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Imaging dopamine release with Positron Emission Tomography (PET) and ^{11}C-raclopride in freely moving animals

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We investigated an imaging strategy that provides simultaneous measurements of radiotracer binding and behavior in awake, freely moving animals. In this strategy, animals are injected intravenously (i.v.) through a catheterized line and permitted to move freely for 30 min during uptake of the imaging agent, in this case ^{11}C-raclopride. After this Awake Uptake period, animals are anesthetized and scanned for 25 min. We tested the utility of this strategy for measuring changes in striatal ^{11}C-raclopride binding under control conditions (awake and freely moving in the home cage) and with several drug challenges: a loading dose of unlabeled raclopride, pretreatment with methamphetamine (METH) or pretreatment with γ-vinyl-GABA [S(+)-GVG] followed by METH. An additional group of animals underwent a stress paradigm that we have previously shown increases brain dopamine. For drug challenge experiments, the change in ^{11}C-raclopride binding was compared to data from animals that were anesthetized for the uptake period ("Anesthetized Uptake") and full time activity curves were used to calculate ^{11}C-raclopride binding. Regardless of the drug treatment protocol, there was no difference in ^{11}C-raclopride striatum to cerebellum ratio between the Awake versus the Anesthetized Uptake conditions. Awake and Anesthetized groups demonstrated over 90% occupancy of dopamine receptors with a loading dose of cold raclopride, both groups demonstrated a ~30% reduction in ^{11}C-raclopride binding from METH pretreatment and this effect was modulated to the same degree by GVG under both uptake conditions. Restraint during Awake Uptake decreased ^{11}C-raclopride binding by 29%. These studies support a unique molecular imaging strategy in which radiotracer uptake occurs in freely moving animals, after which they are anesthetized and scanned. This imaging strategy extends the applicability of small animal PET to include functional neurotransmitter imaging and the neurochemical correlates of behavioral tasks.

Keywords: Positron Emission Tomography (PET); Awake; Methamphetamine; Stress; Dopamine; GABA; Gamma-vinyl GABA (GVG)

Introduction

Functional brain imaging with Positron emission tomography (PET) has opened up new avenues for the study of neurochemical activity in the brain. Initially, studies designed to measure competition between a radiotracer and an endogenous neurotransmitter focused on measuring the effects of specific pharmacologic challenges on radiotracer binding in primates and humans (Dewey et al., 1990, 1991, 1993, 1992). The underlying premise is that a specific pharmacological challenge changes the available synaptic concentrations of a neurotransmitter which then manifests as an increase or decrease in radiotracer receptor occupancy. More recently, this strategy has been expanded to include non-pharmacological challenges, where behavior-induced changes in neurotransmitter activity produce similar alterations in radiotracer receptor occupancy. A host of PET studies have shown changes in the binding of the dopamine D_2 radiotracer, ^{11}C-raclopride, reflective of increases in synaptic dopamine secondary to behaviors including video gaming (Koepp et al., 1998), monetary reward (Zald et al., 2004), cognitive learning (Badgaiyan et al., 2007), the smell and presentation of reinforcing foods (Volkow et al., 2003), and drug craving triggered by contextual cues (Volkow et al., 2006; Wong et al., 2006). Thus, PET imaging in humans has the potential to play a pivotal role in demonstrating objective biological measures that coincide with human behavior.

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At the same time, advances in imaging technology and instrumentation now permit PET studies of small animals, offering similar *in vivo* imaging capabilities in humans, mice and rats. Identical PET outcome measures (e.g. changes in radiotracer binding) provide a common platform to compare and contrast information between different species and also between animal models and human disease. However a fundamental limitation that continues to face the translation of these studies is that anesthesia or immobilization are required to keep animals in the tomograph during scanning. Humans are rarely if ever, scanned under anesthesia. Inasmuch as anesthesia adds a tremendous confound to the interpretation of biochemical data, it also greatly limits the scope of behaviors that can be measured. In turn, this limits exciting and powerful new information about the brain that small animal PET can provide.

Here we present an imaging paradigm in awake, freely moving animals that permits concurrent measurements of animal behavior and ^{11}C-raclopride binding. In this paradigm, freely moving

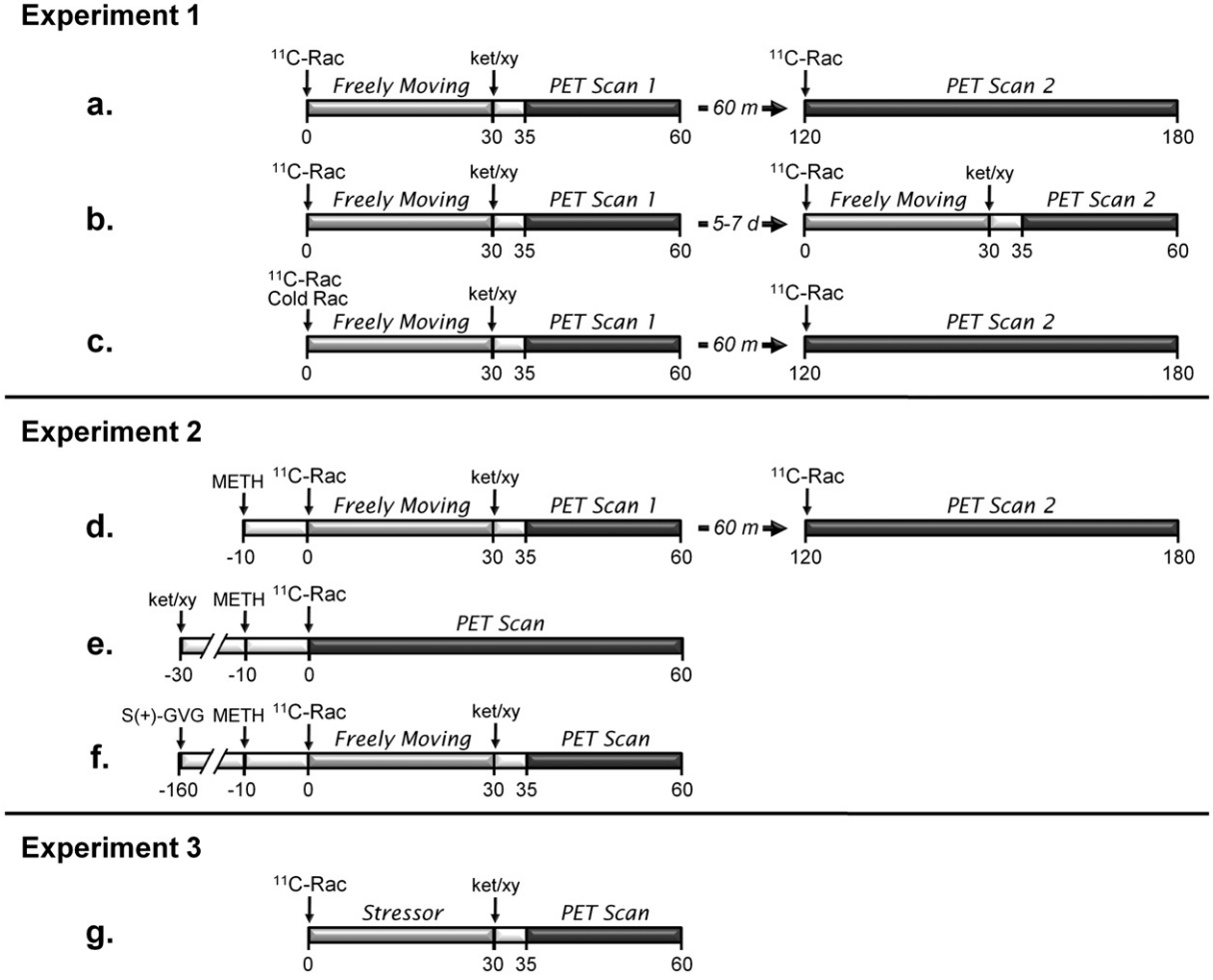

Fig. 1. Graphic depiction of ^{11}C-raclopride (^{11}C-rac) scanning protocols. Protocols are divided by Experiment. Time is depicted in five min intervals, where ^{11}C-raclopride was given at time 0 in all cases. Light grey shading indicates the duration of Awake Uptake and dark grey shading indicates PET acquisition under ketamine/xylazine anesthesia (ket/xy). Experiment 1 was designed to assess the reproducibility and range of ^{11}C-raclopride binding during Awake or Anesthetized Uptake, and three scanning protocols were implemented (a–c). In Experiment 1 (a), the Awake Paired: Control condition is depicted in which animals receive an intravenous injection of ^{11}C-raclopride 30 min prior to ket/xy anesthesia and scanning (PET Scan 1), paired with a second scan under anesthesia where the full time course of ^{11}C-raclopride in the brain could be obtained from the same animals (PET Scan 2; $n=5$). In Experiment 1 (b), the Awake Single: Control condition is depicted where the same animals received two single ^{11}C-raclopride scans using the Awake Uptake protocol separated by five to seven days ($n=6$). In Experiment 1 (c), the Awake Paired: Cold Raclopride protocol is depicted where unlabeled raclopride was co-injected with ^{11}C-raclopride and administered using the Awake Uptake protocol, paired with a second scan 60 min later under the Anesthetized Uptake protocol ($n=4$). Experiment 2 was designed to assess ^{11}C-raclopride displacement by endogenous dopamine during Awake or Anesthetized Uptake, and three protocols were implemented (d–f). In Experiment 2 (d), the Awake Paired: METH condition is depicted in which METH (5 mg/kg, i.p.) was administered to awake animals followed by anesthesia and scanning (PET Scan 1), paired sixty min later with a second scan using the Anesthetized Uptake protocol for PET Scan 2 ($n=4$). In Experiment 2 (e), a separate group of animals received anesthesia followed 20 min later by METH and a subsequent scan ($n=6$). For Experiment 2 (f), five animals were studied using only the Awake Uptake protocol, where each animal received a single administration of the active enantiomer of γ-vinyl-GABA (S(+)-GVG) 2.5 h prior to METH, given 10 min prior to ^{11}C-raclopride. Experiment 3 was designed to assess the effects of handling or restraint stress on ^{11}C- raclopride binding in the Awake Uptake condition. In Experiment 3 (g), a single scan using the Awake Uptake protocol is depicted where during the 30 min of Awake Uptake, animals underwent handling or restraint stress, followed by ket/xy anesthesia and scanning.

animals received the radiotracer through a previously placed jugular vein catheter in their home cage during which they had absolutely no restrictions on their range or degree of movement. Following 30 min of radiotracer uptake in their home cage, animals were anesthetized and scanned within the tomograph for 25 min (depicted in Fig. 1). To test this paradigm, we first characterized the range of responses that can be measured by using high mass, low specific activity injections and low mass, high specific activity injections. Next, we demonstrated that these measurements were reproducible in the same animal scanned on different days. Having established this range and variability, we performed a series of studies using pharmacological and behavioral challenges. Specifically, we demonstrated that this method reliably captures increases in synaptic dopamine (decreases in striatal ^{11}C-raclopride binding) following a methamphetamine (METH) challenge and that this increase can be attenuated by increasing GABA levels following administration of the active enantiomer of gamma vinyl-GABA [S (+)-GVG]. Finally, we demonstrated that handling-induced stress in awake animals also reduced ^{11}C-raclopride binding.

