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## Dopamine uptake dynamics are preserved under isoflurane anesthesia

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### Abstract

Fast scan cyclic voltammetry is commonly used for measuring the kinetics of dopamine release and uptake. For experiments using an anesthetized preparation, urethane is preferentially used because it does not alter dopamine uptake kinetics compared to freely moving animals. Unfortunately, urethane is highly toxic, can induce premature death during experiments, and cannot be used for recovery surgeries. Isoflurane is an alternative anesthetic that is less toxic than urethane, produces a stable level of anesthesia over extended periods, and is often used for recovery surgeries. Despite these benefits, the effects of isoflurane on dopamine release and uptake have not been directly characterized. In the present studies, we assessed the utility of isoflurane for voltammetry experiments by testing dopamine signaling parameters under baseline conditions, after treatment with the dopamine uptake inhibitor cocaine, and after exposure to increasing concentrations of isoflurane. Our results indicate that surgical levels of isoflurane do not significantly alter terminal mechanisms of dopamine release and uptake over prolonged periods of time. Consequently, we propose that isoflurane is an acceptable anesthetic for voltammetry experiments, which in turn permits the design of studies in which dopamine signaling is examined under anesthesia prior to recovery and subsequent experimentation in the same animals.

### Keywords

Fast scan cyclic voltammetry; cocaine; urethane; dopamine transporter; nucleus accumbens

### Introduction

Fast scan cyclic voltammetry (FSCV) is a powerful technique for measuring catecholamine release and uptake kinetics, and is commonly used to study neurochemical, pharmacological, and genetic manipulations in anesthetized animals [3, 6, 17, 19]. FSCV experiments performed under anesthesia have preferentially used urethane, an anesthetic thought to produce its effects through interaction with ligand gated ion channels in a manner

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similar to many others [9, 12, 25]. Importantly, however, urethane is the only anesthetic which has been shown to produce anesthesia without significantly changing basal dopamine (DA) uptake kinetics when compared to awake animals [7, 20], thus it is the preferred anesthesia for use with in vivo voltammetry [8]. Despite this benefit, there are also significant disadvantages to the use of urethane. In particular urethane is a carcinogen, produces cardiomyopathy, and respiratory deficits, and is toxic to the liver, kidney, gastrointestinal and nervous systems [10, 13, 27]; because of these effects urethane can lead to death at any point during an experiment, and is limited to use in terminal studies.

Isoflurane is an inhalation anesthetic that offers several advantages over urethane. Isoflurane is far less toxic than urethane, can be rapidly adjusted to maintain appropriate levels of anesthesia for extended periods, and can be used for recovery experiments. Recent evidence suggests that isoflurane may produce anesthetic hypnosis by interacting with ligand-gated ion channels [12] to promote the activation of endogenous sleep circuitry [15]. A limited number of studies have examined the effects of isoflurane on DA signaling. Unfortunately, many of these studies either administered isoflurane with N<sub>2</sub>O, an agent that produces changes in DA signaling on its own [11, 21], or used concentrations of isoflurane that are higher than necessary to maintain surgical level anesthesia (2–6%). For example, studies in which isoflurane was administered in combination with N<sub>2</sub>O indicate that extracellular DA concentrations are elevated through interactions with the DA transporter (DAT) [16, 22]. Likewise, 2.5% isoflurane elevates extracellular DA levels in vivo [1, 23]) and 4–6% isoflurane has been shown to induce DAT internalization in vitro [23]. By comparison, lower concentrations of isoflurane did not significantly alter extracellular DA levels [1], and were reported as insufficient to reliably produce DAT internalization [23] in these same studies. Together, these observations indicate that low concentrations of isoflurane administered with air may not significantly alter DA signaling, and thus may serve as an acceptable anesthetic for use in FSCV experiments.

We tested the utility of isoflurane as an anesthetic for use in FSCV experiments by characterizing DA release and uptake parameters in the nucleus accumbens core (NAc). We compared baseline DA uptake and release kinetics and the effects of the DAT inhibitor cocaine, *ex vivo*, in tissue prepared from unanesthetized animals or animals that received urethane or isoflurane anesthesia. We then compared baseline DA uptake kinetics and release as well as the effects of cocaine in vivo in animals anesthetized with isoflurane or urethane. Last, we characterized the effects of isoflurane exposure duration and isoflurane concentration on DA release and uptake parameters in vivo.

