-DOPA accumulation increased 4-fold (0.12 ± 0.01 to 0.48 ± 0.06) in control mice ($n = 4$ and 8) and approximately 3-fold (0.22 ± 0.05 to 0.64 ± 0.15) in 22-TNJ mice ($n=8$ and 9) following administration of GBL. There were no significant effects of mouse line, or interaction between groups ($P>0.05$). Since it is known that D2-type autoreceptors in particular can regulate synthesis, the D2-type agonist, quinpirole, was used to inhibit tyrosine hydroxylase and block the accumulation of L-DOPA following administration of NSD-1015 and GBL. Quinpirole significantly (Fig. 6B,

$F_{1,31}=27.41$, $P < 0.0001$) decreased L-DOPA formation in the striatum in both lines of mice in comparison to NSD and GBL administration alone; however, 22-TNJ mice showed an augmented effect of quinpirole in the striatum compared to 1-TNH mice ($F_{1,17}=19.24$, $P < 0.05$).

Discussion

Overall, we conclude that the striatum of the 22-TNJ mutant mouse is hypodopaminergic compared to wildtype controls. Using a variety of neurochemical techniques such as *in vivo* microdialysis, *in vitro* voltammetry, DA synthesis measures and behavioral testing, we have characterized the striatal DA system of the 22-TNJ mutant mouse and demonstrated that the mutant mice display (1) increased DA uptake rates, (2) reduced extracellular DA levels in the CPu, (3) supersensitive presynaptic D2 DA autoreceptors controlling synthesis and release, (4) supersensitive behavioral responses to the DA receptor agonist apomorphine (5) enhanced DA release in response to cocaine or ethanol and (6) potentiated response to the locomotor stimulating effects of ethanol and cocaine. Taken together, these results show that the 22-TNJ mutant mouse can be considered a model of relative reduced extracellular DA levels and consequent supersensitivity to ethanol, cocaine and DA receptor agonists.

Based on these findings, the 22-TNJ mutant mouse represents a model of supersensitive DA receptor function and concomitantly enhanced response to drugs that elevate extracellular DA levels or activate DA receptors. Because supersensitivity occurs with G-protein coupled receptors in response to reduced agonist stimulation, the enhanced responses seen here are consistent with the documented low tonic DA levels. Our findings show that the 22-TNJ mutant mice displayed supersensitivity to the locomotor stimulating and dopamine-elevating effects of acute doses of ethanol and cocaine. Although the increases in dopamine, in particular in response to ethanol, appear to be slower than those found in other models, this difference appears to be neurobiological in origin. Blood ethanol concentrations, measured in different groups of animals, do not differ between the two strains of mice, indicating no change in ethanol metabolism following ENU mutagenesis (Hamre et al, personal communication). In addition, *in vivo* microdialysis demonstrated that 22-TNJ mutant mice have decreased extracellular DA levels in the CPu, though there was no significant difference in extracellular DA levels in the NAc. *In vitro* voltammetry data supported the reduced extracellular DA levels found via *in vivo* microdialysis, providing evidence that the 22-TNJ mutant mice have increased DA uptake rates and supersensitive D2-type autoreceptors controlling DA release in the core of the NAc. Both of these changes could contribute to reduced extracellular DA levels. The findings of no baseline difference in the NAc using microdialysis, but changes in uptake and release regulation measured by voltammetry illustrate the different types of information gathered by the two different techniques. The change in uptake in the NAc might have some influence on the baseline microdialysis data, since with quantitative no-net-flux techniques, it has been shown that extraction fraction is altered by changes in uptake rate.

D2-type autoreceptors located on nerve terminals are also known to inhibit DA synthesis, thus we evaluated the effects of quinpirole on the enzymatic activity of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, and found that autoreceptors regulating synthesis in the 22-TNJ mutant mice were also supersensitive. Finally, apomorphine, a non-selective DA receptor agonist, caused decreased vertical activity responses at an autoreceptor-selective dose (0.3 mg/kg) and caused augmented vertical activity compared to control at the postsynaptic receptor-activating highest dose (3.0 mg/kg) (Becker et al., 1995; Tien et al., 2003), indicating that 22-TNJ mutant mice exhibit supersensitive pre- and post-synaptic D2-type DA receptor function. Taken together, these findings offer sound support for a hypodopaminergic system in the 22-TNJ mouse relative to the 1-TNH control.

The chronically reduced extracellular DA levels and increased DA uptake in the 22-TNJ could be expected to produce supersensitive DA receptors. Indeed, using a variety of methods including voltammetry, synthesis measurements and behavioral analyses, we determined that pre- and postsynaptic DA receptors were supersensitive. Our voltammetry studies shows that D2-type autoreceptors controlling DA release in the NAc core of 22-TNJ mice were supersensitive to the effects of quinpirole, a D2-type receptor agonist. In addition, quinpirole was more effective in decreasing the rate of L-DOPA synthesis following AADC blockade in the 22-TNJ, providing further evidence for D2 autoreceptor supersensitivity.

