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Title: Regional and Sex Differences in Spontaneous Striatal Dopamine Transmission

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Abbreviations: DS: dorsal striatum; NAcc: nucleus accumbens core; NAcs: nucleus accumbens shell; DA: dopamine; VTA: ventral tegmental area; SNc: substantia nigra pars compacta; CIN: cholinergic interneuron; nAChR: nicotinic acetylcholine receptor; RRID: Research resource identifier; acsf: artificial cerebral spinal fluid; Vmax: maximal rate of dopamine uptake; ANOVA: analysis of variance; RM-ANOVA: repeated measures ANOVA; Kv channel: voltage-gated K+ channel; 4AP: 4-aminopyridine; DAT: dopamine transporter; RMP: resting membrane potential; AHP: after-hyperpolarization

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Abstract

Striatal dopamine release is key for learning and motivation and is composed of subregions including the dorsal striatum (DS), nucleus accumbens core (NAcc) and the nucleus accumbens shell (NAcs). Spontaneously occurring dopamine release was compared across these subregions. Dopamine release/uptake dynamics differ across striatal subregions, with dopamine transient release amplitude and release frequency greatest in male mice, and largest signals observed in the DS. Surprisingly, female mice exhibited little regional differences in dopamine release for DS and NAcc regions, but lower release in the NAcs. Blocking Kv channels with 4-aminopyridine enhanced dopamine detection without affecting reuptake. The 4-aminopyridine effects were greatest in ventral regions of female mice, suggesting regional differences in Kv channel expression. The dopamine transporter blocker cocaine also enhanced detection across subregions in both sexes, with greater overall increased release in females than males. Thus, sex differences in dopamine transmission are apparent and likely include differences in Kv channel and dopamine transporter function. The lack of regional differences in dopamine release observed in females indicates differential regulation of spontaneous and evoked dopamine release.

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Introduction

Dopamine (DA) release in the striatum is important for learning cue-associations, habit formation, reinforcement learning and underlies some of the neurosequela of attention deficit disorders, depression, Parkinson's disease and addiction. Striatal DA release is highly dependent upon firing of DA neurons in the ventral tegmental area (VTA) or substantia nigra pars compacta (SNc). However, DA release can also occur in the absence of any DA firing. For instance, in brain slices where DA terminals are no longer intact with their cell bodies, striatal cholinergic interneurons (CINs) can drive DA release by directly depolarizing DA terminals through activation of nicotinic acetylcholine receptors (nAChRs) (Yorgason *et al.* 2017; Zhou *et al.* 2001; Regan *et al.* 2020). Dopamine transients in brain slices appear to be highly dependent upon coordinated CIN firing (Cachope *et al.* 2012; Yorgason *et al.* 2017; Cover *et al.* 2019; Threlfell *et al.* 2012), are enhanced by acetylcholinesterase inhibition (Zhang *et al.* 2004), ethanol activation of nAChRs (Gao *et al.* 2019), dopamine transporter blockade and dopamine D2 receptor inactivation (Yorgason *et al.* 2017). Striatal regional comparisons of spontaneously occurring DA transients have not been made.

Although the present study focuses on DA transients in brain slices, transients have also been observed *in vivo* throughout the striatum with release amplitude for transients *in vivo* ranging from ~30-400 nM (Brodnik *et al.* 2020; Aragona *et al.* 2009; Owesson-White *et al.* 2009; Howard *et al.* 2013) which is similar to that observed *ex vivo* (~5-500 nM)(Yorgason *et al.* 2017; Gao *et al.* 2019; Zhou *et al.* 2001; Regan *et al.* 2020). The kinetics for transients are quite variable, covering a larger temporal range (0.5 to >10 sec) *in vivo* (Owesson-White *et al.* 2009; Brodnik *et al.* 2020; Howard *et al.* 2013), likely due in part to ongoing somatic DA activity during behavior and subsequent irregular patterns of release. Spontaneous DA release signals observed in slices are also variable, covering a range similar to in vivo studies, with s rapid 0.5-2 sec signals (Yorgason *et al.* 2017; Gao *et al.* 2019; Zhou *et al.* 2001), but also signals lasting ~10 seconds and longer (Regan *et al.* 2020), highlighting the complexity of striatal DA terminal release in slices. While DA neuron firing contributes to DA release *in vivo* (Suaud-Chagny *et al.* 1992; Panin *et al.* 2012), spontaneous release in striatal slices is thought to be mainly driven by local cholinergic interneurons (Zhou *et al.* 2001; Yorgason *et al.* 2017). Based on comparisons of release of DA transients *in vivo* and *ex vivo* (for additional discussion see Regan *et al.* 2020), there appears to be some, but not complete overlap in mechanisms underlying these different measures of release.

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The dorsal striatum (DS), nucleus accumbens core (NAcc) and nucleus accumbens shell (NAcs) are functionally distinct striatal subregions with very different regulation of DA release and uptake. The conventional understanding of evoked DA release regional differences has come almost completely from data obtained in brain slices from males. Electrically evoked release in males follow a dorsal (high release/uptake rates) to ventral (low release/uptake) gradient (Siciliano *et al.* 2014; Calipari *et al.* 2012; Cragg *et al.* 2000; Cragg *et al.* 2002; Yorgason *et al.* 2016). Psychostimulant effects follow this same gradient (Siciliano *et al.* 2014; Yorgason *et al.* 2016). In ventral striatal regions, DA release from multiple pulse stimulations (often called "phasic") is greater that observed in the DS (Zhang *et al.* 2009), suggesting that the intensity of release is heavily regulated by local factors, including intrinsic voltage gated Ca^{2+} channels (Brimblecombe *et al.* 2015), K^+ channels (Yorgason *et al.* 2017; Martel *et al.* 2011; Fulton *et al.* 2011), dopamine transporter (DAT) levels (Yorgason *et al.* 2016), heteroreceptor activity at nAChRs (Zhou *et al.* 2001), muscarinic acetylcholine receptors (Yorgason *et al.* 2017; Threlfell *et al.* 2010; Shin *et al.* 2015) and opioid receptors (Britt & McGehee 2008) to name a few (for review see Nolan *et al.* 2020). The inherent differences in DA release across striatal subregions may also confer differential sensitivity to drugs of abuse. Thus, the present work is a characterization of sex and regional differences in spontaneously occurring DA transients.

Materials and Methods

Animals

Male and Female C57Bl/6J (RRID:IMSR_JAX:000664; Jackson Laboratory, Sacramento, CA) mice (>30 days old; 33 mice; 2-6 per cage; sealed polyphenylsulfone cage) and adult female Sprague-Dawley rats (200-250g; RRID:MGI:5651135; Harlan Laboratories, Frederick, Maryland; 10 rats; 2 per cage; static microisolation cage) were given *ad libitum* access to food and water, and maintained on a 12:12-h light/dark cycle. All protocols and animal care procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Brigham Young University (IACUC#18-1202), Wake Forest School of Medicine, and Vollum Institute of Advanced Biomedical Research Institutional Animal Care and Use Committees.

Brain Slice Preparation and Drug Application

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Isoflurane (CHEBI:6015; Patterson Veterinary, Devens, MA) anesthetized rodents were sacrificed by decapitation and brains were rapidly removed, sectioned into coronal or sagittal striatal slices (220-400 µm; Leica VT1000S, Vashaw Scientific, Norcross, GA). Sagittal sections were obtained by blocking off brains at the optic chiasm before slicing the brain along the longitudinal fissure and gluing the medial aspects to a thin section of agar on the cutting stage. This cutting method ensures that there are no dopamine cell bodies are present in the slices. Slices were incubated for 60 minutes at 34 °C in pre-oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid (aCSF) consisting of (in mM): NaCl (126), KCl (2.5), NaH_2PO_4 (1.2), $CaCl_2$ (2.4), $MgCl_2$ (1.2), $NaHCO_3$ (21.4), D-glucose (11). Isoflurane was selected for its rapid and short-lived actions as an anesthetic and for its efficacy as an anesthetic in order to reduce any animal suffering during the cutting procedures. Animals were anesthetized in an isolated chemical vent hood away from other animals, and anesthesia efficacy was confirmed in rodents by lack of responsivity to a toe pinch. Breathing was monitored throughout isoflurane anesthesia and decapitations were only performed in rodents where anesthesia was apparent. Cutting solution also contained either MK801 (0.01 mM; (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; CHEBI:107963; Abcam, Cambridge, UK) or kynurenic acid (2 mM; CHEBI:18344) for blockade of ionotropic glutamate receptors. At the end of the incubation period, the tissue was transferred to aCSF (34 °C) without glutamate receptor blockers. NMDA receptor blockers were included during slice preparation to prevent glutamate induced neurotoxicity and promote slice health (Nakanishi *et al.* 1996; Arias *et al.* 1999; Lipton *et al.* 1995). 4-aminopyradine (4AP; 30 µM; CHEBI:34385) and cocaine (3 µM; CHEBI:613010; acquired from Sigma-Aldrich and Tocris Bioscience) were bath applied for slice voltammetry experiments where specified.

