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Thomas Y. Park  
*Uniformed Services University of the Health Sciences*

Kevin S. Nishida  
*Uniformed Services University of the Health Sciences*

Colin M. Wilson  
*Uniformed Services University of the Health Sciences*

Shalini Jaiswal  
*Uniformed Services University of the Health Sciences*

Jessica Scott  
*Uniformed Services University of the Health Sciences*

*See next page for additional authors*

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**Authors**

Thomas Y. Park, Kevin S. Nishida, Colin M. Wilson, Shalini Jaiswal, Jessica Scott, Andrew R. Hoy, Reed G. Selwyn, Bernard J. Dardzinski, and Kwang H. Choi

## BEHAVIOURAL NEUROSCIENCE

# Effects of isoflurane anesthesia and intravenous morphine self-administration on regional glucose metabolism ( $[^{18}\text{F}]\text{FDG}$ -PET) of male Sprague-Dawley rats

Thomas Y. Park,<sup>1,2</sup> Kevin S. Nishida,<sup>1,2</sup> Colin M. Wilson,<sup>3,4</sup> Shalini Jaiswal,<sup>3</sup> Jessica Scott,<sup>3</sup> Andrew R. Hoy,<sup>3,5</sup> Reed G. Selwyn,<sup>3,4,5</sup> Bernard J. Dardzinski<sup>3,5</sup> and Kwang H. Choi<sup>1,2,6,7</sup> 

<sup>1</sup>Department of Psychiatry, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA

<sup>2</sup>Center for the Study of Traumatic Stress, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

<sup>3</sup>Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

<sup>4</sup>Department of Radiology, University of New Mexico, Albuquerque, NM, USA

<sup>5</sup>Department of Radiology and Radiological Sciences, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

<sup>6</sup>Program in Neuroscience, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

<sup>7</sup>Graduate School of Nursing, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

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## Abstract

Although certain drugs of abuse are known to disrupt brain glucose metabolism (BGLuM), the effects of opiates on BGLuM are not well characterized. Moreover, preclinical positron emission tomography (PET) studies anesthetize animals during the scan, which limits clinical applications. We investigated the effects of (i) isoflurane anesthesia and (ii) intravenous morphine self-administration (MSA) on BGLuM in rats. Jugular vein cannulated adult male Sprague-Dawley rats self-administered either saline (SSA) or morphine (0.5 mg/kg/infusion, 4 h/day for 12 days). All animals were scanned twice with  $[^{18}\text{F}]\text{-fluoro-deoxy-glucose (FDG)-PET/CT}$  at a baseline and at 2-day withdrawal from self-administration. After the IV injection of FDG, one batch of animals ( $n = 14$ ) was anesthetized with isoflurane and the other batch ( $n = 16$ ) was kept awake during the FDG uptake (45 min). After FDG uptake, all animals were anesthetized in order to perform a PET/CT scan (30 min). Isoflurane anesthesia, as compared to the awake condition, reduced BGLuM in the olfactory, cortex, thalamus, and basal ganglia, while increasing BGLuM in the midbrain, hypothalamus, hippocampus, and cerebellum. Morphine self-administered animals exhibited withdrawal signs (piloerection and increased defecation), drug seeking, and locomotor stimulation to morphine (0.5 mg/kg) during the 2 day withdrawal. The BGLuM in the striatum was increased in the MSA group as compared to the SSA group; this effect was observed only in the isoflurane anesthesia, not the awake condition. These findings suggest that the choice of the FDG uptake condition may be important in preclinical PET studies and increased BGLuM in the striatum may be associated with opiate seeking in withdrawal.

## Introduction

### Opiate addiction

Opioid medications are among the most widely used drugs for pain management and continue to dominate the analgesic medication market (Lever, 2007). However, chronic use of opioid medications can lead to abuse, tolerance, physical dependence, and if stopped, withdrawal, which can cause the user to exhibit drug seeking and relapse (Camí & Farré, 2003). Previous preclinical studies have used

the intravenous (IV) opioid self-administration paradigm to study important aspects of opiate addiction in animals such as voluntary drug intake, self-regulation, tolerance, withdrawal, and drug seeking (Panlilio *et al.*, 2000; Le *et al.*, 2014; Sukhtankar *et al.*, 2014; Lucantonio *et al.*, 2015; Nishida *et al.*, 2016). Similar to other drugs of abuse, opiates increase dopamine function, which is fundamental to how these drugs facilitate the reward pathways in the brain (Wise & Bozarth, 1987; Willuhn *et al.*, 2010). It is thought that opiates act on mainly *mu*-opioid receptors, which activate mesolimbic dopaminergic pathways, thereby mediating the behavioral responses of drug addiction (Al-Hasani & Bruchas, 2011). These receptors are heavily distributed in specific regions of the brain including the striatum, thalamus, cerebellum, and brainstem (Kuhar *et al.*, 1973). However,

Correspondence: Kwang H. Choi, as above.  
E-mail: kwang.choi@usuhs.edu

*in vivo* brain mechanisms by which repeated opiate use modulates addiction-like behaviors remain unclear.

### Opiates on regional brain glucose metabolism (BGLuM)

In the past, the 2-deoxyglucose (2-DG) autoradiography method was used to study how cerebral glucose is metabolized in animals (Sokoloff, 1981; Geary & Wooten, 1983, 1985, 1986; Fanelli *et al.*, 1987; Adams & Wooten, 1994; Orzi *et al.*, 1996). Previous 2-DG autoradiography studies have reported the effects of morphine administration on BGLuM in rodents. For instance, an acute morphine injection (15 mg/kg, IP) significantly increased BGLuM in the striatum of mice (Quelven *et al.*, 2004). Additionally, intravenous morphine administration (0.2–0.4 mg/kg) increased BGLuM in the nucleus accumbens of rats (Orzi *et al.*, 1996). However, continuous morphine exposure by morphine pellets (225 mg over 7 days) reduced BGLuM in the striatum of rats (Wooten *et al.*, 1982). Although previous studies reported altered BGLuM following passive administration of morphine, the effects of IV morphine self-administration (MSA) on BGLuM of rodents have not been reported.

