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Chronic Ethanol Exposure Increases Inhibition of Optically Targeted Phasic Dopamine Release in the Nucleus Accumbens Core and Medial Shell ex vivo

James R. Melchior[1] and **Sara R. Jones**[1,*]

[1]Department of Physiology and Pharmacology, Wake Forest School of Medicine, Winston Salem, North Carolina 27157

Abstract

Dopamine signaling encodes reward learning and motivated behavior through modulation of synaptic signaling in the nucleus accumbens, and aberrations in these processes are thought to underlie obsessive behaviors associated with alcohol abuse. The nucleus accumbens is divided into core and shell sub-regions with overlapping but also divergent contributions to behavior. Here we optogenetically targeted dopamine projections to the accumbens allowing us to isolate stimulation of dopamine terminals *ex vivo*. We applied 5 pulse (phasic) light stimulations to probe intrinsic differences in dopamine release parameters across regions. Also, we exposed animals to 4 weeks of chronic intermittent ethanol vapor and measured phasic release. We found that initial release probability, uptake rate and autoreceptor inhibition were greater in the accumbens core compared to the shell, yet the shell showed greater phasic release ratios. Following chronic ethanol, uptake rates were increased in the core but not the shell, suggesting region-specific neuronal adaptations. Conversely, kappa opioid receptor function was upregulated in both regions to a similar extent, suggesting a local mechanism of kappa opioid receptor regulation that is generalized across the nucleus accumbens. These data suggest that dopamine axons in the nucleus accumbens core and shell display differences in intrinsic release parameters, and that ethanol-induced adaptations to dopamine neuron terminal fields may not be homogeneous. Also, chronic ethanol exposure induces an upregulation in kappa opioid receptor function, providing a mechanism for potential over-inhibition of accumbens dopamine signaling which may negatively impact downstream synaptic function and ultimately bias choice towards previously reinforced alcohol use behaviors.

Keywords

Dopamine; Nucleus Accumbens; Ethanol; Optogenetics; Voltammetry; Kappa Opioid Receptors

[*]Corresponding author: Sara R. Jones, Ph.D., Department of Physiology and Pharmacology, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, Tel: 336.716.8533, Fax: 336.716.8501, srjones@wakehealth.edu.

Introduction

Alcohol use disorders (AUD) and drug addiction share a common feature - persistent seeking and use despite delayed negative consequences on both physical and social health. These aberrant actions suggest abnormalities in the neural circuitry governing motivated behavior (Hyman *et al.* 2006; Stuber *et al.* 2010b), and have prompted research into elucidating ethanol induced neuroadaptations that may underlie the switch from casual to obsessive attention towards alcohol. An area of particular interest is the nucleus accumbens (NAc), which integrates diverse cognitive and limbic inputs onto motor plan-initiating output signals of the basal ganglia, implicating this region in motivation and behavioral selection. For instance, repeated drug exposure causes long term synaptic plasticity within the NAc that results in sensitized locomotor responses (Creed *et al.* 2015; Pascoli *et al.* 2011) and incubation of craving during withdrawal (Conrad *et al.* 2008), prompting increased attention on identifying the synaptic manifestations of chronic ethanol exposure in this region (Jeanes *et al.* 2014; Abrahao *et al.* 2013). Dopamine (DA) signaling in the NAc is thought to encode reward learning via modulation of synaptic signaling and gating aspects of synaptic plasticity (Shen *et al.* 2008; Surmeier *et al.* 2011; Tritsch and Sabatini 2012; Creed and Lüscher 2013), processes that are believed to ultimately bias decision making towards previously reinforced behaviors. Indeed, aberrations in DA signaling have been linked to obsessive/compulsive behaviors (Sesia *et al.* 2013) and behavioral rigidity (Beeler *et al.* 2014).

Kappa opioid (κ-opioid) receptors are expressed within the NAc where they inhibit DA signaling (Spanagel *et al.* 1992) upon activation by endogenous dynorphin, providing a negative feedback mechanism to regulate local DA levels (Steiner and Gerfen 1996). Further, the dynorphin/κ-opioid system appears to be upregulated following chronic ethanol exposure (Sirohi *et al.* 2012). Specifically, the κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI) reduces the escalation of intake observed in alcohol dependent animals while having no effect on non-dependent animals (Walker and Koob 2008), suggesting that recruitment of κ-opioid receptor activity contributes to dependence and may provide a potential mechanism for ethanol-induced adaptations in DA transmission and the subsequent modulation of synaptic function (Shippenberg *et al.* 2007).

The NAc is divided into two sub-regions, the core and the shell, which receive unique assortments of afferent inputs and differentially contribute to reward aspects of behavior (Kelley 2004). The core is involved in reinforcement learning and adaptive instrumental behavior, while the shell is connected with viscero-endocrine effector systems involved in reward processing and motivational states (Kelley 1999). For example, by virtue of its afferent innervation from the ventral hippocampus (Britt *et al.* 2012) and efferent projections to the lateral hypothalamus (Kelley 2004) the shell is considered to be a component of the extended amygdala, a collection of structures heavily implicated in exaggerated stress and anxiety states during alcohol withdrawal (Koob 2013; Lovinger and Kash 2015). Both regions receive DA innervation from the ventral tegmental area (VTA); however, DA signaling does not appear to be homogenous across the NAc (Aragona *et al.* 2009). Acute drug and alcohol administration selectively increase DA release in the shell compared to the core (Di Chiara *et al.* 2004; Howard *et al.* 2008), and chronic drug exposure results in

differential dopaminergic adaptations in the core and shell (Saddoris 2016; Saddoris *et al.* 2016). This is coupled with differences in phasic release parameters of DA terminal fields across regions (Jones *et al.* 1996a; Zhang *et al.* 2009), supporting region-specific heterogeneity in DA signaling.

One level of DA signal regulation occurs at the terminals, where expression of a variety of release-regulating heteroreceptors in the terminal membrane (Zhang and Sulzer 2012; Sulzer *et al.* 2016) allows local environmental influence of terminal physiology and results in diverse micro-domains within terminal fields (Wightman *et al.* 2007; Pickel 2000; Zhang *et al.* 2015; Tritsch *et al.* 2012). Fast-scan cyclic voltammetry (FSCV) is often used in *ex vivo* slice preparations to pharmacologically probe terminal receptor regulation of DA release and how terminal activity may be altered following chronic drug and alcohol administration (Ferris *et al.* 2013; Siciliano *et al.* 2015b; Calipari *et al.* 2015). However, relatively few of these investigations probe terminal fields in the medial NAc shell, partially due to the technical difficulties in obtaining robust, reliable DA release in this region. That is, a single pulse-stimulated DA signal is low in amplitude compared to neighboring regions such as the NAc core and dorsal striatum (Jones *et al.* 1996a). This can be overcome by applying multiple pulses in a stimulation train; however, electrical stimulation trains recruit modulation of DA terminals from concurrent excitation of the surrounding non-dopaminergic neuronal types within the tissue (Melchior *et al.* 2015a). This recruitment often distorts the stimulated DA signal and makes assessments of whether heteroreceptor modulation is occurring through direct (terminal receptors) or indirect (multi-synaptic) mechanisms difficult to determine. Here we use optogenetic tools to selectively light-stimulate DA terminals in the NAc, without concurrent electrical excitation of non-dopaminergic cells in the tissue, providing an isolated DA signal *ex vivo*.

The goal of these studies was to extend our investigations of chronic ethanol-induced alterations of DA signaling in the ventral striatum (Karkhanis *et al.* 2015; Rose *et al.* 2016; Siciliano *et al.* 2015a) across three novel parameters. 1) We measured DA signals in the NAc shell to provide a direct comparison to NAc core DA terminal fields in order to determine whether chronic ethanol exposure results in differential adaptations between regions. 2) We used targeted light stimulation, avoiding concurrent modulation of DA release by excitation of non-dopaminergic cells in the DA terminal field, to provide greater resolution with regard to direct vs indirect ethanol-induced DA terminal heteroreceptor adaptations. 3) We applied 5 pulse stimulation trains to approximate the phasic spikes in DA neurons that occur in response to discrete environmental cues *in vivo*.