Taken together, these studies describe a reproducible and meaningful implementation of a novel imaging paradigm that can be used to merge small animal PET imaging with drug challenges and specific behaviors in awake, freely moving animals. As a result, this approach expands the use of small animal PET to a preclinical platform that simultaneously incorporates both neurochemical and behavioral aspects of animal models of human disease.

Materials and methods

Animals

Adult male Sprague–Dawley rats (330–450 g; Taconic Farms, Germantown, NY) were used under an IACUC-approved protocol and with strict adherence to the NIH guidelines. A total of 35 animals received a total of 54 PET scans, while 44 additional animals were used for ex vivo measurements of radiotracer metabolism and biodistribution. Each animal was housed individually on a 12/12 light/dark schedule in temperature controlled rooms. All animals underwent an external jugular vein catheterization using aseptic surgical techniques. Polyethylene tubing (PE-50, Becton Dickinson, MD) was exteriorized from the nape of the neck, providing a percutaneous port used postoperatively for daily flushes of the catheter and for administration of ^{11}C-raclopride to both awake and anesthetized animals. Tubing was flushed every other day with a 10% heparin/saline solution to maintain line patency. Where anesthesia was used, a mixture of ketamine and xylazine (10% xylazine in 100 mg/ml ketamine; ket/xyl) was used for intravenous injections (15 mg/kg ketamine with 1.5 mg/kg xylazine was administered as a 5–10 s bolus) and intraperitoneal injections of 50 mg/kg ketamine with 5 mg/kg xylazine were used where necessary.

Radiosynthesis

^{11}C-Raclopride was synthesized as detailed previously (Farde et al., 1986). The specific radioactivity of ^{11}C-raclopride was 21.2 Ci/μmol (range 5.2–33.8 Ci/μmol) at the end of bombardment (EOB). Injected doses (ID) of ^{11}C-raclopride ranged from 235–2290.27 μCi (mean 904.0 μCi), with a mean injected raclopride mass of 1.63 nmol/kg (range: 0.3–6.0 nmol/kg). Using ^{11}C-raclopride PET in male Sprague Dawley rats, it has been determined that the *in vivo* concentration at which half (50%) of the available D_2 receptors are occupied is 17.1 nmol/kg (Hume et al., 1995). Using this mean ED50 value of 17.1 nmol/kg and the method of Hume et al. (Hume et al., 1998) to calculate receptor occupancy results in occupancy values ranging from 1.8–27%. Differences in

Table 1
Experimental parameters and results

Uptake condition: challenge	*N*	μCi	Injected dose	(ST−CB) CB	(DVR-1)	% Δ in (ST−CB)/CB from:	
			nmol/kg			Scan 1	Awake Control
Mean awake control[a]	11	764±0.63	1.14±0.6	3.66±0.31			
Experiment 1							
Awake paired: control, scan 1	5	857±260	0.82±0.18	3.69±0.26			+1
Anesthetized paired: control, scan 2	5	824±204	1.12±1.03	3.99±0.28	2.74±0.21	+8	+9
Awake single: control, scan 1	6	687±336	1.41±0.75	3.64±0.37			−1
Awake single: control, scan 2	6	638±257	0.77±0.19	3.45±0.50		−5	−6
Awake paired: cold raclopride, scan 1	4	883±193	3.22±2.82	0.39±0.12 *			−89
Anesthetized paired: cold raclopride, scan 2	4	1234±196	1.08±0.32	0.47±0.15 *	0.37±0.11	+21	−87
Experiment 2							
Awake paired: METH, scan 1	4	743±157	2.72±0.13	2.30±0.24 *			−37
Anesthetized paired: METH, scan 2	4	483±135	0.95±0.18	2.37±0.23 *	1.69±0.17	+3	−35
Anesthetized single: METH, scan 1	6	423±203	1.34±1.50	2.53±0.31 *	1.82±0.22	+7[b]	−31
Awake single: GVG+METH	5	730±208	2.84±1.17	3.22±0.49 **		+40[c]	−12
Experiment 3							
Awake single: stress	5	1411±138	3.16±2.29	2.59±0.46 *			−29

[a] Averaged values from Awake Paired: Control Scan 1 and Awake Single: Control Scan 1.
[b] % Δ from Anesthetized Paired: METH Scan 2.
[c] % Δ from Awake Paired: METH Scan 1.
* $p<0.001$, significant difference from Mean Awake Control.
** $p<0.01$, significant difference from Awake Paired: METH Scan 1.

the injected dose and raclopride mass for each group of animals are given in Table 1.

Plasma metabolite analysis

To determine whether the rate of peripheral ^{11}C-raclopride metabolism differed in the Awake versus the Anesthetized Uptake conditions, blood samples were obtained at predefined times to obtain the ratio of unmetabolized ^{11}C-raclopride to total plasma radioactivity using high-performance liquid chromatography (Dewey et al., 1992). The rate of ^{11}C-raclopride metabolism was compared using a two way Analysis of Variance (ANOVA) with factors of condition (Awake Uptake versus Anesthetized Uptake) and time (20, 30–35 or 60 min after injection). A Student's *t*-test was used to assess the significance of the ANOVA with a critical value of $p<0.05$.

Awake Uptake

Eight awake, freely moving animals received intravenous ^{11}C-raclopride through the jugular catheter and underwent 20 min ($n=1$), 30–35 min ($n=4$) or 60 min ($n=3$) of ^{11}C-raclopride uptake in their home cages. At the end of each uptake period, animals were sacrificed and trunk blood was collected in heparinized vials. Vials were centrifuged and 200–400 μL aliquots of plasma were used to determine the percent of unchanged ^{11}C-raclopride according to a modification of previously established methods for non-human primate plasma (Dewey et al., 1992). Briefly, the HPLC system included a Waters Bondapak C-18 column (3.9×300 mm), with a mobile phase of 25% acetonitrile, 75% ammonium formate containing 0.8% glacial acetic acid, at a flow rate of 1.0 ml/min. Samples were spiked with unlabeled raclopride and the peak was detected with ultraviolet (UV) absorption at 254 nm. Percent unchanged raclopride was determined by dividing the decay corrected counts in each fraction containing the raclopride UV peak by the summed decay corrected counts in all collected fractions, and the percent recovered was determined by counting a standard before injection and comparing this to the total counts.

Anesthetized Uptake

Eleven animals were anesthetized with ket/xyl for 60 min prior to ^{11}C-raclopride administration. ^{11}C-raclopride was injected through the jugular catheter and after 20 ($n=1$), 35 ($n=7$) or 60 ($n=3$) min animals were euthanized and trunk blood was collected in heparanized vials. Following these uptake periods, plasma analysis for the amount of unchanged ^{11}C-raclopride was performed using methods referenced above.

Analysis of whole body distribution

To determine whether the Awake Uptake condition influenced the peripheral distribution of radiotracer, ^{11}C-raclopride was measured from the brain, heart, lungs, liver, spleen, kidneys and 0.5 ml whole blood of animals that were awake or anesthetized during uptake.

Awake Uptake

Six animals were injected with ^{11}C-raclopride through the jugular catheter and returned to their home cages. After 35 min, animals were euthanized by decapitation and the brain and peripheral organs were collected into preweighed gamma vials. Organs were weighed and counted for carbon-11 in a gamma well counter.

Anesthetized Uptake

Seven animals were anesthetized with ket/xyl 60 min prior to receiving ^{11}C-raclopride through the jugular catheter. After 35 min of ^{11}C-raclopride uptake under anesthesia, animals were euthanized by decapitation and organs were harvested and counted as described above. An additional ten animals were studied after 15 min of anesthesia ($n=3$), 90 min of anesthesia ($n=4$) or 3.5 h of anesthesia ($n=3$) prior to 35 min of ^{11}C-raclopride uptake and subsequent euthanasia. In the three animals studied after 15 min of anesthesia prior to ^{11}C-raclopride, brains were harvested and the striatum and cerebellum dissected out and counted to ascertain the ratio of ST-CB/CB in these ex vivo measurements.

Data analysis

Radioactivity measurements were normalized to organ weight and injected dose after decay correction to the time of injection. Significant effects of the duration of anesthesia on whole brain uptake were examined using a one-way ANOVA with a factor of time. Data are presented as a percentage of the injected dose in each animal per gram of tissue (% ID/g).

MicroPET imaging

The present studies were designed to examine the validity of an awake animal imaging paradigm to derive measurements of ^{11}C-raclopride binding in freely moving, behaving animals and to evaluate its potential to measure changes in receptor occupancy due to changes in dopamine.