Study Design

The study was not preregistered. No randomization was performed to allocate subjects in the study. Animal subjects (males and females) and brain regions were assessed at random. Experimenter was aware of rodent sex, brain region, and drug concentration applied. Analysis of spontaneous dopamine signals was performed and validated by 3 researchers (different from the experimenter) blinded to all experimental conditions. The study was exploratory. Spontaneous dopamine release was observed in all brain slices and no subjects were excluded from the study. Experiments were performed from 9 am-6 pm, with brain slices obtained at 8-10 am each day. Male and female mice were randomly interspersed throughout the study. Electrodes were placed in random locations throughout the striatum by a student researcher blinded to experimental conditions. A

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photograph of the electrode position was acquired at the end of each experiment. A different researcher blinded to results of voltammetry experiments used photographs to determine electrode positioning using *The Mouse Brain In Stereotaxic Coordinates* brain atlas (Paxinos & Franklin 2004). This atlas delineates the NAcc from the NAcs based on immunoreactivity for acetylcholinesterase (higher in shell) and Timm's sulphide silver technique (higher staining in core). Sagittal locations from the atlas were readily identifiable and locations recorded accordingly. Coronal images used in Figure 1B were adapted from the digital version of the atlas and were selected for unique ability to depict multiple regions in the same section. Similar amounts of spontaneous dopamine transients have been observed in both coronal and sagittal slices (Yorgason *et al.* 2017). In contrast, there are reports of increased transients in horizontal slices (Zhou *et al.* 2001) possibly due to preservation of longer DA axons and/or activity from connections to DAergic soma present in horizontal slices (Patel & Rice 2013). However, transients in horizontal slices are also dependent on nAChR activity (Zhou *et al.* 2001). Regardless, horizontal slices were avoided in order to prevent possible effects from preserving somatic connections. Dopamine signals are influenced by circadian factors (Ferris *et al.* 2014). Thus, in order to control for possible effects from recording from DA signals from different time points throughout the day, brain slices from subjects were obtained at the same time of day, irrespective of sex. Furthermore, there was no experimental preference for obtaining recordings from specific brain regions early vs later in the day and no observed differences between release amplitude and frequency of transients from recordings performed earlier vs later in the day (Pearson r^2=0.0002, p=0.512). Spontaneous DA release was not observed under baseline conditions in all locations, but was observed after 4-AP in each recording. Therefore, Figure 4C and 4D are a visual representation of all locations recorded from in mice, which were partitioned by region by locations depicted in Figure 1B. All four dimensional plots depict locations for the experiments shown on respective figures and highlight the variability in responses by region.

Voltammetry Recordings

Rodent brain slices were transferred to the recording chamber, and perfused with aCSF (34 °C) at a rate of ~1.8 ml/min. Fast scan cyclic voltammetry recordings (Ferris *et al.* 2013) were performed and analyzed using Demon Voltammetry and Analysis software (RRID:SCR_014468). Carbon fiber electrodes used in voltammetry experiments were made in-house. The carbon fiber (7 µm diameter, Thornel T-650, Cytec, Woodland Park, NJ) was aspirated into a borosilicate glass capillary tube (TW150, World Precision Instruments, Sarasota, FL).

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Electrodes were then pulled on a P-87 Horizontal pipette puller (Sutter Instrument Company, Novato, CA) and cut so that ~100 µm of carbon fiber protruded from the tip of the glass. The electrode potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V (Ag vs AgCl) with a scan rate of 400 V/sec, repeated every 100 ms (Yorgason *et al.* 2011). Carbon fibers were advanced completely into the tissue at a 20˚ angle with the entirety of the carbon tip positioned ~85 µm below the slice surface. For experiments measuring stimulated DA release, a glass (30-100 µA, 0.5 ms; mouse experiments) monopolar or tungsten (350 µA, 4 msec, monophasic; rat experiments) bipolar stimulating electrode was placed adjacent to the carbon fiber electrode and DA release was evoked every 1-3 minutes until the DA response was stable before drug application.

Data Analysis

Spontaneous dopamine release was analyzed using Demon Voltammetry software as described previously (Yorgason *et al.* 2017; Yorgason *et al.* 2011). Briefly, dopamine release was measured at peak oxidation currents. Dopamine uptake was measured as the time constant (τ) from a single exponential fit between the peak dopamine current and its return to baseline. If signals did not return to within 5% of pre-release baseline values, those signals were not included in uptake comparisons. This resulted in ~18% inclusion of signals for tau analysis. Factors that contribute to exclusion include ongoing release that occurs during the descending limb of a signal, as well as background noise and electrode drift. Thus, this inclusion criteria represents a conservative bias for uptake estimates. Custom software was written for detecting and analyzing spontaneous dopamine release events. The software performs a running subtraction on recordings to reduce drift and aliasing noise. Post-subtracted data is then compared across time against known cyclic voltammograms for dopamine, with a low threshold r^2 value for initial detection ($r^2>0.3$). The time domain of each putative event (legitimate and spurious) is captured in the software for subsequent manual verification. The program reverts the data back to its non-subtracted form, and performs a new background subtraction (non-running) at the time point preceding the putative event. The resultant color plot around that time point, and cyclic voltammogram at the peak current are then examined, and compared against a known dopamine voltammogram to verify similar oxidation potentials. If signals are smaller than the limit of detection (calculated by multiplying the median standard deviation for each file by 3) they are automatically rejected. The events are simultaneously examined for evidence of a false positive caused by drift and aliasing noise

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coinciding with the oxidation potential. Dopamine concentrations were calculated from calibration values (at 1 μM dopamine). Baseline frequency of dopamine transients was measured during a 10 minute period prior to drug application. Group data from experiments where a drug was applied was measured across a multiple collections. All transient analysis was performed and verified by individuals blinded to the experimental conditions. For measuring the maximal rate of dopamine uptake (Vmax), stabilized DA signals were analyzed using a modified Michaelis-Menten model described elsewhere (Yorgason *et al.* 2011; Wightman & Zimmerman 1990).

Statistics

Statistics were performed using JMP 15 (RRID:SCR_014242; SAS Institute, Cary, NC) and Prism 5 (RRID:CR_002798; Graphpad, San Diego, CA). Analyses were performed using linear regression models. No sample calculation was performed. Animal numbers were selected based on similar experiments performed previously in the NAcc (Yorgason *et al.* 2017; Gao *et al.* 2019). No tests for outliers was used, and thus outliers were not excluded. For baseline comparisons, data from other drug conditions were excluded. Data were assessed for normality by visual inspection of the distribution of the groups. Non-normally distributed groups were log transformed for the statistical analysis. After that, it was decided to perform comparisons of signal amplitude and transient frequency between regions and sex. Thus, the initial model for both response variables contain terms for region and sex. However, it was undetermined whether there was an interaction between sex and region. In order to determine this, an extra sum of squares F-test for the addition of an interaction term between region and sex was performed for each response variable. The interaction term contributed significantly to the model for amplitude, but not for frequency. This means that for frequency, the simplest and clearest model to estimate sex and regional effects does not include a response term. This speaks to a more fundamental sex effect rather than a region-specific sex effect. Therefore, at baseline, the linear regression model for amplitude included terms for region, sex and an interaction term between region and sex, while the model for frequency includes only terms for region and sex but no interaction term. For drug conditions, it was decided to compare changes from baseline condition, region and sex for the response variables signal amplitude and frequency. Interaction terms were included in all drug models to assess region and sex specific changes from baseline.

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Statistical significance was determined for groups of 2 variables using a two-tailed student t-test when no hypothesis was assumed or a one-tailed student for experiments where an effect was already hypothesized. Experiments with more than 2 groups, but only one factor were tested for significance using a one-way analysis of variance (ANOVA) or when data was from multiple time points from the same experiment a repeated measures ANOVA (RM-ANOVA) was performed. For experiments that examined multiple factors, and possible interactions, two-way ANOVA or RM-ANOVAs were used. Tukey's HSD and Bonferroni correction methods were used for post-ANOVA analysis and are indicated in the text. In comparisons where sample sizes were vastly different, Welch's correction was used to avoid heteroskedasticity-dependent confounds.