Methods

Animals

Male Tyrosine Hydroxylase (TH)-internal ribosome entry site (IRES)-Cre Recombinase knock-in mice on a C57Bl/6J background (TH:Cre) were bred, genotyped for positive cre recombinase expression and maintained in group housing in the mouse colony. All animals were maintained according to the National Institutes of Health guidelines and all experimental protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine.

At 8–12 weeks of age mice were anesthetized with 100 mg/kg ketamine and 8 mg/kg xylazine and placed in a stereotaxic frame. A custom-made glass micropipette (80 μm outer diameter) was inserted directly above the VTA (coordinates from Bregma in mm: −3.3 AP, ±0.5 ML, −4.3 DV). Microinjections were administered using an air pressure injection system and consisted of applying small pulses of pressure (30 psi, 40–80 msec duration) to the infusion pipette. Individual injections were performed on each side of the midline resulting in bilateral VTA infusions, with each hemisphere of VTA receiving approximately 0.4 μl of AAV5-EF1α-hChR2(H134R)-eYFP (5.5 × 1012 virus molecules/ml; Virus Vector Core, University of North Carolina). Following surgery, mice were returned to the mouse colony, single housed, and maintained for a minimum of 14 days to recover from surgery and allow expression of channelrhodopsin-2 (ChR2).

Histology

Immunohistochemistry was used to verify ChR2 expression in dopamine axons in the nucleus accumbens as previously described (Melchior *et al.* 2015a). Mice were anesthetized with ketamine (100mg/kg) and xylazine (8 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS) followed by 10% buffered formalin phosphate (Fischer Scientific, Waltham, MA, USA). Brains were then removed, submerged in 10% buffered formalin phosphate for an additional 24–48 hours, and subsequently transferred to 30% sucrose in PBS for 72 hours. Sections (40 μm) were obtained on a microtome (American Optical Company, Buffalo, NY, USA) and stored in PBS for immunohistochemistry.

Sections were permeablized in 0.3% triton (Sigma, St Louis, MO, USA) in PBS (PBS-Tx) for 2 hours, blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS-Tx, and incubated in primary antibody in the blocking solution for 24–48 hours. Primary antibodies include chicken anti-GFP (1 μg : 500 μl; Aves labs, Tigard, OR, USA) and rabbit anti-tyrosine hydroxylase (1 μg : 1000 μl; Cell Signaling, Danvers, MA, USA). Sections were rinsed and transferred to secondary antibody in blocking solution for 1.5 hours. Secondary antibodies include fluorescein-labeled goat anti-chicken IgY (Aves labs, 1 μg : 250 μl) and goat anti-rabbit alexa fluor 594 IgG (1 μg : 250 μl; Molecular Probes, Eugene, OR, USA). Sections were mounted on 1mm slides with Vectashield (Vector Labs) mounting medium and images were obtained with an Olympus BX-51 Microscope and Optronics Microfire digital camera (Goleta, CA, USA). Images were processed in Adobe Photoshop.

CIE

ChR2 expressing mice were administered the chronic intermittent ethanol (CIE) exposure protocol outlined on the website for the INIA-Stress research consortium (http://iniastress.org/dependence). Briefly, mice were injected with a ‘loading’ dose of ethanol (1.6 g/kg) and pyrazole (1 mmol/kg), an alcohol dehydrogenase inhibitor that stabilizes and maintains blood ethanol concentrations, and exposed to ethanol vapor in an air tight vapor chamber for 16 consecutive hours. This procedure is repeated, daily, for four consecutive days, followed by 3 days of exposure to room air with no injections - constituting one cycle of CIE. The ethanol treated group was exposed to 4 cycles of CIE spanning 4 consecutive weeks. An ‘air-treated’ control group was administered saline and pyrazole (1 mmol/kg)

injections and exposed to room air across the 4 weeks with no exposure to ethanol. Following CIE or air-treatment, animals were sacrificed for voltammetry experiments. The CIE and air treatment groups were staggered such that each ethanol-treated animal had a complimentary air-treated control that was scheduled for voltammetry experiments the following day.

Blood ethanol concentrations (BEC) for animals exposed to ethanol vapor were measured at the end of each cycle for each animal to ensure that BECs were physiologically relevant. Blood samples were collected from the mice using the submandibular vein punch immediately after removal from the ethanol vapor chamber. Blood samples were assayed for ethanol concentration as described previously (Karkhanis *et al.* 2015).

Ex Vivo Voltammetry

Slice preparation and FSCV was performed as described previously (Ferris *et al.* 2012). On the morning following completion of the final cycle of CIE (72 hours after final ethanol exposure), animals were anesthetized with isoflurane, decapitated, and the brain rapidly removed and cooled in ice-cold, pre-oxygenated (95% O2/5% CO2) artificial cerebral spinal fluid (aCSF) consisting of (in mM): NaCl (126), KCl (2.5), NaH2PO4(1.2), CaCl2(2.4), MgCl2(1.2), NaHCO3(25), glucose (11), L-ascorbic acid (0.4) and pH was adjusted to 7.4. Multiple coronal slices (300 μm thick) containing the NAc (NAc) were prepared from each animal with a vibrating tissue slicer (Leica VT1000S; Leica Instruments, Nussloch, Germany). Slices were maintained in oxygenated aCSF at room temperature for 1 hour before transfer to a submersion recording chamber through which 32°C oxygenated aCSF was perfused at a rate of 1 ml/min. Two voltammetry rigs were used; slices were divided along the midline and each hemisphere was transferred to the recording chamber of the corresponding voltammetry rig. Thus, NAc core and shell recordings were occurring simultaneously, across two voltammetry rigs, from separate hemispheres of the same slice.

Carbon fiber microelectrodes (100–150 μM length, 7 μM diameter) were fabricated with a waterproof epoxied seal which results in reduced noise and increased stability, allowing a single electrode to be used across experiments. A total of 6 electrodes were used in this study, and the same electrode was used on consecutive days, for each corresponding animal in the experimental and control groups. The carbon fiber electrode was placed into the core or shell of the NAc approximately 100 μM below the surface. Light stimulation was delivered from an optic fiber coupled to a 100 mW, 473 nm blue laser. The optic fiber was positioned in the slice bath, above the tissue, and aimed to deliver light to the area of tissue immediately surrounding the recording electrode. DA was evoked by an optical pulse (~5 mW, 4 ms duration) applied as a single pulse or a 5 pulse train (20 Hz) every 5 min. Extracellular DA was monitored at the carbon fiber electrode every 100 msec using fast-scan cyclic voltammetry (Wightman et al., 1988) by applying a triangular waveform (−0.4 to +1.2 to −0.4 V vs Ag/AgCl, 400 V/s). Following experiments, recording electrodes were calibrated by measuring responses (in electrical current; nA) to a known concentration of DA (3 μM), using a flow-injection system. This was used to convert electrical current to DA concentration.

Experiments began with single pulse stimulations which were repeated every 5 minutes until the DA signal reached stability, defined by less than 10% variation in magnitude and not trending up or down across three successive recordings; stability occurs in approximately 90 minutes. Once stable single pulse evoked DA signals were obtained, 5 pulse (20 Hz) phasic stimulations were applied once every 5 minutes. In control slices, we found that signals remained stable for approximately 2 hours using this stimulation protocol, after which the peak amplitude would slowly decline (< 1%) with each successive stimulation. Once 5 pulse phasic stimulations were stable, the κ-opioid opioid receptor agonist U50,488 (30–1000 nM) or the D2 receptor agonist quinpirole (10–100 nM) was applied cumulatively to the brain slice. Recordings for each drug concentration were continued until the magnitude of DA release was again stable, before proceeding to the next concentration. Following U50,488, slices were washed in aCSF containing the κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI; 30 μM) to reverse the U50,488 inhibition of DA release. To account for potential deterioration of baseline amplitude resulting from prolonged application of phasic light stimulations, the nor-BNI reversal for each group was normalized to the baseline magnitude and this correction was applied to each drug dose response accordingly.

