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Phenotype-dependent inhibition of glutamatergic transmission on nucleus accumbens medium spiny neurons by the abused inhalant toluene

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Abstract

Abused inhalants are voluntarily inhaled at high concentrations to produce intoxicating effects. Results from animal studies show that the abused inhalant toluene triggers behaviors, such as self-administration and conditioned place preference that are commonly associated with addictive drugs. Little is known however about how toluene affects neurons within the nucleus accumbens (NAc), a brain region within the basal ganglia that mediates goal-directed behaviors and is implicated in the development and maintenance of addictive behaviors. Here, we report that toluene inhibits a component of the after-hyperpolarization potential (AHP), and dose-dependently inhibits NMDA-mediated currents in rat NAc medium spiny neurons (MSN). Moreover, using the multivariate statistical technique, partial least squares discriminative analysis (PLS-DA) to analyze electrophysiological measures from rat NAc MSNs, we show that toluene induces a persistent depression of AMPA-mediated currents in one subtype of NAc medium spiny neurons, and that the electrophysiological features of MSN neurons predicts their sensitivity to toluene. The CB1 receptor antagonist AM281 blocked the toluene-induced long-term depression of AMPA currents, indicating that this process is dependent on endocannabinoid signaling. The neuronal identity of recorded cells was examined using dual histochemistry and shows that toluene-sensitive NAc neurons are dopamine D2 MSNs that express *preproenkephalin* mRNA. Overall, the results from these studies indicate that physiological characteristics obtained from NAc MSNs during whole-cell patch clamp recordings reliably predict neuronal phenotype, and that the abused inhalant toluene differentially depresses excitatory neurotransmission in NAc neuronal subtypes.

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Author Contributions:

JTB and JJW designed the electrophysiology experiments. JTB and BAH performed the electrophysiology experiments. RJS and PWK designed the biochemistry experiment. RJS performed the biochemistry experiment. JTB and PKR analyzed the data. JTB and JJW wrote the manuscript.

Keywords

Abused Inhalants; D2R Medium Spiny Neurons; Endocannabinoids; Nucleus Accumbens; Toluene; Whole-Cell Patch Clamp

Introduction

Abused inhalants are substances that are voluntarily inhaled for intoxication effects, such as behavioral disinhibition and euphoria (Beckley and Woodward, 2013). The most commonly abused inhalants are volatile solvents, such as toluene, that are contained in a wide range of household and industrial solutions. The abuse of volatile solvents is prevalent worldwide, and young adolescents have the highest rates of abuse (Lubman *et al*, 2008). Overall, the state of research on the addictive properties of volatile solvents lags behind that of other drugs of abuse, but there is emerging evidence that solvents like toluene share similar pharmacological and behavioral properties as other addictive substances. For example, dopamine (DA) release is increased in the nucleus accumbens (NAc) following acute exposure of rats to abuse-level concentrations of toluene (Apawu *et al.*, 2015), similar to that produced by other drugs of abuse (Di Chiara and Imperato, 1988). Furthermore, rats exhibit a conditioned place preference (CPP) to an environment paired with toluene exposure (Lee *et al*, 2006), and mice and non-human primates will self administer intravenous or vaporized toluene, respectively (Blokchina *et al*, 2004; Weiss *et al*, 1979). Although currently unknown, these findings predict that similar to other drugs of abuse, toluene likely alters the activity of neurons in the NAc, a brain region that encodes the salience and valence of both natural and exogenous reinforcers (Wise, 2004).

The NAc is the ventromedial component of the striatum and is critically involved in shaping goal-directed behavior. The vast majority (>90%) of projection neurons in the NAc are GABAergic medium spiny neurons (MSNs; Gerfen, 1992) and fall into two distinct subpopulations. One type expresses D1 DA receptors and the peptides substance P and dynorphin (D1 MSN) and the second type expresses D2 receptors, adenosine A2a receptors and enkephalin (D2 MSN) (Aubert *et al*, 2000). These neurons have opposing actions on thalamocortical activity, with D1 MSNs promoting goal-directed behavior and D2 MSNs generally suppressing action (Haber and Knutson, 2010; Kravitz *et al*, 2012; Parent and Hazrati, 1995), while both D1 MSNs and D2 MSNs in the striatum are required for movement initiation (Cui *et al*, 2013). NAc neurons receive inputs from other brain regions implicated in the response to drugs of abuse including glutamatergic neurons in the prefrontal cortex (PFC) and dopaminergic neurons in the ventral tegmental area (VTA) (Sesack and Grace, 2010). In previous studies, we showed that the volatile solvent toluene alters glutamatergic signaling in accumbens-projecting VTA DA neurons (Beckley *et al.*, 2013) and induces an endocannabinoid (eCB) mediated long-term depression (LTD) of glutamatergic transmission in deep layer pyramidal neurons of the medial prefrontal cortex (mPFC) (Beckley and Woodward, 2011). Endocannabinoid modulation of glutamatergic neurotransmission is also present in NAc neurons (Robbe *et al.*, 2002; Wang *et al.*, 2010), but appears to be restricted to D2 MSNs (Kreitzer and Malenka, 2007) suggesting that toluene may induce LTD only in these neurons. In the present study, we combined whole-

cell patch-clamp electrophysiology with multivariate statistical methods and show that rat NAc core MSNs can be reliably stratified based on their basal electrophysiological properties into two populations that accurately predict whether a neuron will be sensitive to toluene-mediated LTD.

Methods

Preparation of Brain Slices

All procedures were carried out according to Medical University of South Carolina's Institutional Animal Care and Use Committee protocols. Male Sprague-Dawley rats (postnatal day 22–35) were anesthetized with isoflurane and decapitated. Brains were rapidly removed and placed in an ice-cold sucrose solution that contained (in mM): sucrose (200), KCl (1.9), NaH₂PO₄ (1.4), CaCl₂ (0.5), MgCl₂ (6), glucose (10), ascorbic acid (0.4) and NaHCO₃ (25); osmolarity 310–320 mOsm. The solution was bubbled with 95% O₂/5% CO₂ to maintain physiological pH. Coronal slices (300 µm) containing the NAc core (NAcc) prepared using a Leica VT1000S vibrating microtome (Buffalo Grove, IL) with a double-walled chamber through which cooled (2–4°C) solution was circulated. Slices were collected and transferred to a warmed (32–34°C) chamber containing a carbogen-bubbled aCSF solution containing (in mM): NaCl (125), KCl (2.5), NaH₂PO₄ (1.4), CaCl₂ (2), MgCl₂ (1.3), glucose (10), ascorbic acid (0.4) and NaHCO₃ (25); osmolarity 310–320 mOsm. Slices were warmed for 30 minutes and then kept at room temperature in carbogen-bubbled aCSF for at least 45 minutes before beginning recordings.