Results

Regional comparison of DA release under baseline conditions

Voltammetry was used to measure DA transients throughout the striatum of mouse brain slices, with regional locations determined by atlas references. A timeline of the study is shown in **Figure 1A.** Striatal regional distinctions are depicted in **Figure 1B**. Example DA transients under non-drug treated conditions from striatal subregions are shown (**Figure 1C**). Dopamine transients were characterized (**Figure 2**) and varied widely in amplitude across the striatum, with most transients occurring at lower amplitudes (**Figure 2A**; typically 5-10 nM, but with some > 100 nM). Cumulative probability distributions followed clear regional differences, with greatest release in dorsal regions (**Figure 2B**). Transient release amplitude varied across regions (F-Test; $F(2,258)=4.86$; $p=0.0085$) and by sex (F-Test; $F(1,258)=9.21$; $p=0.0026$), and with an interaction between these variables (F-Test; $F(2,258)=13.49$; $p<0.0001$) (**Figure 2C**). Therefore, transient amplitude was compared separately across regions within sex. In females there was no significant difference in transient amplitude between the DS and NAcc (Tukey's HSD; $q(258)=-2.53$; $p=0.1206$), the DS and NAcs (Tukey's HSD; $q(258)=-0.09$; $p=1.0$) or the NAcc and the NAcs (Tukey's HSD; $q(258)=0.85$; $p=0.9579$), with average transient amplitude of 16.3±1.75 nM and range of 6.7-56 nM. In contrast, for males, amplitude varied greatly across regions and was 133.42±13.96% larger in the DS than the NAcc (Tukey's HSD; $q(258)=8.03$; $p<0.0001$) and 78.99±14.36% larger in the NAcs than the NAcc (Tukey's HSD; $q(258)=4.84$; $p<0.0001$). There is no significant difference in the amplitude of DA transients in males between the DS and the NAcs (Tukey's HSD; $q(258)=1.60$; $p=0.5966$), with an average amplitude of 27.45±1.2 nM and range of 6.85-102.5 nM across regions. Transient amplitude was further visualized in relation to each recording's location in 3-dimensional

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space (**Figure 2D**), though no clear pattern of average release amplitude was observed, likely due to the large number of small release events. In order to determine if the lack of regional differences observed in females is specific to spontaneous DA release, or if electrically evoked DA release follows a similar trend, electrically evoked DA release and reuptake were examined in female rats in the DS and NAcc (**Supplementary Figure 1**). Evoked DA signals in females exhibited a similar lack of regional differences in evoked DA release (NAcc 597±10nM, DS 651±12nM; $t(18)=0.3369$, $p=0.7401$) and uptake (NAcc 2.06±0.297 μM/s, DS 2.3±0.39 μM/s; $t(18)=0.5915$, $p=0.5615$) across NAc and DS subdomains. Thus, regional DA release is sex dependent, and the dorsal to ventral release gradient reported previously (Yorgason *et al.* 2016; Calipari *et al.* 2012) and observed herein is specific to males and not observed in females.

Regional and Sex Differences in DA Transient Frequency

Dopamine transient frequency followed a regional distribution with highest frequency observed in the DS and lowest frequency in the NAcs (**Figure 3A**; F-Test; $F(2,694)=9.72$; $p<0.0001$). Interestingly, females exhibited a ~46% lower release frequency than males (**Figure 3B**; Tukey's HSD; $q(694)=-3.44$; $p=0.0006$). Frequency collapsed across sex in the DS is greater than in the NAcs by 185±4% (Tukey's HSD; $q(694)=4.28$; $p<0.0001$) greater in the NAcc than in the NAcs 126±4% (Tukey's HSD; $q(694)=3.21$; $p=0.0039$). Although the difference between DS and NAcc is not significant (Tukey's HSD; $q(694)=0.77$; $p=0.7188$), there is a clear trend in both sexes for greater transient frequency in dorsal to ventral gradient. However, it is important to note that since spontaneous and evoked DA release do not follow a similar gradient in females, this frequency dependent gradient is likely dependent on other mechanisms that differ by region. Variability for transient frequency appeared lower in females (**Figure 3C**), and higher in the NAcs, though these comparisons were also highly variable experiments across regions (**Figure 3D**) and not significant for either comparison. Frequency of release events across striatal regions is plotted (**Figure 3E**).

Blocking Kv channels increases dopamine transients

Even though DA transients can be observed under baseline conditions, release amplitude and frequency are low, making it difficult to study underlying mechanisms regulating spontaneous DA transient release. We have previously demonstrated that inactivation of voltage-gated K+ (Kv) channels greatly enhances DA terminal release probability, and DA transient release amplitude and frequency in the NAc (Yorgason *et al.* 2017). Thus, the effects of Kv channel blockade were examined across regions and by sex using the non-selective Kv channel blocker 4-aminopyridine (4AP; 30 μM; **Figure 4**).

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Application of 4AP increased average DA transient release amplitude across all regions (**Figure 4A-C**; BL vs 4AP: Welch's correction: t(754)=5.175, p<0.0001, F2406,261=6.908, p<0.0001), with similar release amplitude observed across regions after 4AP (**Figure 4E**; F(2,2401)=1.889, p=0.1514) and sex (F(1,2401)=1.252, p=0.2633), but an interaction between sex and region (F(2,2401)=8.558, p=0.0002) and a ~51% greater mean DA release in the NAcs in males over females (Tukey's HSD; q(4627)=6.84; p<0.0001). Analysis of the upper quartile of DA release (**Figure 4F**) revealed no regional effect (Two Way ANOVA: F(2,489)=1.413, p=0.2443), but revealed a sex effect (F(1,489)=4.566, p=0.0331) and an interaction between sex and region (F(2,489)=4.143, p=0.0164), with greater DS release in females over males.

Application of 4AP resulted in increased average instantaneous frequency of DA transients (F(2,5473)=51.61; p < 0.0001; **Figure 4D,G**) with greater increases generally observed in females than males. In females alone, 4AP increased transient frequency across subregions to ~225% in DS, ~268% in NAcc, and ~2.4k% in the NAcs (**Figure 4G**). Since frequency was disproportionately increased in females over males, the averaged frequency across regions appeared somewhat normalized after 4AP, though sex differences (F(2,5473)=4.42 p=0.036) across regions (F(2,5473)=3.9, p=0.0204) were still observed (**Figure 4H**; Tukey's HSD; F vs M DS q=3.64, p=0.03; F vs M NAcc q=5.04, p<0.0001; F vs M NAcs q=3.87, p=0.01). Thus, 4AP effects vary both by striatal subregion and by sex, possibly due to inherent differences in Kv channel subtypes and DA readily releasable pools.

If blocking Kv channels results in changes in DAT function, this could potentially explain the observed increases in DA transients across the striatum. In order to get a good estimation of uptake rates, and effects of Kv channel blockers on DA uptake, DA release was electrically evoked (Yorgason *et al.* 2011), and the effects of Kv channel blockade were examined using two different Kv channel blockers (4AP and Barium). Dopamine clearance for transients as measured using the single exponential decay measure tau (Yorgason *et al.* 2017). The tau measure was used in this instance instead of Vmax in order to avoid violating Michaelis-Menten assumptions of DAT saturation by small DA signals (Yorgason *et al.* 2011). These blockers were examined in the NAcc (4AP, 30 µM; **Figure 5A-D**) and DS (Barium 100 µM; **Figure 5E-F**) of female and male mice (collapsed across sex). Evoked NAcc DA release was significantly increased with 4AP from 0.75±0.1 to 1.08±0.13 µM (two tailed t-test t(4)=6.669, p=0.0026), but tau clearance rates were unchanged (two tailed t-test t(4)=0.424, p=0.6934). Barium also increased evoked DA release in the DS from 1.45±0.3 to 1.89±0.39 µM (two tailed t-test t(4)=7.145, p=0.002), but did not affect DA tau clearance rates (two tailed t-test t(4)=0.1919,

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p=0.8571). Thus, blocking Kv channels increases DA release probability without any apparent effect on DAT function.

Dopamine clearance for transients was next measured and compared separately across regions and sex (**Figure 5G**). Sex differences were apparent (Two way ANOVA; Sex: $F(1,485)=208.2$, $p<0.0001$) and different across regions by sex (Sex vs Region: $F(2,485)=10.78$, $p<0.0001$), though a main effect of region was not detected by the ANOVA (Region: $F(2,485)=1.613$, $p=0.2004$). Interestingly, the range for clearance values was much larger in females (tau: 0.2626-3.731 sec) than males (tau: 0.1214-1.441 sec), indicative of the wide variability in clearance measures in females for DA transients. This variability may be due to estrus effects on DA clearance described elsewhere (Calipari *et al.* 2017), though this will need to be tested explicitly and DA clearance from females may actually reflect differences in terminal populations that are not dependent upon estrus effects.

Dopamine Release Amplitude after Cocaine Differ by Region and Sex

Cocaine's effects on evoked DA transmission vary by region in males (Yorgason *et al.* 2016), which may be due to underlying differences in DAT levels across the striatum. Therefore, cocaine's effects on DA transients were examined throughout striatal subregions in 4AP treated slices. Cocaine-induced decreases in DA clearance were readily observable from raw data (**Figure 6**). Indeed, cocaine increased reuptake time as measured by tau in all brain regions by 35±11% (Welch's t-test; $t=-3.20$; $p=0.0015$). No regional differences in reuptake after cocaine were discovered (ANOVA; $F(7,17)=0.1949$; $p=0.8239$), suggesting that other measures may be more reliable at detecting cocaine-induced effects than just effects on clearance.