All voltammetry data were collected and modeled using Demon Voltammetry and Analysis Software (Yorgason *et al.* 2011). Parameters of evoked levels of DA are determined based on Michaelis–Menten kinetics (Wightman *et al.* 1988), following standard voltammetric modeling procedures (Wu *et al.* 2001).

Statistics

Graph Pad Prism (version 5, La Jolla, CA, USA) was used to statistically analyze data sets and create graphs. Planned comparisons in DA release and uptake measures were analyzed using an unpaired two-tailed t-test. Drug concentration response curves were subjected to a two-way repeated measures ANOVA; concentration was the within subjects factor and experimental group was the between subjects factor. Differences between groups were tested using a Bonferroni post hoc test. All p values of < 0.05 were considered to be statistically significant.

Results

Histological analysis of ChR2 expression

In order to target ChR2 expression in DA terminal fields in the nucleus accumbens, we used a viral construct with DNA encoding ChR2 expression in a manner dependent on cre-recombinase expression, and a transgenic mouse line that endogenously expresses cre-recombinase based on the transcription of TH mRNA (Tsai *et al.* 2009); thus, when used together, ChR2 will be targeted to dopamine precursor expressing neurons. Indeed, we found viral transfection of the VTA of TH:Cre mice resulted in ChR2 expression in midbrain DA neurons and their axonal projections to the NAc, as in previous studies (Melchior *et al.* 2015a). Figure 1 shows representative immunohistochemical images of ChR2 expression 6 weeks after viral injection. In the VTA, TH expression was immunolabeled with red fluorescence, allowing identification of midbrain DA neurons (Fig. 1A). ChR2 expression was immunolabeled with green fluorescence and robustly overlapped the TH expressing

neurons in the VTA. Note that ChR2 expression did not extend laterally to the neighboring substantia nigra compacta, indicating accurate targeting of viral injections. In the ventral striatum, ChR2 expression was seen in the terminal projections of VTA DA neurons, which densely innervate the NAc core and shell regions (Fig. 1B).

An important consideration when using the TH:Cre mouse model is the ectopic expression of Cre-driven transgenes in midline VTA neurons that are not dopaminergic (Lammel *et al.* 2015; Stuber *et al.* 2015). This results from a population of cells within the medial VTA nuclei which express low levels of TH mRNA, sufficient to induce endogenous Cre recombinase expression, but do not translate detectable levels of TH protein and are not dopaminergic (Yamaguchi *et al.* 2015; Lammel *et al.* 2015). Thus, the population of ChR2-expressing projections to the accumbens may include glutamate- or GABA-releasing axons (Yamaguchi *et al.* 2015; Brown *et al.* 2012; Taylor *et al.* 2014). For the purposes of these studies, in which we are interested in presynaptic regulation of dopamine terminal release, our goal was to eliminate excitation of local accumbens elements which may profoundly modulate dopamine release such as acetylcholine and GABA interneurons, or retrograde release of neuropeptides or reactive oxygen species from repeated excitation of medium spiny neurons (Zhou *et al.* 2001; Steiner and Gerfen 1996; Patel and Rice 2012). While VTA glutamatergic projections may be included in the stimulation field, dopamine terminal fields do not receive direct glutamatergic input (Sulzer *et al.* 2016; Rice *et al.* 2011). Further, optogenetically targeted dopamine release is insensitive to blockade of nicotinic acetylcholine receptors and $GABA_B$ receptors ($GABA_A$ receptors are not expressed on dopamine terminals) in *ex vivo* preparations (Melchior *et al.* 2015), which suggests that non-dopaminergic projections from VTA are not cross-modulating dopamine terminals, either directly or indirectly through cholinergic interneurons (Kosillo *et al.* 2016) in this model.

Light-evoked DA release measured with voltammetry

Coronal slices containing the NAc were examined using voltammetry to measure light stimulated DA release (Melchior *et al.* 2015a). A single slice was divided into separate hemispheres and signals were measured at the same time using two separate voltammetry rigs. Within the slice, NAc core measurements were sampled at a placement slightly ventral and lateral to the anterior commissure. In the opposite hemisphere (randomly assigned as right or left), NAc shell measurements were sampled from the medial shell region, also slightly ventral to the level of the anterior commissure, on the same lateral plane as core samples. These locations are represented by the labels ‘core’ and ‘shell’ in Fig. 1B. Light stimulations applied to both regions resulted in increases in current (Fig. 2A) with peak changes in current detected at 0.6 V and −0.2 V on the voltage scan (Fig. 2A inset); these peaks are consistent with the oxidation and reduction profile of DA (Wightman *et al.* 1988). Color plot representations show the changes in current (z-axis) with respect to voltage (y-axis) and time (x-axis). Representative traces of 5 pulse (20 Hz) light stimulated DA release show characteristic signals obtained in the core and shell regions (Fig. 2A).

DA release parameters: core versus shell

Stimulated DA signals in the NAc shell were smaller in amplitude compared to signals in the core (n = 60 total slices across 25 animals; 60/25). Single pulse light stimulations resulted in

1.08 ± 0.08 μM DA release in the core compared to 0.31 ± 0.03 μM DA release in the shell, which were significantly different between regions ($t_{(57)}$ = 9.0, p < 0.001; Fig. 2B). Similarly, 5 pulse light stimulations resulted in 3.22 ± 0.22 μM DA release in the core and 1.25 ± 0.10 μM DA release in the shell, which were also significantly different between regions ($t_{(58)}$ = 8.0, p < 0.001). Further, there was a significant interaction between region and pulse number (two-way anova; $F_{(1,115)}$ = 20.73, p < 0.001).

It has been reported that the shell shows a greater magnitude of phasic activation compared to the core (Zhang *et al.* 2009). That is, the ratio of multi-pulse stimulated release levels to single pulse stimulated release levels is enhanced in the shell. Here we calculated the ratio of 5 pulse (phasic) release magnitude to single pulse (tonic) release magnitude (5 p:1p ratio). The phasic release ratio was 3.10 ± 0.09 in the core and 4.09 ± 0.09 in the shell, thus the shell shows significantly greater phasic activation than the core ($t_{(57)}$ = 7.7, p < 0.001; Fig. 2C) in agreement with previous reports. In order to assess possible differences in release probability, we analyzed DA release per stimulus pulse (DAp) across groups (Fig. 2D). We found that DAp was significantly higher for a single pulse stimulation 1006 ± 70.8 nM compared to release per pulse across a 5 pulse stimulation train (814.4 ± 50.1 nM) in the core ($t_{(59)}$ = 2.2, p < 0.05). Conversely, in the shell, a single pulse stimulation results in a similar magnitude of DA release (293.2 ± 26.6 nM) compared to each pulse in a stimulation train (296.4 ± 24.0 nM; p > 0.05). There was a significant interaction between region and pulse number (two-way anova; $F_{(1,115)}$ = 4.20, p < 0.05).

Chronic ethanol effects on DA release

Next we wanted to determine whether chronic ethanol exposure resulted in changes in DA terminal parameters, specifically DA release, uptake rates and modulation by hetero-receptors. To this end, mice expressing ChR2 in DA neurons were exposed to chronic intermittent ethanol (CIE) exposure using ethanol vapor chambers (Becker and Lopez 2004). Mice were exposed to ethanol vapor for 16 hours a day, 4 days a week, for 4 consecutive weeks. BECs were measured at the end of each week and showed an average of 231.6 ± 14.7 mg/dL (mean ± SEM; n = 13). A separate group of ChR2 expressing mice were exposed to the identical protocol as the CIE treated animals except they were exposed to normal room air instead of ethanol vapor.

