Functional neuroimaging of amphetamine-induced striatal neurotoxicity in the pleiotrophin knockout mouse model

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ABSTRACT

Amphetamine-induced neurotoxic effects have traditionally been studied using immunohistochemistry and other post-mortem techniques, which have proven invaluable for the definition of amphetamine-induced dopaminergic damage in the nigrostriatal pathway. However, these approaches are limited in that they require large numbers of animals and do not provide the temporal data that can be collected in longitudinal studies using functional neuroimaging techniques. Unfortunately, functional imaging studies in rodent models of drug-induced neurotoxicity are lacking. The aim of this study was to evaluate *in vivo* the changes in brain glucose metabolism caused by amphetamine in the pleiotrophin knockout mouse (PTN-/-), a genetic model with increased vulnerability to amphetamine-induced neurotoxic effects. We showed that administration of amphetamine causes a significantly greater loss of striatal tyrosine hydroxylase content in PTN-/- mice than in wild-type (WT) mice. In addition, [^{18}F]-FDG-PET shows that amphetamine produces a significant decrease in glucose metabolism in the striatum and prefrontal cortex in the PTN-/- mice, compared to WT mice. These findings suggest that [^{18}F]-FDG uptake measured by PET is useful for detecting amphetamine-induced changes in glucose metabolism *in vivo* in specific brain areas, including the striatum, a key feature of amphetamine-induced neurotoxicity.

KEYWORDS: [^{18}F]-FDG-PET, amphetamine, pleiotrophin, neurotoxicity, Parkinson's disease, midkine.

INTRODUCTION

Amphetamine and its derivatives are widespread drugs of abuse that lead to addictive behavior and severe neurological damage [7, 39]. As a drug of abuse, amphetamine activates the reward pathway of the brain. Most of the mesolimbic and mesocortical projections involved in reward are located in the ventral tegmental area. Its dopaminergic neurons send their axons to nucleus accumbens, the striatum and prefrontal cortex. On the other hand, hallmarks of neurotoxicity induced by these drugs include proliferation of astrocytes in striatum [19], apoptosis of striatal neurons, and destruction of striatal dopaminergic terminals [2, 19]. In addition, amphetamine derivatives have been shown to induce dopaminergic cell loss in the substantia nigra, which is also a feature of Parkinson's disease (PD) [11]. The prevalence of PD was recently shown to be increased in users of amphetamine-type drugs [5], suggesting that these drugs increase the risk of developing PD. However, the authors also showed that a high proportion of the 40,000 methamphetamine users enrolled did not develop PD during the 15 years of the study, thus suggesting the possible existence of genetic factors underlying individual vulnerability to PD after consumption of this type of psychostimulants. These genetic factors include the neurotrophic factor pleiotrophin (PTN), which has recently received significant attention [12]. PTN has been shown to limit the neurotoxic effects of amphetamine [9, 10]. Accordingly, genetic inactivation of PTN confers increased vulnerability to amphetamine-induced neurotoxic effects in the nigrostriatal pathway. Administration of amphetamine in mice that are genetically deficient in PTN (PTN-/-) results in increased astrocytosis in the striatum and substantia nigra, increased loss of striatal dopaminergic terminals,

and, surprisingly, loss of tyrosine hydroxylase (TH)–positive neurons in the substantia nigra, an effect that is not observed in WT mice [10]. These different responses to amphetamine in PTN-/- and WT mice suggest this model could be useful for identifying the remaining unknown mechanisms through which amphetamine exerts its neurotoxic effects and for identifying druggable targets within those mechanisms [12].

Traditional studies characterizing amphetamine-induced neurotoxic effects are subject to significant limitations that prevent more efficient approaches. Immunohistochemistry and other post-mortem techniques have proven invaluable in our investigations of amphetamine-induced dopaminergic damage in the nigrostriatal pathway. However, they require large numbers of animals and do not provide the temporal data that can be collected in longitudinal studies. Functional neuroimaging techniques, such as positron emission tomography (PET), single-photon emission computed tomography, functional magnetic resonance imaging (fMRI), and electro-encephalography, have been extensively applied to study the emotional and cognitive-behavioral components of addiction [27]. These techniques are ideally suited for studying the potential neurotoxic effects of drugs in the living brain and make it possible to characterize the functional effects of parkinsonian toxins in the rodent brain [16]. MRI has been used in humans to investigate *in vivo* tissue abnormalities caused by the neurotoxic effects of drugs [36]; however, functional imaging studies in animal models are lacking. In this study, we used the PTN-/- mouse, a genetic model with increased vulnerability to amphetamine-induced neurotoxic effects, to validate [^{18}F]-fluoro-2-deoxy-d-glucose ([^{18}F]-FDG) PET as a useful neuroimaging marker of neurotoxicity in mice.