Cocaine (3 µM) increased transient amplitude (**Figure 7A-C**; 4AP vs Coc: Welch's correction: $t(3521)=5.769$, $p<0.0001$; $F(1973,2406)=1.878$, $p<0.0001$), with differences in release amplitude observed across regions after cocaine (**Figure 7E**; Two-way ANOVA, $F(2,1968)=10.9$, $p<0.0001$), no sex effect ($F(1,1968)=1.3$, $p=0.2543$), but a significant interaction between region and sex variables ($F(2,1968)=14.2$, $p<0.0001$). A comparison of the upper quartile of signals revealed a main effect of region (**Figure 7F**; Two-way ANOVA, $F(2,543)=3.163$, $p=0.0431$) with a trend for differences in sex ($F(1,543)=3.376$, $p=0.0667$) and a trend for an interaction ($F(2,543)=2.458$, $p=0.0865$), suggesting that the highly variable nature of larger amplitude DA transients may influence regional and sex comparisons.

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Cocaine increased the overall frequency across regions and sexes by ~53% (**Figure 7D,G,H**; $F(1,4644)=54.88$, $p<0.0001$), with no clear differences in sex or region variables (**Figure 7G**; $p>0.05$). The post cocaine DA transient frequencies were compared and exhibited a dorsal to ventral gradient with highest frequencies observed DS vs NAcs of females (**Figure 7H**; Region: $F(2,2137)=2.0581$, $p=0.1279$; Sex: $F(1,2137)=3.08$, $p=0.0021$; Region vs Sex: $F(2,2137)=30.16$, $p<0.0001$). Interestingly, in males, the opposite gradient was apparent, with highest frequencies observed in the NAcs. Thus, cocaine increases transient frequency differentially across regions and sexes. Differences in cocaine effects may be due to a number of factors including non-homogenous expression of D2 autoreceptors and effectors, and other non-specific effects of cocaine on local circuitry.

Discussion

Regional and sex differences in DA release/uptake may drive increased risk for illicit drug seeking behavior observed in males. The present studies highlight the complexity in a novel form of spontaneously occurring DA release across regions. In males, DA transients follow a dorsal-ventral gradient, but this gradient was absent in females. Blocking Kv channels and DATs resulted in increased DA transient detection, which was greater in females. Previous studies have focused on stimulated DA release to measure DA clearance. Stimulated signals are larger and more likely to satisfy Michaelis-Menten enzymatic assumptions, but also likely recruit entire populations of terminals. Since spontaneously occurring DA transients exhibit wide variability in amplitude and clearance, DA transients may reflect asynchronous activity from a diverse sample of terminals, which may differ in their function. Therefore, spontaneous DA transients may provide useful information regarding variability in terminals within a region, including release/uptake machinery, and auto- and heteroreceptor regulation. Differential effects of 4AP and cocaine on DA transients indicate two powerful dissociable regulators of DA release and clearance, Kv channels and DATs. Release/uptake regulators differ by sex and striatal subregion, which has implications for observed differences in drug reinforcement learning and craving.

The mechanisms underlying spontaneous DA release across the striatum are largely unknown. However, several studies have indicated that transients are dependent on local CIN activity onto nAChRs (Yorgason *et al.* 2017; Zhou *et al.* 2001; Zhang *et al.* 2004; Gao *et al.* 2019). Cholinergic interneurons typically fire at ~1-3Hz and increases in CIN firing are associated with increased DA transient detection (Yorgason *et al.* 2017).

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Dopamine neurons follow similar firing patterns, driven by intrinsic ion channel function, including the hyperpolarization activated (Ih) channel and potassium channels (Wolfart *et al.* 2001; Zhang *et al.* 2010). Recent patch-clamp experiments of DA terminals have determined that DA striatal axons have a resting membrane potential (RMP) of ~-71 mV (compared to DA soma ~-62 mV) and axonal action potentials have a shorter duration (half-width in axon: 0.89 ms, soma: 1.24 ms), both measures indicative of a greater K contribution under RMP and firing conditions (Kramer *et al.* 2020). Furthermore, Kv1.1, 1.2, 1.3 and 1.6 channels have all been implicated in DA terminal function in the DS (Fulton *et al.* 2011; Martel *et al.* 2011). However, the relative contribution of different Kv channels in DA terminal function across regions has not been studied. Furthermore, the present results indicate the necessity of considering sex as an essential variable when examining K channel contributions to DA terminal function. It was previously suggested that DA transients would not be observed in slices where DA somatic connections were not maintained (Patel & Rice 2013). This idea seems to have originated in part from observations where transients were observed by another research group in horizontal but not coronal slices (Zhou *et al.* 2001). We and others have recently reported DA transients in coronal and sagittal slices where somatic connections are not maintained (Yorgason *et al.* 2017; Gao *et al.* 2019; Regan *et al.* 2020). This demonstrates that somatic activity is not required for DA transients. Interestingly, many DA terminals still exhibit tonic firing despite being severed from the soma and sitting at a hyperpolarized potential (Kramer *et al.* 2020). This finding is really surprising since DA transients in the NAcc are completely abolished by nAChR blockers (Yorgason *et al.* 2017), and suggests that there may be ongoing release not being detected by voltammetry. Therefore, although DA terminals and CINs depolarize spontaneously at ~1-3 Hz, either DA release from this activity is not sufficient for driving DA release, or detection is too low to pick up ongoing release. If there is insufficient release, this could possibly be due to overactive K+ channels near release sites, including Kv and SK channels, resulting in a dampening effect on action potential mediated depolarization of voltage dependent calcium channels. Furthermore, DA transient frequency is correlated with evoked DA release variability (Yorgason *et al.* 2017), suggesting that undetected transient tone may shunt DA release during DA cell firing (Yorgason *et al.* 2017). However, this correlation is largely driven by cases of high frequency (5-30/min) transient release (Yorgason *et al.* 2017). It will thus be informative to know if firing observed in DA terminals of brain slices is dependent upon intrinsic channel activity (i.e. Ih activated by low RMPs) or if action potentials are driven solely by local CINs through nAChR dependent DA terminal depolarization. If Ih is present in DA terminals and not just cell bodies, this will likely have big implications for regional differences, especially since Ih is already a reliable regional marker in

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midbrain DA neurons (Zhang *et al.* 2010). Therefore, the location and composition of various ion channels is extremely important when considering the source of DA transients and effects of potassium channel blockers. Presently, blocking Kv channels did not affect DA uptake, but greatly enhanced detection of release events, likely due to a combined effect of increased DA terminal depolarization and increases in CIN firing and subsequent acetylcholine release on DA terminals (Yorgason *et al.* 2017). This makes 4AP an extremely useful tool for examining regulators of DA transients but also for studying DA clearance from diverse populations of terminals.

Since DA release appears to be dampened at terminals by K channels, an important K+ channel contributor to normal DA cell firing is the small conductance calcium activated SK channel. SK channels help establish tonic DA firing by providing a slow K+ conductance to drive after-hyperpolarizations (AHPs) in an action potential. Thus, DA terminals may not actively release DA at 1-3 Hz due to this dampening effect. While it is currently unknown if SK channels are expressed at DA terminals, metabotropic glutamate receptors activate SK in DA cell bodies (Kramer & Williams 2016) and inhibit striatal DA release through an apamin sensitive channel (Zhang & Sulzer 2003). Furthermore, blockade of SK channels via apamin alters CIN firing from tonic to phasic burst modes and appears to mildly increase DA transient frequency and variability in the NAcc (Yorgason *et al.* 2017). SK channels are activated by calcium released from internal stores (Kramer & Williams 2016). However, blocking calcium release from internal stores has no apparent effect on DA release under baseline conditions (Yorgason *et al.* 2020). Furthermore, methamphetamine application induces DA release that can be attenuated by blocking intracellular calcium release (Yorgason *et al.* 2020) and sigma receptor dependent mechanisms (Hedges *et al.* 2018). Therefore, intracellular calcium release effects are not constitutively active and require external stimulation. Activation of an SK conductance should result in DA inhibition. However, blocking intracellular calcium release attenuates DA increases. This suggests that there are other calcium dependent effects in DA terminals that can override potential SK effects. In relation to regional differences, in the midbrain both SK channels and AHP are larger in SNc than VTA DA neurons (Wolfart *et al.* 2001), suggesting that larger SK effects may be observed in DS terminals. Although this wasn't tested explicitly, this may explain the smaller 4AP induced increases observed in the DS.