In order to determine if chronic ethanol exposure reduced DA terminal function, we assessed release using single or 5 pulse stimulations following CIE. We found no differences in light stimulated release in either the core or shell regions in ethanol treated animals. In the NAc core, single pulse light stimulations resulted in 1.01 ± 0.08 μM DA release in CIE treated animals (n = 16/13) which was not different from the 1.16 ± 0.15 μM DA release measured in air treated animals (n = 14/12; $t_{(28)}$ = 0.9, p > 0.05; Fig. 3A). Similarly, 5 pulse light stimulations resulted in 3.23 ± 0.25 μM DA release in CIE treated animals (n = 16/13) and 3.21 ± 0.37 μM DA release in air-treated animals (n = 15/12; $t_{(29)}$ = 0.03, p > 0.05; Fig. 3B). In the NAc shell, single pulse light stimulations resulted in 0.30 ± 0.03 μM DA release in CIE treated animals (n = 15/12) and 0.31 ± 0.03 μM DA release in air treated animals (n = 14/11; $t_{(27)}$ = 0.05, p > 0.05; Fig. 3A). Similarly, 5 pulse light stimulations resulted in 1.23

± 0.14 μM DA release in CIE treated animals and 1.27 ± 0.15 μM DA release in air-treated animals ($t_{(27)}$ = 0.17, p > 0.05; Fig. 3B).

Chronic ethanol effects on uptake rates

It has been reported that DA uptake rates are slower in the NAc shell compared to the NAc core in rodents (Jones *et al.* 1996b; Zhang *et al.* 2009). In order to examine this in our model we measured maximal uptake rates (Vmax) using 5 pulse light stimulations, which produced the largest amplitude DA release signals in each region. Consistent with previous reports, we found that uptake rates in the shell were slower than those in the core. In control animals (n = 15/12) Vmax in the core was 2172 ± 223 nM/s whereas in the shell (n = 14/11) Vmax was 726 ± 79 nM/s, which was significantly less compared to the core ($t_{(27)}$ = 5.9, p < 0.001; Fig. 4A).

Following chronic ethanol exposure we found that there was there was a significant interaction of treatment and region on uptake rates ($F_{(1,56)}$ = 4.18, p < 0.05). Specifically, we found that in ethanol treated animals (n = 16/13), Vmax in the core was 2780 ± 175 nM/s which was significantly increased compared to air treated animals in which the Vmax was 2172 ± 223 nM/s; ($t_{(29)}$ = 2.2, p < 0.05; Fig. 5A). Figure 4B shows representative traces of 5 pulse light stimulated DA signals in the NAc core of air and CIE treated animals, which produced similar magnitudes of release. There is a clear increase in steepness of slope on the descending limb of the DA signal, highlighted by the colored lines (blue, air-treated; red, CIE treated). In the NAc shell, however, we found no differences in Vmax between CIE treated animals (n= 15/12; 697 ± 78 nM/s DA/s) and air-treated controls (n = 14/11; 726 ± 79 nM/s; $t_{(27)}$ = 0.26, p > 0.05; Fig. 4A).

Chronic ethanol effects on autoreceptors

Regulation of DA signaling is tightly controlled by D_2/D_3-type DA receptors located on DA terminals which act as autoreceptors, providing feedback-inhibition of DA release. Autoreceptor sensitivity has been shown to be altered following chronic exposure to stimulants (Lee *et al.* 1999; Mateo *et al.* 2005; Calipari *et al.* 2014). Here we wanted to assess autoreceptor function in both the NAc core and shell regions following chronic ethanol exposure.

To test autoreceptor sensitivity, we bath applied the D_2/D_3 receptor agonist quinpirole (30–300 nM); 5 pulse light stimulations were applied every five minutes and DA release was measured following incubation with each concentration of quinpirole. Quinpirole concentration effects were measured within-slice as a percentage of pre-drug baseline release in control animals (n = 7). Quinpirole induced a dose-responsive inhibition of 5 pulse light stimulated DA release across regions (concentration: $F_{(2,24)}$ = 1127, p < 0.001; Fig 5A). Surprisingly, we found a regional difference in the effect of quinpirole, with the core being significantly more sensitive than the shell ($F_{(1,24)}$ = 17.04, p < 0.01), including an interaction between concentration and region ($F_{(2, 24)}$ = 3.9, p < 0.05). Bonferroni post hoc analyses of individual concentrations revealed a significant regional difference in response to quinpirole at all doses tested: 30nM (p < 0.05), 100 nM (p < 0.001) and 300 nM (p < 0.001). These

results showed that the NAc core was significantly more sensitive to autoreceptor regulation than the shell.

Next, we wanted to determine whether chronic ethanol exposure altered autoreceptor function in either region (Fig. 5B). The quinpirole induced inhibition of DA release in NAc core did not differ between CIE (n = 8) and air treated (n = 7) groups ($F_{(1,26)} = 0.01$, $p > 0.05$). Similarly, in the NAc shell, quinpirole induced inhibition of DA release did not differ between CIE and air treated animals ($F_{(1,26)} = 0.74$, $p > 0.05$). These results show that DA terminal autoreceptor regulation of optogenetically-evoked release is not altered 72 hours after chronic ethanol exposure using a CIE vapor paradigm.

Chronic ethanol effects on kappa opioid receptors

Chronic ethanol exposure has been suggested to upregulate the dynorphin/kappa opioid system receptor *in vivo*, a process that could include increased expression of dynorphin, the endogenous ligand for κ-opioid receptors (Sirohi *et al.* 2012; Shippenberg *et al.* 2007), or changes in sensitivity of κ-opioid receptors expressed within the NAc where activation decreases DA release (Spanagel *et al.* 1992; Sirohi *et al.* 2012). We used our model of targeted light stimulation of DA terminals to assess κ-opioid receptor modulation of DA release and the effects of chronic ethanol exposure on κ-opioid receptor sensitivity.

To test κ-opioid receptor inhibition of DA release we bath applied the κ-opioid receptor agonist U50,488 (0.03–1.0 μM) and subsequently reversed U50,488 effects with aCSF containing the κ-opioid receptor antagonist nor-BNI (30 μM). 5 pulse light stimulations were applied every 5 minutes and DA release was measured following incubation with each concentration of U50,488. U50,488 concentration effects were measured within-slice as a percentage of pre-drug baseline release and nor-BNI reversal.

In the NAc core, U50,488 inhibited DA release in a dose response manner ($F_{(3, 42)} = 56.3$, $p < 0.001$). We found that CIE treated mice (n = 8) showed increased sensitivity to the κ-receptor agonist U50,488 ($F_{(1, 42)} = 7.55$, $p < 0.05$) compared to air treated controls (n = 8; Fig. 6A). *Post-hoc* analysis revealed that there was a significant difference between the groups at the 0.3 μM ($p < 0.05$) and 1.0 μM ($p < 0.01$) concentrations of U50,488. Further, there was an interaction effect between group and drug dose ($F_{(3, 42)} = 3.82$, $p < 0.05$) with the largest difference between groups being observed at the highest dose of U50,488 tested.

In the NAc shell, U50,488 also inhibited 5 pulse light stimulated DA release ($F_{(3, 42)} = 104.5$, $p < 0.001$). Similarly, we found that CIE treated mice showed increased sensitivity to the κ-opioid receptor agonist U50,488 ($F_{(1, 42)} = 6.94$, $p < 0.05$) compared to air treated controls (Fig. 6B). *Post-hoc* analysis revealed that there was a significant difference between the groups at the 0.3 μM and 1.0 μM ($p < 0.05$) doses of U50,488. There was also an interaction effect between group and drug dose ($F_{(3, 42)} = 2.12$, $p < 0.05$) with the largest difference between groups being observed at the highest dose of U50,488 tested.