MATERIAL AND METHODS

PTN-/- mice

PTN-/- mice were generated as previously described [1]. We used male PTN-/- and PTN+/+ (WT) animals aged 9-10 weeks (20-25 g). All experimental animal procedures complied with European Union Council Directive 2010/63/EU and were approved by the Institutional Animal Care and Use Committee.

Drug protocol

Animals received 4 consecutive intraperitoneal injections of amphetamine (10 mg/kg) or saline (10 ml/kg) with an interval of 2 hours between administrations. This “binge” regimen is known to cause significant damage to striatal dopaminergic terminals [20]. For both immunohistochemistry (IH) and PET imaging studies, mice were divided into 4 groups according to the drug protocol applied: A) PTN-/- + saline (PTN-/- control); B) PTN-/- + amphetamine; C) WT + saline (WT control); and D) WT + amphetamine.

Our study sample comprised 22 PTN-/- and 22 WT mice. The animals used for the IH studies were different from those used in the PET studies in order to test for striatal TH loss (IH) and FDG uptake (PET) at the same time point after the last amphetamine or saline administration. The sample size for both IH and PET studies was 5-6 animals per group.

Immunohistochemistry studies

One of the consequences of amphetamine administration is the loss of dopaminergic terminals in the striatum [3]. We previously demonstrated significantly greater striatal dopaminergic denervation in PTN-/- than in WT mice [8]. In confirmation of those studies, and as part of the validation process of [^{18}F]-FDG-PET to assess the neurotoxic effects of amphetamine, expression of

TH in the striatum of PTN-/- and WT mice treated with either amphetamine or saline was analyzed using immunohistochemistry.

Animals were euthanized 4 days after the last administration of amphetamine or saline because at this time point we have previously found significant between-genotype differences in amphetamine-induced striatal TH loss [9]. The brains were removed immediately and conserved in p-formaldehyde for 7 days before being transferred to a solution of 0.1 M phosphate buffer containing 0.02% sodium azide for storage at 4ºC. The brains were cut into 30 µm sections using a vibratome (Leica, Wetzlar, Germany), and striatal free-floating sections were processed following a protocol previously described [9]. Striatal TH-positive fiber staining was assessed using optical density (OD) measurements after digitalized images of TH-immunostained striatal sections were collected. ODs were measured using Image-Pro Plus software (Version 3.0.1; Media Cybernetics, Silver Spring, Maryland, USA). For each animal, the nonspecific background correction in each section was made by subtracting the OD value of the corpus callosum from the striatal OD value obtained from the same section.

PET-CT imaging studies

All animals were scanned 4 days after the end of the drug administration protocol using a small-animal PET/CT scanner (ARGUS, SEDECAL, Madrid, Spain) under isoflurane anesthesia (3% induction and 1.5% maintenance in 100% O_2). This anesthesia was used because of its lower effect on brain glucose metabolism compared to other anesthetics [32], although the ideal situation would have been to perform the imaging study at consciousness [25]. [^{18}F]-FDG (~0.5 mCi) was injected into the tail vein, and, after an uptake period

of 45 minutes, animals were imaged for 60 minutes. Images were reconstructed using a 2D-OSEM algorithm. The spatial resolution for the scanner was 1.45 mm full width at half maximum (FWHM). The energy window was 400-700 keV. Decay and deadtime corrections were applied.

Computed tomography (CT) studies were acquired using the above-mentioned PET/CT scanner. The images were reconstructed using a Feldkamp algorithm. These anatomical images did not require registration with their corresponding PET scans thanks to the intrinsic alignment of the PET/CT device.