The validation of this approach was organized into three experiments with the following objectives: one, to establish the reproducibility of the measurement and determine the range of ^{11}C-raclopride binding potential when radiotracer uptake occurs in the awake versus the anesthetized condition, two, to determine whether ^{11}C-raclopride can be displaced by endogenous dopamine in the awake condition and compare this to displacement under anesthesia, and finally to establish that a behavioral challenge alone is sufficient to displace ^{11}C-raclorpide binding. For the first two experiments, the same animals were studied in both awake and anesthetized conditions using a paired bolus paradigm where ^{11}C-raclopride uptake in the first of two sequential scans occurred in the awake state, after which the animals were anesthetized and scanned and remained in the tomograph for the second radiotracer delivery, which was used to assess ^{11}C-raclopride binding when the same animals were anesthetized for the whole duration of radiotracer uptake. General procedures are described below for the Awake and the Anesthetized Uptake conditions. Table 1 presents the number of animals, injected dose and injected mass for each group of animals while Fig. 1 presents individual protocols used for each experiment.

General procedures

Awake Uptake

On the day of scanning, catheterized animals were brought to the PET imaging facility 3 h prior to the first scheduled radiotracer delivery. The room was illuminated with standard laboratory fluorescent light. One hour prior to the scheduled ^{11}C-raclopride delivery, polyurethane tubing was connected to the jugular vein catheter port (positioned in the infrascapular dorsal midline) in unrestrained animals as they were allowed free movement in their

home cage, a standard 48 cm × 25 cm × 22 cm (length × width × height) acrylic plastic rodent cage (Nalgene, Inc., Rochester, NY, USA) with a floor area of 1200 cm^2. The size of the tubing was fixed (0.64 mm inner diameter, 30 cm length, ~100 µl volume) to maintain a constant dead volume and to provide sufficient slack to allow the animals free movement. The tubing was attached at one end to a 23G blunt luer hub (Hamilton Kel-F Hub, Point Style 3 with dimensions of 0.64 mm outer diameter, 51 mm length and a volume of 4.3 µl; Hamilton Company, Reno NV, USA) and at the other end to 23 G stainless steel tubing, 0.5 cm in length which inserted into the animal's catheter port. A syringe containing heparinized saline was attached to the luer hub and when not in use, the whole unit was taped to the side of the home cage. Because the line extended from the infrascapular dorsal midline of the animal up through the slatted cage lid, animals were not encumbered by the line and only rarely became tangled or tried to chew the tubing.

At the time of injection, the heparinized saline syringe was replaced with a syringe containing a predetermined dose of ^{11}C-raclopride in a fixed volume of 0.3–0.6 ml. Injected doses of radioactivity and the associated mass for each group are given in Table 1. In all cases, ^{11}C-raclopride was administered as a 5–10 s i.v. bolus injection flushed immediately after with 0.2 ml heparinized saline. Rats were allowed to sit undisturbed for 30 min of radiotracer uptake, after which the heparinized saline syringe was replaced with a syringe containing a mixture of ketamine and xylazine (10% xylazine in 100 mg/ml ketamine; ket/xyl). A dose of 15 mg/kg ketamine with 1.5 mg/kg xylazine was administered as a 5–10 s bolus and animals were non-responsive to tail pinch and eyeblink reflex in 15–30 s. Animals were immediately positioned in a nylon stereotaxic head holding device (Kopf Instruments, Tujunga, CA, USA) adapted for small animal PET to eliminate attenuation artifact (Schiffer et al., 2006). A catheter was introduced into the intraperitoneal cavity for maintenance doses of ket/xyl required for paired studies, detailed below. The unit and the animal were advanced into the center of the PET field of view (automated to 160 mm forward). Precisely five min after the induction of anesthesia, a dynamic PET acquisition (five 300 s time frames) commenced for 25 min.

Anesthetized Uptake

For paired studies, animals remained in the tomograph 60 min after the end of the first scan, described above. Maintenance doses of ket/xyl (50 mg/kg ketamine with 5 mg/kg xylazine) were given at 30 min intervals through an intraperitoneal catheter. With the second delivery of ^{11}C-raclopride, the heparinized saline syringe was replaced with a syringe containing a predetermined dose of radiotracer which was injected as a 5–10 s bolus and flushed with heparinized saline as described above. A dynamic, 60 min PET acquisition commenced with radiotracer delivery.

For single acquisition experiments, animals were anesthetized 60 min prior to the scheduled radiotracer delivery as described above. All procedures were identical to the paired studies with exceptions noted below for specific drug challenge conditions.

Experiment 1: Reproducibility and range of ^{11}C-raclopride binding during Awake or Anesthetized ^{11}C-raclopride uptake

In our first set of imaging experiments, established the reproducibility of the measurement and determined the range of ^{11}C-raclopride binding potentials when radiotracer uptake occurs in the awake versus the anesthetized condition. We examined the reproducibility of ^{11}C-raclopride binding using a test/retest experimental protocol that involved repeated scans on the same (Fig. 1a) or different days (Fig. 1b) with high specific activity, no carrier added ^{11}C-raclopride injections. For these studies, there were two groups of animals: one group that received paired scans according to the experimental timeline in Fig. 1a and a second group that received two single scans using the Awake-Uptake protocol separated by 5–7 days (Fig. 1b). To determine the range of available binding, we used carrier-added, low specific activity injections where a predetermined amount of cold raclopride was added to the ^{11}C-raclopride and co-injected as a loading dose (Fig. 1c).

According to the protocol illustrated in Fig. 1a, five animals received paired ^{11}C-raclopride scans where the only difference between scans was that the animals were awake for uptake during the first scan and anesthetized for uptake during the second. For the first scan, ^{11}C-raclopride was injected as described above and uptake occurred as animals were undisturbed in the home cage. After 30 min, the animals were anesthetized, placed on the tomograph bed and scanned for 25 min. Animals remained in this position for the second scan, in which uptake occurred in the anesthetized state. Data obtained from these studies gave full time activity curves for within-animal comparisons of specific binding in the awake and anesthetized conditions. Kinetic information from the second scan only was used to calculate binding potential using the graphical method to obtain a Distribution Volume Ratio (DVR; Logan et al., 1990). The DVR is a more rigorous estimate of specific ^{11}C-raclopride binding than a simple ratio (Logan et al., 2007) and can thus be used to validate measurements obtained with the (ST − CB)/CB ratio obtained from the first scan in freely moving animals.

To determine the reproducibility within the same animal over time under our awake scanning protocol, six freely moving animals were administered ^{11}C-raclopride according to the Awake Uptake protocol described above. After the 25 min scan, they were returned to their home cage and allowed to recover. Five to seven days later, these same animals were scanned again using the same protocol.

To assess the range of available binding and determine the degree of non-specific binding, (Fig. 1c), we compared binding in animals that were awake but received a loading dose of cold raclopride co-injected with ^{11}C-raclopride, with that from the same animals that were anesthetized but not moved from the tomograph bed between scans. For the Awake Uptake studies, four animals were given stable raclopride (2 mg/kg or 5762 nmol/kg raclopride) co-injected with the ^{11}C-raclopride injection. ^{11}C-raclopride uptake occurred in the home cage for 30 min, followed by anesthesia and scanning. For the second of the paired scans, animals remained in the tomograph and received a second injection of ^{11}C-raclopride. Previous studies have shown that blocking conditions are stable up to 4 h after a single dose (Kohler et al., 1985). The dose of cold raclopride was chosen to provide a D_2 occupancy in excess of 80% (Kapur et al., 2001, 2000).

Experiment 2: ^{11}C-raclopride displacement by endogenous dopamine during Awake or Anesthetized Uptake

To determine whether ^{11}C-raclopride can be displaced by endogenous dopamine in the awake condition and compare this to displacement under anesthesia, animals in the Awake Uptake condition were predosed with methamphetamine (METH) 10 min

prior to the first ^{11}C-raclopride injection (Fig. 1d) and remained in the tomograph bed for a second ^{11}C-raclopride scan. For these experiments, METH was administered using the i.p. route of administration because we have previously established the time course of METH-induced changes in dopamine using this route of administration at the same dose (Gerasimov et al., 1999). Ten minutes after the METH injection, ^{11}C-raclopride was injected as described above (dosing details are given in Table 1). As before, animals had 30 min of unrestrained movement in their home cage during the Awake Uptake period. Ket/xyl was administered intravenously after the 30 min uptake period and animals were moved to the tomograph bed for the first Awake Uptake scan. Following this scan, animals were not moved from the gantry for the second, Anesthetized Uptake scan. The stability of amphetamine-induced dopamine release to displace ^{11}C-raclopride binding has been well established (Carson et al., 1997; Endres et al., 1997; Laruelle, 2000), and the addition of a methyl group to amphetamine, making methamphetamine, is most likely responsible for the enhanced effects of METH on extracellular dopamine (Fleckenstein et al., 2007). Although the continued displacement of ^{11}C-raclopride by amphetamine-induced increases in dopamine reflects the degree to which endogenous dopamine displacement of ^{11}C-raclopride binding occurs (Houston et al., 2004), it is most likely due to factors other than competition with synaptic dopamine, such as receptor internalization (Sun et al., 2003). For comparison, an additional group of animals (n=6) received a methamphetamine challenge 10 min prior to ^{11}C-raclopride as a single Anesthetized Uptake scan according to the protocol depicted in Fig. 1e.

To assess the malleability of the dopaminergic response to METH in the Awake Uptake condition, we pretreated animals with a suicide inhibitor of GABA-transaminase, γ-vinyl GABA (GVG) to indirectly increase brain GABA levels before administering the same METH challenge (Fig. 1f). We have previously shown with *in vivo* microdialysis in awake animals that prior administration of GVG will attenuate METH-induced increases in extracellular dopamine (Gerasimov et al., 1999). Here we used the active enantiomer of GVG [S(+)-GVG] at a dose we have shown in primate PET and rodent microdialysis studies to be comparable to the racemic compound (Schiffer et al., 2000). Five freely moving animals were pretreated with S(+)-GVG (150 mg/kg) 2.5 h prior to METH (5.0 mg/kg i.p.), which was given 10 min before ^{11}C-raclopride using the Awake Uptake protocol modified for METH administration as described above. Following 30 min of Awake Uptake, animals were anesthetized with ket/xyl (i.v.) and scanned using the same procedures.