### Small animal FDG-PET/CT

Positron emission tomography (PET), which quantifies the *in vivo* distribution of radiolabeled compounds in the body, has been used extensively to study how drugs of abuse affect cerebral energy utilization in humans (Volkow *et al.*, 2003). Over the years, there has been an effort to improve the capabilities of PET imaging for small animal studies, which would allow researchers to control for many confounding variables that are unavoidable in clinical studies (Matsumura *et al.*, 2003; Jagoda *et al.*, 2004; Shimoji *et al.*, 2004; Riemann *et al.*, 2008; Dalley *et al.*, 2009; Casteels *et al.*, 2013). One emerging point of concern in preclinical [<sup>18</sup>F]-fluoro-deoxyglucose (FDG)-PET studies is the use of anesthesia during the PET scan. It has been reported that anesthesia can significantly affect the BGLuM of rodents (Alkire *et al.*, 1997; Shimoji *et al.*, 2004; Mizuma *et al.*, 2010; Prieto *et al.*, 2011; Spangler-Bickell *et al.*, 2016). These studies found that isoflurane anesthesia generally reduces BGLuM in cortical regions, but its effect on sub-cortical regions is unclear. Thus, it is important to compare the effects of isoflurane anesthesia on BGLuM in the cortical and sub-cortical regions of animals.

### Rationale of the study

Although previous 2-DG studies reported altered BGLuM with passive administration of morphine, the effects of voluntary morphine intake on *in vivo* BGLuM have not been reported. Furthermore, it is not clear whether different FDG uptake conditions (anesthesia vs. awake) may influence the pattern of BGLuM in morphine self-administered animals. We chose a 2-day withdrawal time point because we were interested in chronic rather than acute effects of morphine and to avoid any potential interaction between residual morphine and isoflurane anesthesia during the PET/CT scan. Therefore, the main goal of the study was to investigate the effects of (i) isoflurane anesthesia during the FDG uptake period and (ii) 2 day withdrawal from chronic MSA on *in vivo* BGLuM of rats. We hypothesized that spontaneous withdrawal from chronic MSA may increase BGLuM in the regions of the mesolimbic dopamine pathway.

## Materials and methods

### Animals

Adult male Sprague-Dawley rats weighing between 250–275 g (7 weeks old) were obtained from Taconic Farms (German Town, NY, USA). The animals were housed two per standard rat cage (42.5 × 20.5 × 20 cm) on hardwood chip bedding (Pine-Dri) with free access to food (Harlan Teklad 4% Mouse/Rat Diet 7001) and water in a temperature controlled room with 40% humidity and a reversed 12 h light-dark cycle (lights off at 6:00 am). The experimental protocol and treatment of the animals were approved and conducted in full compliance with the USUHS Institutional Animal Care and Use Committee (IACUC).

### Catheter surgery

After 1 week of acclimation to the facilities, the animals were anesthetized with a cocktail of ketamine/xylazine (80 and 10 mg/kg, IP), and a small portion of the animal's back and neck was shaved. A catheter was threaded subcutaneously over the animal's shoulder from its back to the neck and inserted into the right jugular vein as described previously (Le *et al.*, 2014). In order to maintain catheter patency, it was flushed daily with a solution of sterile saline, heparin (10 USP units/mL), and gentamycin (1 mg/mL). Afterwards, all animals were given a 1-week recovery period during which they were single housed to maintain the integrity of the indwelling catheter.

### Morphine self-administration

Each animal was placed in an individual operant conditioning chamber (Med Associates Inc., St. Albans, VT, USA) and connected to a Razel Model A infusion pump (Stamford, CT, USA) with a 10 mL glass syringe via a fluid swivel and Teflon tubing. Each chamber was equipped with two levers (a drug-paired and an inactive), a cue light, house light, and two infrared photobeams, which quantified the locomotor activity of the animals (Med Associates Inc., St. Albans, VT, USA). The cue light turned on when the rat pressed the drug-paired lever, followed by both lights turning off signifying a 15 s time-out period in which the drug was not available. The inactive lever did not have any programmed consequences. This setup allowed the animals to self-administer morphine (0.5 mg/kg/infusion, 0.1 mL across 5 s) on a one lever press/injection (Fixed Ratio 1) reinforcement schedule in a daily 4 h session (5 days per week) for 12 days. The animals were tested between 8:00 am and 12:00 pm during the weekdays. The maximum number of morphine infusions was set at 30 (15 mg/kg) to prevent any accidental overdose of morphine. The number of active lever and inactive lever presses, infusions, and locomotor activity were recorded, and after the daily sessions, the number of fecal boli in the chambers was counted. Morphine sulfate was obtained from Medisca Inc. (Plattsburgh, NY) and dissolved in 0.9% sterile saline. All drug doses were expressed as the weight of the salt.

### PET/CT imaging

Two FDG-PET/CT scans were obtained for each animal at a baseline and at 2 day withdrawal from the last self-administration. PET/CT images were acquired using an Inveon multimodality preclinical scanner (Siemens Medical Solutions, Malvern, PA, USA) in the small animal PET/CT facility of the Translational Imaging Core, Center for Neuroscience and Regenerative Medicine (CNRM) as

described previously (Brabazon *et al.*, 2016; Selwyn *et al.*, 2016). Animals were anesthetized with isoflurane (4% induction; 1.5–2% maintenance) and injected intravenously with 1–2 mCi (37–74 MBq) FDG. After the IV injection of FDG, one batch of animals (MSA: 8 and SSA: 6) was anesthetized with isoflurane (Experiment 1) and the other (MSA: 8 and SSA: 8) kept awake (Experiment 2) during the FDG uptake period (45 min). For Experiment 2, the animals were kept awake in their home cages (single housed) in a quiet room adjacent to the room with PET/CT scanners. They were undisturbed and exhibited minimum movement in the cages during the FDG uptake. After FDG uptake, all animals were anesthetized with isoflurane to perform a PET/CT scan (30 min). Physiologic monitoring included measurements of temperature, respiration rate, heart rate, and oxygen saturation. A three-bed CT scan was acquired for attenuation correction and anatomical localization (80 kVp, 500  $\mu$ As, 420 msec, 195–220° rotation in 120–220 steps). CT data were reconstructed in real time using a modified Feldkamp algorithm (bilinear interpolation, Shepp-Logan filter) and corrected for beam hardening. The CT image dimensions were 384  $\times$  384  $\times$  594 with a voxel size of 0.22 mm isotropic.

PET data were acquired with a coincidence-timing window (Dt) of 3.432 ns and energy window (De) of 350–650 keV in list mode for 30 min following the 45-min uptake period. PET sinograms were reconstructed as a single, high resolution static frame using a 3D-OSEM/MAP algorithm (2 OSEM iterations, 18 MAP iterations, requested resolution: 0.5 mm) with scatter, attenuation, and decay corrections applied. The intrinsic resolution of the PET scanner is  $\sim$ 1.4 mm full width at half maximum (FWHM) at the center of the field of view. PET image dimensions were 256  $\times$  256  $\times$  159 with a voxel size of 0.39  $\times$  0.39  $\times$  0.80 mm.