Since regional and sex differences observed herein may be explained by a diversity of Kv and SK channels in DA and CIN terminals, these studies highlight the importance of K+ channels in regulating striatal DA release, an avenue which remains largely unexplored and has huge implications in the use of pharmacological agents

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that modulate Kv channel activity. Kv blockers are already clinically used for treating symptoms associated with multiple sclerosis, spinal cord injury (Hsu *et al.* 2020; Goodman *et al.* 2010; Judge & Bever 2006), and may be effective therapeutic treatment for other immune-related disorders (Wulff *et al.* 2009). Thus, knowing about regional and sex differences in Kv channel function may inform on use of Kv channel blockers in humans with these disorders and may also have implications for treating DA related disorders such as attention deficit disorder and Parkinson's disease in a sex dependent manner. Interestingly, the release gradient observed in males herein does not follow the simple dorsal-ventral gradient for electrically evoked release reported elsewhere (Yorgason *et al.* 2011; Calipari *et al.* 2012). This is highlighted by the fact that the NAcs is not always ventral to the NAcc, as shown in the diagram in Figure 1B. These previous studies do not show the exact location of each recording, making it difficult to compare locations of recordings and relative expression of circuit specific markers. The regional boundaries used herein are based on delineations described previously which were informed by immunoreactivity for acetylcholinesterase, which is higher in the NAcs and lower in the NAcc (Paxinos & Franklin 2004; Paxinos & Watson 2007). Acetylcholinesterase is known to affect DA transients in slices (Zhang *et al.* 2004; Zhou *et al.* 2001), with cholinesterase inhibitors enhancing transients at low concentrations but inhibiting at high concentrations (Zhang *et al.* 2004), a higher acetylcholinesterase expression in the NAcs could partly explain the lower DA transient frequency in the shell for males and females. However, acetylcholinesterase expression is also highly variable in the DS, differing greatly between matrix (high expression) and patch (low expression) compartments (Graybiel *et al.* 1981). Therefore, acetylcholinesterase expression alone cannot explain differences between release frequency in the DS vs the NAcc and NAcs regions. Future studies will need to characterize this dependency across regions. This divergent expression of acetylcholinesterase is only one example of the complexity of the circuits underlying spontaneous DA release, which needs further characterization in females in order to interpret circuit specific activity.

Spontaneous DA signals are highly variable in amplitude, and seem to represent asynchronous release from diverse populations of terminals. In contrast, electrically evoked signals are inherently non-selective, low in variability and represent synchronous DA release a population of diverse terminals. Thus, there are likely many other factors that affect the gradient of release from evoked signals that are not contributing to spontaneously occurring release. Examining evoked DA release is extremely valuable when measuring uptake kinetics (i.e. Michaelis-Menten measures of DAT function, Yorgason *et al.* 2013), psychostimulant effects on proteins expressed in DA terminals (Hedges *et al.* 2018; Yorgason *et al.* 2020; Torres *et al.* 2021), and

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examining circuitry from direct auto- and hetero-receptor activity on DA terminals (Gao *et al.* 2019; Torres *et al.* 2021; Yorgason *et al.* 2013). However, since regional activity differs between non-stimulated (spontaneous) and evoked conditions, further pharmacological and/or genetic manipulations are necessary to describe circuit specific effects recruited during evoked release experiments. In the context of the present study, future studies examining sex dependent differences in types of K+ channels expressed on DA vs Acetylcholine releasing terminals would greatly enhance our understanding of how spontaneous release is regulated. The present results indicate that females have greater 4AP sensitivity, which could be due to higher expression of Kv channels on either DA and/or Acetylcholine terminals. It is also unknown if the DA transients observed herein are dependent on glutamate terminals (which may also differ in their Kv channel expression) onto DA terminals or cholinergic interneurons.

Regulation of CIN firing, acetylcholine release and nAChR activation are all involved in modulation of DA transients. Indeed, cholinesterase inhibitors increase acetylcholine transients (Mamaligas & Ford 2016) and DA transients at low concentrations, but eventually cholinesterase inhibitors reduce DA transients (Zhou *et al.* 2001; Zhang *et al.* 2004), presumably due to nAChR desensitization. Interestingly, ethanol acts as a positive allosteric modulator at α6*nAChRs, reducing receptor desensitization and increasing DA transient frequency and amplitude (Gao *et al.* 2019). This effect is highly concentration dependent since ethanol at extremely high concentrations inhibits evoked DA release in a nAChR dependent manner (Hedges *et al.* 2020; Yorgason *et al.* 2015; Yorgason *et al.* 2014). Interestingly, the sex hormone estradiol is also a known positive allosteric modulator for α4*nAChRs (Wang & Lindstrom 2018). It is therefore somewhat surprising that in females DA transients were lower in amplitude and frequency, which could be explained by pre-desensitized (though still functional) nAChRs in females. Estradiol has many other effects, including inhibitory effects on Kv channel activity (Li *et al.* 2014; Li *et al.* 2013), which we would predict would enhance spontaneous release. Therefore, it is unclear if estrus effects in female mice used here in contributing to spontaneous DA release since estradiol could have opposing effects on the same circuit. We and others have observed DA transients previously in the midbrain (Gantz *et al.* 2013), accumbens (Yorgason *et al.* 2017; Gao *et al.* 2019), and dorsal striatum (herein). Considering the diversity of ways DA terminal activity is regulated in these different regions, the present studies set the foundation for future studies examining sex differences amongst these regions which could vary greatly depending on the cellular components regulating release in these disparate regions.

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The DAT is a powerful regulator of DA clearance that changes across the estrus cycle (Calipari *et al.* 2017; Zachry *et al.* 2020). While the present study did not examine estrus effects, DA clearance was more variable in female mice, likely due in part to estrus effects. Interestingly, striatal DAT function as measured by voltammetry is higher in female vs male rats (Walker *et al.* 2000). In this previous study, function was assessed by Michaelis-Menten kinetics and reported higher maximal velocity in DAT activity measured in females. This is supportive of higher DAT levels in females vs males, which has been corroborated by binding (Morissette *et al.* 1990; Morissette & Di Paolo 1993) and expression data (Calipari *et al.* 2017). In contrast, acutely, estradiol has also been shown to decrease DAT function (Dluzen & McDermott 2008; Disshon *et al.* 1998; Disshon & Dluzen 1999; Thompson 1999; Thompson & Certain 2005). This is more consistent with the present observation of slower clearance for DA transients in females. Therefore, estradiol may be acting as a negative modulator of DA transport which may be obscured by the increased DAT levels and resultant increases in maximal rates of uptake. Overall drug effects and mechanisms driving release appear similar in males and females since 4AP and cocaine increase DA transients, and transients are blocked by nAChR antagonists in both sexes (Yorgason *et al.* 2017).

Conclusions

Regional and sex differences in DA release are apparent throughout the striatum. It was surprising that regional differences in DA transient amplitude were not apparent in females. Given the large host of possible mechanisms through which DA release in the striatum is modulated, further work is needed to determine the underlying mechanisms for driving sex differences. A major contributor to these sex effects is K+ channels which are expressed everywhere. The Kv channel blocker 4AP is extremely non-selective, but has no apparent effects on uptake, supporting it as a tool for examining transients with the important caveat that regional differences in Kv expression can affect drug sensitivity. The highly variable nature of DA transients suggests that mechanisms release involve intrinsic regulators to dampen activity. These regulators may become dysregulated in disease states, highlighting the importance of understanding CIN mediated DA release.

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Figure Legends

Figure 1: Example dopamine (DA) transients measured across striatal subregions. A) Timeline of experimental procedures. Spontaneous DA (sDA) was measured in mice across subregions at baseline (figures 1- 3), after 4-aminopyridine (4AP; Kv channel blocker; figures 4,5) and after cocaine (DAT blocker; figures 6,7). Evoked DA (eDA) was measured in mice (figure 5) in dorsal and ventral striatum and the effects of Kv channel blockers (4AP and Ba2+) on eDA clearance were tested (figure 5). **B**) Schematic indicating the locations used to determine regional location of DA transients in brain slices. **C**) Example traces and color plots for DA transient events in the dorsal striatum (DS), Nucleus Accumbens core (NAcc) and Nucleus Accumbens shell (NAcs).

Figure 2: Regional and sex differences in dopamine (DA) transient amplitude. A) Distribution of transient release amplitude under baseline (no drug) conditions. Inset (**a1**) represents the top 25% of amplitude values across regions. **B**) Cumulative release probability distribution of DA amplitudes indicates directional trends when only region is investigated. **C**) Quantification of DA release by region and sex under baseline conditions indicates clear sex differences in regional release, with no apparent regional differences for female mice. **D**) 3-dimensional visualization of average amplitude by anatomical location. Bar plots indicate Mean±SEM values. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n=264 release events from 60 brain slices from 23 mice.