Electrophysiology

As previously described (Beckley and Woodward, 2011), slices were transferred to a recording chamber and perfused with aCSF at approximately 2 ml/min. Experiments were conducted at a bath temperature of 32°C controlled by in-line bath heaters (Warner Instruments, Hamden, CT). Neurons were visually identified under infrared light using an Olympus BX51WI microscope with Dodt gradient contrast imaging (Luigs and Neumann, Ratingen, Germany). Whole-cell recordings were carried out using thin-wall borosilicate glass electrodes (OD 1.5 mm, ID 1.1mm; R = 1.5–4 MΩ; Warner Instruments). For most experiments, pipettes were filled with internal solution containing (in mM): K-gluconate (130), KCl (10), HEPES (10), MgCl₂ (2), EGTA (1), Na₂ATP (2), NaGTP (0.3), 0.2% biocytin; osmolarity 295 mOsm, pH = 7.3. The liquid junction potential with the K-gluconate internal solution was previously empirically determined to be +12.2 mV (Beckley and Woodward, 2011). Values presented in the results section are uncorrected for this measure. For the experiment examining NMDA-mediated EPSCs, K-gluconate and KCl were replaced with CsCl (120 mM). Series resistance (R_s) was monitored throughout the recording, and an experiment was discontinued if R_s went over 30 MΩ or changed by more than 25% over the course of the recording.

For experiments involving measures of intrinsic excitability and NMDA responses, baseline values were collected until they were stable. A starting concentration of HPLC grade toluene (0.3, 1, or 3 mM, Sigma-Aldrich, Saint Louis, MO) was added to pre-gassed aCSF and immediately perfused into the recording bath using Teflon tubing to minimize solvent loss.

Previous studies in our laboratory showed that the concentration of toluene in the bath 15 min after dilution was $77.9 \pm 15\%$ (mean $\pm$ SEM) of baseline value (Beckley and Woodward, 2011; Cruz et al., 1998). Concentrations of toluene reported in the results section are not corrected for this loss. For measuring NMDA-mediated currents, picrotoxin and NBQX were added to the aCSF to block GABA-A and AMPA receptors, respectively, and the neuron was held at +40mV to alleviate the Mg^{2+} block.

For experiments examining toluene's effect on AMPA-mediated EPSCs, each recording began with a battery of electrophysiology protocols in order to build a statistical model that segregates neurons based on their physiological profile. A set of 24 neurons was used for this experiment only and these neurons received the battery of protocols as a training set for the model. The physiological battery went as follows: first, a current step protocol (0.7 s recording electrode stimulation from -40 to 300 pA with 20 pA interval) was used to determine the input/output relationship and x-intercept, or rheobase for action potential (AP) generation. We then selected a current pulse that elicited about 8 APs over 1.5 s, and from this protocol calculated resting membrane potential, membrane resistance, absolute AP height, AP threshold, magnitude of AHP first component (AHP1), and magnitude of AHP second component (AHP2). Following these current clamp protocols, picrotoxin (100 μ M) was added to the aCSF to block GABA_A currents, the amplifier was switched to voltage clamp (-80 mV), and spontaneous EPSC (sEPSC) currents were recorded for at least 100 seconds. Finally, as described previously (Wang et al, 2010), we tested whether each neuron displayed depolarization-induced suppression of excitation (DSE), an endocannabinoid mediated form of short-term plasticity. To monitor DSE, EPSCs between 150–300 pA were evoked every 4 seconds for 25 seconds, followed by a depolarizing step to 0 mV for 5 seconds and a return to evoked EPSCs. The average of the two EPSCs prior to the depolarization step was used as the baseline value, while the second and third EPSC following the depolarizing step were used to assess the extent of DSE.

In the test set of neurons (n = 15 neurons), we recorded AMPA-mediated currents following the battery of protocols described above. In these neurons, after measuring DSE, 100 μ M DL-APV (Abcam Biochemicals) was added to the aCSF to block NMDA receptors. Baseline AMPA currents were collected until the responses were stable, and then 3 mM toluene was perfused into the recording bath. In a third set of neurons (n = 7), baseline AMPA-mediated currents were recorded and then the CB1 receptor antagonist AM-281 (1 μ M, Tocris) was added prior to toluene perfusion. For these recordings, 0.5% bovine serum albumin (BSA) was added to the aCSF to aid delivery of the highly lipophilic AM-281 and BSA containing aCSF was used in the control recordings.

In all experiments, responses were collected at a rate of 0.05 Hz. AMPA- and NMDA-mediated currents were evoked using a concentric bipolar electrode placed adjacent to the recorded neuron, and a 0.2 ms stimulus pulse was delivered at a current of 8 to 500 μ A, and elicited a reliable, sub-maximal response from the recorded neuron. Data were acquired using an Axon MultiClamp 700B amplifier (Molecular Devices, Union City, CA) and an ITC-18 digital interface (HEKA Instruments, Bellmore, NY) controlled by AxographX software (Axograph Scientific, Sydney, NSW, Australia). Recordings were filtered at 4 kHz, acquired at 10 kHz and analyzed offline using AxographX software. Spontaneous EPSC

events (at least 100 per cell) were detected and analyzed using the template matching event detection algorithm in AxographX. Detection parameters were set at amplitude > 5 pA and acquired events were visually inspected before averaging.