#### Volume of interest (VOI) analysis

Image processing and analysis of the FDG-PET data were performed using VivoQuant software (version 2.1, inviCRO; LLC Boston, MA, USA). FDG-PET data was resampled to match CT voxel size (0.22 mm isotropic) and dimensions (384  $\times$  384  $\times$  594). The PET data was converted to units of activity ( $\mu$ Ci) and registered to the CT image (six parameter, rigid-fast). Coregistered PET/CT images were uniformly cropped to a region surrounding the brain (170  $\times$  170  $\times$  240), which were manually reoriented ( $x$ ,  $y$ ,  $z$  rotation) and automatically registered to a 13-region rat brain atlas using an algorithm that combines a rigid transformation of the data and scaling of the atlas. The 13 regions include basal ganglia, thalamus, amygdala, cerebellum, cortex, hypothalamus, midbrain, corpus callosum, olfactory, hippocampus, septal area, white matter, and other (ventricles). All images were reviewed for quality assurance. The uptake concentration for each VOI was normalized to the uptake concentration of the entire atlas (whole-brain normalization) for inter-subject comparison. A further analysis of the basal ganglia including the nucleus accumbens, caudate putamen, globus pallidus, and stria terminalis was performed using the brain atlas with 54 regions (VivoQuant, version 2.1; inviCRO, LLC Boston) as shown in Fig. S2.

#### Voxel-based analysis (VBA)

The VBA was performed using the statistical parametric mapping (SPM12) and the Small Animal Molecular Imaging Toolbox (SAMIT) (Wellcome Department of Cognitive Neurology, University College London, UK) (Garcia *et al.*, 2015). Initial preprocessing (reorientation, registration, resampling, and cropping) of PET

data was performed in VivoQuant (ver 2.1; inviCRO). The toolbox was used to create a study-specific FDG-PET template in Paxinos space from baseline PET data. The template was evaluated in terms of registration error and mean uptake. Spatial normalization of the PET data for all time points to the template was performed using an affine registration algorithm. The standard uptake value (SUV) in each image was normalized to the mean uptake of the whole brain (SUV<sub>w</sub>). Normalized PET images were masked to remove extra-cerebral signal and smoothed with a 1.2 mm isotropic Gaussian kernel. For VBA, a flexible factorial design was used to explore the mean effect of anesthesia and time post treatment. A two-sample *t*-test analysis was used for group comparisons (awake vs. anesthetized uptake and MSA vs. SSA). For the interpretation of statistical differences, T-map data was interrogated at  $P = 0.001$  (uncorrected) and clusters were defined with a threshold of  $k = 200$  voxels. Only clusters with  $P < 0.05$  corrected for family wise error (FWE) were considered significant. Brain regions were assigned to suprathreshold cluster coordinates (medial-lateral, anterior-posterior, dorsal-ventral) using the rat brain atlas (Paxinos and Watson, 4th Edition, Academic Press, 1998).

#### Withdrawal signs and addiction-like behavior following MSA

The number of fecal boli in the home cages was counted in the morning of self-administration day 12, and withdrawal day 1 and 2. Each animal was also observed in its home cage for morphine withdrawal signs such as wet dog shakes, piloerection, ptosis, diarrhea, teeth chatter, and salivation as described previously (Cobuzzi & Riley, 2011). In withdrawal day 2, drug seeking behavior was measured in the self-administration chambers by allowing animals to press levers for 2 h (8:00–10:00) while morphine was not available (extinction condition). Open field activity (OFA) following a bolus morphine (0.5 mg/kg, IV) was measured using the Omnitech Electronics Digiscan infrared photocell system (Omnitech Electronics, Columbus, OH), located in a dedicated room. Animals were individually placed in the clear Plexiglas boxes (40  $\times$  40  $\times$  30 cm). A photocell array measured horizontal locomotor activity using 16 pairs of infrared photocells located front-to-back in a plane 2 cm above the floor of the arena. Data were automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. Animals were habituated to the OFA boxes for 60 min and spontaneous locomotor activity was monitored following IV saline (0.2 mL) and morphine (0.5 mg/kg) administration with a 60-min interval (12:00–15:00).

#### Statistical analysis

All data were analyzed using SPSS (ver 21, Chicago, IL, USA) and GRAPHPAD PRISM (ver 7.0, GraphPad Software, Inc.) with  $P$  values  $< 0.05$  considered significant. Self-administration behavioral data were analyzed with two-way analysis of variance (ANOVA) with morphine and day as factors. FDG-PET data were analyzed with two-way ANOVA with morphine and brain region as factors. Significant interaction or main effect was followed up by Holm-Sidak's multiple comparisons tests to reveal statistically significant differences between the groups. Defecation during the self-administration and piloerection during the withdrawal were analyzed with a Mann-Whitney *U* test because those data were not normally distributed by D'Agostino & Pearson normality test.

## Results

### IV Morphine self-administration

An overall experimental design is illustrated in Fig. 1A. After the IV injection of FDG, one batch of animals was isoflurane anesthetized (Experiment 1) and another batch kept awake (Experiment 2) during the FDG uptake period (45 min). After FDG uptake, all animals were anesthetized with isoflurane in order to perform a PET/CT scan (30 min). A third batch of animals was used to collect behavioral data only during spontaneous withdrawal from self-administration (Experiment 3). The self-administration data between experiments 1 and 2 were comparable, and so the data was combined and presented as the MSA and SSA groups in Fig. 1. The MSA group maintained a stable morphine intake pattern across the 12-day period with an average amount of morphine intake of 6.75 mg/kg on the last day of self-administration. The number of drug-paired lever presses was significantly higher in the MSA group as compared to the SSA group (Fig. 1B). A two-way ANOVA indicated a significant interaction between morphine and day ( $F_{11,336} = 3.01$ ,  $P = 0.001$ ), and main effect of morphine ( $F_{1,336} = 74.26$ ,  $P < 0.0001$ ). *Post hoc* tests revealed that self-administration day 3, 4, 5, 7, 8, 9, 10, 11, and 12 were significantly different between the MSA and SSA groups ( $P < 0.05$ ). Moreover, locomotor activity levels during the self-administration sessions were significantly higher in the MSA group as compared to those of the SSA group (Fig. 1C). A two-way ANOVA indicated significant main effects of morphine ( $F_{1,336} = 307.1$ ,  $P < 0.0001$ ) and day ( $F_{11,336} = 2.11$ ,  $P = 0.019$ ). *Post hoc* tests revealed that all self-administration days were significantly different between the MSA and SSA groups ( $P < 0.05$ ). Morphine self-administered animals exhibited constipation during the daily sessions. The number of

fecal boli was smaller in the MSA group as compared to that of the SSA group (Fig. 1D). A Mann-Whitney  $U$  test revealed a significant effect of morphine on defecation ( $U = 0$ ,  $P < 0.0001$ ). Interestingly, there was no sign of tolerance to this morphine-induced constipation over the 12 days of self-administration. Taken together, these results indicate that the animals actively self-administered IV morphine and experienced behavioral and physiological changes over the 12 day period.