## Methods

### Animals

Male Sprague-Dawley rats (350–420g, Harlan, Frederick, MD) were given *ad libitum* access to food and water and kept on a 12:12hr light:dark cycle (lights on at 7:00h). All protocols and animal care procedures were maintained in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals: Eighth Edition (The National Academies Press, Washington, DC, 2011) and approved by the Institutional Animal Care and Use Committee at Drexel University College of Medicine.

### Ex vivo voltammetry

Striatal slices were prepared from animals that were sacrificed by decapitation alone, or by decapitation after being fully anesthetized with urethane or isoflurane ( $n = 6$  for all groups). Animals treated with urethane received an intraperitoneal (i.p.) injection of 1.5–1.7 g/kg urethane (dissolved in water; Sigma-Aldrich, St. Louis, MO), while animals treated with isoflurane were placed in a gas chamber and exposed to 2.5% isoflurane (99.9%, Piramal Critical Care, Bethlehem, PA) flushed with air (~21% O<sub>2</sub>) for 3 min to induce anesthesia, and then maintained at 1.5% isoflurane for an additional 30 min.

Brains were removed following decapitation and then transferred into oxygenated ice-cold artificial cerebral spinal fluid (aCSF) containing (in mM) NaCl (126), KCl (2.5), NaH<sub>2</sub>PO<sub>4</sub> (1.2), CaCl<sub>2</sub> (2.4), MgCl<sub>2</sub> (1.2), NaHCO<sub>3</sub> (25), glucose (11), l-ascorbic acid (0.4), pH adjusted to 7.4. A vibrating microtome was used to produce 300  $\mu\text{m}$  thick coronal sections containing the NAc, which were then transferred into a testing chamber and flushed with aCSF (32°C) flowed at 1 ml/min. Following 30 min of equilibration, a carbon fiber microelectrode (150–200  $\mu\text{m}$  length  $\times$  7  $\mu\text{m}$  diameter) and a bipolar stimulating electrode (Plastics One, Roanoke, VA) were placed in the NAc core. DA release was elicited every 5 min using a single electrical pulse (400  $\mu\text{A}$ , 4 msec, monophasic), and was recorded as previously described [3]. After recording three stable responses (less than 10% variation) cocaine (obtained from the National Institute on Drug Abuse) was cumulatively applied (0.3–30  $\mu\text{M}$ ) to the tissue.

### In vivo voltammetry

For one group of animals, anesthesia was induced with an i.p. injection of 1.5–1.7 g/kg urethane which produced stable anesthesia throughout the duration of experiment. For a second group of animals, anesthesia was induced by placing animals in a gas chamber perfused with 2.5% isoflurane flushed with air for 3 min and then maintained at a constant 1.5% isoflurane which is the lowest concentration that consistently maintained a surgical level of anesthesia (indicated by loss of paw withdrawal reflex).

Rats used to investigate the effects of cocaine were implanted with an intravenous (i.v.) catheter into the jugular vein, while rats used to investigate the effects of various isoflurane concentrations were not. Rats were placed into a stereotaxic apparatus and implanted with a bipolar stimulating electrode aimed at the ventral tegmental area (VTA; 5.3 P, +1.0 L), a carbon fiber microelectrode aimed above the NAc core (+1.3 A, +1.3 L) and a reference electrode in contralateral cortex as described previously [4, 18].

Following surgery, the carbon fiber microelectrode was initially positioned in the caudate nucleus (–4.5 V), and the stimulating electrode was driven into the VTA (–7.2 to –7.6 V) until a 1 sec, 60 Hz, 4 ms monophasic (~600  $\mu\text{A}$ ) stimulation train elicited a robust DA signal. Once stimulator and carbon fiber electrodes were positioned to achieve adequate dopamine release the carbon fiber recording electrode was lowered into the NAc (–6.5 V) which yields lower and slower dopamine release and uptake [2–4]. Release was then elicited every 5 min. Three baseline responses were recorded (less than 10% variation), and treatment began immediately after baseline collections were completed.