Dual histochemistry for enkephalin mRNA and biocytin

A plasmid containing a 937-bp cDNA insert for preproenkephalin (kindly provided by laboratory of Dr. Kristen Keefe, University of Utah) was linearized with EcoRI-HF restriction enzyme (New England Biolabs), followed by purification via phenol-chloroform extraction. In vitro transcription of digoxigenin (DIG)-labeled antisense RNA probes was carried out using a DIG RNA labeling kit and SP6 RNA polymerase (Roche). Sections containing a recorded neuron were acetylated in 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min, followed by rinses in PBS. Sections were transferred to 2× saline sodium citrate (SSC) at 55°C for 20 min, and then placed in hybridization buffer for 1 hr at 55°C. Hybridization buffer consisted of 50% formamide, 20 mM Tris, 1 mM EDTA, 300 mM NaCl, 5% dextran sulfate, 1× Denhardt's solution, and 0.1% each of sodium thiosulfate and sodium dodecyl sulfate (SDS). Sections were then placed into hybridization buffer containing denatured preproenkephalin RNA probe (~100 ng/ml), as well as 100 ug/ml salmon sperm DNA and 250 ug/ml each of yeast total RNA and yeast tRNA, for 24-hr incubation at 55°C. After three rinses in 2× SSC, sections were placed in post-hybridization stringency washes for 30 min each, consisting of 4× SSC/50% formamide/0.1% SDS (two washes at 70 °C), 2× SSC/50% formamide/0.1% SDS (two washes at 65 °C), 2× SSC/0.1% SDS (one wash at 60 °C), and 0.2× SSC/0.1% SDS (one wash at RT). Sections were then rinsed in Tris-buffered saline (TBS) three times and placed in TBS with 0.3% triton-X (TBST) and 2% normal donkey serum (NDS) for 1 hr. Alkaline phosphatase (AP)-conjugated sheep anti-DIG (Roche) was diluted 1:3000 in TBST with 2% NDS for overnight incubation at RT. After rinses in TBS hybridization, the DIG signal was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche) diluted 1:50 in AP buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5 with 0.3% Triton-X, 5% polyvinyl alcohol, and 1 mM levamisole) to yield a purple reaction product and once the color had developed for about 1 hr, sections were rinsed several times in PBST and incubated in Vectastain ABC Elite (Vector Labs) for 2 hr. The biocytin signal was then visualized by placing sections into 3,3'-diaminobenzidine (0.025%) and hydrogen peroxide (0.015%) in PBST for 10 min, yielding a brown reaction product. Following rinses in PBS, sections were then fixed in 4% formaldehyde for 20 min, mounted onto glass slides, and coverslipped with Permount.

Statistics

Data from the stimulus-evoked NMDA EPSC and intrinsic excitability experiments were tested for statistical significance using repeated-measures ANOVA (i.e. baseline × toluene × washout) with Bonferroni's multiple comparison test for determining group differences. Changes from baseline following toluene application were determined by averaging the peak current amplitude in episodes from the final four minutes of each condition and comparing them to baseline values. Evoked current amplitudes were measured as a percentage of the pre-drug baseline and expressed as means ± SEM. Physiological differences between NAc neuronal subtypes were tested by t-tests or Welch-corrected t-test when the groups had

unequal variances. We used the nonparametric Kruskal-Wallis test with Dunn's multiple comparison test, due to unequal variances between groups, to measure statistical difference in the experiment examining toluene-induced LTD. Tests of statistical significance for these experiments were computed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

For neurons that underwent the battery of protocols, correlations between different parameters were tested, and statistical significance was computed with GraphPad Prism software. Logistic regression was run using SPSS (IBM, Armonk, NY) with toluene induced LTD defined categorically as the dependent variable (DV) and each individual parameter as the independent variable (IV). Toluene sensitivity was defined as a binary function as toluene-induced LTD had a bimodal distribution.

Partial Least Squares – Discriminant Analysis (PLS-DA) were conducted using SOLO, a standalone version of the PLS-ToolBox (Eigenvector Research, Wenatchee, WA). PLS is an extension of multiple regression that is used as a means of exploratory analysis and variable selection when faced with a large number of independent variables, single or multiple dependent variables, and often a relatively small number of samples. It is similar to Principal components regression in that it decomposes a predictor matrix into components according to a criterion. However, as opposed to Principal components analysis that only derives latent variables in order to explain all the variance in the set of independent variable, PLS derives latent variables so as to maximize the relationship between IVs and DV (Datta, 2001). In this application, there is a single DV (toluene sensitivity) so components of the IVs (i.e. neuronal parameters) are derived that optimally predict toluene-induced LTD. Furthermore, because we defined neurons as either sensitive or insensitive to neurons, we utilized PLS-DA, that is optimized to categorize the DV into classes.

The scores on these latent variables are then used as IVs similar to multiple regression. Each IV has a loading on each latent variable reflecting the weight given to each variable in the calculation of the factor score. Factor scores were plotted for each neuron and each factor was run individually and together through a logistic regression using SPSS. We also used Variable Importance Projection (VIP) score and Selectivity ratio as a measure of the importance of each IV to the overall model fit. The VIP reflects the importance in the overall model fit, (i.e. how well does the model do with and without the independent variable in question), while the Selectivity ratio reflects the proportion of variation in the predictor that is associated with the dependent variable variation (Tran et al., 2014).

Like multiple regression, the PLS model can be tautological to some extent since it is calculated to maximally predict the DV's. This problem is greatly attenuated by utilizing a "training" and "test" set of observations that drastically reduces the degree to which prediction is dependent on idiosyncratic statistical error in the original data set. In constructing our model, we used recordings that only received the battery of electrophysiological protocols as the training set and recordings with toluene perfusion represented the test set.