### FDG-PET/CT imaging

FDG-PET and CT images were analyzed with a three-dimensional rat brain atlas (VivoQuant ver.21) for the VOI analysis. A representative FDG-PET/CT image of a rat with an indwelling catheter implanted in the jugular vein is shown in Fig. 2A. High FDG uptake levels are prominent in the harderian gland of the eye and the heart of the rat. Representative images of FDG-PET and CT of the head (sagittal, horizontal and coronal sections) before (Fig. 2B) and after the brain atlas registration (Fig. 2C) are shown. The PET/CT images were co-registered to the rat brain atlas using the VIVOQUANT software. The major brain regions quantified using the brain atlas method are shown in Fig. 2D. These regions include 1: Olfactory, 2: Cortex, 3: Basal ganglia, 4: Septal area, 5: Corpus Callosum, 6: Hypothalamus, 7: Thalamus, 8: Amygdala, 9: Hippocampus, 10: Midbrain, and 11: Cerebellum.

### Isoflurane anesthesia on BGLuM

Isoflurane anesthesia, as compared to the awake condition, induced dramatic effects on the BGLuM of rats (Fig. 3A). A two-way ANOVA indicated a significant interaction between anesthesia and brain

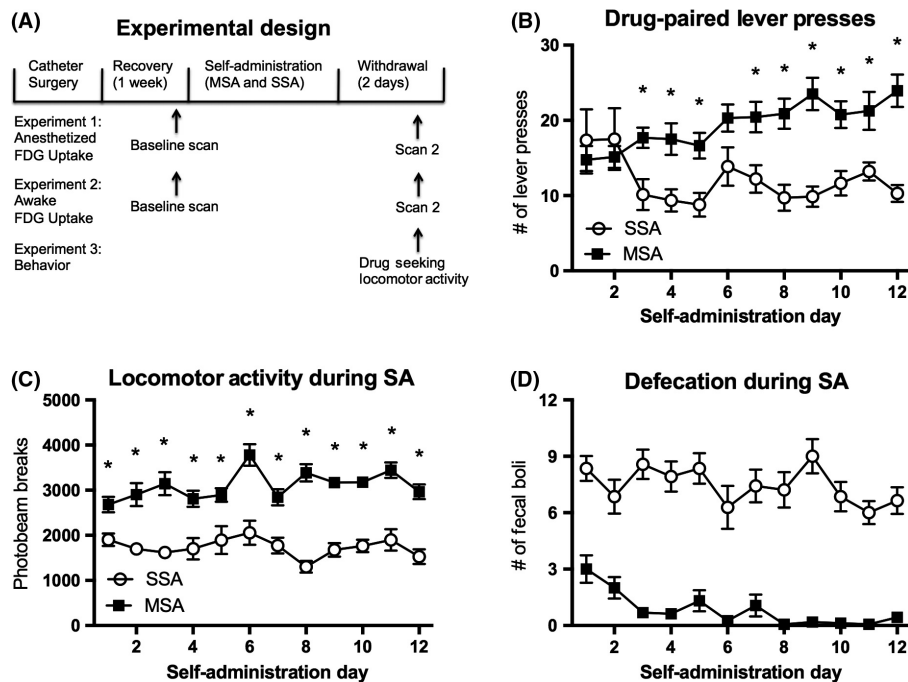


FIG. 1. Chronic intravenous morphine self-administration in rats (0.5 mg/kg, 4 h/day). (A) An experimental design. In experiment 1, animals were anesthetized with isoflurane during the FDG uptake (45 min) and PET/CT scan (30 min). In experiment 2, animals were kept awake during the FDG uptake (45 min) and then anesthetized with isoflurane during the PET/CT scan (30 min). In experiment 3, animals were tested for behaviors in 2 day withdrawal from self-administration. (B) The number of drug-paired lever presses during the daily self-administration sessions. (C) Locomotor activity levels during the daily self-administration sessions. (D) The number of fecal boli produced during the daily self-administration sessions. SSA: saline self-administration ( $n = 14$ ), MSA: morphine self-administration ( $n = 16$ ). Data are presented as MEAN  $\pm$  SEM. \*significantly different between the MSA and the SSA ( $P < 0.05$ ).