MRI study

An MRI study of one WT mouse was acquired with a 7-Tesla Biospec 70/20 scanner (Bruker, Ettlingen, Germany). The animal was anesthetized with sevoflurane (4.5% induction and 2.5% maintenance in 100% O_2). A T2-weighted spin echo sequence was acquired with TE=33 ms, TR=4281 ms, and a slice thickness of 0.4 mm (33 slices). Matrix size was 256 x 256 pixels at an FOV of 1.6 x 1.6 cm^2. The inhomogeneity artifact caused by the use of the surface antenna was corrected.

This single study was used as an anatomical template for the PET images.

Analysis of PET data

PET data were analyzed using regions of interest (ROI). All CT studies were automatically co-registered with a reference CT scan that was chosen randomly from among the animals [28]. Since the PET/CT small animal scanner provides aligned CT and PET images for each animal, the spatial transformation obtained for each CT image was subsequently applied to the

corresponding PET image. The ROIs drawn on coronal sections in the MRI image were striatum, prefrontal cortex, thalamus, cerebellum (CB), background, and whole brain (WB). Two regions of reference (ROI_{ref}) were used to normalize [^{18}F]-FDG uptake, the whole brain (ratio to WB) and the cerebellum (ratio to CB). WB is the preferred reference region in most studies with small animals [32, 34, 35] (provided there are no between-group differences in this region [6], as it is our case). The PET data assessment included the analysis of mean Standardized Uptake Value (SUV), which takes into account the tissue radioactivity concentration, the injected activity and the body weight.

Statistical analysis

Data from IH and PET studies are presented as mean ± standard deviation (SD). The hypothesis of normality of the distributions and homogeneity of variance were assessed using the Kolmogorov–Smirnov and Levene tests, respectively. Intra-subject variability was assessed through the coefficient of variation (COV), which shows a range of 15-30% in other studies on neurodegenerative disorders characterized by striatal dopaminergic damage [14]. Data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc tests (Tukey). All statistical analyses were performed using the SPSS 15.0 software package.

The threshold for statistical significance was set at $p < 0.05$.

RESULTS

Immunohistochemistry

ANOVA of TH expression in the striatum revealed a significant group effect ($F=30.68$, $p<0.0001$, d.f.=3). The striatal TH levels in amphetamine-

treated WT animals (0.49±0.07 arbitrary units [au]) were significantly lower than those of WT controls (0.90±0.06 au) ($p<0.01$) (Fig. 1A). The striatal TH levels in amphetamine-treated PTN-/- animals (0.18 ± 0.04 au) were also significantly lower than those of PTN-/- controls (0.84 ± 0.09 au) ($p<0.001$). Interestingly, we also found that striatal TH levels in amphetamine-treated PTN-/- mice were significantly lower than those in amphetamine-treated WT mice, whereas TH levels in control animals were similar in both genotypes (Fig. 1A).

PET

Measurements based on absolute value of SUV for each ROI resulted in no significant statistical differences between groups (Fig. 1C), probably due to the high COV (range: 5-30%). When PET data were normalized, COV decreased to 2-9%, thus allowing us to detect statistically significant differences. We performed the analysis of [^{18}F]-FDG uptake data using two ROI_{ref} for normalization, the WB and CB. One pre-requisite to use WB as the reference region is to make sure there are no between-group differences in global uptake [6], as it is our case. The tendency with both normalizations was similar, although statistical significance was achieved when normalizing to WB. We decided to use WB as our ROI_{ref}, as it is the case in most studies with small animals [32, 34, 35].

ANOVA of [^{18}F]-FDG uptake in the prefrontal cortex and striatum revealed a significant group effect ($F=4.924$, $p=0.011$, d.f.=3 and $F=6.747$, $p=0.003$, d.f.=3 respectively). No statistically significant between-group differences were found in the thalamus and cerebellum (Fig. 1B,C).

Striatum: Uptake in amphetamine-treated PTN-/- animals (1.12±0.06) was significantly lower than in amphetamine-treated WT mice (1.27±0.08)

(p=0.009) (Fig. 1B,C). We also found that striatal [^{18}F]-FDG uptake in amphetamine-treated PTN-/- mice was significantly lower compared with saline-treated WT mice (p=0.004), whereas [^{18}F]-FDG uptake in control animals was relatively similar in both genotypes (Fig. 1B,C).