Results

Toluene and Intrinsic Excitability

We first examined whether toluene altered the intrinsic excitability of NAc neurons. We tested the effect of 0.3 and 3 mM toluene on excitability, comparing the measures immediately at the end of the toluene perfusion and those following a prolonged washout to baseline measurements. Toluene at a concentration of 0.3 mM did not alter any of the measures of intrinsic excitability collected ($n = 5$ neurons, 3 rats; data not shown), including: resting membrane potential (RMP) (Repeated Measures (RM) ANOVA: $F(2,14) = 0.8763$, $p = 0.4528$) membrane resistance (RM ANOVA: $F(2,14) = 0.9638$, $p = 0.2471$), action potential (AP) threshold (RM ANOVA: $F(2,14) = 0.5211$, $p = 0.6127$), or number of spikes during a constant current injection (RM ANOVA: $F(2,14) = 0.0960$, $p = 0.9095$). Furthermore, 0.3 mM toluene had no effect on either the fast or slow component of the after-hyperpolarization potential (AHP; data not shown; RM ANOVA: fast component – $F(2,14) = 0.8295$, $p = 0.4706$; slow component – $F(2,14) = 0.6095$, $p = 0.5671$). At a higher concentration (3 mM), toluene produced a mixed effect on these measures (Figure 1; $n = 6$ neurons, 4 rats). There was no effect of 3 mM toluene on membrane resistance (RM ANOVA: $F(2,17) = 0.5132$, $p = 0.6135$), AP threshold (RM ANOVA: $F(2,17) = 2.272$, $p = 0.1537$), or number of spikes (RM ANOVA: $F(2,17) = 1.418$, $p = 0.2870$). However, at the end of the washout period, RMP was significantly more hyperpolarized (RM ANOVA: $F(2,17) = 18.57$, $***p = 0.0004$, Figure 1B), albeit the magnitude of this effect was modest (baseline RMP: -78.81 ± 1.56 mV; washout RMP: -80.63 ± 1.73 mV). Furthermore, 3 mM toluene altered the AHP, with a selective effect on the fast AHP component (RM ANOVA: fast component – $F(2,17) = 4.079$, $*p = 0.0388$; slow component – $F(2,17) = 1.859$, $p = 0.2058$, Figure 1D). Using pharmacological blockers, we determined that the fast AHP component was blocked by iberiotoxin (100 nM), while the slow component was sensitive to apamin (200 nM), indicating that the fast component reflects, at least in part, activity of the Big Potassium (BK) channel. Overall, these data indicate that toluene, even at a relatively high concentration only modestly alters neuronal excitability of NAc MSNs, with the largest magnitude effect on the fast AHP component.

Toluene and NMDA-mediated Currents

Toluene has been previously shown to dose-dependently inhibit NMDA-mediated currents both in recombinant expression systems (Cruz *et al*, 1998) and in deep-layer mPFC neurons (Beckley and Woodward, 2011). Using slices containing the NAc, we examined the effects of toluene stimulus-evoked NMDA currents in NAc MSNs. Toluene at 0.3 mM ($n = 5$ neurons, 4 rats) did not affect NMDA current amplitude immediately during toluene perfusion but a significant inhibition developed during the washout period (Figure 2A; RM ANOVA: $F(2,14) = 5.063$, $*p = 0.0379$). The decrease in NMDA current in the washout period was likely due to the 0.3 mM toluene but might also reflect a non-specific rundown of the NMDA signal, as responses in the sham recording, where no toluene was introduced ($n = 4$ neurons, 2 rats), also showed a trend toward a reduction in NMDA currents during washout ($F(2,11) = 4.498$, $p = 0.0640$). At higher concentrations, both 1 mM toluene ($n = 7$ neurons, 7 rats) and 3 mM toluene ($n = 5$ neurons, 4 rats) robustly inhibited NMDA-mediated currents, and inhibition persisted following the washout (RM ANOVA: 1 mM

toluene – $F(2,20) = 14.27$, *** $p = 0.007$, 3 mM toluene – $F(2,14) = 20.05$, *** $p = 0.008$, Figure 2B). When the effects of 1 and 3 mM toluene on NMDA currents were examined in individual neurons, two responses were seen with some neurons showing recovery to control responses while others remained significantly inhibited following the washout, (Figure 2C,D). Since previous studies suggest that only D2 MSNs show eCB-mediated LTD (Kreitzer and Malenka, 2007), these results suggest that toluene may selectively alter glutamatergic signaling in a subset of MSNs. To test this, we employed a combined physiological and statistical approach to stratify NAc neuron subpopulations based on their electrophysiological properties. To avoid complications associated with the direct effect of toluene on NMDA receptors, we monitored responses mediated by AMPA receptors that are resistant to the direct effects of even very high concentrations of toluene (Cruz *et al*, 1998).

Toluene and AMPA

In these experiments, each neuron was recorded under current and voltage clamp conditions and a range of physiological parameters were collected ($n = 39$ neurons, 19 rats). These included AP height, AP threshold, rheobase, membrane resistance (R_{membrane}), fast AHP component (AHP1), differences between AHP components (AHP2-1), mean sEPSC amplitude, max sEPSC amplitude, sEPSC > 50 pA, mean sEPSC frequency, sEPSC decay constant and the presence or absence of depolarization induced suppression of excitation (DSE), an endocannabinoid-mediated form of short-term plasticity that is expressed in NAc MSNs (Wang *et al*, 2010). A correlation matrix was then used to determine the interrelationships between all measured variables. As shown in Table 1, there are statistically significant correlations between parameters, with AP height, AP threshold, R_{membrane} and sEPSC amplitude each correlating with several other measures. On their own, these correlations were unable to determine whether there were different neuronal subpopulations that are clearly defined by electrophysiological properties. However, because these parameters appeared highly interrelated, we determined whether the response of AMPA EPSCs to a toluene challenge could be predicted by the basal properties of the neuron.

While 24 recordings underwent a battery of electrophysiology protocols in the absence of toluene, in an additional 15 recordings, all parameters were measured, followed by a recording of AMPA currents during an exposure to toluene (3mM). In this group, toluene produced a prolonged inhibition of AMPA-mediated currents in only a subset of NAc MSNs (Figure 3), with 7 of 15 neurons showing inhibition (<88% of baseline peak amplitude) and 8 of 15 showing no effect. Logistic regressions were used to determine the ability of individual neuronal parameters to predict toluene sensitivity, as defined by a reduction in evoked AMPA EPSC amplitude to below 90% of the pre-drug baseline following an 8-minute perfusion of 3 mM toluene. Six parameters significantly predicted toluene-induced LTD: AP threshold, R_{membrane} , rheobase, DSE, and sEPSC amplitude (Table 2). By itself, DSE predicted toluene-induced LTD perfectly ($\chi^2 = 20.728$, $p < 0.0001$), whereas the other 5 parameters that predicted LTD above chance were at most 80% correct.