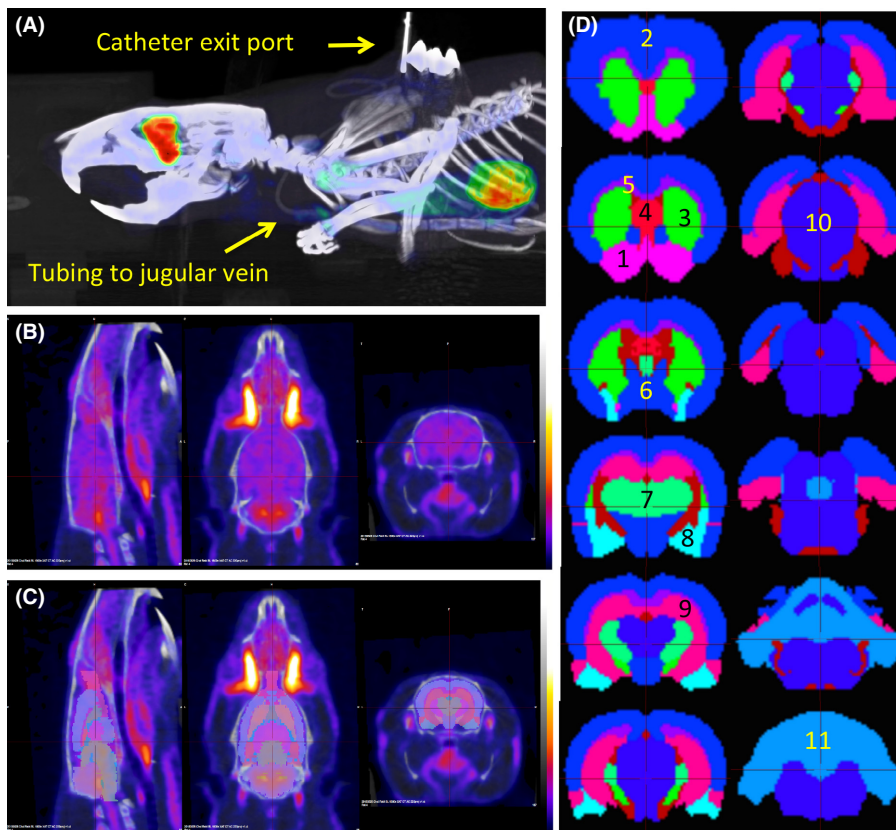


FIG. 2. A volume of interest analysis of FDG-PET/CT using the rat brain atlas (VivoQuant ver 2.1). (A) Representative FDG-PET/CT images of a rat with an indwelling catheter implanted in the jugular vein. (B) Representative FDG-PET/CT images of a rat brain (sagittal, horizontal, and coronal sections). (C) Representative FDG-PET/CT images registered to the rat brain atlas for the VOI analysis (VivoQuant ver 2.1). (D) Major brain regions quantified with the rat brain atlas. 1: olfactory, 2: cortex, 3: basal ganglia, 4: septal area, 5: corpus callosum, 6: hypothalamus, 7: thalamus, 8: amygdala, 9: hippocampus, 10: midbrain, 11: cerebellum.

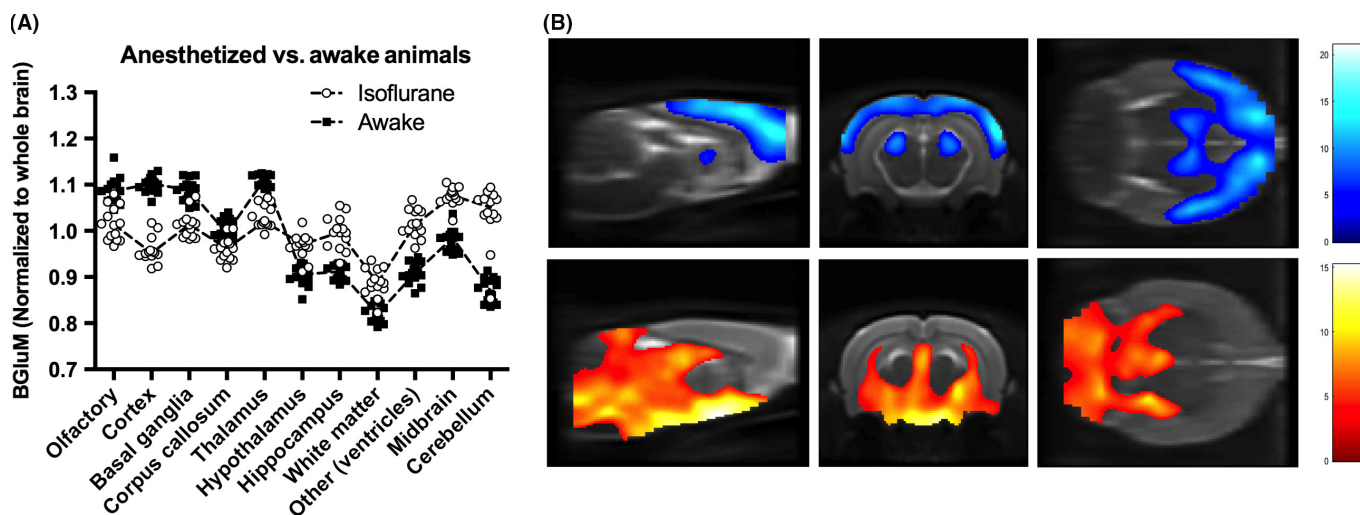


FIG. 3. Effects of isoflurane anesthesia on BGLuM of rats. (A) Regions with significant differences in BGLuM between the isoflurane anesthesia and the awake conditions. The BGLuM is lower in the olfactory, cortex, basal ganglia, corpus callosum, and the thalamus, while higher in the hypothalamus, hippocampus, white matter, midbrain, and the cerebellum in the isoflurane anesthesia condition. The median of each group is indicated by a dotted line. (B) Representative SPM images of BGLuM between the animals kept under isoflurane anesthesia or awake during the FDG uptake period. A single slice is shown in all three imaging planes (sagittal, coronal, and horizontal). SPM T-map data is depicted using the Mango software (<http://ric.uthscsa.edu/mango/index.html>) as a multi-slice coronal MRI T2 template (Schwarz *et al.*, 2006). The color bar has been set to Min: 0 Max: 15 for increases in glucose uptake and Min: 0 Max: 20 for decreases in glucose uptake for anesthetized subjects ( $n = 14$ ) as compared to awake subjects ( $n = 16$ ).

region ( $F_{12,364} = 73.61$ ,  $P < 0.0001$ ) and main effects of brain region ( $F_{12,364} = 150.5$ ,  $P < 0.0001$ ) and anesthesia ( $F_{1,364} = 32.05$ ,  $P < 0.0001$ ). *Post hoc* tests revealed that isoflurane anesthesia reduced BGLuM in the olfactory, cortex, basal ganglia, corpus callosum, and thalamus, while increasing BGLuM in the hypothalamus, hippocampus, white matter, other (ventricles), midbrain, and cerebellum ( $P < 0.05$ ). Representative images of the voxel-based SPM analysis confirmed reduced BGLuM in major cortical regions (upper panel of Fig. 3B) and increased BGLuM in major sub-cortical regions (lower panel of Fig. 3B) in isoflurane anesthesia as compared to the awake condition. A serial coronal slices from the SPM analysis is shown in Fig. S1.

Consistent with the brain atlas-based VOI analysis, the voxel-based SPM analysis revealed robust differences between the isoflurane anesthesia and awake conditions. Table 1 shows that isoflurane anesthesia reduced BGLuM in cortical regions and increased BGLuM in the hypothalamus and brainstem. These regions were identified based on the coordinates from the rat brain atlas (Paxinos & Watson, 1998).

#### Withdrawal from chronic MSA on BGLuM

The effects of 2 day withdrawal from chronic MSA on BGLuM were investigated in the isoflurane anesthesia and the awake condition. A two-way ANOVA indicated significant main effects of morphine ( $F_{1,156} = 8.29$ ,  $P = 0.005$ ) and brain region ( $F_{12,156} = 5.35$ ,  $P < 0.0001$ ) in the isoflurane anesthetized condition (Fig. 4A and B). *Post hoc* tests revealed that MSA increased BGLuM in the basal ganglia and corpus callosum as compared to the SSA group ( $P < 0.05$ ), shown in Fig. 4A. A further analysis of the sub-regions of basal ganglia using the brain atlas with 54 regions (Fig. S2) indicated that the caudate putamen and the nucleus accumbens were the significant regions between the MSA and SSA groups ( $P < 0.05$ ). This indicates that 2 day withdrawal from chronic MSA selectively increased BGLuM in the striatum of rats. However, in the awake FDG uptake condition, none of the regions were significant between the MSA and SSA groups (Fig. 4C and D). A two-way ANOVA indicated significant main effect of brain region ( $F_{12,182} = 13.58$ ,  $P < 0.0001$ ), but not morphine ( $F_{1,182} = 3.27$ ,  $P > 0.05$ ).