The first set of in vivo voltammetry experiments was designed to compare the effects of cocaine in animals anesthetized with urethane or isoflurane. Cocaine injections (1.5 mg/kg) were experimenter-delivered (5 sec, ~200  $\mu$ l i.v. bolus) after the final baseline recording in the NAc. Electrically-stimulated DA responses were acquired at 30 and 60 sec post-cocaine injection, and every 5 min thereafter [4].

A second set of in vivo voltammetry experiments was designed to test DA uptake kinetics and release under increasing concentrations of isoflurane. After recording stable baselines of electrically stimulated DA release at 1.5% isoflurane, the concentration of isoflurane was increased by 0.5% and signals were monitored until 30 min of stable responses were observed (less than 10% variation). This was repeated such that 30 min of stable DA responses were recorded for 1.5, 2.0, 2.5 and 3.0% isoflurane in each animal. Average values recorded from the final three stimulations were used for statistical analysis.

## Data Analysis

The total concentration of electrically-stimulated DA release and transporter-mediated uptake kinetics, including maximal uptake rate ( $V_{max}$ ) and apparent affinity of endogenous DA ( $K_m$ ) for the DA transporter were monitored. DA overflow curves were fitted to a Michaelis–Menten-based kinetic model [24] using Demon Voltammetry and Analysis Software [26]. Changes in uptake and release were obtained by setting baseline  $K_m$  values (prior to any treatment) to 0.17 – 0.19  $\mu$ M and establishing a baseline  $V_{max}$  individually for each subject. For cocaine experiments,  $V_{max}$  was held constant for the remainder of the experiment and alterations in uptake were attributed to changes in apparent  $K_m$ . For studies on the concentration effects of isoflurane,  $K_m$  values were held constant throughout the experiment and thus alterations in uptake were attributed to changes in  $V_{max}$ . The concentration of stimulated DA release was assessed by comparing the current at the peak oxidation potential for DA in voltammograms with electrode calibrations of 3  $\mu$ M of DA.

Statistics were carried out using SPSS version 22 (IBM, Armonk, NY). Ex vivo baseline measurements for all groups were compared using one-way ANOVA, and in vivo baseline measurements were compared with independent samples t-tests. The effects of cocaine for both ex vivo and in vivo experiments were tested with two-way ANOVA with repeated measures. Correlation for duration of exposure to isoflurane and  $V_{max}$  was tested with Pearson's correlation test, and the concentration effects of isoflurane were tested with one-way ANOVA with repeated measures. All results were verified with retrospective power analysis using G-Power [5]. Alpha was set to 0.05. Effect size was estimated based on the previous reports of high concentration isoflurane effects described above and the standard deviation observed in the current studies. All null results hold greater than 80% power.

## Results

### Urethane and isoflurane do not alter baseline dopamine signaling or the effects of cocaine ex vivo

We first sought to determine if isoflurane or urethane alters terminal mechanisms of DA release and uptake. To this end, we compared baseline DA uptake kinetics and release and

cocaine-induced DA uptake inhibition in the NAc of tissue sections prepared from unanesthetized rats or from rats that were anesthetized with either urethane or isoflurane (Fig. 1). These experiments showed no significant difference in baseline uptake rates ( $n = 6$  per group,  $F(2, 15) = 0.354$ ,  $p=0.707$ ) or DA release ( $n=6$  per group,  $F(2,15) = 0.455$ ,  $p=0.643$ ) across conditions. Further, we observed no differences in cocaine-induced DA uptake inhibition ( $n = 6$  per group,  $F(2,15) = 0.912$ ,  $p = 0.423$ ), or changes in DA release ( $n = 6$  per group,  $F(2,15) = 0.892$ ,  $p = 0.431$ ) between groups. Additionally, no interactions were observed between anesthetic group and cocaine effects on DA uptake inhibition ( $n=6$  per group,  $F(8,60) = 0.703$ ,  $p = 0.688$ ) or DA release ( $n = 6$  per group,  $F(8,60) = 0.906$ ,  $p = 0.517$ ).