PLS-DA—Following the results obtained with the logistic regression, a PLS-DA regression was performed in order to more accurately determine whether the multivariate dataset of neuron parameters could predict toluene sensitivity. Discriminant analysis was used due to

the binary nature of toluene sensitivity. The 24 neurons that received a battery of physiology protocols were used as training cases to build the model, while the 15 neurons that underwent all physiological tests and received 3mM toluene were the test set. PLS-DA recognized two clear components among the predictors (Figure 4), with factor 1 consisting largely of intrinsic excitability characteristics along with DSE, while factor 2 mostly consisting of sEPSC components. Both Rmembrane and sEPSC amplitude loaded onto factors 1 and 2 and all variables had a loading value of greater than |0.25| except for AHP1 (Figure 4, 5). We assessed the relative importance of each variable with the variable importance in projection (VIP) score and the selectivity ratio. The six variables that showed predictive power with logistic regression also had a VIP score > 1 and included AP threshold, Rmembrane, rheobase, DSE, sEPSC amplitude, and sEPSC decay. DSE had a selectivity ratio of above 4, dwarfing all other variables (Figure 5B). This is in agreement with the logistic regression showing that DSE predicted toluene-sensitivity with 100% success. On its own, factor 1 fully predicted toluene sensitivity, likely because of the DSE contribution, while factor 2 was irrelevant (Figure 5C, D). This was confirmed by running a logistic regression on the two factors, which showed that factor 1 predicted LTD with 100% success ($\chi^2 = 20.728$, $p < 0.0001$), and factor 2 explaining no additional variance in the model (Table 3).

Because both toluene-induced LTD and DSE are likely mediated by eCB signaling, DSE alone should predict toluene-sensitivity. To test if all other variables were also predictive of toluene sensitivity, we ran a PLS-DA with all IVs except DSE. Just like the PLS-DA with DSE included, two components were extracted, with factor 1 containing intrinsic excitability factors, and factor 2 containing sEPSC parameters, with mean sEPSC amplitude and R membrane loading on both (Figure 6A). VIP scores revealed that AP threshold, Rmembrane, rheobase, sEPSC amplitude, and sEPSC decay were all significantly important in the model, just like in the PLS-DA with DSE included (Figure 6B). Factor 1 alone correctly predicted toluene sensitivity in 14 out of 15 neurons (Figure 6C), and combined with factor 2, the model correctly predicted toluene sensitivity of all cells (Table 3). This was confirmed with logistic regression, showing that factor 1 had significant predictive value of toluene sensitivity ($\chi^2 = 10.067$, $p = 0.002$), but factor 2 explained variance that went unexplained in factor 1 ($\chi^2 = 4.510$, $p = 0.034$) resulting in a 100% success rate for the overall model ($\chi^2 = 20.728$, $p < 0.0001$; Table 3). These two models indicate that DSE is far and away the best predictor of toluene-sensitivity, but it is not a necessary variable because the combination of other basal neuronal properties accurately predicts toluene sensitivity.

NAC neuronal properties—We compared physiological properties between Type I neurons that are toluene-insensitive ($n = 8$ neurons, 7 rats) and Type II neurons that are toluene-sensitive ($n = 7$ neurons, 5 rats). Similar to previous studies (Cepeda et al., 2008), type II neurons had a more hyperpolarized AP threshold ($t(13) = 2.231$, $*p = 0.0431$; Figure 7A), a higher rheobase ($t(13) = 2.230$, $*p = 0.0440$; Figure 7A) and a lower membrane resistance that was nearly statistically significant (Welch-corrected $t(7) = 2.105$, $p = 0.073$; not shown). Spontaneous EPSCs from type II MSNs had larger amplitude ($t(13) = 2.448$, $*p = 0.0293$, Figure 7D) and faster decay kinetics (Welch-corrected $t(8) = 2.606$, $*p = 0.0313$). Reflecting the superior predictive value of DSE on toluene sensitivity, type II neurons

displayed DSE ($83.09\% \pm 2.080$ of evoked EPSC amplitude; Figure 7E) whereas type I neurons did not ($97.58\% \pm 1.399$, $t(13) = 5.914$, $***p < 0.0001$). Finally, as expected, type II neurons showed robust inhibition of EPSCs during exposure to toluene (to $68.13\% \pm 6.063$ of peak EPSC amplitude), whereas type I neurons did not ($97.89\% \pm 1.336$). To confirm that toluene-induced LTD is mediated by eCB signaling, we repeated experiments in the presence of the CB1 antagonist AM-281. Under conditions where CB1 receptors are blocked, neurons predicted to be type II ($n = 6$ neurons, 4 rats) showed no alteration in EPSC amplitude during exposure to toluene (following toluene: $104.43\% \pm 3.23$). Furthermore, AMPA EPSCs from type II neurons in the absence of AM281 were significantly lower after 3 mM toluene compared to type I neurons or type II neurons in the presence of AM281 (Kruskal-Wallis; $H(2) = 11.92$, $p < 0.01$, Figure 7F). Overall, these results show that NAc neurons have two distinct electrophysiological phenotypes, with differences in intrinsic excitability, spontaneous EPSC activity, and eCB-mediated short- and long-term plasticity.

Verification of Neuron type—Based on the finding that toluene inhibits AMPA EPSCs in an eCB-dependent manner and previous results showing that eCB signaling is limited to D2 MSNs (Kreitzer and Malenka, 2007), we hypothesized that type II neurons are D2 MSNs and type I neurons are D1 MSNs. To test this hypothesis, we selected slices containing a biocytin-filled recorded neuron with the phenotype predicted from the PLS-DA protocol and used in situ hybridization to label *preproenkephalin* (*Enk*), whose expression is restricted to D2 MSNs (Le Moine *et al*, 1990; Lu *et al*, 1998; Smith *et al*, 2013). The PLS-DA model predicted neuronal subtype for all recorded neurons, including those that did not receive 3 mM toluene (Total: $n = 46$ neurons, 23 rats; physiology battery only – 24 neurons; battery and toluene – 15 neurons; battery, AM281, and toluene – 7 neurons). We tested 10 slices, 5 that contained Type I classified neurons and 5 that contained Type II neurons. We found that all 5 type II neurons showed *Enk* mRNA labeling, while 4 out of 5 type I neurons did not express *Enk*, (Figure 8B). The model estimated a probability of phenotype classification that was greater than 97% (i.e. probability of Type I or Type II $> 97\%$) in 40 of 46 neurons recorded (87%), suggesting a high level of certainty of subtype classification for the vast majority of neurons recorded (Figure 8C). The *Enk+* neuron that was misclassified as a Type I neuron was one of four cases where the model calculated the probability of a given phenotype as below 80%.