#### Withdrawal signs and addiction-like behaviors

In the current study, morphine self-administered animals did not exhibit severe signs of morphine withdrawal except piloerection and increased defecation observed in the 2 day withdrawal. The MSA group produced significantly greater number of feces on withdrawal day 2 as compared to previous MSA day 12 and

withdrawal day 1 (Fig. 5A), indicating a rebound effect from morphine-induced constipation during the self-administration period. A one-way ANOVA on defecation indicated a significant effect of day ( $F_{1,539,10,77} = 31.01$ ,  $P < 0.0001$ ). Newman-Keuls *post hoc* tests revealed that withdrawal day 2 was significantly different from both MSA day 12 and withdrawal day 1 ( $P < 0.05$ ). The number of daily feces in the SSA group was not significantly different between these days. The MSA group exhibited piloerection as compared to the SSA group when observed on withdrawal day 2 (Fig. 5B). A Mann-Whitney *U* test indicated significant effects of morphine on piloerection ( $U = 4$ ,  $P = 0.001$ ). Despite mild withdrawal signs, morphine self-administered animals showed robust addiction-like behaviors when tested on withdrawal day 2. The MSA group showed drug seeking as compared to the SSA group under an extinction condition (Fig. 5C). A two-way ANOVA indicated a significant interaction between morphine and lever presses ( $F_{1,28} = 18.39$ ,  $P < 0.001$ ), and main effects of morphine ( $F_{1,28} = 31.76$ ,  $P < 0.0001$ ) and lever presses ( $F_{1,28} = 18.01$ ,  $P < 0.001$ ). *Post hoc* tests revealed significant differences between the MSA and SSA groups on drug-paired lever presses. The MSA group also exhibited increased locomotor activity to a bolus morphine (0.5 mg/kg, IV) as compared to that of the SSA group (Fig. 5D). A two-way ANOVA indicated a significant interaction between morphine and dose ( $F_{1,28} = 14.58$ ,  $P < 0.001$ ), and main effects of morphine ( $F_{1,28} = 27.88$ ,  $P < 0.0001$ ) and dose ( $F_{1,28} = 12.43$ ,  $P = 0.002$ ). *Post hoc* tests revealed significant differences between the MSA and SSA groups on morphine (0.5 mg/kg). These results indicate that morphine self-administered animals exhibited mild withdrawal symptoms and robust addiction-like behaviors in the 2 day withdrawal from MSA.

#### Discussion

The current study presents two main findings: (i) isoflurane anesthesia, as compared to the awake condition, induced opposite effects on the BGLuM between cortical and sub-cortical regions of rats, and (ii) spontaneous withdrawal from MSA increased BGLuM in the striatum of rats, under the isoflurane anesthesia condition. To our knowledge, this is the first study reporting the effects of chronic MSA on BGLuM of rodents using FDG-PET.

During the 12-day period of self-administration (4 h/day), the animals maintained a stable level of morphine intake with an average intake of 6.75 mg/kg on the last day of self-administration. The animals experienced spontaneous withdrawal (20 h) in their home cages following 4 h of daily MSA. This intermittent and limited access to IV morphine induced robust locomotor hyperactivity and constipation during the self-administration sessions as previously reported (Lee

TABLE 1. The voxel-based SPM analysis of glucose uptake between the isoflurane anesthetized and the awake  $^{18}\text{F}$ -FDG uptake conditions

| Cluster level    |        | Peak level       |       | Paxinos coordinates |       |      | Region                     | Change    |
|------------------|--------|------------------|-------|---------------------|-------|------|----------------------------|-----------|
| $P_{\text{FWE}}$ | $k_E$  | $P_{\text{FWE}}$ | $T$   | ML                  | AP    | DV   |                            |           |
| < 0.0001         | 84 846 | < 0.0001         | 21.02 | -4.9                | 0.4   | -3.0 | S1DZ, Sensory Cortex       | Decreased |
|                  |        | < 0.0001         | 17.09 | 4.5                 | 1.4   | -2.4 | S1j, Sensory Cortex        | Decreased |
|                  |        | < 0.0001         | 8.78  | -2.7                | -7.4  | -1.2 | VIM, Primary Visual Cortex | Decreased |
| < 0.0001         | 83 835 | < 0.0001         | 5.21  | -0.1                | -1.6  | -9.2 | AHC, Anterior Hypothalamus | Increased |
|                  |        | < 0.0001         | 12.41 | -3.7                | -11.2 | -7.8 | Icp, Brainstem             | Increased |
|                  |        | < 0.0001         | 11.80 | 4.7                 | -10.2 | -9.0 | I8, Brainstem              | Increased |

This table summarizes the clusters ( $k_E > 200$ ) with voxels that are significant at  $P < 0.001$  (uncorrected at voxel level). Height threshold:  $T = 3.42$ ,  $P = 0.001$ ,  $P_{\text{FWE}}$ , Family wise error; ML, medial-lateral; AP, anterior-posterior; DV, dorsal-ventral.