In order to determine if there was a regional difference in sensitivity to κ-opioid receptor agonism we collapsed grouped data to compare U50,488 inhibition of dopamine release between the nucleus accumbens core and shell (Fig. 6C). We found that the NAc shell was

more sensitive to U50,488 compared to the core ($F_{1,56} = 4.7$, $p < 0.05$), with post-hoc analysis of individual doses finding a significant difference at the highest dose tested (1 μM; $p < 0.05$). These data suggest that κ-opioid receptor modulation of DA release is greater in the NAc shell compared to the core; however, both regions show a similar magnitude of increased sensitivity to κ-opioid receptor modulation following chronic ethanol exposure.

Finally, we wanted to determine if κ-opioid receptor mediated inhibition differentially effects DA release resulting from single (tonic) stimulation or a 5 pulse train (phasic) stimulation. Receptor mediated modulation of DA terminal release is often overcome by increases in pulse number and/or frequency of stimulation (Zhang and Sulzer 2012). Here we wanted to see if κ-opioid receptor modulation of DA terminals follows those same principles. We ran a single dose of U50,488 on NAc core slices from naïve animals (n = 5) and compared the magnitude of inhibition between single pulse and 5 pulse light stimulation-induced DA release. Baseline DA release was established using 1 and 5 pulse stimulations in the same location in the slice, resulting in average of 1.1 ± 0.2 and 3.1 ± 0.8 μM DA signals respectively. U50,488 reduced single pulse light stimulated DA release by 26.6% ±1.8% and reduced 5 pulse stimulated release by 15.7% ± 2.1% of baseline in the same DA terminal fields (Fig. 6D). Thus, the inhibition produced by U50,488 was significantly greater on single pulse stimulated compared to multi-pulse burst stimulated release (paired $t_{(4)} = 3.9$, $p < 0.05$).

Discussion

Selectively targeting DA terminals for isolated stimulation *ex vivo* has advanced our understanding of DA signaling in the striatum, revealing unique characteristics previously masked by electrical stimulation (Stuber *et al.* 2010a; Tritsch *et al.* 2012). For example, DA release can be significantly enhanced by co-stimulation of local acetylcholine interneurons (Threlfell *et al.* 2012; Cachope *et al.* 2012; Melchior *et al.* 2015a) via nACh receptors on DA terminals. These receptors modulate tonic to phasic DA release ratios, which affect the amplitude of transient DA spikes in relation to basal DA levels (Zhang *et al.* 2009). Here we used optogenetic DA terminal stimulation, removing the influence of nACh activation that occurs with electrical stimulation (Melchior *et al.* 2015a), to compare signaling parameters between the NAc core and shell. We used single pulses and five-pulse trains to measure tonic and phasic DA release. We found that the core had greater amplitude DA release, but the shell has greater phasic-to-tonic DA release ratios. This is due, in part, to the core showing a paired pulse-like depression (McCool 2011), in which release probability is higher for a single pulse stimulation than for the pulses that follow it in a train. In the shell, the release per pulse is more equally distributed across pulses, suggesting a lower initial release probability compared to the core. This occurs in the absence of local microcircuit regulation, and instead suggests that the mechanisms are intrinsic to the terminals, likely due to differences in releasable pools and sensitivity to calcium (Sulzer *et al.* 2016; Brimblecombe *et al.* 2014). This highlights the different terminal properties of core- and shell-projecting DA axons (Jones *et al.* 1996b; Zhang *et al.* 2009). These differences also include increased uptake rates and increased sensitivity to autoreceptor regulation in the core. Uptake rates were measured using 5 pulse stimulated release, which results in large amplitude signals which allow accurate estimation of ‘maximal’ uptake rates at saturating

DA concentrations (Wu *et al.* 2001). There were clearly reduced uptake rates in the shell, which is consistent with reduced DA transporter (DAT) expression (Coulter *et al.* 1996) in this region. We also measured D_2-type autoreceptor function in the absence of concurrent stimulation of D_2 expressing post synaptic neurons using optogenetic stimulation and found increased inhibition of presynaptic release in the core region. To our knowledge this is the first report of differences in autoreceptor sensitivity between accumbens sub-regions. Together, the increased tonic release, increased autoreceptor function and increased uptake rates suggest that DA signaling is more tightly regulated in the core. The shell has lower initial release probability but greater phasic-to-tonic release ratios, suggesting that DA release amplitude in this region tracks changes in DA neuron activity with more fidelity than the core.

We examined whether DA terminal characteristics were altered following chronic ethanol exposure. We have previously reported decreased electrically stimulated DA release in NAc core slices from mice following chronic intermittent ethanol exposure (Rose *et al.* 2016; Karkhanis *et al.* 2015); however, this finding did not extend to rats (Budygin *et al.* 2003) under a similar ethanol exposure paradigm, and there were regionally divergent effects in primates with a history of chronic daily ethanol self-administration (Siciliano *et al.* 2015a). These inconsistencies suggest that species, region and pattern of ethanol administration may be important in determining ethanol-induced changes in release parameters. Further, terminal neuroadaptations may develop from ethanol effects within divergent cellular microenvironments across regions (Robinson *et al.* 2009) in addition to direct ethanol actions on terminals, which would presumably apply uniformly across regions. Here we targeted only DA terminal fields with light stimulation and found no differences in stimulated release in either the NAc core or shell region following chronic ethanol treatment. An important caveat in the current study is that basal DA release magnitudes resulting from light stimulations are dependent on the amount of expression of ChR2 in the fields being stimulated. However, the variability of release across slices was comparable to studies using electrical stimulation (Rose *et al.* 2016), suggesting that there was consistency in the viral injection and expression of ChR2 between animals. Another consideration is that super-physiological Ca^{2+} entry during light stimulation (Zhang and Oertner 2007) occludes potential ethanol-induced decreases in terminal Ca^{2+} signaling and transmitter release. We previously demonstrated that in the presence of Na^{2+} channel blockade, Ca^{2+} entry during ChR2 activation is insufficient to induce measurable dopamine release (Melchior *et al.* 2015). Alternatively, due to the relatively slower kinetics of ChR2, the depolarization width is increased, which may result in increased Ca^{2+} entry and enhanced transmitter release (Zhang and Oertner 2007). However, using light stimulated release, we were able to detect differences in release parameters between the accumbens core and shell, supporting that this model is sufficiently sensitive to detect differences in Ca^{2+}-mediated release events between populations. Therefore, we believe that potential ethanol-induced alterations in terminal Ca^{2+} signaling would be detected using light-stimulated release in ethanol-treated and control groups. Nonetheless, as the understanding of presynaptic ethanol targets advances, the caveats associated with light-stimulated transmitter release should be considered.

The lack of ethanol-related differences in stimulated DA release suggests no differences in basal terminal release capability or terminal field density. We hypothesize that the previously

reported differences in stimulated DA release following chronic ethanol involve adaptations in the microcircuitry within the terminals fields. Electrical stimulation recruits local microcircuit modulation of dopamine release whereas targeted optical stimulation does not (Melchior *et al.* 2015a); thus, electrical stimulation may detect ethanol-induced changes in striatal modulation of dopamine release. For example, chronic ethanol may affect the release probability of GABAergic or cholinergic interneurons in the NAc, the simultaneous excitation of which inhibits and augments stimulated DA release, respectively (Melchior *et al.* 2015a). Prior ethanol exposure has been suggested to augment GABA interneuron inhibition of DA neurons in the ventral tegmental area (Melis *et al.* 2002). A similar mechanism may be occurring in the NAc where populations of GABAergic interneurons (Tepper *et al.* 2010) may modulate DA release via terminal $GABA_B$ receptors when using electrical stimulation (Pitman *et al.* 2014). Similarly, concurrent stimulation of local acetylcholine interneurons significantly augments stimulated DA release (Threlfell *et al.* 2012; Cachope *et al.* 2012), providing a means through with ethanol-induced inhibition of cholinergic transmission would be detected in the DA signal (Dohrman and Reiter 2003; Boutros *et al.* 2016). An increased number of GABAergic interneurons and a decreased density of cholinergic interneuron varicosities has been reported in the NAc of rats after chronic ethanol administration (Pereira *et al.* 2014). In light of this, the variability in release differences reported across species using local electrical stimulation may reflect ethanol-induced changes in indirect modulation of DA release probability through a multi-synaptic mechanism. This hypothesis is supported by a lack of direct effect of acute ethanol on DA terminals when applied using physiologically relevant doses and stimulation parameters (Budygin *et al.* 2001). Instead, long multi-pulse electrical stimulation trains are needed to detect ethanol inhibition of DA release (Yorgason *et al.* 2014), reinforcing the idea that recruitment of local circuitry is required. Together, these possibilities support the use of targeted stimulation methods, as utilized in this study, to probe specific mechanisms of ethanol induced release modulation in the NAc.