### Dopamine signaling is similar in urethane- and isoflurane-anesthetized rats in vivo

Given that exposure to isoflurane does not alter DA signaling *ex vivo*, we next compared baseline DA uptake kinetics and release as well as changes in DA uptake in response to cocaine in animals anesthetized with urethane or 1.5% isoflurane (Fig. 2).  $V_{max}$  and DA release in urethane-anesthetized animals were similar to previously reported values [4, 18]. Similar to the *ex vivo* studies, no significant differences were observed across anesthetic conditions for  $V_{max}$  (urethane  $n = 6$ , isoflurane  $n = 13$ ,  $t(17) = -0.341$ ,  $p = 0.0.737$ ) or DA release (urethane  $n = 6$ , isoflurane  $n=13$ ,  $t(17) = -0.971$ ,  $p = 0.344$ ). Likewise, no differences were observed between urethane- and isoflurane-treated animals for cocaine-induced uptake inhibition ( $n = 6$  per group,  $F(1,22) = 0.1774$ ,  $p = 1.0$ ) or changes in DA release ( $n = 6$  per group,  $F(1,22) = 0.2258$ ,  $p = 0.99$ ).

### In vivo DA signaling is stable after prolonged exposure to isoflurane but changes across isoflurane concentration

Previous studies have shown that changes in DA signaling occur when rats are administered concentrations of isoflurane that are higher than those used herein [1, 23]. To determine if the degree of isoflurane exposure alters DA release and uptake *in vivo*, we examined the effects of isoflurane exposure duration and isoflurane concentration on DA signaling (Fig. 3). We tested if the duration of exposure to 1.5% isoflurane produced changes in maximal DA uptake rate by comparing  $V_{max}$  after various exposure times across separate animals, and observed no correlation between exposure time and  $V_{max}$  ( $n = 13$ ,  $R^2 = 0.008$ ,  $p = 0.770$ ). We also examined the effect of varying isoflurane concentrations on DA uptake and release. We found that increasing concentrations of isoflurane significantly decreased  $V_{max}$  ( $n = 6$ ,  $F(3, 15) = 3.674$ ,  $p = 0.036$ ), although no significance was seen at any individual concentration (Dunnett's post hoc with 1.5% isoflurane as a control). Moreover, increasing isoflurane concentration did not significantly alter DA release ( $n = 6$ ,  $F(3, 15) = 0.621$ ,  $p = 0.612$ ).

## Discussion

Our observations indicate that maintenance concentrations of isoflurane are an acceptable anesthetic for use in *ex vivo* and *in vivo* voltammetry experiments. First, we found that DA release and uptake kinetics, *ex vivo*, are similar between untreated rats and rats anesthetized with urethane or 1.5% isoflurane under baseline conditions and in response to cocaine. We

then verified that baseline DA uptake kinetics and release as well as changes in DA uptake in response to cocaine are comparable between animals anesthetized with 1.5% isoflurane or with urethane. Last, we determined that DA uptake remains stable over extended exposure to 1.5% isoflurane, but is altered by increasing concentration of isoflurane.

### **Anesthetic effects on baseline dopamine signaling**

Our *ex vivo* results indicate that neither 1.5% isoflurane nor urethane significantly alters terminal mechanisms of DA release and uptake, however *in vivo* studies have indicated that although urethane does not alter DA uptake kinetics it does reduce stimulated DA release [7, 20]. This *in vivo* effect may be mediated by altered inhibitory balance at DA cell bodies, and urethane and isoflurane may produce this effect through similar actions at ligand gated ion channels in the central nervous system [9, 12]. Accordingly, we found no differences in DA uptake rates or release between urethane- and isoflurane-anesthetized animals. Together, these observations suggest the possibility that urethane and maintenance concentrations of isoflurane may alter DA signaling through similar actions at DA cell bodies. It should be noted, however, that our observations are limited to a comparison between acute isoflurane and urethane exposure, and thus our findings do not provide any evidence regarding DA signaling following recovery from anesthesia.

### **Anesthetic interaction with cocaine**

FSCV experiments using urethane anesthesia have often been used to investigate DA changes in response to cocaine [2, 3, 14, 18], but the extent to which urethane itself alters cocaine's effect on DAT remains unknown [8]. Here we demonstrate that neither urethane nor isoflurane alters cocaine's effects *ex vivo*. This may appear to be in contrast to previous investigations that suggested isoflurane enhances the effect of DAT inhibitors, including cocaine [16, 22]. Those studies, however, used isoflurane mixed with 70% N<sub>2</sub>O, an agent that has been shown to alter DA signaling on its own [11, 21], and thus the extent to which these effects are attributable to N<sub>2</sub>O versus isoflurane remains unclear. Whether urethane or isoflurane alter the effects of cocaine *in vivo* remains an open question, however, our *ex vivo* results indicate that exposure to these anesthetics does not alter the action of cocaine on DA terminals and our *in vivo* results suggest that any changes in cocaine's action are similar between urethane and isoflurane.