Figure 3: Regional and sex differences in dopamine (DA) transient frequency. A) Regional comparison of DA transient frequency. Frequency was highest in the DS, followed by the NAcc and finally the NAcs. **B**) Comparison of DA transient frequency between males and females. Female mice generally have lower frequency of release. **C**) Plot of the variance of the frequency between males and females and **D**) across regions did not differ significantly across the sex and region variables. **E**) 3-dimensional visualization of frequency by anatomical location. Bar plots indicate Mean±SEM values. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n=60 brain slices from 23 mice.

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Figure 4: Kv channel blockade increases dopamine (DA) transient amplitude and frequency, with greater increases observed in females. A) Distribution of transient release amplitude in the presence of 4AP (30 µM). Inset (a1) represents the top 25% of amplitudes. **B**) Cumulative release amplitude shows an increase in amplitude in each region following 4AP administration. 3-dimensional visualization of **C**) amplitude and **D**) frequency by anatomical location. **E**) overall amplitude and **F**) upper quartile amplitude following 4AP administration. **G**) Within subject changes in frequency compared to baseline conditions after 4AP indicated clear increases in release frequency that were more pronounced in females. Regional and sex comparison of **H**) transient frequency. Bar plots indicate Mean±SEM values. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n=2407 events from 60 brain slices from 23 mice.

Figure 5: Clearance for dopamine (DA) transients varies by sex and is not influenced by Kv channel blockade. Example evoked DA traces and example curve fit from single exponential decay model (blue) under **A**) baseline and **B**) 4AP (30 µM) conditions. **C**) 4AP increases evoked DA release in the Nucleus Accumbens core (NAcc). However, **D**) 4AP has no apparent effect on DA clearance in the NAcc. The non-selective K channel blocker barium (100 µM) increased DA release (**E**) but had no effect on DA clearance (**F**) in the Dorsal Striatum (DS). Clearance for DA transients was measured after 4AP and **G**) females demonstrated greater variability and higher mean tau values than males in all regions. Bar plots indicate Mean±SEM values. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n=5 brain slices from mice for each evoked experiment. n=1111 events from 60 brain slices from 23 mice for spontaneous release experiments.

Figure 6: Dopamine (DA) transients have reduced clearance after cocaine. A,B) Examples of cyclic voltammograms (CV; **a1**), current traces (**a2,3**) and color plots (**a4,5**) of DA transients after administration of the Kv channel blocker 4AP (30 µM). These peaks are used as baseline comparison for reuptake findings. **B**) Example CV (**b1**), current traces (**b2,3**) and color plots (**b4,5**) for DA transients after cocaine (3 µM) administration. Arrows in **a2,3** and **b2,3** indicate the peak for the color matched CVs depicted in **a1** and **b1**.

Figure 7: Cocaine increases dopamine (DA) transient amplitude and frequency. A) Distribution of transient release amplitude in the presence of cocaine (3 µM). Inset (**a1**), the top 25% of amplitudes. **B**) Cumulative

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release amplitude indicates increases in amplitude across regions following cocaine. 3-dimensional visualization of **C**) amplitude and **D**) frequency by anatomical location. **E**) overall amplitude and **F**) upper quartile amplitude following 4AP administration. **G**) Increases in transient frequency after cocaine indicate clear increases in release frequency across all regions. **H**) Regional and sex comparison of transient frequency. In general, sex and regional relationships observed in Figure 7 were maintained with cocaine. Bar plots indicate Mean±SEM values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n=1974 events from 60 brain slices from 23 mice.

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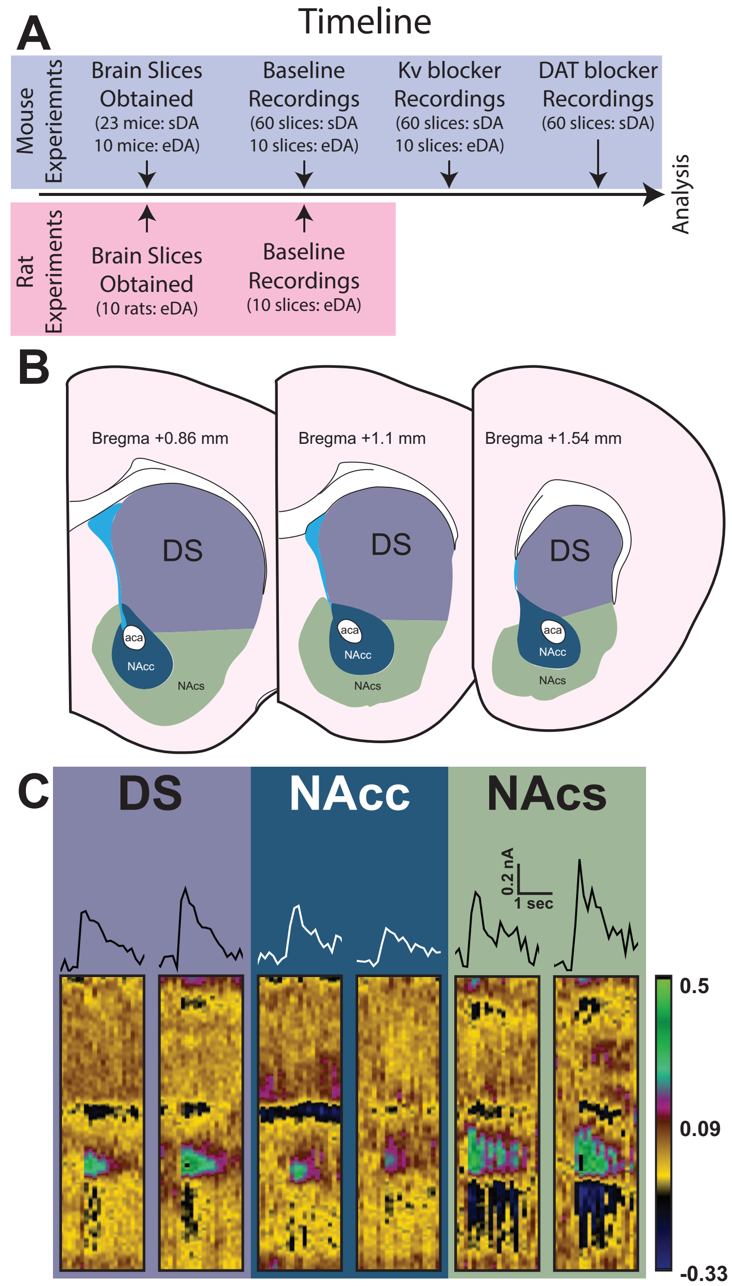

Fig1

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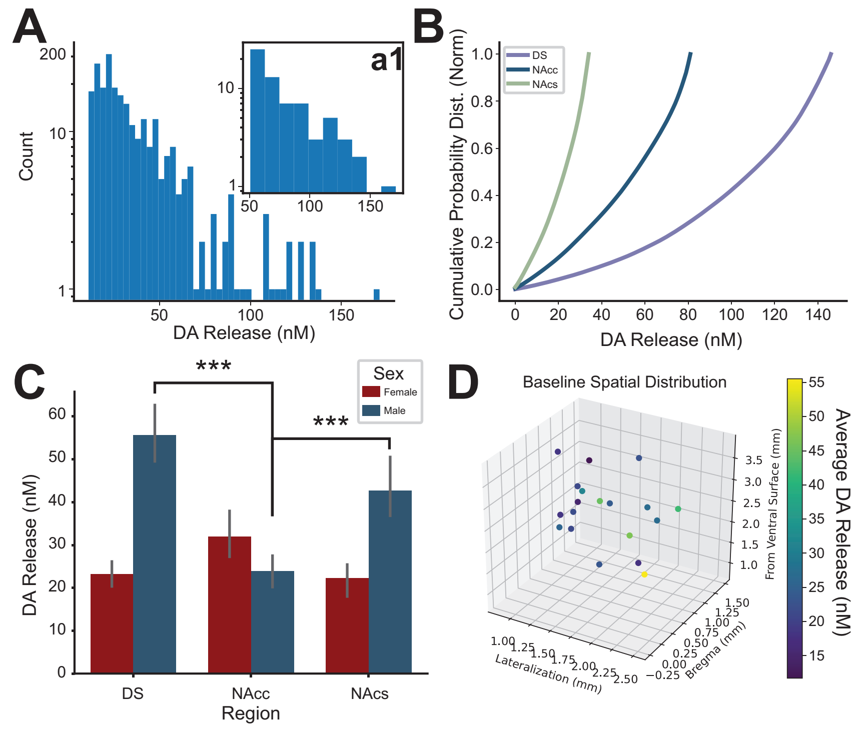