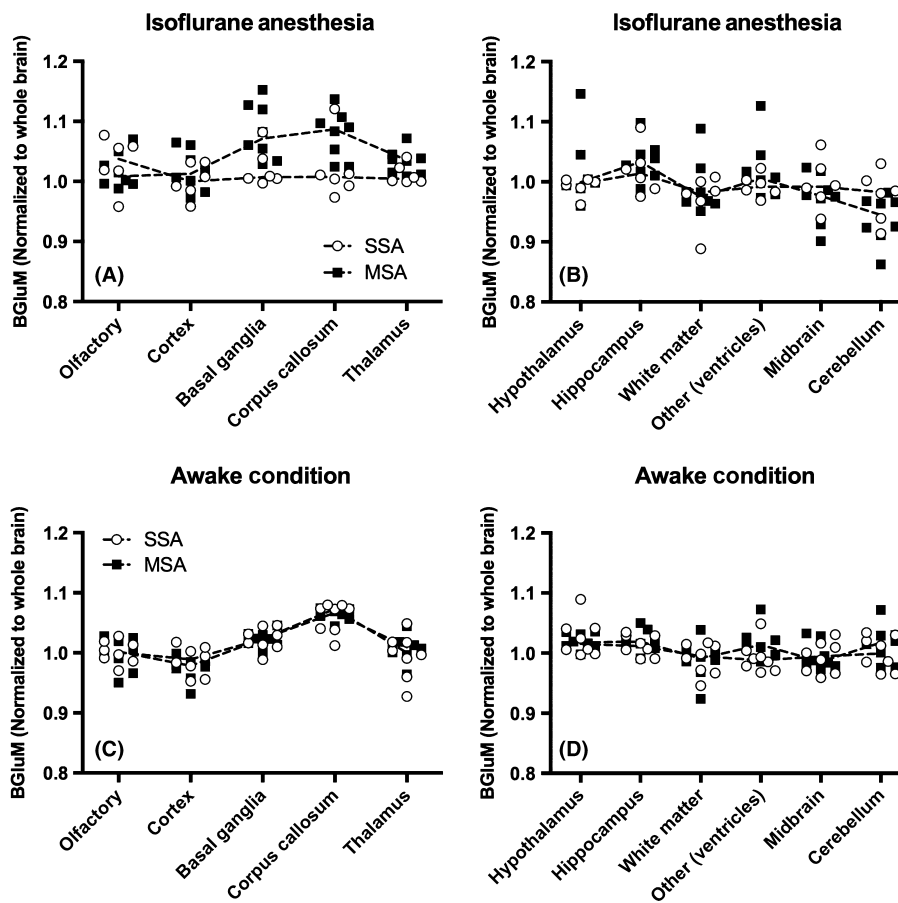


FIG. 4. Effects of two day withdrawal from MSA on BGLuM of rats. (A) Increased BGLuM in the basal ganglia and corpus callosum in the MSA group as compared to those of the SSA group in isoflurane anesthesia. (B) No effects of MSA on BGLuM in sub-cortical regions in isoflurane anesthesia. (C) No effects of MSA on BGLuM in cortical regions in the awake condition. (D) No effects of MSA on BGLuM in sub-cortical regions in the awake condition. The median of each group is indicated by a dotted line. Isoflurane anesthesia (MSA: 8 and SSA: 6) and awake condition (MSA: 8 and SSA: 8).

*et al.*, 2016). The morphine intake pattern also corresponds with a previous study suggesting that the second week of drug self-administration may be relevant to a transition from casual use to drug addiction in humans (Ahmed & Koob, 2005). During 2-day withdrawal from self-administration, the MSA group exhibited mild withdrawal signs (increased defecation and piloerection) and robust addiction-like behaviors (drug seeking and locomotor stimulation to morphine). In the current study, severe withdrawal signs such as wet dog shakes, teeth chattering, and diarrhea were not evident in the morphine self-administered animals. This may be due to the nature of the IV drug self-administration paradigm, which allows each animal to regulate its own drug intake during the limited amount of time. Thus, the animals are likely to experience the reinforcing effects of morphine rather than its aversive and stressful effects by self-regulating morphine intake. This was evident because the animals showed robust drug seeking and locomotor stimulation to a bolus morphine despite the lack of severe withdrawal signs during 2 day withdrawal. Taken together, these results indicate that the animals actively self-administered intermittent morphine and exhibited behavioral and physiological changes over the self-administration period.

It has been well established that the choice of anesthesia can have a significant impact on BGLuM in small animal FDG-PET studies. In the current study, isoflurane was used because it allows for better control over the length and depth of the anesthesia than injectable anesthetics such as barbiturates, ketamine, and medetomidine. Isoflurane is the most commonly used inhalation anesthesia with good

bioavailability and fast recovery from anesthesia for preclinical PET studies [for a review see (Hildebrandt *et al.*, 2008; Alstrup & Smith, 2013)]. However, because isoflurane anesthesia is known to suppress overall BGLuM in many brain regions, it is necessary to compare its effects with the awake condition. The current study found that isoflurane anesthesia during the FDG uptake (45 min) decreased BGLuM in the olfactory, cortex, basal ganglia, and thalamus which are mainly cortical areas of the brain. This appears to be a general characteristic of isoflurane anesthesia, which has been reported when comparing isoflurane anesthetized and conscious rats with FDG-PET (Shimoji *et al.*, 2004). Reduced BGLuM in the basal ganglia and thalamus are consistent with a previous FDG-PET study that reported significantly reduced glucose metabolism in the cortex, striatum, and thalamus of mice under isoflurane anesthesia (Mizuma *et al.*, 2010). Thus, these findings suggest that isoflurane anesthesia reduces the neuronal activity of specific brain regions involved in sensory and motor function, which are not as vital to maintain in the unconscious state.

Interestingly, the current study also found that isoflurane anesthesia increased BGLuM in sub-cortical regions such as the hypothalamus, hippocampus, white matter, and midbrain. These regions are generally related to learning and memory (hippocampus), and necessary for basic physiological functions such as the breathing and thermoregulatory control (midbrain and hypothalamus). A 2-DG autoradiography study investigated the effects of isoflurane on the BGLuM and found widespread decreases in the majority of regions

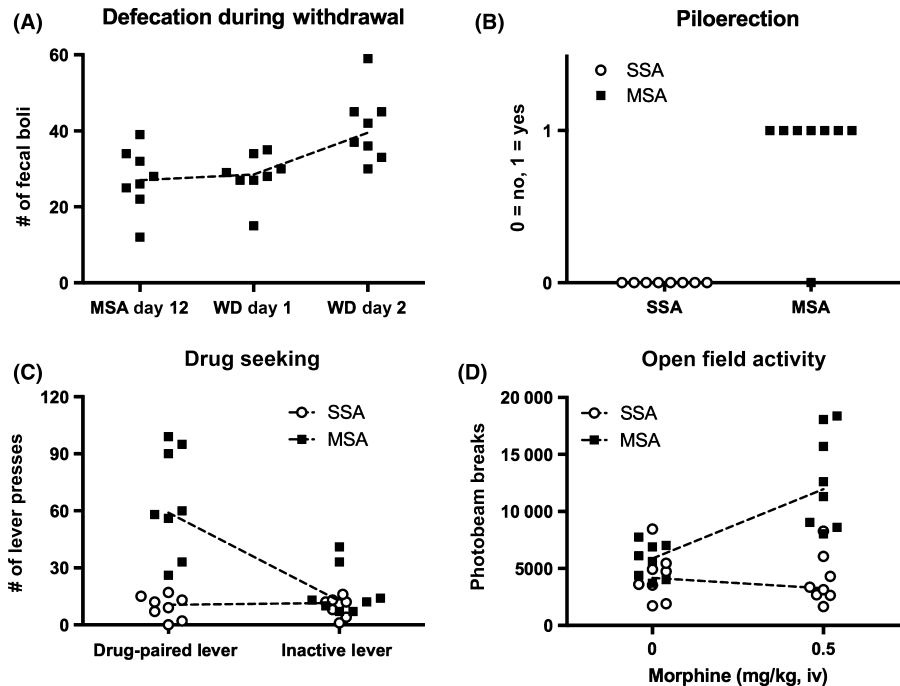


FIG. 5. Withdrawal signs and addiction-like behaviors in two day withdrawal from MSA. (A) Increased defecation on withdrawal day 2 as compared to MSA day 12 and withdrawal day 1 in morphine self-administered animals. (B) Piloerection in the MSA group as compared to the SSA group observed on withdrawal day 2. (C) Robust drug seeking behavior (active lever presses while morphine is not available) in the MSA group as compared to that of the SSA group. (D) Increased morphine-induced locomotor activity (0.5 mg/kg, IV) in the MSA group as compared to that of the SSA group. The median of each group is indicated by a dotted line. MSA:  $n = 8$  and SSA:  $n = 8$ .