### **Concentration effects of isoflurane on dopamine signaling**

It is important to note that the utility of isoflurane for FSCV is likely restricted to low maintenance concentrations. High concentrations of isoflurane (4 – 6%) have been shown to directly induce the internalization of DAT *in vitro* [23], which should correspond with decreases in  $V_{max}$  [6]. Indeed, we found modest decreases in  $V_{max}$  with increasing concentration of isoflurane. The highest concentrations tested herein (2.5 and 3.0%), however, are typically lethal over prolonged exposure, and thus are inappropriate and unnecessarily high for use with FSCV. Therefore, these results indicate that FSCV experiments that use isoflurane anesthesia should be performed with the lowest concentration of isoflurane that produces a surgical level of anesthesia.

## Conclusion

Our results demonstrate that DA release and uptake kinetics monitored under isoflurane anesthesia are similar to those observed under urethane, and thus isoflurane is a reasonable alternative anesthetic for FSCV studies. Using isoflurane for FSCV experiments increases the safety and reliability of these studies and, further, will allow investigators to design experiments in which DA signaling can be assessed in an acute anesthetized preparation prior to recovery and further experimentation.

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## Abbreviations

<b>FSCV</b>	fast scan cyclic voltammetry
<b>DA</b>	dopamine
<b>DAT</b>	dopamine transporter
<b>NAc</b>	nucleus accumbens
<b>aCSF</b>	artificial cerebral spinal fluid
<b>VTA</b>	ventral tegmental area
<b>i.p</b>	intraperitoneal
<b>i.v</b>	intravenous

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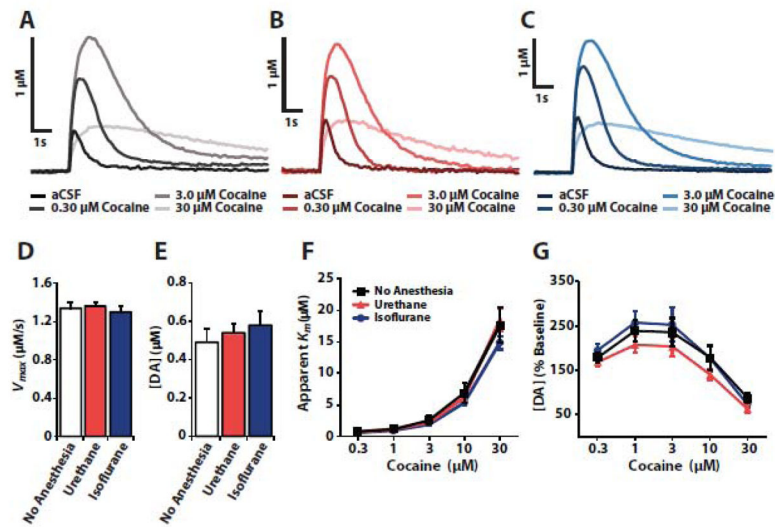
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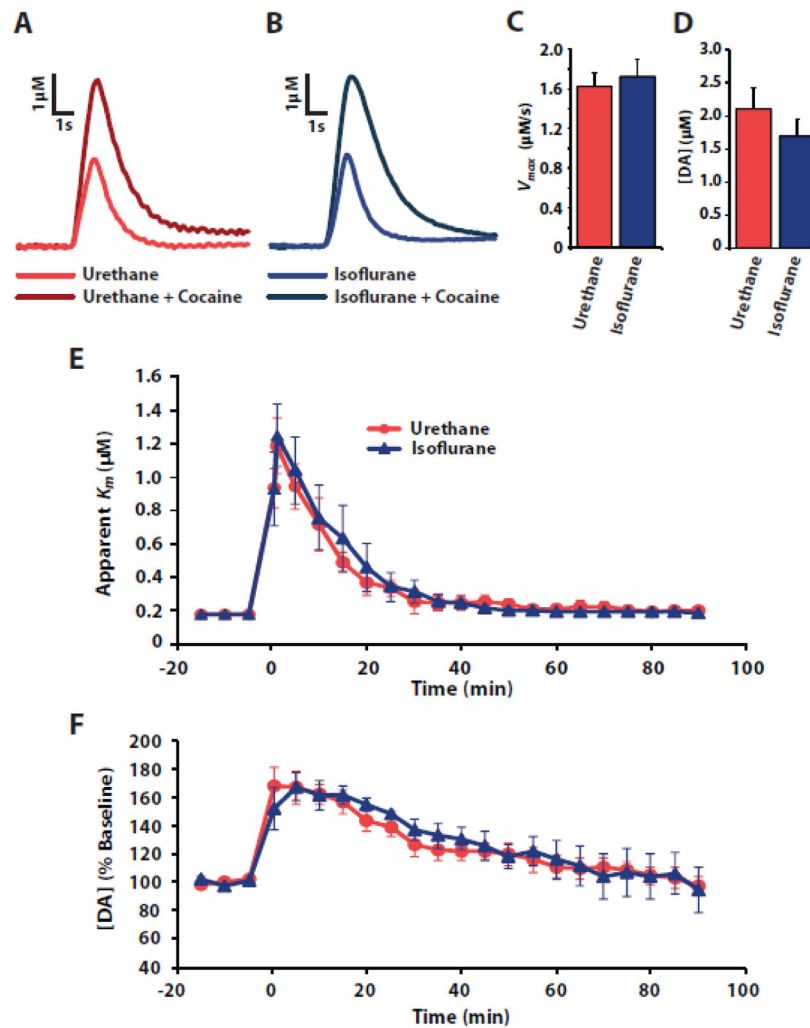
### Highlights