Prefrontal cortex: [^{18}F]-FDG uptake in amphetamine-treated PTN-/- mice (1.02±0.06) was significantly lower than in amphetamine-treated WT mice (1.16±0.05) (p=0.012), whereas [^{18}F]-FDG uptake in control animals was relatively similar in both genotypes (Fig. 1B,C).

DISCUSSION

To our knowledge, this is the first report to demonstrate that striatal damage caused by amphetamine treatment correlates with significantly decreased striatal [^{18}F]-FDG uptake in PTN-/- mice and can therefore be assessed using [^{18}F]-FDG-PET. Also, a significant decrease in glucose metabolism was found in the prefrontal cortex in amphetamine-treated PTN-/- mice compared to WT mice.

Despite existing evidence based on the results of imaging techniques in humans [31, 36], few studies have used [^{18}F]-FDG-PET to explore the effects of drugs in animal models [13, 35]. This gap in knowledge is significant, considering that the most remarkable advances in the characterization of the mechanisms underlying psychostimulants-induced neurotoxicity have been achieved in rodent models. In addition, these models have enabled identification of critical genetic factors modulating the toxic effects of psychostimulants and potential pharmacological targets to prevent the brain damage they induce [12].

The recent development of PET scanners for laboratory animals enables detailed *in vivo* study of the neurotoxic effects of drugs in rodent models by providing insights into the biochemical and molecular processes involved and noninvasive follow-up with neuromodulatory approaches [35]. Because of the close relationship between energy expenditure (glucose metabolism) and functional activity, PET may provide an alternative way to explore the underlying mechanisms of amphetamine induced neurotoxicity using the minimum number of animals [15]. In the present study, [^{18}F]-FDG was used as a marker of cerebral glucose consumption resulting from neuronal entrapment and accumulation of [^{18}F]-FDG-6-PO4, which indicates neuronal activity [32]. [^{18}F]-FDG can be considered a broad-spectrum tracer, since its uptake in the brain is by all existing cell types, independently of the neurotransmission system involved. Thus, [^{18}F]-FDG is a good candidate tracer for screening when the neurotransmission system affected is not known. Another advantage of [^{18}F]-FDG is that it has been efficiently applied in humans to demonstrate changes in relative regional cerebral glucose metabolism in users of this type of psychostimulant [17, 18, 23, 38]. Finally, the much wider availability of FDG (as compared to more specific dopaminergic tracers) makes validation of this tracer more likely to become useful in clinical medicine and animal research. Our [^{18}F]-FDG PET data showed a slight, statistically non-significant, decrease in striatal and prefrontal cortex glucose metabolism in the WT mice, whereas glucose uptake was significantly reduced in the striatum and prefrontal cortex of PTN-/- mice.

Cortico-striato-thalamic Pathway

The [^{18}F]-FDG method enables simultaneous visualization of metabolic changes throughout the entire nervous system, making it possible the identification of complex neuronal circuits which mediate the response to a pharmacological manipulation, similarly to [^{14}C]-deoxyglucose [33]. In this respect, we found changes in [^{18}F]-FDG uptake in a different brain area in addition to the striatum, which highlights that not only dopamine cells are affected, as other authors have already pointed out [38]. The prefrontal cortex is part of the mesolimbic system and crucial for drug reward. Its dopaminergic innervation is limited compared to striatum. The thalamus, with poor dopaminergic innervation, is an important component of the cortico-striato-thalamo-cortical loops. It constitutes a central link between the basal ganglia (striatum) and cerebral cortices and mediates information flow between cortical networks [24]. Imaging studies in methamphetamine abstinents have provided evidence of functional abnormalities in the thalamus and frontal cortex [37, 38, 40]. Even a dose-dependent frontal hypometabolism has been described in methamphetamine abusers [18]. Kim and colleagues found significantly lower metabolism in the prefrontal cortex in methamphetamine abusers, which was associated to frontal executive dysfunction [17, 18].