Experiment 3: Effects of handling or restraint stress on ^{11}C-raclopride binding

To establish that a behavioral challenge alone is sufficient to displace ^{11}C-raclorpide binding, we adapted a handling stress protocol that we have previously shown increases cortical dopamine levels using *in vivo* microdialysis in awake animals (Fig. 1g; Marsteller et al., 2002). In the protocol adapted for these experiments, animals were handled continuously for 30 min during ^{11}C-raclopride uptake. ^{11}C-Raclopride administration coincided with the onset of handling stress, with one investigator handling the animals (SLD) while another performed the injection (DEL or VDP). Injected doses of radioactivity and mass of stable raclopride are given in Table 1. Handling sessions consisted of partial removal from the home cage, during which animals were continually restrained. Continuous restraint included picking up the animal by the front shoulders, hips, or tail, and changing positions of the animal in the hand throughout this time so that it was not allowed to escape. Animals were returned to their home cage immediately prior to administration of the anesthetic.

Image acquisition

Images were acquired using a microPET R4 tomograph (Concorde Microsystems, Knoxville, TN) which has a transaxial field of view of 11.5 cm. All animals were positioned in the center of the field of view. Each PET scan included subtraction of random coincidences collected in a delayed time window. Three dimensional sinograms were converted into two dimensional (2D) sinograms before image reconstruction. Data were corrected for photon scatter using the method of tailfitting of the projections. The measured attenuation correction method available for this system used a ^{68}Ge point source requires very long acquisition times (~30–60 min) to minimize image noise introduced by transmission scanning, and therefore was not carried out. In general, attenuation correction factors are constant over time and should not change the shape of the time activity curves. Further, because the data analysis methods proposed here rely on a ratio of ^{11}C-raclopride in a receptor-rich region (the striatum) with a receptor-poor region (the cerebellum), and it has been determined previously that the striatum and cerebellum have about the same attenuation factor (see discussion in Alexoff et al., 2004), thus a constant attenuation correction factor for the striatum and cerebellum was not reasoned to be necessary. Finally, we have previously demonstrated that attenuation correction using calibrated segmented image data had no effect on the measured DVR (Alexoff et al., 2004). Scatter-corrected sinograms were reconstructed using the maximum likelihood expectation maximization (MLEM) algorithm, which with the 20 iterations employed here yields an image resolution of ~1.5 mm FWHM (Full Width at Half Maximum) at the center of the field of view. The image pixel size in MLEM reconstructed images was 0.8 mm transaxially with a 1.21 mm slice thickness.

Image processing

A requirement for whole-brain analysis is that the images of different brains must be spatially normalized into a standard space, which we identified as Paxinos and Watson stereotaxic space. As a template, we used the MRI atlas provided by Schweinhardt et al. (2003), which is in stereotaxic space and for which we have previously developed ^{11}C-raclopride reference images for spatial preprocessing (Schiffer et al., 2005). Software packages used for the procedures described below were: Pixelwise Modeling Software Package (PMOD, version 2.85, www.pmod.com) for resampling dynamic and static microPET image volumes into atlas space, including image rotation, resizing and generating time activity curves that were also fit to the Simplified Reference Tissue Model (SRTM) in PMOD after spatial preprocessing; Statistical Parametric Mapping (SPM2; http://www.fil.ion.ucl.ac.uk/spm/software/spm2/) for spatial preprocessing including coregistration and normalization, although newer versions of PMOD are equally effective for coregistration and normalization of rodent PET studies, and MRIcro (http://www.sph.sc.edu/comd/rorden/mricro.html) for volumetric visualization and skull stripping. Data voxel size in the Schweinhardt atlas (2003) is scaled by a factor of 10 to enable a

one-to-one relationship between the coordinates of the Paxinos atlas and the voxel display in the SPM software package. This also approximates the human brain size, allowing minimal modifications to the default parameter settings in each of the three image processing software packages. The first steps were performed in PMOD and included isotropic interpolation and rotation of the raw PET images in space to roughly match the Paxinos atlas. This included sorting each image from left to right, making the z axis perpendicular to the coronal (y) slice, and a flip of the x-axis 180° around the z axis. While in PMOD, the images were also scaled by a factor of 10 and resampled into a volume ("bounding box") that encompassed the spatial extent of the scaled Schweinhardt atlas, which was −80 to +80 mm in the x-dimension (negative to the left of the midline and positive to the right), −120 to 10 mm in the y-dimension (posterior to anterior) and −156 to +60 mm in the z-dimension (ventral to dorsal). The template provided by Schweinhardt et al. (2003) swaps the y and z dimenstions to provide a layout that accommodates the SPM2 software package. In this template, the zero-reference plane was set to bregma, resulting in an image origin at $40.5 \times 78.5 \times 60.5$ ($x \times y \times z$) in pixel space. The scaled voxel size was set to $2 \times 2 \times 2$ mm (with an actual voxel size of $0.2 \times 0.2 \times 0.2$ mm), resulting in an image volume of $80 \times 63 \times 108$ voxels.

For these experiments and for blocking studies in particular, it was necessary to generate two reference images which we used as templates; one of the initial five min of a dynamic scan which we used for data where the striatum was not readily discernable and a second for those scans where the striata were clearly visible. For these reference images, we chose one control, dynamic ^{11}C-raclopride scan with a high degree of symmetry and consistent alignment. From this one dynamic study, an averaged reference image was created from the first five min of dynamic ^{11}C-raclopride data using the PMOD software package. This image contained a mixture of blood flow and specific striatal binding (early reference image), while an averaged image of the last five time frames (25 min; late reference image) contained primarily specific striatal binding. The late reference image was smoothed with a Gaussian kernel, full width at half maximum (FWHM) of 6 mm (approximately three times the voxel dimensions), co-registered and spatially normalized to our existing ^{11}C-raclopride template using the sum-of-squared differences minimization algorithm and 12-parameter affine transformations provided by SPM2. The same spatial transformation and normalization parameters were then applied to the early-frame reference image to obtain a reference image for preprocessing blocking studies with little or no specific binding.

ROI analysis was performed using a modified version of the WFU Pickatlas tool (http://www.fmri.wfubmc.edu/cms/software) integrated into the SPM software package, modified for rats by Schwarz et al. (Schwarz et al., 2006). ROIs for PET studies were chosen based on previous guidelines provided for primates by Black et al. (2004) and recently described for rodent PET data (Dalley et al., 2007). Rather than outlining the entire structure on the MRI template, this approach minimizes the effects of spillover by using spheres placed at the stereotaxic center of the dorsal and ventral striata, measuring 2 mm in diameter (left and right striata were combined, coordinates for the center of the dorsal striatal ROIs were ±2.5 mm lateral to bregma, +0.5 mm anterior and 4 mm below bregma while coordinates for the ventral striatal ROI were ±0.5 mm lateral to bregma, +1.0 mm anterior and 6 mm below bregma). Combining the left and right dorsal and ventral striatal ROIs gave two striatal regions with volumes of 8336 mm3 each, containing 1042 voxels. A single bilateral cerebellar region (volume, 9232 mm^3 with center coordinates at ±1.9, −12.5, −6.0 mm in the x, y and z dimensions) was used as a reference region due to its low D_2 receptor density (Farde et al., 1986; Wagner et al., 1983).

Quantitation

One of the main objectives of this study was to use a method of receptor occupancy determination in rats that was analogous to human studies (Farde et al., 1988). Human studies involve the intravenous injection of ^{11}C-raclopride commensurate with PET scanning, which measures radioactivity in different brain regions. In our previously published studies and in pilot data for these experiments, we injected ^{11}C-raclopride in rats and scanned them to obtain the time course of specific and non-specific binding (Schiffer et al., 2005). These data showed that the specific binding (i.e. striatum minus cerebellum over cerebellum) reached equilibrium between 20 and 30 min, as reported previously (Hume et al., 1992; Kohler et al., 1985). Therefore we chose 30 min post injection as the optimal time for scanning. To examine the feasibility of imaging dopamine release in awake, freely moving animals, we examined striatal ^{11}C-raclopride binding following several challenge conditions previously shown using *in vivo* microdialysis to either stimulate dopamine release or prevent it (Dewey et al., 1991, 1993, 1992; Gerasimov et al., 1999; Marsteller et al., 2002).

The ratio of counts in the striatum and cerebellum (striatum minus cerebellum/cerebellum; [ST−CB]/CB) was used to estimate the binding potential of ^{11}C-raclopride for dopamine D_2 receptors. In this case, the cerebellar counts reflected non-specific binding and free ligand whereas the striatal counts reflected specific binding of the ligand to D_2 receptors in addition to the non-specific and free ligand binding. Ratios from all animals were calculated using the average radioactivity during the last 25 min of scanning (data collected in 5 min bins). These ratios were compared for both awake and anesthetized animals.

The occupancy of D_2 receptors in each rat was determined with the same formula used in human (Farde et al., 1988) and in animal studies (Gatley et al., 1995; Wadenberg et al., 2000):

% Occupancy = 100 * (D2BPcontrol − D2BPindividual/D2BPcontrol) where BP is the binding potential established using the ratio of (ST−CB)/CB.