Finally, we used apomorphine to assess presynaptic autoreceptor (Becker et al., 1995; Bradbury et al., 1984; Radhakishun and van Ree, 1987) and postsynaptic receptor (Tien et al., 2003) function. Apomorphine is a non-selective DA D1- and D2-type agonist, and thus elicits a U-shaped behavioral dose response curve in which low doses produce behavioral inhibition through presynaptic receptor feedback inhibition, while high doses produce behavioral stimulation due to post-synaptic receptor activation. In our studies, the 22-TNJ mutant mice were more susceptible to both the enhancing (3.0 mg/kg) and the inhibiting (0.3 mg/kg) effects of apomorphine than the 1-TNH mice, indicating supersensitivity of DA receptors in both locations. The 1-TNH mice did not demonstrate significant locomotor stimulation, even at the highest dose of apomorphine given. They appeared less sensitive to the locomotor stimulating effects of apomorphine than other mouse strains such as the C57BL/6J (Cabib and Puglisi-Allegra, 1991; Archer et al., 2003), however the important comparison is between the 1-TNH controls and the 22-TNJ mutants, and the 22-TNJ mice were clearly more sensitive to both locomotor-inhibiting and –stimulating apomorphine effects. Although DA receptor function was measured, we were unable to distinguish between up-regulation, defined as an increase in the number of presynaptic D2 receptors, and individually supersensitive receptors with no change in number. Future studies could include the quantitative analysis of D2-like receptors using autoradiography.

Since the 22-TNJ mutant mice have relatively reduced extracellular DA levels, it may appear paradoxical to call the mutant mice hyper-responsive; however, they are supersensitive to the acute effects of drugs that elevate DA levels or activate DA receptors. Although our data clearly supports compensatory changes in the D2-type receptor system, we have made no attempt to distinguish between D2, D3 and D4 receptors. The D3 and D4 receptors have been difficult to study due to their low abundance and lack of specific agonists. 22-TNJ mutant mice and the genetic knockouts of the DA D3 receptor and D4 receptor mice all show increased sensitivity to ethanol (McQuade et al., 2003; Rubinstein et al., 1997). This finding makes examining D2-type receptor subtypes in the 22-TNJ a potentially interesting avenue to explore.

These data in the 22-TNJ mouse strain provide an interesting complement to other published works of ethanol supersensitivity as a function of genotype. Genotypic differences in locomotor activity as a function of genotype are not unknown (Liljequist and Ossowska, 1994), and some of these differences have been well-characterized with regard to neurotransmitter system alterations. For example, the FAST and SLOW mouse lines have shown supersensitivity to the locomotor stimulating effects of ethanol, and these findings are thought to correlate with changes in their dopaminergic responses (Bergstrom et al., 2003). Additionally, the findings in the 22-TNJ mouse strain appear to bear a marked similarity to recent findings in the VMAT2 heterozygous mouse. VMAT2 heterozygotes show similar locomotor sensitivity to ethanol and psychostimulants, an effect directly related to deficiencies in the storage and release of monoamines (Wang et al., 1997). Finally, P and NP rats are also known to have locomotor changes in response to ethanol with correlate with changes in the dopaminergic system (Engleman et al., 2006; McKinzie et al., 2002). Thus it is not surprising that the locomotor changes associated with the 22-TNJ mouse should be correlated with deficiencies in the

dopaminergic system, especially with the specific changes in monoamine release known to underlie the behavior of such animal models as the VMAT2 heterozygous mouse.

In published works examining ethanol-related behaviors, many differences in DA system parameters have been reported; however, very few have been linked to the overall status of the DA system. When only one, or a few, aspects of the DA system are measured, it is difficult to make conclusions concerning the functional status of the overall system. For example, measured in isolation, a finding of increased numbers of D2 receptors could be interpreted as greater signaling through the DA system, whereas in the case of the 22-TNJ mice, it is more likely an adaptive response to chronically decreased DA. Using a wide variety of methods to measure different aspects of DA neurobiology, we have described here a classic example of a low functioning DA system, producing compensatory hyper-responsiveness to drugs. The results we have obtained could be considered hallmarks of a hypodopaminergic system: supersensitivity to stimulants, D2 receptor supersensitivity, and decreases in synthesis, which culminate in low basal levels of DA and increased uptake by the dopamine transporter. The findings from these studies clearly show hallmarks of a low-functioning DA system, and thus this mouse could serve as a model for future investigations into the consequences of chronically altered dopaminergic tone.

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Abbreviations

ENU, N-ethyl-N-nitrosourea
 DA, Dopamine
 NAc, Nucleus Accumbens
 CPu, Caudate-Putamen
 i.p., Intraperitoneally
 HPLC, High Performance Liquid Chromatography
 L-DOPA, L-3,4-dihydroxyphenylalanine
 NSD-1015, 3-(hydrazineomethyl)phenol dihydrochloride
 GBL, γ -butyrolactone
 P, Alcohol-preferring rat
 NP, Alcohol non-preferring rat

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Figure 1.