(Maekawa *et al.*, 1986). However, several sub-cortical regions such as the hippocampus, interpeduncular nucleus, and substantia nigra either resisted anesthetic depression or increased metabolic rate with an increasing dosage of isoflurane. Moreover, another 2-DG study found that BGLuM was decreased in the cortex and thalamus, while increased in the midbrain, hippocampus, and interpeduncular nucleus of rats (Ori *et al.*, 1986). A recent FDG-PET study reported that the delivery of odorants during awake FDG uptake increased BGLuM in the posterior lobe of the cerebellum of rats (Litaudon *et al.*, 2017). This is interesting because the current study found that isoflurane anesthesia increased BGLuM in the anterior lobe of the cerebellum. Thus, it is likely that different parts of the cerebellum are active depending on the states such as under sensory stimulation or anesthesia. These findings on the effects of isoflurane anesthesia in sub-cortical regions parallel the results of electrophysiological studies, which point to the possibility of elevation, not depression, of cerebral glucose metabolism in sub-cortical regions, such as the mid-brain and hippocampus (MacIver & Roth, 1988; Becker *et al.*, 2012). Therefore, the choice of the FDG uptake condition (anesthesia vs. awake condition) may be critical in small animal FDG-PET studies because higher or lower basal levels of BGLuM may confound the biological effects that researchers are interested in.

Currently, there is a lack of information on how voluntary use of opiates and its withdrawal affects *in vivo* BGLuM in a controlled setting. Thus, the current study investigated the effects of chronic MSA on BGLuM using FDG-PET. The BGLuM was increased in the basal ganglia of MSA animals as compared to SSA animals: observed under the isoflurane anesthesia, not in the awake condition. It is likely that basal BGLuM levels in the basal ganglia were higher in the awake condition as compared to the isoflurane anesthesia, making it more difficult to detect MSA-induced increased BGLuM in the awake condition (ceiling effects). Therefore, it is important to

determine the FDG uptake condition (awake vs. anesthetized) in small animal PET studies based on the regions of interest and the hypothesis of the study.

Previous *ex vivo* 2-DG autoradiography studies reported increased BGLuM in the striatum of rodents following an acute morphine injection (15 mg/kg, IP) (Quelven *et al.*, 2004) or IV morphine bolus administration (0.2–0.4 mg/kg) (Orzi *et al.*, 1996). However, high doses of continuous morphine exposure by morphine pellets failed to alter BGLuM in the striatum of rats (Wooten *et al.*, 1982). The current FDG-PET study also found increased BGLuM in the striatum of morphine self-administered animals. The importance of the striatum in opiate addiction has been demonstrated previously. For instance, electrical lesions in the striatum selectively reduced intravenous morphine self-administration in rodents (Glick *et al.*, 1975). Another study showed that turnover rates of several neurotransmitters including dopamine, serotonin, noradrenaline, glutamate, and GABA in the striatum were correlated with morphine self-administration in rats (Smith *et al.*, 1980). A recent study demonstrated that the regulator of G-protein signaling 7 (RGS7) protein regulates opiate reward by controlling mu-opioid receptors in the striatum of mice (Sutton *et al.*, 2016). The authors reported that RGS7 in striatal neurons was selectively responsible for determining the sensitivity of the reinforcing effects of morphine without affecting its other effects such as analgesia, tolerance, and withdrawal. Thus, the current study supports the previous findings suggesting that the striatum plays a crucial role in modulating the reinforcing effects of opiates.

## Conclusions

The current study found that (i) isoflurane anesthesia suppressed BGLuM in cortical regions while increasing BGLuM in sub-cortical

regions and (ii) 2 day withdrawal from chronic MSA selectively increased BGluM in the striatum of rats. These findings are significant because the choice of the FDG uptake condition (anesthetized vs. awake) may be critical in detecting drug-induced changes of BGluM in preclinical PET studies. Moreover, increased neuronal activity in the striatum during 2 day withdrawal from MSA may be associated with craving and drug seeking in addicted individuals. The current study demonstrated the utility of combining an IV morphine self-administration paradigm with non-invasive PET/CT imaging to enhance our understanding of the *in vivo* brain mechanisms of opiate addiction.

## Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. Comparison of subjects at baseline (controls) with and without the influence of anesthesia during uptake period.

Fig. S2. A brain atlas analysis of basal ganglia regions including nucleus accumbens (NA), caudate putamen (CP), stria terminalis (ST), and globus pallidus (GP).

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## Conflict of interests

The authors declare no conflict of interest. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

## Authors contributions

T.P., A.H., R.S., B.D., and K.C. were responsible for the study concept and design. T.P., K.N., C.W., S.J., and J.S. contributed to the acquisition of animal data. T.P., C.W., A.H., R.S., B.D., and K.C. assisted with data analysis and interpretation of findings. T.P. and K.C. drafted the manuscript. K.N., C.W., and B.D. provided critical revision of the manuscript for important intellectual content.

## Data accessibility

The article's supporting data and materials can be accessed upon request to the corresponding author. Moreover the supporting information were uploaded on the journal website.

## Abbreviations

2-DG, 2-deoxyglucose; ANOVA, analysis of variance; BGluM, brain glucose metabolism; CT, Computed tomography; FDG, [<sup>18</sup>F]-fluorodeoxyglucose; FEW, family wise error; FWHM, full width at half maximum; IP, intraperitoneal; IV, intravenous; MSA, morphine self-administration; PET, positron emission tomography; RGS7, regulator of G-protein signaling 7; SAMIT, Small Animal Molecular Imaging Toolbox; SPM, statistical parametric mapping; SSA, saline self-administration; SUV, standard uptake value; USP, United States Pharmacopeia; VBA, voxel-based analysis.

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