Since the ratio of ST−CB/CB is at best an approximation of ^{11}C-raclopride specific binding following a bolus injection, we also validated results with kinetic analysis where possible. In order to assess the suitability of the ratio method to estimate binding potential, a graphical analysis (Logan et al., 1990) was applied. This kinetic analysis was carried out using the time course of radioactivity concentrations available from Anesthetized Uptake animals, anesthetized for the duration of uptake, for which full time activity curves were available. This was compared to the same data where the last five time points were used in calculations of the (ST−CB)/CB ratio. The graphical analysis, designed specifically for reversible systems, allows a direct calculation of the steady state distribution volume ratios between a region rich in receptor binding sites and a reference region, devoid or with negligible receptor concentrations. The DV ratio is not sensitive to changes in radiotracer delivery due to changes in the input function or regional cerebral blood flow (Holthoff et al., 1991; Logan et al., 1990).

Ratio estimates of specific binding from awake animals, described above, were compared to the measured binding potential (distribution volume ratio-1; DVR-1) from the same anesthetized animals scanned for 60 min using the paired bolus protocol described above, where receptor availability was derived using the full time activity curves and a well established graphical technique specifically designed for reversible systems (Logan et al., 1990). While the use of a ratio of counts at equilibrium in a selected region compared with those in a reference region devoid of specific binding may be successful in some circumstances, the importance of measuring the kinetics of ligand-receptor binding rather than static analyses has recently been stressed by Logan et al. (2007).

Statistical analysis

The statistical analysis was designed to address the hypothesis that receptor availability measured with ^{11}C-raclopride was different for the challenge conditions relative to the unperturbed, awake state. Receptor availability data for the (ST−CB)/CB ratio from awake animals that received drug challenges and loading doses of raclopride were compared with data from a pooled sample of Awake Control groups using a one-way ANOVA, with a post-hoc Bonferroni *t*-test to determine the significance of the ANOVA. For paired studies in which the same animals were scanned either on the same day or later in the week, a two-way repeated measures ANOVA was used to compare the results from the first scan with the second scan. For this analysis, the two factors were treatment (control, raclopride or METH) and condition (Awake Uptake or Anesthetized Uptake). Where there appeared to be a difference, a Bonferroni *t*-test was used to assess the significance set to a critical value of 0.05. Time activity data were quantitated using both a graphical analysis method and the ST−CB/CB ratio method and compared using a regression analysis based on the F statistic, which gauges the contribution of the independent variable (in this case, the ratio of ST−CB/CB) in predicting the dependent variable (in this case, DVR-1). The F statistic represents the ratio of regression variation from the dependent variable (DVR-1) to the residual variation around the regression line. A similar analysis was performed on ratio data from awake animals and the DVR-1 from same animals scanned under anesthesia. In this case, the ratio of ST−CB/CB also comprised the independent variable and DVR-1 was the dependent variable in an identical F-test. A final regression compared the ST−CB/CB ratio data from anesthetized animals with that from the same animals scanned just before during the Awake Uptake condition. Here, the dependent variable was still the Awake Uptake ST−CB/CB and the independent variable was the ST−CB/CB ratio from the same animals scanned under anesthesia. All statistical analyses were performed using the Systat Software package (Sigmastat 3.5, San Jose, CA, USA).

Results

Plasma metabolite analysis

The rate of ^{11}C-raclopride metabolism was measured in the awake and anesthetized animals. Fig. 2 shows the relative amount of parent compound as a function of time from the ^{11}C-raclopride injection. The clear overlap of the two datasets indicates that the rate of peripheral metabolism was nearly identical under both conditions (awake vs. anesthetized). This was substantiated by a two-way ANOVA (factors of time and condition), with a post-hoc Student's *t*-test indicating a

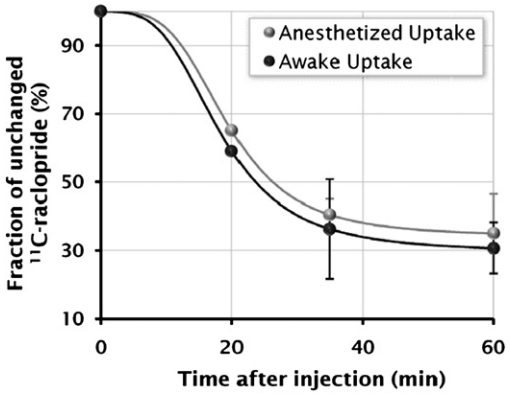

Fig. 2. Fraction of total radioactivity attributed to ^{11}C-raclopride in the plasma of anesthetized (●) or awake (●) animals. Plasma samples were taken at 20, 30–35 and 60 min. Data represent mean±standard deviation for each time point. Lines represent the curve fitted to a Hill equation, according to methods described by (Gunn et al., 1998). There was a close agreement between the parent fraction obtained from animals that were awake and anesthetized during ^{11}C-raclopride uptake, and no significant difference in the rate of metabolism between the two conditions.

significant effect of time (F=6.02, P=0.014) but not of condition (awake versus anesthetized, F=0.50, P=0.49). These data demonstrate that the difference in uptake between awake and anesthetized conditions was not due to the rate of peripheral ^{11}C-raclopride metabolism.

Analysis of whole body distribution

Fig. 3 depicts the accumulation of carbon-11 in brain and peripheral organs of awake or anesthetized animals. When dose corrected, accumulation was significantly higher (p<0.05) in the brain (132%), lung (90%) and spleen (52%) of anesthetized compared to awake animals. In the brain of animals that were awake during uptake and euthanized after 35 min without anesthesia, uptake represented 0.075±0.014% of the injected dose, while in animals that were anesthetized 60 min prior to the ^{11}C-raclopride injection, whole brain uptake was 0.163±0.006% ID/cm^3. Comparisons with the *in vivo* PET data from awake and anesthetized animals are presented in Supplementary Table 1. The effects of the duration of anesthesia on whole brain uptake of ^{11}C-raclopride indicated a significant effect of anesthesia duration on brain uptake (F=15.442, p<0.001), however the sample sizes were small and further studies are needed to fully characterize this effect. In animals where the striatum and cerebellum were dissected and counted, the mean ST−CB/CB ratio was 3.29±0.17, while PET measurements from the same animals gave a ratio of 3.07±0.17.

microPET imaging

The average time between ^{11}C-raclopride injection and anesthesia was 31±2 min, while the average time between the ^{11}C-raclopride injection and the microPET acquisition was 36±3 min.

Experiment 1: Reproducibility and range of ^{11}C-raclopride binding during awake or anesthetized ^{11}C-raclopride uptake

In paired test/re-test studies in which the same animals underwent Awake Uptake prior to scanning followed by Anesthetized Uptake, a whole-brain ROI was used to compare total brain uptake

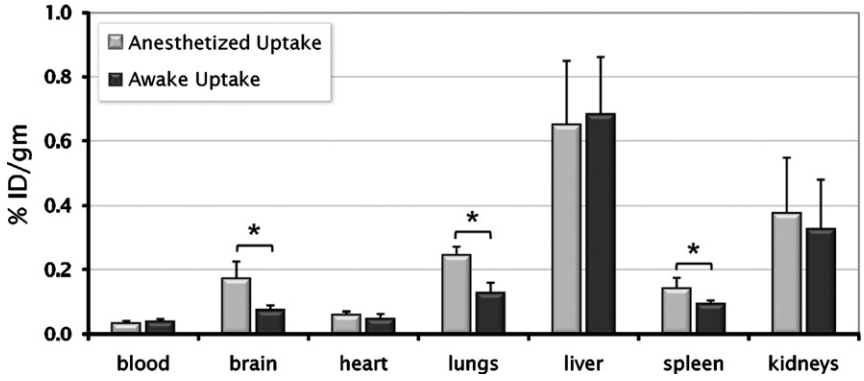

Fig. 3. Whole body organ distribution of carbon-11 in anesthetized or awake animals after 35 min of ^{11}C-raclopride uptake. Data are expressed as a percent of the injected ^{11}C-raclopride dose per gram of organ weight and represent mean±standard deviation for 7 anesthetized animals and 6 awake animals. Significantly more radioactivity was detected from the brain, lungs and spleen of anesthetized animals (*$p<0.05$).

of ^{11}C-raclopride measured with the PET with whole brain ROI uptake measured from ex vivo biodistribution experiments. From the PET, proportional uptake from a whole brain ROI extracted at 35 min after injection from animals in the Awake Uptake control condition was 0.036±0.011% ID/cm^3 (mean±standard deviation) while from the same animals scanned immediately after under conditions of Anesthetized Uptake, the whole brain uptake was 0.15±0.03% ID/cm^3 (mean±standard deviation). Comparisons between these values and the ex-vivo biodistribution data are presented in Supplementary Table 1.

In the test/retest studies (Fig. 1a), the difference in ST−CB/CB ratio was, on average, less than 5% when the same animals were scanned on separate days under identical conditions in the awake state. This magnitude of variation in repeated measures without intervention is consistent with our reported test/retest measurements in anesthetized rats. Pooled data from awake and anesthetized control animals (in the absence of a pharmacological or behavioral challenge) indicated that the (ST−CB)/CB obtained in the awake or anesthetized state was virtually identical (Table 1). D_2 receptor occupancy in the four animals treated with unlabeled raclopride ranged from 88 to 98%. Despite the five-fold difference in uptake noted previously, there were no significant differences in binding potential between awake or anesthetized animals in the control, non-challenged condition (Table 1, comparison of Awake vs. Anesthetized) nor was there an effect of anesthesia in animals who received a loading dose of unlabeled raclopride, evident in the measures of specific ^{11}C-raclopride binding also detailed in Table 1.

In animals co-injected with cold raclopride (Fig. 1c), striatal ^{11}C-raclopride uptake at 35 min was 0.033±0.012% ID/cm^3 in the Awake Uptake condition and 0.166±0.01% ID/cm^3 in the same animals scanned under anesthesia. Cerebellar uptake values at 35 min in the blocked condition were 0.022±0.007 in awake animals and 0.12±0.008 in the same animals under anesthesia. These studies indicated that in the blocked state, there was five times the ^{11}C-raclopride uptake in anesthetized versus awake animals.