Discussion

The major finding from this study is that toluene differentially inhibits glutamatergic synaptic transmission depending on the NAc neuronal subtype. When examining NAc neurons as a single group, the data show that toluene inhibits the fast AHP component and dose-dependently inhibits NMDA-mediated currents, as has been previously shown (Beckley and Woodward, 2011; Cruz *et al*, 2000). When separating NAc neurons into two subtypes based on their physiological properties, toluene induced a long-lasting inhibition of AMPA-mediated currents in only D2 MSNs. This is the first study that we are aware of that separates rat NAc MSNs into their two primary subpopulations based on a statistical analysis of their neurophysiological properties.

The effects of toluene on the AHP is in line with previous findings showing that toluene dose dependently inhibits BK channels (Del Re *et al*, 2006). In this experiment, we show that toluene selectively inhibits the fast component of the AHP that is sensitive to the BK inhibitor iberiotoxin. There was high variability in NAc neuronal AHP shape, which reflected individual neuronal differences in the fast and slow component of the AHP. Interestingly, in the PLS-DA analysis, the AHP components were two of the least informative parameters in terms of loading value and VIP score and contributed very little to the model. This indicates that the variation in AHP magnitude cannot be explained by the two PLS-DA defined models, and suggests that AHP likely does not differ between the two neuronal subtypes. This is in line with previous work showing that D1 and D2 MSN AHPs do not differ in amplitude in mice NAc neurons (Ma *et al*, 2012).

The dose-dependent inhibition of NMDA currents by toluene is consistent with data from our previous reports (Bale *et al.*, 2005; Cruz *et al.*, 2000; Cruz *et al.*, 1998). Specifically, we show here that toluene produces a long-lasting inhibition of NMDA currents, but in contrast to the inhibitory effect of toluene on NMDA currents in mPFC neurons (Beckley and Woodward, 2011), this persistent reduction of NMDA currents is observed in only a subset of NAc neurons. This suggests that toluene may differentially affect NAc neuronal subtypes and this finding was a major impetus for attempting to determine NAc neuron phenotype using a multivariate statistical model. While we speculate that PLS-DA analysis would show that the long-lasting inhibition of NMDA currents by toluene is restricted to D2 MSNs and requires eCB signaling, we decided to restrict the use of PLS-DA analysis to AMPA-mediated currents because of toluene's additional direct effect on NMDA receptors. Furthermore, in experiments where NMDA currents were recorded, a Cs⁺ based internal solution that blocks K⁺ channels was used and would be expected to alter measures of intrinsic excitability that contribute to the utility of the PLS-DA model.

The results of this study show that PLS-DA accurately identified the two major sub-populations of MSNs in the NAc and predicted the outcome of a specific dependent variable that in this case was sensitivity to toluene-mediated LTD. PLS regression has seen more extensive use in the field of chemometrics in order to establish quantitative structure-retention relationships, or the relationship between physicochemical properties of compounds and their retention on a chromatograph (Li *et al*, 2007; Put and Vander Heyden, 2007). In neurobiology, fMRI studies have also utilized PLS to examine the latent structure of regional brain activation as a means to predict a phenotype or an outcome (Andersen *et al*, 2012; Salimpoor *et al*, 2013). In addition, large genomic, proteomic, and metabolomic studies have used PLS to predict an outcome based on the pattern of gene, protein, or metabolite activity, respectively (Datta, 2001; Kaddurah-Daouk and Krishnan, 2009; Rozen *et al*, 2005). However, this present study appears to be the first to utilize PLS in combination with in vitro whole-cell patch-clamp electrophysiology. Electrophysiology is particularly well suited to PLS analysis as once whole-cell configuration is achieved, it is relatively easy to collect a wide variety of neuronal parameters in a short period of time. While these datasets are usually analyzed by multiple regression techniques, PLS is superior because it is less affected by co-linearity that is inherent in several important neuronal properties such as rheobase and membrane resistance. Nonetheless, PLS is likely most effective at predicting neuronal phenotype when there are measurements obtained in different recording

configurations, i.e. current and voltage-clamp, that reduce the degree of co-linearity. PLS is also most effective when recording from neurons in a region where individual neuronal physiological features may reflect membership in discrete neuronal sub-populations that serve important and unique physiological roles. This is particularly true for the striatal sub-regions due to the nearly binary distribution of D1-containing and D2-containing MSNs that make up the vast majority of neurons within this region.

Consistent with results from our previous study with mPFC neurons (Beckley and Woodward, 2011), toluene persistently inhibited AMPA responses in NAc neurons in a CB1R-dependent manner. The effect was restricted to D2 MSNs in line with previous research showing that only D2 MSNs in the NAc show eCB-mediated forms of plasticity (Grueter et al., 2010). Because of this selectivity, the eCB-dependent short-term plasticity protocol DSE is an independent variable that is D2-selective, making it an excellent indicator of MSN cell phenotype. Furthermore, our results suggest that any drug that interacts with the eCB system, such as toluene or alcohol (Pava and Woodward, 2012), will likely alter D2 MSNs differently than D1 MSNs. In some individuals, binge drug use can transition to compulsive drug abuse when control over drug-seeking behavior is lost, and basal activity in D2 MSNs in the NAc may explain some of the variance in response to drugs. In a mouse model of compulsive drug taking, mice who had potentiated activity onto D2 MSNs showed resilience to an escalating compulsive drug response pattern, while selective inhibition of D2 MSNs made subjects more vulnerable to compulsive self-administration (Bock *et al*, 2013). While the current study involved *in vitro* approaches, we predict that the abused inhalant toluene selectively and persistently inhibits D2 MSNs *in vivo*, although this needs to be tested. During repeated exposures to toluene, D2 MSNs may become progressively more difficult to stimulate, that in turn would increase the odds of transitioning to compulsive drug taking by attenuating the normal ability of D2 MSNs to counter D1-driven goal-directed behavior. In this light, the results of the present study complement those of others showing that D1 and D2 MSNs encode different aspects of normal and diseased states.