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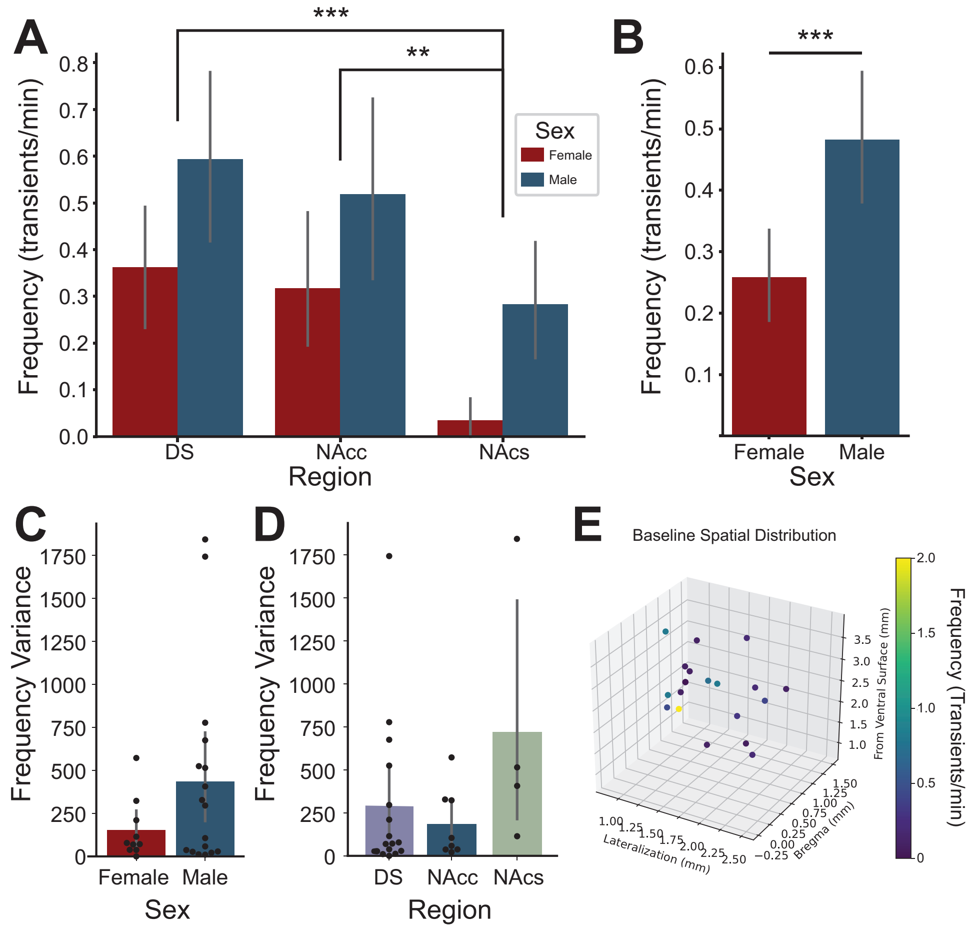

Fig3

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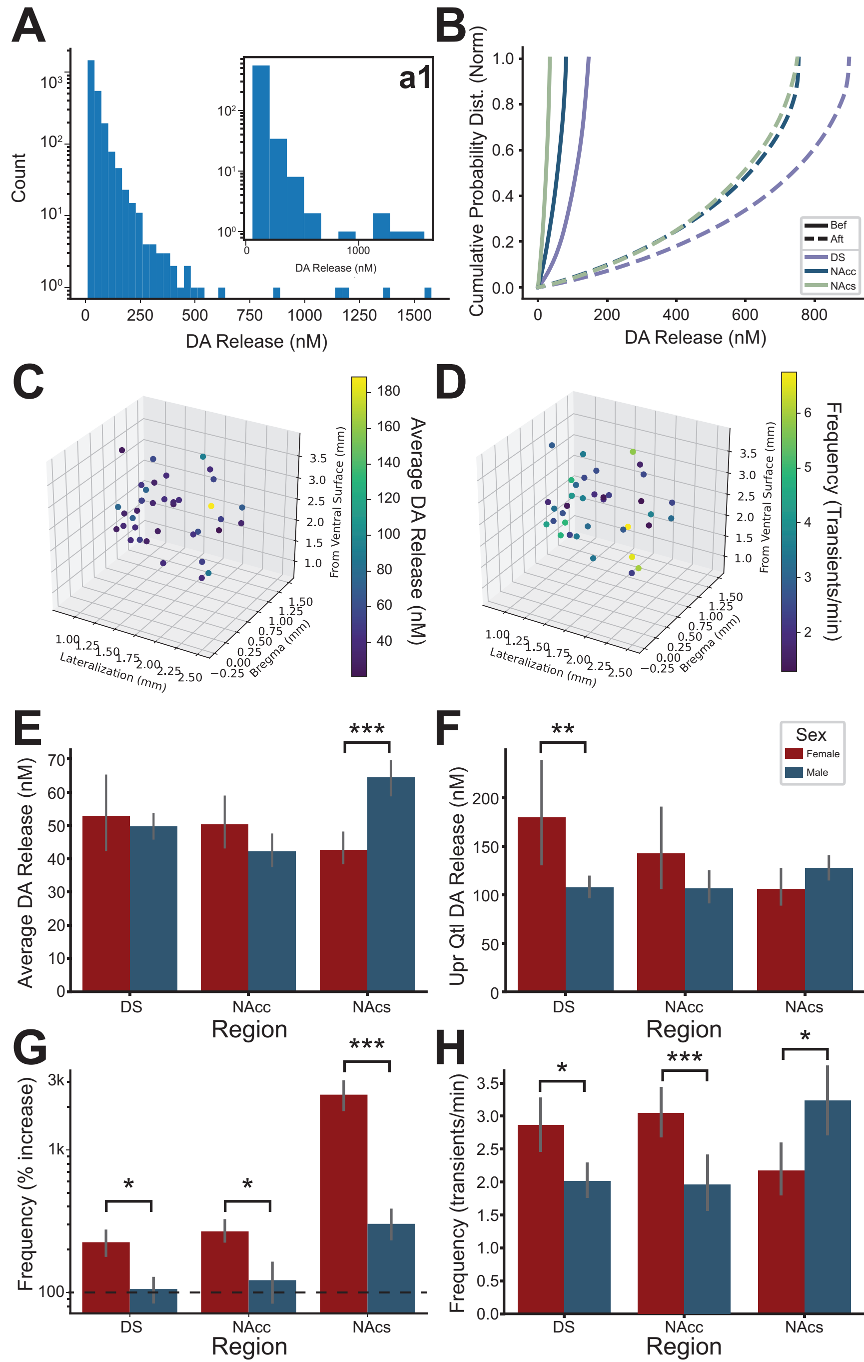

Fig4

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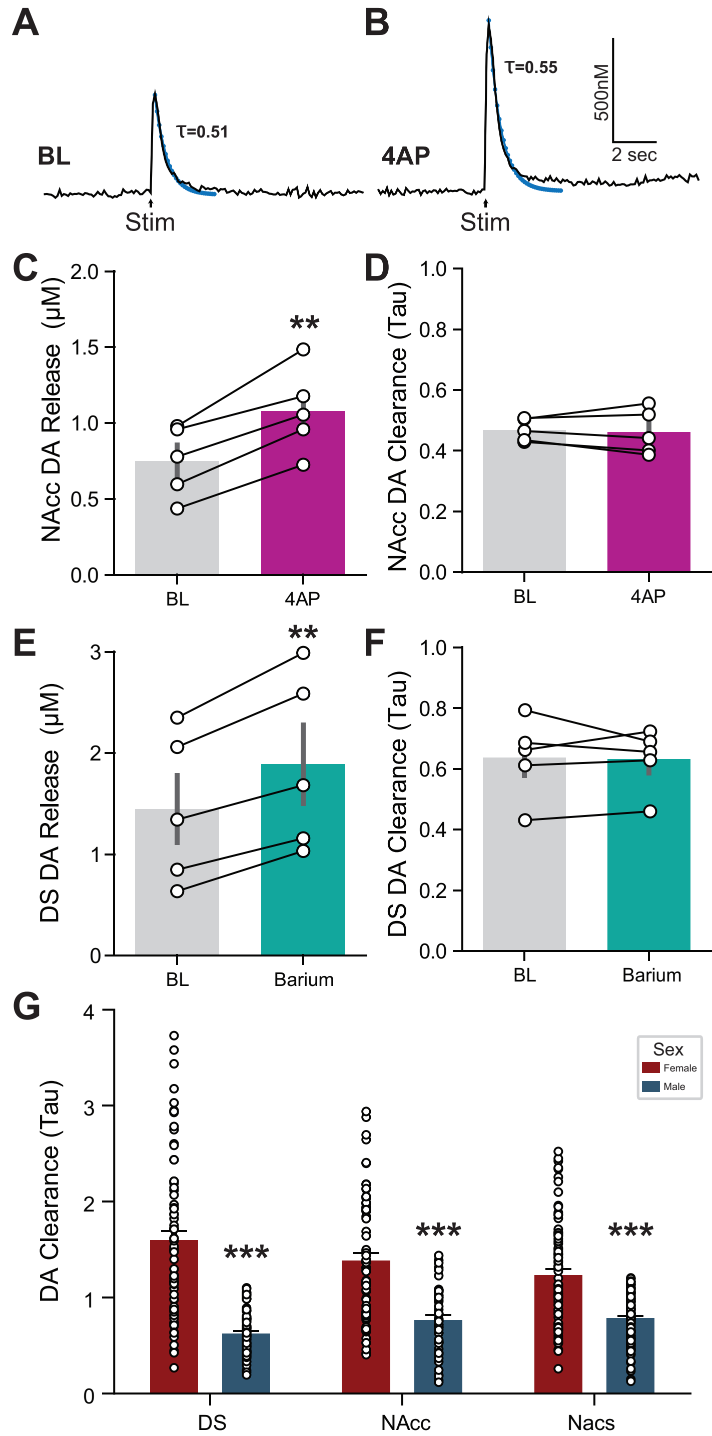