An additional mechanism through which chronic ethanol exposure may regulate DA signaling is modulation of DA uptake rates, which alters the magnitude and duration of DA signaling. Increased uptake rates have been reported in the NAc of mice (Rose *et al.* 2016), rats (Budygin *et al.* 2007) and monkeys (Siciliano *et al.* 2016) following chronic ethanol exposure, and these findings are supported by increased DAT protein tissue expression (Healey *et al.* 2008). Here we add to the consensus by showing increased uptake rates in the NAc core following a CIE vapor exposure paradigm. However, this effect did not extend to the medial shell of the same animals. A possible technical consideration is that DA release elicited in the shell was below the saturation level needed to accurately define changes in 'maximal' uptake rate (Wu *et al.* 2001); indeed, there was a 3 fold difference in release between the core and shell in the current study. However, ethanol-induced changes in uptake were detected in the core *ex vivo* using single pulse stimulations (Karkhanis *et al.* 2015) where DA release was of similar magnitude to that reported here in the shell (using phasic stimulations). Therefore, it appears more likely that there is a regional difference in ethanol-induced increases in uptake rates. For example, D_2 autoreceptor activation can potentiate DAT trafficking to the membrane (Ford 2014; Wu *et al.* 2002), and may explain differential modulation of uptake rates across regions. The larger DA signals, and increased regulation

by D_2 autoreceptors in the core may translate into increased sensitivity of DAT trafficking in response to chronic ethanol insult.

Kappa opioid receptors are expressed in the NAc where they inhibit DA release from DA terminals (Spanagel *et al.* 1990), however, κ-opioid receptor modulation of afferent synaptic input can be cell-type specific. For example, in the bed nucleus of the stria terminalis, dynorphin expressing cells modulate afferent terminals arising from the basolateral amygdala but not those arising from the prefrontal cortex (Crowley *et al.* 2016). Conversely, in the ventral tegmental area, κ-opioid receptor agonists selectively decrease activity in DA neurons projecting to the prefrontal cortex but not those projecting to the NAc (but do regulate terminal release in NAc) (Margolis *et al.* 2006), further highlighting cell specific regulation by κ-opioid receptors and DA neuron heterogeneity based on projection region. Here we demonstrate that κ-opioid receptors have direct effects on dopamine terminals in the nucleus accumbens core and medial shell. Further, the κ-opioid receptor-mediated inhibition of dopamine release was sensitive to phasic stimulations, although showing reduced efficacy in attenuating 5 pulse-stimulated compared to single pulse-stimulated dopamine release. This is consistent with most heteroreceptor-mediated inhibitory regulation of dopamine terminals, which can be overcome with increases in pulse number or frequency (Zhang and Sulzer 2012; Melchior *et al.* 2015a). The inhibition of dopamine release produced by a κ-opioid receptor agonist on single pulse light-stimulated release was also less than what has been previously reported using electrical stimulation (Rose *et al.* 2016). Electrical stimulation likely recruits both direct and indirect effects of κ-opioid receptors on dopamine release. For example, it has been suggested that κ-opioid receptors are expressed on cholingergic interneurons (Schoffelmeer *et al.* 1997); therefore κ-opioid receptors may inhibit both dopamine terminal release and acetylcholine augmentation of dopamine release when both cell types are excited using electrical stimulation.

Within the NAc, κ-opioid receptor activation results in divergent reward-related behaviors dependent on the specific sub-region, i.e. hot-spot, being targeted (Al-Hasani *et al.* 2015; Castro and Berridge 2014) and suggests possible differential regulation of DA terminals. For example, κ-opioid receptor activation in the shell, but not the core, contributes to escalation of drug intake (Whitfield *et al.* 2015), a hallmark of dependence. Our results show that κ-opioid receptor-mediated inhibition of DA release is greater in the medial shell compared to the NAc core. This is in contrast to Ebner et al., who showed that salvinorin A caused greater decreases in DA release in the core versus the shell (Ebner *et al.* 2010); however, that study applied salvinorin A systemically in combination with electrical stimulation *in vivo*, thus incorporating multiple facets of potential κ-opioid receptor modulation of DA output in NAc. Here we provide a direct assessment of κ-opioid receptor inhibition of DA release in each region. Our detection of increased efficacy of κ-opioid receptor agonists in the shell is supported by κ-opioid receptor binding assays, which suggest a marginally higher density of κ-opioid receptor expression in the shell versus the core in rats (Mansour *et al.* 1995) and prairie voles (Resendez *et al.* 2016). However, κ-opioid receptors are distributed unevenly across cell types, and how that translates specifically to DA terminals across regions is unknown and may vary across species, sex and social housing arrangements. Indeed, our data suggest that κ-opioid receptor expression may be flexible and responsive to environmental influences.

Following chronic ethanol exposure, κ-opioid receptor sensitivity was increased in terminal fields of both the core and shell, and to a similar magnitude, suggesting that the mechanism regulating κ-opioid receptor functionality is local to the NAc and generalized across regions. Dynorphin is generated in the D_1 receptor-expressing medium spiny neurons (D_1-MSN) in response to D_1 receptor activation and is released in order to provide feedback inhibition of over-excitation of D_1-MSNs (Gerfen and Surmeier 2011; Steiner and Gerfen 1996). That is, dynorphin peptides are transported to recurrent collateral axons within the NAc in order to decrease DA or glutamate transmitter release via presynaptic κ-opioid receptors (Steiner and Gerfen 1993). κ-opioid receptors also desensitize in response to high levels of dynorphin, including those induced by a single injection of amphetamine, subsequently increasing DA signaling on MSNs *in vivo* (Xia *et al.* 2008). Chronically elevated DA levels across the daily 16 hour ethanol exposure that occurs with CIE (for 4 weeks) may induce repeated κ-opioid receptor desensitization, resulting in a subsequent upregulation of κ-opioid receptors in a manner similar to nicotine-induced nACh receptor upregulation (Fenster *et al.* 1999). An alternative compensatory mechanism to receptor upregulation may be changes in receptor coupling and efficacy, which were not tested here. Conversely, increased receptor function may also result from chronic antagonism of receptor activity (Jacobson *et al.* 1996; MacLennan *et al.* 1988). Acute ethanol actions on DA signaling in the NAc are biphasic, with increases in DA levels occurring with low-to-moderate doses of ethanol and decreases in DA levels occurring with high doses of ethanol that coincide with sedation (Imperato and Di Chiara 1986). Therefore, chronic exposure to high doses of ethanol may result in prolonged decreases in DA signaling and subsequent dynorphin signaling, which prime the system to be more sensitive once ethanol exposure has stopped. In order to fully understand the interplay between DA and dynorphin/κ-opioid systems in the NAc it will be important to have a clearer picture of temporal changes in DA activity across the duration of exposure. Other stress-inducing factors such as intracranial virus injection surgeries, subsequent social isolation housing and non-contingent ethanol exposure in the vapor chambers need also be considered with respect to alterations in dynorphin/κ-opioid systems.