Experiment 2: ^{11}C-raclopride displacement by endogenous dopamine during Awake or Anesthetized Uptake

In animals injected with METH (Fig. 1d) 10 min prior to ^{11}C-raclopride administration, striatal ^{11}C-raclopride uptake at 35 min was in anesthetized animals was 0.36±0.17% ID/cm3 and 0.085±0.009% ID/cm3 in the striatum of awake animals. Cerebellar ^{11}C-raclopride uptake at 35 min after injection was 0.136±0.036% ID/cm3 in anesthetized animals and 0.026±0.003% ID/cm3 in the same animals scanned under the Awake Uptake protocol. Again, there was a five-fold higher uptake in anesthetized versus awake animals, regardless of the region.

Nevertheless, the ratio of ST−CB/CB showed a similar inhibition in both awake and anesthetized animals. Results are detailed in Table 1. In awake animals compared to the mean control group, there was a significant decrease in occupancy of D_2 receptors. ST−CB/CB estimates of binding potential were similar when the same animals were scanned awake and then under anesthesia (3% difference between the two scans), and the anesthetized animals showed a similar reduction in D_2 receptor occupancy when compared with the mean awake control group (Table 1). This demonstrates reliable, reproducible effects of synaptic dopamine on ^{11}C-raclopride binding when animals are scanned under conditions of awake versus Anesthetized Uptake. When both striata were combined, pretreatment with S(+)-GVG significantly attenuated the effects of METH. There was no significant difference in ^{11}C-raclopride binding between control animals and those treated with S(+)-GVG prior to METH (Table 1), while there was a significant difference when this group was compared to awake animals that received METH alone (Table 1).

Experiment 3: Effects of handling or restraint stress on ^{11}C-raclopride binding

Animals were subjected to lifting and hand restraint stress during ^{11}C-raclopride uptake. (Fig. 1f) This condition produced a significant decrease in ^{11}C-raclopride binding (Table 1). When dorsal and ventral striata were combined, the effects of restraint stress were not significantly different than the effects of METH. However unlike METH, the effects of restraint stress on dopamine release were higher in the dorsal compared to the ventral striatum. Thus, all animals, regardless of experimental condition, showed a considerably higher uptake in the anesthetized state versus the awake state. In Fig. 4, the mean time activity of ^{11}C-raclopride in the striatum and cerebellum is shown for animals scanned in both the awake and anesthetized states under control conditions (Fig. 4a), when a loading dose of cold raclopride was given (Fig. 4b), and when METH was administered prior to the first of two paired ^{11}C-raclopride injections (Fig. 4c). In all three sets of time activity curves, there was a notable difference in ^{11}C-raclopride uptake between the awake and anesthetized state.

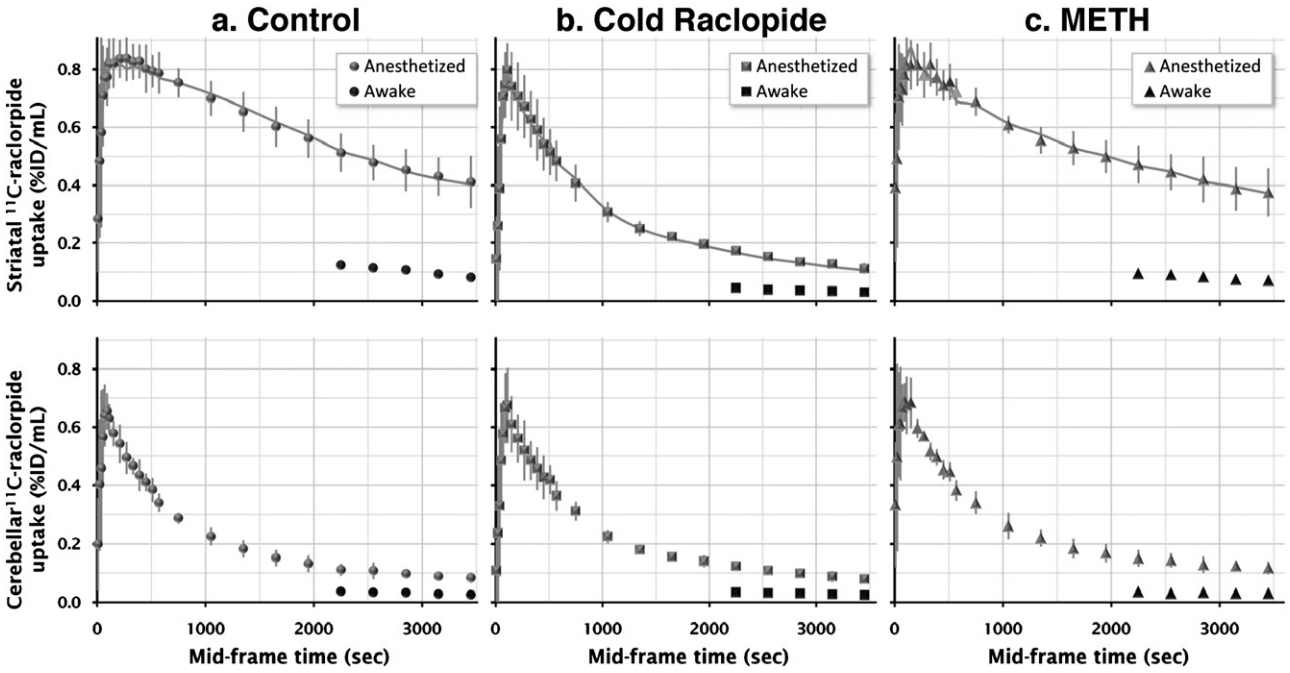

Fig. 4. Mean time activity of ^{11}C-raclopride in the striatum and cerebellum of paired studies in which the first Awake Uptake scan was followed by an Anesthetized Uptake scan in the same animals. Fig. 4a comprises data from the striatum (top) and cerebellum (bottom) of five control animals, where Anesthetized Uptake (●) is plotted against data from the Awake Uptake condition from the same animals (●). In Fig. 4b, the effects of cold raclopride co-injection in the striatum (top) and cerebellum (bottom) are plotted from four animals in the Anesthetized Uptake condition (■) and from the same animals scanned using the Awake Uptake protocol (■). In Fig. 4c, the effects of METH pretreatment (5 mg/kg) are shown for the striatum (top) and cerebellum (bottom) from four animals under the Anesthetized Uptake protocol (▲) or the same animals under the Awake Uptake protocol (▲). Data are presented as a percent of the injected ^{11}C-raclopride dose in nanocuries. Error bars represent standard deviation from the mean. Solid lines are best fits to the striatal data using the cerebellum as the input function in a reference tissue model (Hume et al., 1992). Estimated binding potential values from the fit were 2.6 for the Control group (Fig. 4a), 0.34 for the Cold raclopride group (Fig. 4b) and 1.71 for the METH pretreatment group (Fig. 4c).

Quantitative comparisons

Distribution volume ratios (DVRs) were calculated for anesthetized animals using the graphical analysis method (Logan et al., 1990) where binding potential is represented by DVR-1. First, this data was compared to the same time activity data that was quantitated using the (ST−CB)/CB ratio from animals that received either METH or a loading dose of raclopride, or no challenge at all. The results of the F-test indicate that there was a significant correlation between the DVR and the (ST−CB)/CB ratio values, with a correlation coefficient of 0.96. Average (ST−CB)/CB ratios from anesthetized animals were 45% higher than the average DVR-1 values obtained from the same anesthetized animals while the DVR-1 was 34% higher than the ST−CB/CB ratio from the same animals scanned in the awake state (Fig. 5a), a significant difference ($p<0.001$, paired t-test). This increase in the (ST−CB)/CB compared to DVR is expected due to the small but persistent decrease in blood radioactivity concentrations expected after a bolus injection (Logan et al., 2007). Finally, data from the same animals scanned awake and anesthetized and quantitated in both states using the (ST−CB)/CB ratio are presented in Fig. 5b. The strong correlation between outcome measures is maintained here, supporting the use of both our quantitative method and our experimental protocol for measuring ^{11}C-raclopride binding in freely moving animals.

Discussion

In the present study, we developed and validated a new imaging paradigm where ^{11}C-raclopride was injected into awake, freely moving animals prior to anesthesia and a subsequent microPET scan. In order to examine the feasibility of imaging dopamine release in these animals, we examined the striatal binding of the D_2 ligand, ^{11}C-raclopride following several challenge conditions. The conditions included both pharmacological and behavioral challenges that we have previously shown, using *in vivo* microdialysis in awake, freely moving (albeit tethered) animals, to increase and attenuate extracellular dopamine levels. In the first series of experiments, we examined the reproducibility of ^{11}C-raclopride binding using a test/retest experimental protocol where each animal served as its own control.