Like previous studies (Cepeda et al, 2008; Kreitzer and Malenka, 2007), the present findings show that D1 and D2 MSNs possess distinctly different physiological properties. However, some of the neuronal group differences that we observed were not reported previously. For example, the sEPSC profile observed in previous studies showed that sEPSCs in mouse D2 MSNs had a higher frequency compared to D1 MSNs (Cepeda et al, 2008; Grueter et al, 2010). In contrast, rat D2 MSNs in the present study had larger sEPSC amplitude and faster decay kinetics compared to D1 MSNs. There are two primary interpretations of this discrepancy. First, it is possible that there are species- and strain-dependent differences in sEPSC profile in D1 and D2 MSNs. Further experiments regarding species-specific differences in striatal neurophysiology are needed, but it is evident that mice and rats display differences in learning and behavioral outcomes (Frick *et al*, 2000; Snyder *et al*, 2009) that may be indicative of differences in neuronal physiology. In addition, it is possible that overexpression of proteins in BAC transgenic mice may affect sEPSC characteristics. For example, (Kramer et al., 2011) showed that *Drd2-eGFP* mice have increased membrane expression of D2 receptors in the striatum and exhibit behavioral and physiological

hypersensitivity to a D2 agonist. However the behavioral differences seen in Drd2-eGFP mice have not been consistent (Nelson *et al*, 2012).

In conclusion, we show that toluene persistently inhibits AMPA receptors via an endocannabinoid-mediated mechanism selectively in NAc D2 MSNs identified by analyzing patch clamp electrophysiology data with PLS regression. This approach can be done with relatively few recordings that include multiple measures that are commonly collected during electrophysiological recordings. Unlike other statistically based approaches, PLS reduces problems associated with co-linearity, and once validated, establishes a predictive set of variables that can be applied in real time thus permitting efficient and relevant analysis of drug or treatment effects in selective neuronal populations. Our experiments indicate that rat NAc neurons are physiologically distinct enough to segregate recordings by neuronal subpopulation.

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Figure 2.

Toluene dose-dependently inhibits NMDA-mediated currents. A) Time course of effect of toluene (black horizontal line) on NMDA mediated currents: 0.3 mM toluene (gray triangle, $n = 5$ neurons, 4 rats), 1 mM toluene (dark gray square, $n = 7$ neurons, 7 rats), 3 mM toluene (black diamond, $n = 5$ neurons, 4 rats), sham (Open circle, $n = 4$ neurons, 2 rats). B) Summary of effect of toluene on NMDA-mediated currents. Repeated-measures ANOVA, Bonferroni's multiple comparison test, *washout (after 0.3 or 3 mM toluene) v. baseline, $p < 0.05$; **washout (after 1 mM toluene) v. baseline, $p < 0.01$; ***toluene (1 or 3 mM) v. baseline, $p < 0.001$. C) Representative NMDA traces with a reversible (top) or non-reversible (bottom) inhibition due to toluene, at baseline (black), at the end of 3 mM toluene perfusion

(light gray), and at the end of a washout period (dark gray). Calibration: 25ms, 25pA. D) Effect of 1 mM toluene (gray square) or 3 mM toluene (black diamond) on NMDA currents at the end of toluene perfusion and following a washout period. While many neurons display a persisting inhibition due to toluene, some recover and return to baseline values.

Figure 3.

Long lasting inhibition of AMPA currents by 3 mM toluene in a subset of NAc neurons. Top: Histogram showing AMPA EPSC inhibition due to 3 mM toluene (15 neurons, 10 rats). 7 neurons showed inhibition due to toluene (AMPA peak amplitude < 90% of baseline), while 8 neurons showed displayed no inhibition. Middle: time course of effect of 3 mM toluene on AMPA EPSC amplitude in a toluene-insensitive neuron (open circle) and toluene-sensitive neuron (closed square). Bottom: Representative traces from two neurons, one that

is insensitive to 3 mM toluene (gray), and the other that shows pronounced inhibition following toluene perfusion (black), calibration 10 ms, 20 pA.

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Figure 4.

Schematic illustrating how each recording measurement loads onto PLS-DA factor 1 and factor 2. The variance that each factor explains in the model is listed in the F1 and F2 circles. The loading score for each variable is listed under the recording parameters. Minimum loading score – 0.25. *Neuronal parameters that had a variable importance in projection (VIP) score > 1. **Neuronal parameters with VIP score and selectivity ratio > 1.

Figure 5.

PLS-DA regression predicts toluene sensitivity by projecting recording variables onto a latent structure. A) Loading plot for neuron recording parameters. B) Variable importance in projection (VIP) score (top) and selectivity ratio (bottom) for each variable. At a threshold of 1, AP threshold, R membrane, rheobase, sEPSC amplitude, sEPSC decay and DSE were the most important variables in the model, with DSE showing a selectivity ratio nearly 8-fold higher than every other variable. C) Scattergram showing observed effect (LTD) versus predicted response as determined by factor 1 (top) and factor 2 alone (bottom). D) Scatter

plot showing individual neuron's factor scores. Symbols: Open circle (toluene insensitive); closed square (toluene sensitive).

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Figure 6.

PLS-DA regression predicts toluene sensitivity when DSE is removed. A) Loading plot for neuron recording parameters. B) VIP score (top) and selectivity ratio (bottom) for each variable. AP threshold, R membrane, rheobase, sEPSC amplitude, sEPSC decay were the most important variables, with VIP score > 1. C) Scatter plot showing observed effect (LTD) versus predicted response as determined by factor 1 (top) and factor 2 alone (bottom). D) Scatterplot showing individual neuron's factor scores. Symbols: open circle – toluene insensitive; closed square – toluene sensitive.

Figure 7.

PLS-DA reveals two subpopulations based on their electrophysiological properties. A) Summary of measures of AP threshold (left) and rheobase (right). *p < 0.05. B) Relationship between firing frequency and current injection magnitude for type I and type II neurons. C) Representative example of averaged sEPSC event for type I and II neurons. D) Summary of measures of sEPSC parameters amplitude (left) frequency (center) and decay τ (right) in type I and type II neurons. *p < 0.05. E) Time course of AMPA EPSCs before and after a 5s depolarizing pulse. Inset: the summary of effect of DSE on EPSC amplitude (right). ***p < 0.001. F) Left: Time course of AMPA EPSCs with 3 mM toluene perfusion (black line). Right: Summary of effect of 3 mM toluene on AMPA EPSC amplitude. **Kruskal-Wallis,

Dunn's Multiple Comparison test, type II compared to other two groups, $p < 0.01$ for each comparison. Symbols: white (open circles, bar)-Type I; black (closed squares, bar)-Type II; gray (triangle, bar-Type II with AM281 (1 μM) included in aCSF.