- Neither isoflurane nor urethane alter terminal dopamine release and uptake *ex vivo*
- Dopamine release and uptake are similar under isoflurane or urethane *in vivo*
- Cocaine effects on dopamine signaling are similar under isoflurane or urethane
- Dopamine uptake rate remains stable after prolonged exposure to isoflurane
- Isoflurane is a reasonable alternative anesthetic for use in voltammetry studies



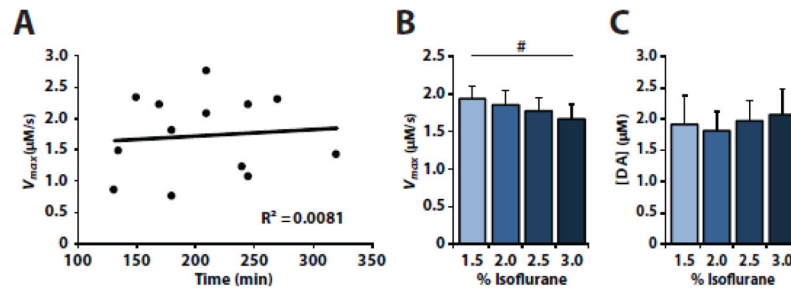
**Figure 1.**

Urethane and isoflurane do not alter baseline dopamine uptake kinetics and release or the effects of cocaine ex vivo. Representative traces recorded from tissue prepared from (A) unanesthetized rats and rats anesthetized with (B) urethane or (C) isoflurane. Shown are mean  $\pm$  SEM of (D) baseline  $V_{max}$ , and (E) baseline DA release, as well as cocaine effects on (F) apparent  $K_m$  and (G) DA release.



**Figure 2.**

In vivo baseline dopamine uptake and release as well as the effects of cocaine are similar between rats anesthetized with urethane or isoflurane. Representative traces recorded before and after cocaine in animals anesthetized with (A) urethane or (B) isoflurane. Shown are mean  $\pm$  SEM of (C) baseline  $V_{max}$ , (D) baseline DA release, and cocaine effects on (E) apparent  $K_m$  and (F) DA release across time.



**Figure 3.**

In vivo dopamine uptake kinetics are stable under isoflurane after prolonged exposure but not across increasing concentration. (A) A scatterplot with linear regression of baseline  $V_{max}$  recorded from different animals at after various isoflurane exposure times. Shown are (B)  $V_{max}$  and (C) DA release after increasing concentrations of isoflurane. # indicates a significant main effect ( $p < 0.05$ ).