Dopamine neurons encode learning and motivation via burst activity in response to unconditioned rewards and cues predicting conditioned rewards (Schultz 2002), and the amount of bursting scales with the magnitude of an expected reward (Morris *et al.* 2004; Eshel *et al.* 2016). Acutely, moderate doses of ethanol increase DA signaling in the NAc via increased activity in DA neurons in the ventral tegmental area. These acute increases in NAc DA gate plasticity at glutamatergic synapses encoding converging information about environmental contexts and cues paired with alcohol reward and strengthen context-associated alcohol use behaviors (Shen *et al.* 2008; Reynolds *et al.* 2001). Conversely, a general increase in κ-opioid inhibition of phasic dopamine responses in the NAc may result in reward-predicting stimuli being undervalued, negatively impacting behavioral flexibility. Thus, in the early period of abstinence following chronic ethanol exposure, a hypo-dopaminergic state occurs in the NAc (Diana *et al.* 1993; Volkow *et al.* 1996; Martinez *et al.* 2005; Volkow *et al.* 2007) and this is hypothesized to result in deficits in reward processing which may simultaneously be anhedonic (Schulteis *et al.* 1995; Danjo *et al.* 2014) and bias choice towards previously reinforced behaviors (Twining *et al.* 2015) over novel behaviors (Pierce *et al.* 1990; Rebec *et al.* 1997), resulting in continued and persistent alcohol use.

In summary, we have used an optogenetic model to target dopamine terminal stimulation in isolation within the heterogeneous environment in nucleus accumbens slices. We demonstrate that this model results in robust and reliable dopamine release that may be probed pharmacologically, providing a direct assessment of dopamine terminal function following chronic ethanol treatment. Using this model we show novel data that support intrinsic differences in release and regulation of dopamine terminals across accumbens sub-regions. Further we show that chronic ethanol exposure results in adaptations in distinct aspects of terminal regulation that may be uniform across the nucleus accumbens in some parameters and regionally selective in others. As the heterogeneity in dopamine neuron phenotype and accumbal field responses becomes more apparent (Juarez and Han 2016; Wightman *et al.* 2007), similar genetically targeted approaches to dissecting cell type-specific neuroadaptations will be useful to elucidate the molecular mechanisms that underlie neuronal plasticity and to develop accurately targeted pharmacotherapeutics.

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Abbreviations

aCSF	artificial cerebral spinal fluid
AUD	alcohol use disorder
BEC	blood ethanol concentration
ChR2	channelrhodopsin-2
CIE	chronic intermittant ethanol
DA	dopamine
FSCV	fast scan cyclic voltammetry
NAc	nucleus accumbens
nor-BNI	nor-binaltorphimine
PBS	phosphate buffered saline
TH	tyrosine hydroxylase
Vmax	maximal rate of uptake
VTA	ventral tegmental area
κ-opioid	kappa opioid receptor

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Highlights

- Optogenetic stimulation induces phasic dopamine release in NAc slices.
- Dopamine terminals in core and shell show intrinsic differences in release regulation.
- Chronic ethanol increases dopamine uptake rates in the NAc core.
- Chronic ethanol increases kappa opioid receptor inhibition of dopamine release.

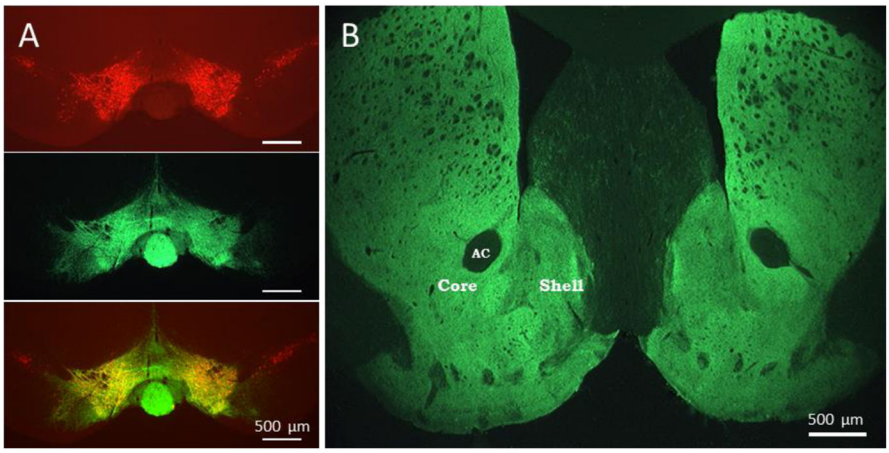

Figure 1. Expression of ChR2 in ventral tegmental area and striatum

A. Coronal midbrain section immunolabeled to show location of tyrosine hydroxylase expressing neurons in the VTA (red, top), ChR2-eYFP expression (green, middle) and a merged overlay showing the ChR2 expression throughout the site of injection in the VTA (bottom). **B.** Coronal section of striatum showing expression of ChR2 in the terminal fields of midbrain dopamine neurons (A). The ventral striatum shows robust ChR2 expression in both the nucleus accumbens core and shell regions. AC; anterior commissure. Scale bar = 500 μm.

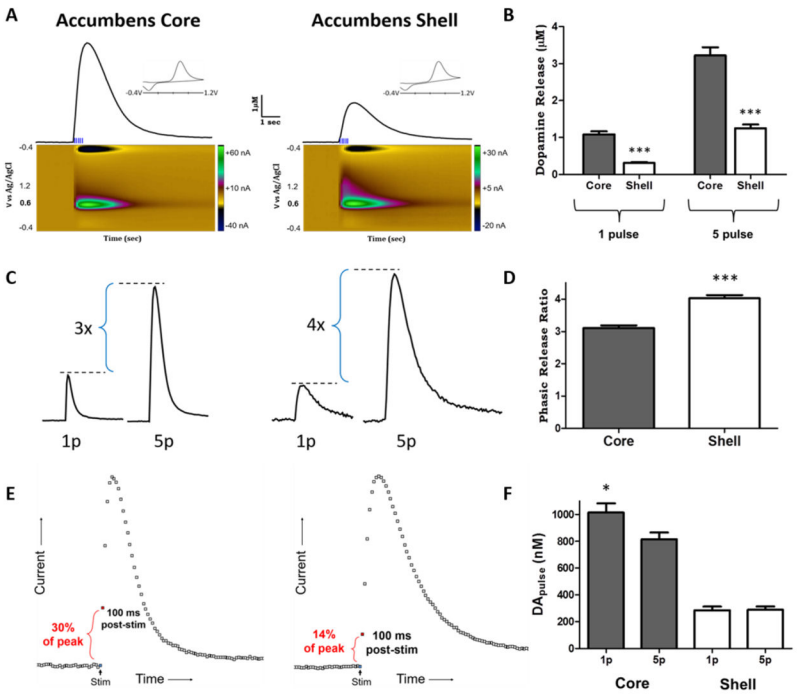

Figure 2. Light stimulated dopamine release parameters in the nucleus accumbens core and shell

A. Representative traces of light-stimulated dopamine release in the ventral striatum. Five pulse, 20 Hz (phasic) light stimulations resulted in robust dopamine release in the nucleus accumbens core and shell. Color plots (below) display signals as current vs. voltage vs. time. Dopamine was identified by characteristic oxidation peaks at 0.6 V and reduction peaks at −0.2 V represented for both regions in the current vs. voltage plot (inset). Nucleus accumbens shell terminal fields show reduced stimulated dopamine release (peak height) and reduced dopamine uptake rates (descending portion of curve) compared to terminal fields in the nucleus accumbens core. Phasic light stimulations resulted in > 1 μM dopamine release magnitude in the accumbens shell. **B.** Grouped data showing dopamine release magnitude following a tonic (1 pulse) or phasic (5 pulse) light stimulation in the nucleus accumbens core and shell. Dopamine release was significantly less in the shell compared to the core for tonic and phasic stimulations. **C.** Representative traces show the relationship between 1p and 5p light stimulated dopamine release within nucleus accumbens core (left) and shell (right) terminal fields. **D.** Grouped data show phasic release as a factor of tonic release in the same field (5 pulse release / 1 pulse release). Phasic release ratios are significantly greater in the shell compared to the core. **E.** Representative traces show the difference in initial release velocity between the accumbens core (left) and shell (right). Dopamine measured 100 ms following the shell (14% of peak release). **F.** Grouped data showing that dopamine release per pulse was greater with a single pulse stimulation compared to 5 pulse stimulation in the core. Dopamine release per pulse was more uniform across stimulations in the shell. ***p < 0.05, ****p < 0.001