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Figure 8.

PLS-DA-based classification of MSN subtype and *Enk* mRNA expression. Expression of *Enk* was used as an indicator of D2R MSN phenotype. A) Left: Two examples of type I neurons that do not express *Enk*. Right: Two examples of type II neurons that express *Enk*. White arrow: *Enk* mRNA labeling. Yellow Arrow: Patched neuron (biocytin+) that does not express *Enk*. Black arrow: patched neuron (biocytin+) that expresses *Enk*. B) Summary of neuron classification and *Enk* expression C) Top: Factor scores (Factor 1 vs. Factor 2) of neurons. Open circles represent cells classified as type I and black squares are neurons classified as type II. All Type II neurons (n=5) expressed *Enk* (purple), and 4 out of 5 Type I neurons did not express *Enk* (yellow). Neurons within green circle were classified as Type I (n=28), and neurons within red circle were classified as Type II (n=18). Bottom: Probability of type II phenotype (black square) versus probability of type I phenotype (open circle) for all neurons (n=46). Neurons with x>0.97 (probability of type I > 97%) are represented by large open circle (n=25), and neurons with y>0.99 (probability of type II > 99%) are represented by large black square (n=15). *Enk*+: purple, *Enk*-: yellow.

Table 1

Correlation Matrix of NAc MSN recording variables

Variable	1	2	3	4	5	6	7	8	9	10	11
1 AP height (mV)											
2 AP threshold (mV)	-0.399 ^{**}										
3 V membrane (mV)	-0.296 [*]	0.367 [*]									
4 Rheobase(pA)	0.105	-0.270	-0.913 ^{**}								
5 AHP1 (mV)	0.329 [*]	-0.059	-0.297 [*]	0.228							
6 AHP(2-1)(mV)	-0.035	0.116	-0.101	0.104	-0.541 ^{**}						
7 DSE inhibition	-0.240	0.478 ^{**}	0.269	-0.118	-0.051	0.051					
8 sEPSC Freq (Hz)	0.166	-0.174	-0.243	0.200	-0.292 [*]	0.555 ^{**}	-0.147				
9 sEPSC Amp (pA)	-0.243	-0.400 ^{**}	-0.119	0.250	-0.293 [*]	0.052	-0.283	0.175			
10 sEPSC Max Amp (pA)	-0.132	-0.214	0.162	-0.072	-0.388 ^{**}	0.042	-0.212	0.158	0.613 ^{**}		
11 sEPSC > 50 pA	-0.155	-0.245	-0.043	0.158	-0.193	-0.063	-0.151	-0.130	0.794 ^{**}	0.699 ^{**}	
12 sEPSC tau (ms)	-0.300 [*]	0.415 ^{**}	0.332 [*]	-0.217	-0.278	0.101	0.448 ^{**}	0.119	-0.245	-0.182	-0.234

Note: n = 46

*p < 0.05

**p < 0.01

Table 2

Logistic Regressions for predicting toluene sensitivity

Variable	LTD observed	LTD Predicted			χ^2	p
		N	Y	% Correct		
AP Height	N	6	2	75.0	2.525	0.112
	Y	3	4	57.1		
	Overall			66.7		
AP Threshold	N	6	2	75.0	4.152	0.042
	Y	1	6	85.7		
	Overall			80.0		
Vmembrane	N	4	4	50.0	4.861	0.027
	Y	2	5	71.4		
	Overall			60.0		
Rheobase	N	7	1	87.5	4.592	0.032
	Y	2	5	71.4		
	Overall			80.0		
AHP1	N	5	3	62.5	0.165	0.685
	Y	5	2	28.6		
	Overall			46.7		
AHP2-1	N	6	2	75.0	0.104	0.708
	Y	6	1	14.3		
	Overall			46.7		
DSE	N	8	0	100.0	20.728	< 0.0001
	Y	0	7	100.0		
	Overall			100.0		
sEPSC Frequency	N	6	2	72.0	0.198	0.656
	Y	4	3	42.9		
	Overall			60.0		
sEPSC Amplitude	N	6	2	75.0	6.119	0.013
	Y	3	4	57.1		
	Overall			66.7		

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Variable	LTD observed	LTD Predicted			χ^2	p
		N	Y	% Correct		
sEPSCMax Amp	N	5	3	62.5	1.127	0.289
	Y	4	3	42.9		
	Overall			53.3		
sEPSC>50pA	N	7	1	87.5	0.035	0.852
	Y	5	2	28.6		
	Overall			60.0		
sEPSC Decay	N	6	2	75.0	5.698	0.017
	Y	2	5	71.4		
	Overall			73.3		

Table 3

Logistic Regressions with PLS-DA factors for predicting toluene sensitivity

Variable	Step	LTD observed	LTD Predicted			χ^2	p
			N	Y	% Correct		
PLSDAwithDSE2		N	6	2	75.0	1.544	0.214
		Y	4	3	42.9		
		Overall			60.0		
PLSDAwithDSE1 & PLSDAwithDSE2	Step 1	N	8	0	100.0	20.728	<0.0001
		Y	0	7	100.0		
		Overall			100.0		
	Step 2	N	8	0	100.0	0.000	0.999
		Y	0	7	100.0		
		Overall			100.0		
	Model					20.728	<0.0001
PLSDA _{no} DSE2		N	6	2	75.0	2.014	0.156
		Y	4	3	42.9		
		Overall			60.0		
PLSDA _{no} DSE1 & PLSDA _{no} DSE2	Step 1	N	7	1	87.5	10.067	0.002
		Y	0	7	100.0		
		Overall			93.3		
	Step 2	N	8	0	100.0	4.510	0.034
		Y	0	7	100.0		
		Overall			100.0		
	Model					20.728	<0.0001