Fig5

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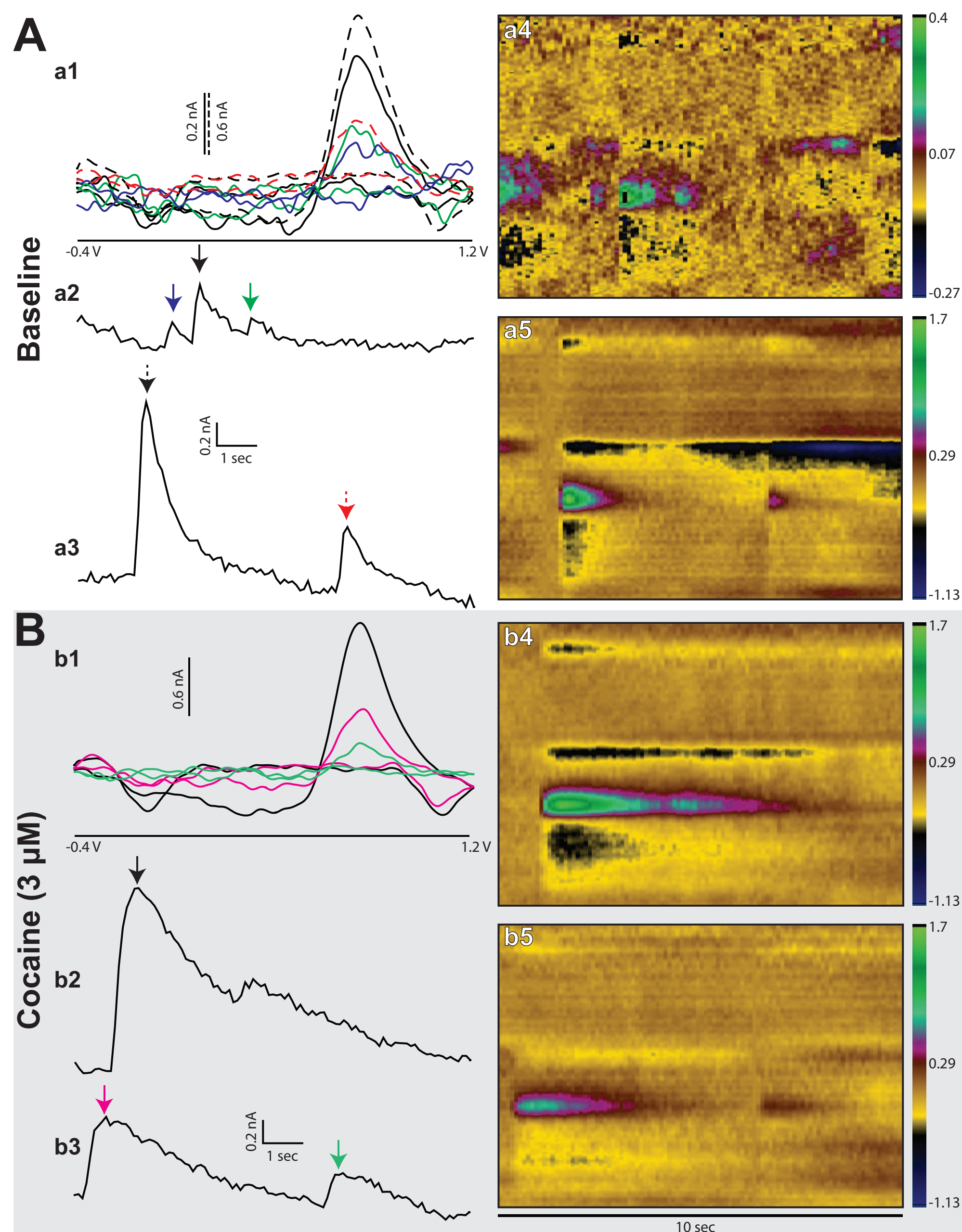

Fig6

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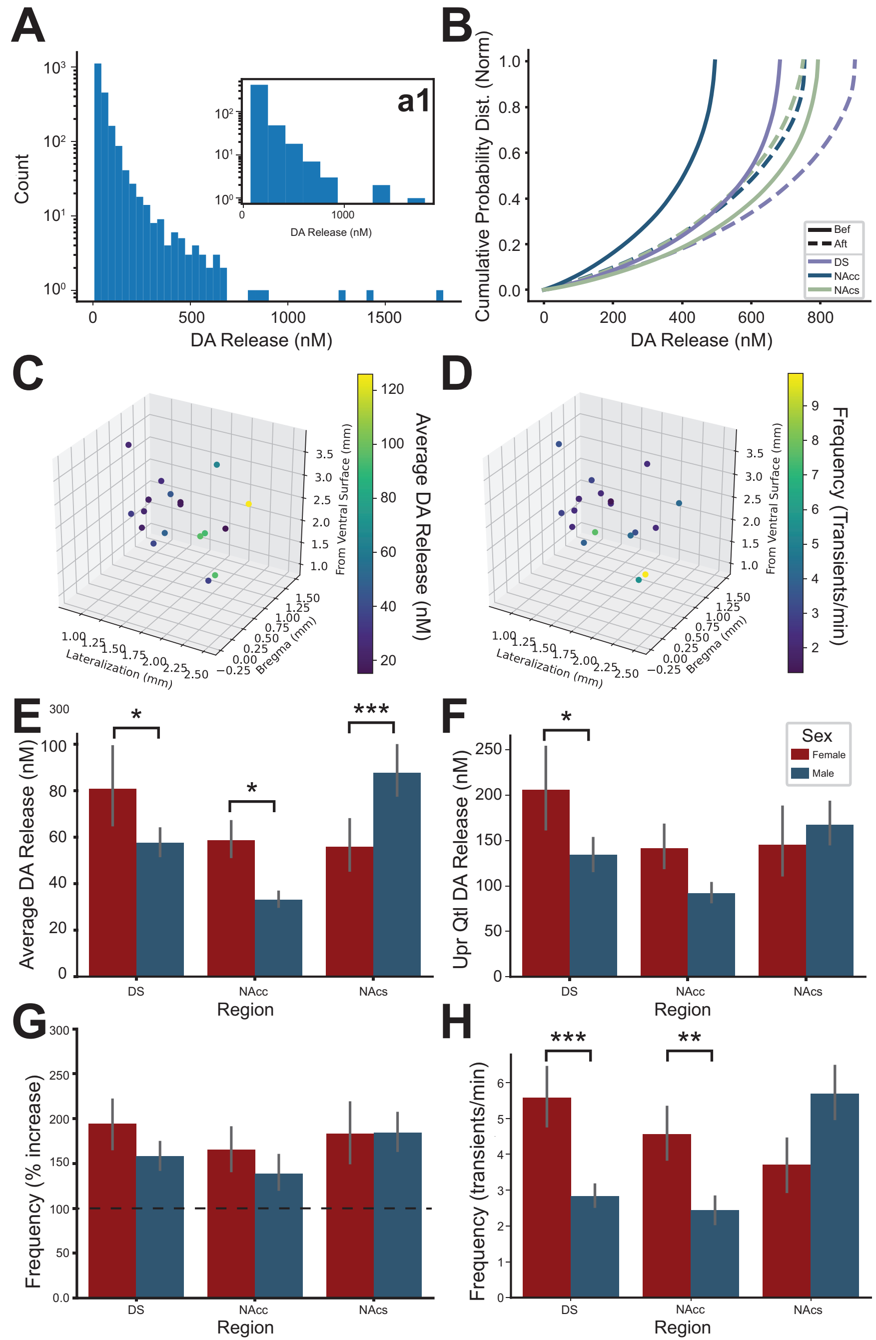

Fig7

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