In the first of these experiments (Fig. 1a), animals underwent the Awake Uptake protocol followed by an Anesthetized Uptake scan which occurred without moving animals from the tomograph bed after the first Awake Uptake scan. This provided a baseline measurement upon which the effects of Awake Uptake could be directly compared to a traditional Anesthetized Uptake protocol, and allowed us to evaluate the effects of estimating binding potential as a ratio versus calculations of binding potential using a graphical method. These data demonstrated an 8% variation in ^{11}C-raclopride binding between the Awake and Anesthetized Uptake protocols and a significantly higher estimate of binding potential using the ratio method versus the graphically derived Distribution Volume Ratio (where binding potential is equivalent to DVR-1). Nevertheless, a regression analysis (Fig. 5) demonstrated a high correlation between these two outcome measures when all of the data between the Awake and Anesthetized Uptake conditions were pooled. We also wanted to evaluate the stability of striatal ^{11}C-

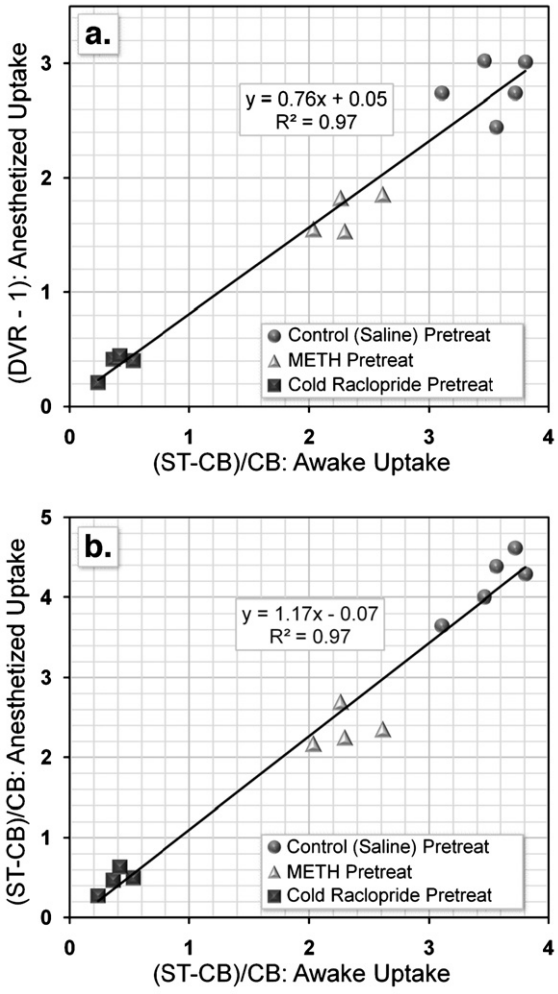

Fig. 5. Correlation between different methods of quantitation paired studies in which animals underwent the Awake Uptake protocol paired with a second scan under anesthesia. The same animals were scanned first awake and then anesthetized, under control conditions (●), after a METH challenge (▲) or with a cold raclopride co-injection (■). Each point represents data from paired bolus injections where the animals remained in the tomography between the awake-uptake acquisition and the anesthetized-uptake acquisition. In (a), the ratio approximation of binding potential in the awake-uptake protocol (ST−CB/CB; ordinate) is correlated with Distribution Volume Ratio approximation of binding potential (DVR-1; abscissa) obtained from the same animals using the anesthetized-uptake protocol in a paired-bolus design. In (b), DVR data are plotted against ratio data from the same animals obtained from a different scanning session in the awake state. In (c), ratio values were compared from the same animals in both awake and anesthetized states.

raclopride binding using the Awake Uptake protocol in the same animals over time, which indicated only a 5% variation when the same animals were scanned twice separated by five–seven days (Fig. 1b, Table 1). When a loading dose of unlabeled raclopride was co-administered with ^{11}C-raclopride (Fig. 1)c, ^{11}C-raclopride was markedly reduced compared to the Mean Control Group (Table 1) consistent with an estimated D_2 receptor occupancy of 89 and 87% in awake and anesthetized animals, respectively.

In a second series of experiments, two pharmacologic strategies were selected to modulate dopaminergic neurotransmission. In the first, a separate group of freely moving animals were given METH 10 min prior to ^{11}C-raclopride. Following 30 min of radiotracer uptake, animals were anesthetized with ketamine and scanned for 25 min, followed by a second scan under the same anesthesia. In awake animals, METH significantly reduced ^{11}C-raclopride binding compared to the Awake Control group (Table 1), a magnitude in agreement with previous small animal PET studies in rats (Houston et al., 2004; Pedersen et al., 2007). While previous studies have shown a persistent effect of amphetamine on ^{11}C-raclopride binding that is dose dependent for up to 5 h after drug administration (Houston et al., 2004), it was still necessary to ensure that METH-induced decreases in ^{11}C-raclopride binding were maintained when given 130 min prior to the Anesthetized Uptake ^{11}C-raclopride scan (Fig. 1d, PET Scan 2). To assess this, a separate group of animals received METH 10 min prior to ^{11}C-raclopride under anesthesia (Fig. 1e), and these results were compared to the Anesthetized Uptake scan that was paired to follow the Awake Uptake scan in the same animals. There was a 7% increase in ^{11}C-raclopride binding potential in these animals compared to anesthetized animals that received METH 130 min prior ^{11}C-raclopride. These experiments demonstrated that the duration of time following the second paired METH experiments did not diminish METH induced decreases in ^{11}C-raclopride.

For the second strategy, S(+)-GVG, a suicide inhibitor of GABA transaminase (a GABA-catabolizing enzyme), was administered 2.5 h prior to the METH challenge. We previously used PET imaging in primates and *in vivo* microdialysis in awake rats to show that stimulant-induced dopamine release is particularly sensitive to GABAergic inhibition by GVG and S(+)-GVG (Gerasimov et al., 1999; Schiffer et al., 2000). The (ST−CB)/CB ratio in animals pretreated with the active enantiomer of S(+)-GVG 2.5 h prior to a METH challenge was reduced by only 12%; a decrease that was not significantly different from control animals (Table 1), however it was significantly different from the Awake Uptake animals given METH alone (40% higher, Table 1). These data further support our previous findings that GVG inhibits METH-induced increases in synaptic dopamine and contributes to a considerable body of preclinical and clinical evidence that GVG may be an effective drug for the treatment of METH addiction (Brodie et al., 2005).

Finally, we also explored whether changes in the specific binding of ^{11}C-raclopride were also present in response to a non-pharmacological challenge. In a third series of experiments, performed under awake conditions, animals underwent restraint stress throughout the 30 min ^{11}C-raclopride uptake period. This condition significantly reduced ^{11}C-raclopride binding, an effect that was comparable to METH-induced reductions in ^{11}C-raclopride binding (Table 1 and Fig. 6b). This effect was more pronounced in the dorsal versus ventral striatum (data not shown). Substantial reductions in ^{11}C-raclopride binding have been reported using a social stress challenge (Pruessner et al., 2004), however stress-induced changes in response to difficult mental arithmetic were not observed with ^{11}C-raclopride in the presence of cardiovascular, hormonal and subjective responses of stressful conditions (Montgomery et al., 2006). We have previously shown that the paradigm used here significantly increases cortical dopamine release in awake animals using *in vivo* microdialysis (Marsteller et al., 2002). Because of the complexity of this particular paradigm, we were not able to measure behavioral or biochemical correlates of this response such as hormone levels associated with stress or the behavior of the animals themselves. This may present a limitation to the awake animal imaging protocol, or at least emphasize the need for an additional arterial catheter from which blood can be readily sampled (although this may in itself induce changes in blood pressure if sampled from a

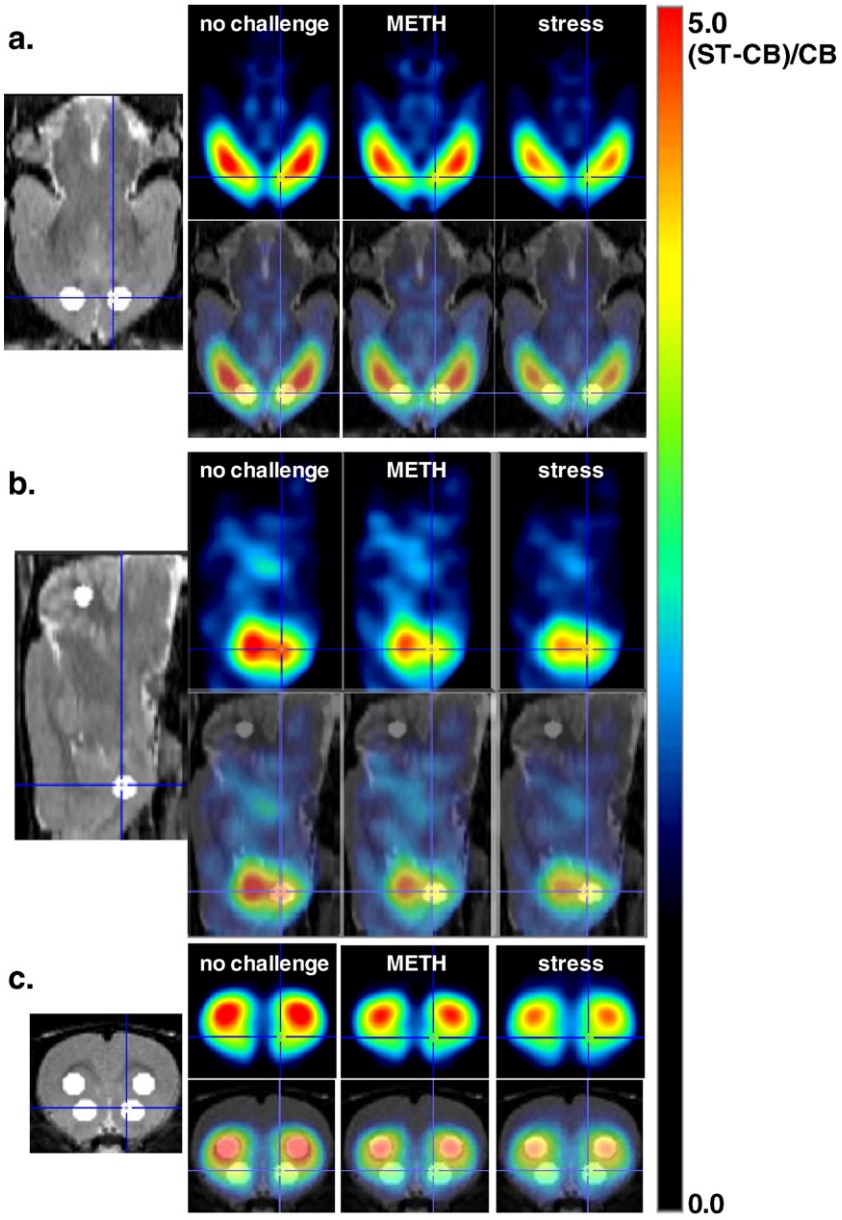

Fig. 6. Mean binding potential images from freely moving animals injected with ^{11}C- raclopride during the control condition ("no challenge"), after 5.0 mg/kg methamphetamine ("METH") or while undergoing mild restraint stress ("stress"). Parametric images are overlayed on the atlas from Schweinhardt et al. (2003), where the horizontal (a), sagittal (b) and coronal (c) planes are shown. Crosshairs pass through the ventral striatum at 1.4, 1.6 and −7.3 mm from bregma in *x*, *y*, and *z* dimensions, respectively. On the atlas image, we have also masked out the regions of interest used for dorsal striatum (shown in c), ventral striatum (shown in a, b and c), and cerebellum (shown in b). Data from dorsal and ventral striata were combined for analysis.

carotid artery, Schiffer et al., 2007). These data demonstrate the ability to image behavior-induced changes in brain dopamine in awake freely moving animals and provide evidence that physical restraint stress significantly increases dopamine and displaces ^{11}C-raclopride binding.