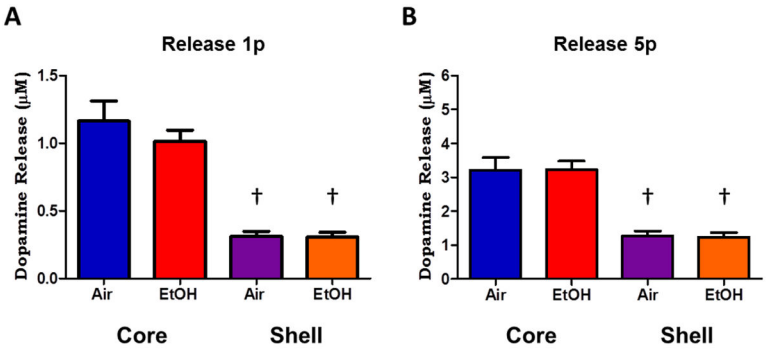

Figure 3. Light stimulated dopamine release following chronic ethanol exposure

A. Grouped data showing dopamine release magnitude following a single pulse of light stimulation. Dopamine release was significantly greater in the core (red, blue) compared to the shell (purple, orange) regions; however there was no significant difference in stimulated release between ethanol-treated (red, orange) and air-treated controls (blue, purple) in either region. **B.** Stimulated dopamine release following five pulses of light stimulation show greater magnitude of dopamine release compared to single pulse stimulations (A). Dopamine release in the core is significantly greater than release in the shell, however, there were no differences in stimulated release between ethanol-treated and air-treated controls in either region. $^{\dagger}p < 0.05$ compared to core release.

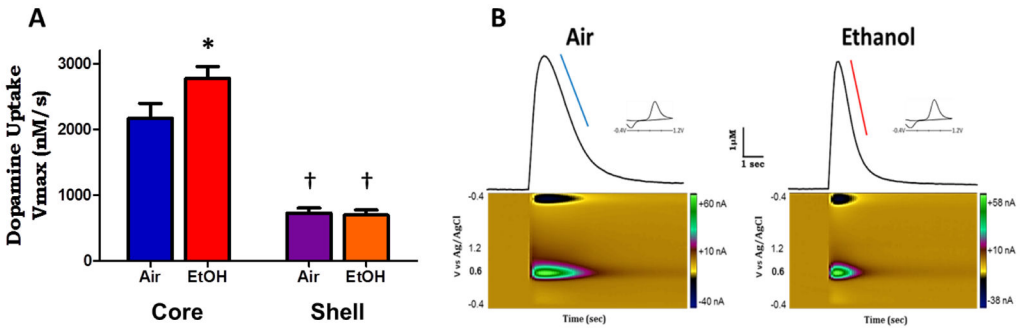

Figure 4. Chronic ethanol results in increased uptake rates in accumbens core

A. Grouped data showing maximal rate of uptake (Vmax) of dopamine (nM/s). Vmax is significantly faster in the core (blue, red) compared to the shell (purple, orange). Following ethanol-treatment the Vmax is significantly enhanced in the core compared to air-treated controls. However, ethanol treatment had no effect on Vmax in the shell compared to air-treated controls. **B.** Representative traces of five-pulse stimulated release in the nucleus accumbens core region of air-treated and ethanol-treated mice. Ethanol treatment had no effect on stimulated release magnitude, however, there was a significant increase in the uptake rates in the core of ethanol treated animals (right, red line) compared to air-treated control (left, blue line), evidenced by the increased steepness of slope in the descending curve of the measured dopamine signal. $^{\dagger}p < 0.05$ compared to core; $^{*}p < 0.05$ compared to air.

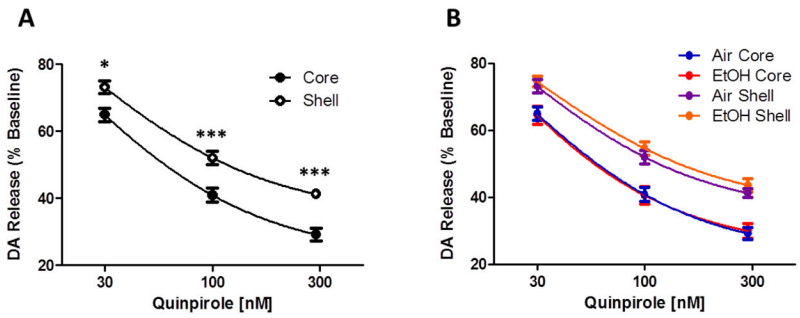

Figure 5. Chronic ethanol effects on dopamine autoreceptor function

Dopamine terminal autoreceptor sensitivity is different between nucleus accumbens core and shell but not altered by chronic intermittent ethanol exposure. **A.** The D_2/D_3 agonist quinpirole dose dependently decreases 5 pulse light stimulated dopamine release to a greater extent in the nucleus accumbens core (open circle) compared to the shell (closed circle). **B.** Chronic intermittent ethanol exposure did not alter sensitivity to quinpirole in either the accumbens core or shell region. *$p < 0.05$, ***$p < 0.001$ compared to core. Air = air treated group, EtOH = CIE treated group.

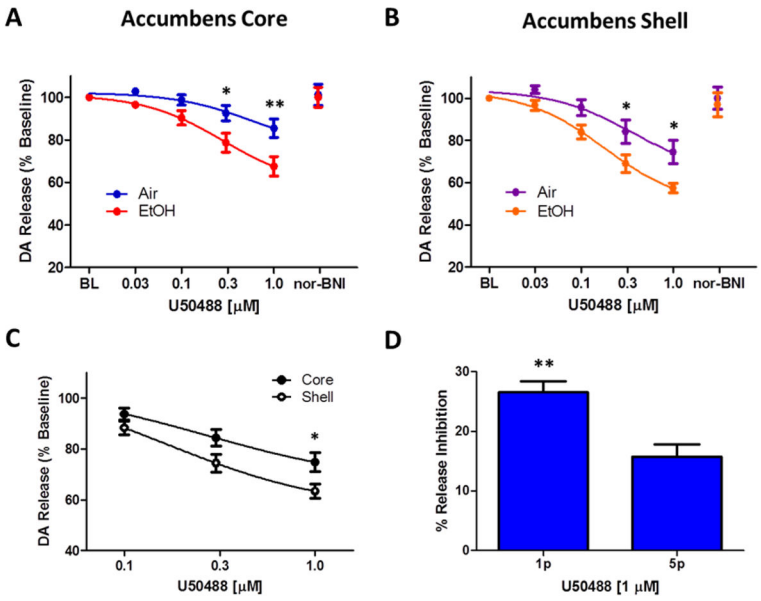

Figure 6. Chronic ethanol exposure increases kappa opioid receptor function in the nucleus accumbens core and shell

The kappa opioid receptor agonist U50,488 decreases stimulated dopamine release in a dose dependent manner (0.03–1.0 μM), presented as the percent inhibition from baseline (BL) stimulated release; and this effect is reversed upon wash with the kappa opioid receptor antagonist nor-BNI (30 μM). **A.** In the nucleus accumbens core, chronic ethanol exposure (red) resulted in increased sensitivity to U50,488 compared to air-treated controls (blue). **B.** In the nucleus accumbens shell, chronic ethanol exposure (orange) resulted in increased sensitivity to U50,488 compared to air-treated controls (purple). **C.** The nucleus accumbens shell (open circle) is more sensitive to U50,488 than the accumbens core (closed circle). **D.** In the nucleus accumbens core of naïve animals, U50,488 (1 μM) inhibits single pulse light stimulated dopamine release significantly more than 5 pulse phasic stimulated release. *$p < 0.05$, **$p<0.01$, Air = air treated group, EtOH = CIE treated group.