22-TNJ mice are more sensitive to the stimulating properties of cocaine and ethanol. Locomotor activity measured total distance traveled (cm) after an i.p. injection of cocaine or ethanol at time zero, represented by the arrow. (A) Locomotor activity measured after 10 mg/kg cocaine injection ($n = 24$ per mouse strain). (B) Locomotor responses of 22-TNJ ($n = 14$) and 1-TNH ($n = 13$) mice to 1.0 g/kg ethanol injection. (C) Locomotor activity measured after 2.25 g/kg ethanol injection ($n = 17$ per mouse strain). Data points represent mean $\pm$ S.E.M, *** = $p < 0.0001$ difference from control strain at time point indicated.

Figure 2.

Representative traces of electrically-stimulated endogenous DA overflow in the NAc core of 1-TNH (control, left trace) and 22-TNJ mutant (right trace) mice. Dots are data points, sampling extracellular DA levels, plotted every 100 ms. A single electrical pulse (300 μ A, 2 ms) was applied where trace begins to rise. Electrically stimulated DA release (DA_p) and uptake rates ($V_{\max}$) were taken from the best fit of the data points to a Michaelis-Menten based kinetic model. Release was not different between the two strains of mice, but DA uptake was significantly faster (* $P < 0.05$) in 22-TNJ mutant mice compared to the 1-TNH.

Figure 3.

DA release-regulating autoreceptors are supersensitive in the NAc of 22-TNJ mice. Concentration-response relationship of quinpirole and maximal evoked DA release in NAc core in control (n = 6) and 22-TNJ mice (n = 8). 22-TNJ mice showed a supersensitized response of inhibitory D2 autoreceptors on DA release (*** $P < 0.001$). This decrease is greatest at 10 and 100 nM (points -8 and -7, on the graph) between the controls and 22-TNJ mice; * $P < 0.05$ and ** $P < 0.01$ controls vs 22-TNJ.

Figure 4.

Effect of ethanol (2.25 g/kg, i.p.) on extracellular concentrations of DA in the NAc of control (□) or 22-TNJ (■) mice. Ethanol significantly elevated extracellular concentration of DA in 22-TNJ ($P = 0.0001$; $n = 7$) and in the control mice ($n = 7$); DA peaked to ~ 30 % above average baseline levels at 20 minutes for the control mice, while the 22-TNJ mice showed a delayed effect and peaked at 100 minutes to ~ 50 % above baseline.

Figure 5.

The effect of cocaine on extracellular DA concentration in the CPu and NAc in control and 22-TNJ mice, as measured by microdialysis. Three baseline samples were collected and then cocaine was administered i.p. during the start of the last baseline sample (arrow). **(A)** In the CPu cocaine increased DA levels ~ 130 % in control (□) and ~ 315 % 22-TNJ (■) mice. Cocaine significantly elevated extracellular concentration of DA in 22-TNJ mice ($P < 0.01$; $n = 7$) compared to 1-TNH ($n=5$) in the CPu. **(B)** In the NAc cocaine increased DA levels ~ 40 and 70% in control and 22-TNJ mice, respectively. Similarly in the NAc, cocaine significantly elevated extracellular concentration of DA of 22-TNJ ($P < 0.01$; $n = 8$) compared to 1-TNH ($n = 12$) mice.

Figure 6.

Vertical activity as assessed by the non-specific D2 receptor agonist apomorphine. At the highest dose known to inhibit presynaptic receptors apomorphine (0.3 mg/kg) significantly decreased vertical activity in the 22-TNJ mouse strain compared to their controls ($P < 0.05$, $n = 12$ per mouse line). In addition, at the highest dose (3.0 mg/kg) known to predominantly stimulate post-synaptic receptors, apomorphine significantly increased vertical activity in 22-TNJ mouse strain compared to their controls ($P < 0.05$, $n = 12$ /mouse line). Results are group means $\pm$ S.E.M.

Figure 7.

Tissue levels of L-DOPA accumulation in the striatum of 22-TNJ and control mice measured by HPLC-electrochemical detection. (A) L-DOPA accumulation measured after inhibition of L-aromatic amino acid decarboxylase with NSD-1015 showed no difference between control (0.11 ± 0.01 ng/mg, $n=4$) and 22-TNJ (0.17 ± 0.02 ng/mg, $n=8$) mice. Treatment with GBL significantly ($P < 0.01$) increased L-DOPA accumulation in the striatum of both control (0.48 ± 0.06 ng/mg; $n=8$) and 22-TNJ (0.65 ± 0.14 ng/mg; $n=9$) mice, with no difference between strains ($P > 0.05$). (B) Modulation of striatal L-DOPA accumulation by quinpirole. Quinpirole (1 mg/kg, i.p.) significantly reduced L-DOPA accumulation in both control and 22-TNJ mice ($P < 0.05$); however, this effect was greater in the 22-TNJ (~ 80 % decrease, $n=10$) mice compared to their controls (~ 62 % decrease, $n=8$). Results are presented as means $\pm$ S.E.M.