Perhaps most importantly, we observed that ^{11}C-raclopride uptake in the striatum and cerebellum of awake, freely moving animals was considerably lower than ^{11}C-raclopride uptake in anesthetized animals (roughly four times lower; Fig. 4). This is consistent with other rodent studies demonstrating significantly less radiotracer uptake in awake versus anesthetized states (Honer et al., 2006; Momosaki et al., 2004). It was suggested that a faster rate of peripheral metabolism might underlie lower brain uptake of several other dopamine targeted radiotracers in awake versus anesthetized mice (Honer et al., 2006), although there was no assay of the rate of metabolism in that study. Our results indicate that, at least with ^{11}C-raclopride, there is no difference in the rate of peripheral metabolism between animals that are awake or anesthetized for radiotracer uptake (Fig. 2). Nevertheless, it is evident from Fig. 2 that the rate of ^{11}C-raclopride metabolism is more variable in awake versus anesthetized animals at the time of the scan (35 min after ^{11}C-raclopride injection). Thus, while there may be other contributions to the stark difference in brain uptake between awake and anesthetized animals (such as clearance of ^{11}C-raclopride from the blood), changes in the peripheral rate of ^{11}C-raclopride metabolism clearly did not contribute.

Ex vivo measurements of carbon-11 concentrations in the brain and peripheral tissue supported the much higher brain uptake of carbon-11 in anesthetized versus awake animals (Fig. 3 and Supplementary Table 1), however the magnitude of this difference is much less than that observed in our PET measurements (two-fold increase measured with tissue counting versus four-fold measured from the tomograph). A more detailed analysis indicated that the values for brain uptake in anesthetized animals were virtually identical between ex vivo biodistribution and *in vivo* PET measurements (Supplementary Table 1), while there was a significant difference in values obtained from animals in the Awake Uptake protocols. Granted, the ex vivo Awake Uptake protocol differed from the *in vivo* PET protocol in that animals studied ex vivo were euthanized 35 min after the ^{11}C-raclopride injection without anesthesia, while animals studied *in vivo* with PET were anesthetized 35 min after the ^{11}C-raclopride injection. Given the global effect observed here, that the Anesthetized Uptake condition is associated with significantly higher brain uptake (Figs. 3 and 4, Supplementary Table 1), one might expect that ex vivo animals under the Awake Uptake protocol (who received no anesthesia) would have lower brain uptake than animals under the *in vivo* PET Awake Uptake protocol (who received 30 min of anesthesia). In fact, we observed a significantly higher brain uptake in the ex vivo animals that received no anesthesia compared to the *in vivo* animals under the Awake Uptake PET protocol that required 30 min of anesthesia (Supplementary Table 1).

If anesthesia-induced increases in blood flow played a significant role, this would facilitate the delivery of ^{11}C-raclopride from plasma to brain tissue, resulting in an increase in brain uptake. In fact, PET studies using ^{15}O-H2O to measure regional cerebral blood flow (rCBF) in cats demonstrated that anesthetic doses of ketamine produced only slight increases in rCBF compared to the awake state (Hassoun et al., 2003). In agreement, ketamine is a vasodilator that decreases systolic and diastolic blood pressure as well as heart rate in rats (Rodrigues et al., 2006), and it also significantly decreases cerebral vascular resistance (Takeshita et al., 1972). However, this is a problematic explanation for two reasons; one, the late portion of the time-activity curves are only mildly affected by blood flow values. Second, a hypothesized increase in blood flow and/or other parameter effecting tracer delivery due to anesthesia occurring ~35 min after tracer administration would effectively increase delivery out of the brain in the same way it would increase delivery into the brain during uptake. In this way the difference in protocols between the awake PET measures (which include the effects of anesthesia after 35 min uptake) and the awake ex-vivo measures (void of all anesthesia) could explain in part the discrepancy between the two assays.

It is important to note that despite the significant difference in ^{11}C-raclopride uptake in awake and anesthetized animals, there was no significant difference in specific binding using the (ST−CB)/CB ratio ($p>0.1$; see Table 1). Thus, it is possible that the cardiovascular and/or hemodynamic effects of different anesthetics may uniquely alter radiotracer binding, but this will only impact PET studies which use radiotracers and quantitation methods that are sensitive to blood flow effects, which are not likely to affect the late portion of ^{11}C-raclopride uptake measured here. Taken together, there are important differences between the awake and anesthetized animals that may or may not affect measurements of specific radiotracer binding, emphasizing the need to carefully consider potential interactions between radiotracers and anesthetics (Elfving et al., 2003).

Anesthesia may profoundly affect the outcome of any PET measurement. In fact, the use of anesthesia can attenuate and even block normal neurochemical responses (Ginovart et al., 2002). Halothane has been reported to convert D_2-receptors to a lower affinity state (Ginovart et al., 2002) as well as increase striatal dopamine levels (Ford and Marsden, 1986; Keita et al., 1999; Mantz et al., 1994; Miyano et al., 1993; Osborne et al., 1990; Savaki et al., 1986; Shiraishi et al., 1997; Spampinato et al., 1986; Stahle et al., 1990). Isoflurane potentiates the effect of dopamine enhancer drugs (Tsukada et al., 1999) as well as significantly lowers ^{11}C-*N*-methylspiperone binding to D_2 receptors (Kobayashi et al., 1995). Similarly, ketamine significantly effects dopamine transmission when used at subanesthetic doses (Breier et al.,1998; Smith et al., 1998). However, when used as a form of anesthesia at higher doses ketamine shows no significant effect on dopamine transmission (Irifune et al., 1997; Koshikawa et al., 1988; Lannes et al., 1991; Mantz et al., 1994; Micheletti et al., 1992; Onoe et al., 1994; Tsukada et al., 2000; Ylitalo et al., 1976). Consistent with these data, we observed no significant increases in extracellular dopamine in animals given anesthetic doses of ket/xyl using *in vivo* microdialysis (unpublished results). It is also important to note that effects of needle pokes and handling from conventional anesthesia administration were negated by administering anesthesia via an exteriorized jugular catheter which was extended outside the home cage. Moreover, the animal was gently moved to the scanning bed only after complete anesthesia was induced.

The anesthesia required for scanning in the Awake Uptake protocol described here might have physiological consequences that obscure changes in radiotracer binding. If changes in blood flow or radiotracer clearance selectively altered ^{11}C-raclopride uptake in the cerebellum, then we would expect differences in the ratio of striatal to cerebellar activity between animals that are awake versus those who were anesthetized during uptake. This is not the case. Further, neither the window of available binding potentials provided by blocking studies nor the responsiveness of dopamine to a stimulant challenge significantly differ between the two experimental conditions (Awake versus Anesthetized Uptake), although there is more variability in blocking studies, see Table 1. All of this evidence points toward the utility of this method for imaging dopaminergic transmission in behaving animals, which is our ultimate goal. While a limitation of the present strategy is that ^{11}C-raclopride binding is measured during a single block of time following radiotracer administration, which may affect the sensitivity of ^{11}C-raclopride binding to subtle perturbations of animal behavior. These experiments provide evidence that this method can be used to image dopamine transmission – perhaps with the same anesthetic confounds as dynamic PET studies – but now we can simultaneously measure animal behavior using a neurotransmitter-specific radiotracer. This greatly extends the scope and clinical relevance of small animal PET experiments.

One approach that has been employed to minimize confounding effects of anesthesia is to adapt animals to the physical restraint required for awake animal PET experiments by behavioral conditioning or repetitive training regimens. However, these strategies may impact imaging studies in two fundamental ways. First, food deprivation required for most training protocols can alter the responsiveness of neurochemical systems to challenge (Pothos et al., 1995). Second, not only does acute handling stress alter neurotransmitter levels (Abercrombie et al., 1989; Cabib and Puglisi-Allegra, 1996; Enrico et al., 1998; Feenstra et al., 1995; Imperato et al., 1990; Kawahara et al., 1999), but these alterations can persist for weeks to months, even with daily episodes designed to adapt animals to immobilization (Hauger et al., 1988; Rusnak

et al., 1998; Zelena et al., 2004, 2003). Thus, while several groups have successfully used PET to measure dopamine-induced changes in ^{11}C-raclopride binding in awake monkeys and cats (Hassoun et al., 2003; Tsukada et al., 2002), these pioneering studies involved protracted training sessions and required a range of behavioral responses restricted to those which could be performed under restraint.

The present method of imaging ^{11}C-raclopride binding in freely moving animals minimizes the time under anesthesia, effects of stress, and the constraints imposed by rigorous and often protracted training paradigms. Thus, it provides an increased flexibility to measure the effects of various drug and/or behavioral challenges in brain dopamine in awake, freely moving animals. For behaviors that can be sustained on the order of 20–30 min, this approach opens the possibility for exploration of specific molecular events which underlie complex behaviors (i.e. conditioned place preference, drug self-administration) that have remained largely inaccessible to other brain mapping techniques. Moreover, this novel paradigm can serve as the experimental basis to study other neurotransmitter-specific radiotracers for conscious small animal imaging, and it may also be useful to investigate other behavioral states linked to dopamine such as sex, food or novelty.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2008.02.065.

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