Striatum

[^{18}F]-FDG results in the striatum are consistent with those of previous studies by our group [10] where we detected moderate depletion of TH content (~40%) in the striatum of amphetamine-treated WT mice compared with saline-treated WT mice, and a significant loss in TH content (~75%) in the striatum of amphetamine-treated PTN-/- mice compared with saline-treated PTN-/- mice. Overall, the amphetamine-induced changes in striatal glucose metabolism

presented here are consistent with the trend in the most well-established amphetamine-induced neurotoxic effect in animal models, namely, striatal TH loss [10, 23].

Although striatal glucose metabolism might be expected to dramatically decrease after administration of amphetamine owing to reduced dopaminergic activity in the nigrostriatal pathway [21], it is important to note that other factors, such as neuroinflammation, could compensate for this reduction, since [^{18}F]-FDG uptake increases in neuroinflammation processes [29]. In this respect, a correlation has been found between [^{18}F]-FDG and [^{11}C]-PK11195, a marker of activated microglia, in the seizure focus of patients with epilepsy [4]. Microglia acts as the resident macrophages in brain tissue and represents the initial responder to tissue damage or foreign pathogens. Activated microglia releases a variety of cytotoxic compounds intended to attack these pathogens, but which may also cause neuronal damage. Although methamphetamine abuse leads to the most profound neurotoxicity in the dopamine-rich basal ganglia [37], it also increases proliferation of cortical microglia and astrocytes [22], which in turn may increase the global cerebral metabolic rate [30]. Therefore, Volkow et al. [38] suggested that higher parietal activity in methamphetamine abusers could reflect reactive gliosis after excitotoxic damage to parietal neurons.

In addition, amphetamine treatment induces the appearance of reactive glial cells and astrocytes in the striatum of PTN-/- mice, as we showed in a previous study by our group [9]. Interestingly, the number of glial fibrillary acidic protein (GFAP)-positive astrocytes in the striata was higher after amphetamine treatment in PTN-/- compared with WT mice, and also the astrocytes in the PTN-/- mice developed large densely stained cell bodies and long extensive

processes compared to the WT mice. These findings could mask a more significant decrease in glucose uptake from dopaminergic neurons than that reflected in the PET assays. Nevertheless, it is noteworthy that the amphetamine-induced decrease in striatal glucose metabolism in our murine model is quantitatively comparable to that observed in the striatum of human addicts to this type of psychostimulant [38]. Volkow and colleagues found significantly lower metabolism in the striatum (~10%) of abstinent methamphetamine users [38], similar to that obtained by us in the mice striatum. Reduced striatum and amygdala [^{18}F]-FDG uptake was observed in ecstasy users when compared to controls [26]. In this regard, our data suggest that [^{18}F]-FDG PET may be useful for detecting amphetamine-induced striatal damage in longitudinal studies in mice.

CONCLUSION

Our findings support the use of PET for testing of [^{18}F]-FDG uptake to evaluate amphetamine-induced damage *in vivo* in specific brain areas, including the striatum, in rodent models. PET could help to overcome the limitations of more traditional approaches by making it possible to perform longitudinal studies in reduced numbers of animals.

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FIGURE LEGENDS

Figure 1. A) TH-immunostained striatal sections of mice, 4 days after administration of saline (SAL) or amphetamine (AMPH) in PTN-/- and WT mice. The photomicrographs illustrate amphetamine-induced TH loss. The graph shows OD measured in arbitrary units (au) of TH- immunoreactive fibers in the striatum. Data are shown as mean±SD (n=5-6 animals per group). **p<0.01 vs. SAL. ***p<0.001 vs. SAL. $^{\#}$p<0.05 vs. WT. Scale bar=200 μm. B) PET study in the striatum of PTN-/- and WT mice after administration of saline or amphetamine. [^{18}F]-FDG-PET overlaid on the MR_{ref} revealed differences in brain glucose metabolism in the striatum. The graphs show the ratio to WB in the striatum, prefrontal cortex, thalamus and cerebellum. Data are shown as mean±SD (n=5-6 animals per group). $^{\#}$p<0.05 vs. WT, $^{\#\#}$p<0.01 vs. WT. C) [^{18}F]-FDG uptake: absolute value of SUV, ratio-to-WB and ratio-to-CB for each ROI in the four groups of study. Data are shown as mean±SD (n=5-6 animals per group). $^{\#}$p<0.05 vs. WT, $^{\#\#}$p<0.01 vs. WT.