PET imaging of cholinergic deficits in rats using [18F]fluoroethoxybenzovesamicol ([18F]FEOBV)

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A B S T R A C T

[18F]fluoroethoxybenzovesamicol ([18F]FEOBV) is one of the most promising radioligands for imaging the vesicular ACh transporter (VACHT) with positron emission tomography (PET). We report here that this method can detect subtle cholinergic terminals losses such as those associated with aging, or those following a partial lesion of the nucleus basalis magnocellularis (NBM). Twenty-one adult rats were evenly distributed in three groups including 1) aged rats (18 months); 2) young rats (3 months); and 3) rats with unilateral lesion of the NBM. In both normal and lesioned rats, our results revealed the highest [18F]FEOBV binding to be in the striatum, followed by similar values in both frontal cortex and thalamus, while lower values were observed in both hippocampus and temporo-parietal cortex. This binding distribution is consistent with the known anatomy of brain cholinergic systems. In the lesioned rats, [18F]FEOBV binding was found to be reduced mostly in the ventral frontal cortex on the side of the lesion, but some reductions were also observed in the homologous region of the contralateral hemisphere. Aging was found to be associated with a [18F]FEOBV binding reduction limited to the hippocampus of both hemispheres. [18F]FEOBV appears to be a very promising marker for the in vivo quantification of the brain VACHT. PET imaging of this agent allows in vivo detection of both physiological and pathological reductions of cholinergic terminals density.

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Introduction

Cholinergic neurotransmission plays a key role in numerous and varied functions, from cognition to regulation of autonomic systems. Presynaptic components of the cholinergic neurons such as choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT) are recognized markers of those cells, which are vulnerable targets of the aging process and of many neurodegenerative conditions (Mufson et al., 2008; Quirion, 1993). For instance, loss of presynaptic cholinergic terminals constitutes an early aspect of the neuropathological process present in Alzheimer’s disease (AD), as well as in progressive supranuclear palsy (PSP), and other Parkinsonian syndromes. Post-mortem studies have shown that cholinergic markers depletion strongly correlates with symptoms severity in AD (Dournaud et al., 1995; Prohovnik et al., 2006).

There is currently a lack of radiolabelled imaging agents for the in vivo quantification of cholinergic deficits. The need for such a tool is regularly emphasized (Thal et al., 2006; Ward, 2007) as a potential biomarker for AD and other neurodegenerative diseases. Current imaging agents for the brain cholinergic systems provide information on a wide range of acetylcholine receptors as well as on its synthesizing enzyme, but those remain indirect surrogate markers of the cholinergic fibers per se. Direct imaging of the presynaptic cholinergic terminals is still a challenge in nuclear medicine, although some radiolabeled compounds have been studied with uneven successes. Vesamicol may be considered as the prototype radiotracer for quantifying VACHT (Efname, 2000). Amongst vesamicol derivatives for in vivo imaging, [18F]fluoroethoxybenzovesamicol ([18F]FEOBV) was found to be the most promising agent for the purpose of quantifying VACHT with positron emission tomography (PET). It displays fast kinetics, readily crosses the blood brain barrier, and binds with high affinity and selectivity to VACHT (Mulholland et al., 1998). In addition, peripheral [18F]FEOBV metabolism does not generate metabolites capable of crossing the blood–brain barrier (Landry St-Pierre et al., 2008b). [18F]FEOBV is also stable in the brain and plasma, and does not generate significant amounts of free [18F]fluoride in pri-mates, as opposed to other vesamicol derivatives (Giboureau et al.,...
2007; Kilbourn et al., 2009; Soucy et al., 2010). Despite slow in vitro kinetics (Mulholland et al., 1998), reversible kinetics of [18F]FEOBV can be observed during a 60 min dynamic PET scan (Kilbourn et al., 2009; Landry St-Pierre et al., 2008a). This discrepancy between in vivo and in vitro kinetic speeds is a relatively common feature of PET radiopharmaceuticals, a notable example being [3H]SCH 23390 (Gifford et al., 2000).

VACHT may be quantified using reference tissue models due to its absence in the cerebellum (Ichikawa et al., 1997). It has previously been imaged in the living human brain with single photon emission computed tomography (SPECT) and [123I]IBVM, a radio-iodinated benzovesamnicol analog. However, quantification on VACHT using [123I]IBVM is complex due to its slow kinetics and other quantification issues (Mazer et al., 2008).

PET [18F]FEOBV allows mapping of VACHT concentrations present in distinct brain cholinergic projections in both rodents (Mulholland et al., 1998) and primates (Kilbourn et al., 2009; Soucy et al., 2010). The highest binding has been invariably found to be in the striatum, corresponding to the local cholinergic interneurons (Mesulam et al., 1992). Significant [18F]FEOBV binding has also been detected in the cortex, the hippocampus and the thalamus, corresponding to projections arising respectively from the nucleus basalis of Meynert (NBM), the septal area, and the pontomesencephalic cholinergic nuclei (Mesulam and Geula, 1988; Mesulam et al., 1983; 1989). These data have been obtained in the normal healthy brain, but the potential clinical applications of [18F]FEOBV would rest mainly on its ability to detect pathological VACHT changes in the living brain. How sensitive is [18F]FEOBV in detecting the slight cholinergic declines associated with aging, or the specific topographic denervation subsequent to a cholinergic nucleus lesion remains to be established, and the current study aims to address these questions.

We hypothesized that decreases in binding of the radioligand to VACHT will be observed both in rats with a NBM lesion and in aged rats, when compared with controls. NBM lesions should result in a [18F]FEOBV binding reduction in specific neocortical subregions (Pizzo et al., 1999), whereas in aged rats [18F]FEOBV binding should be reduced more diffusely in allocortical areas (Ypsilanti et al., 2008).

Methods

All experiments followed the Canadian Council on Animal Care guidelines. Twenty-one adult male Long-Evans rats were used for this study. They were evenly distributed in three groups of 1) Old rats, average age of 18 months (750–800 g); 2) young rats, average age of 3 months (250–300 g); and 3) rats undergoing unilateral lesion of the NBM (average age of 3 months, 250–300 g). All rats were housed under standard conditions in a 12 h–12 h light-darkness cycle, with ad libitum access to water and food.

Imaging procedures

On each scanning day, [18F]FEOBV was synthesized using a modified method (Mzengeza et al., 2007) originally described by Mulholland et al. (1993). A levo enantiomerically pure precursor (ABX advanced biochemical compounds GmbH) was used, labeled with [18F] using a GE TRACERlab module, resulting in (−)-[18F] FEOBV, which is the only enantiomer showing affinity for VACHT (Mulholland et al., 1998).

All rats (n = 21) were scanned using a CTI Concorde R4 microPET for small animals (Siemens Medical Solutions). Each PET session consisted in a 5 min transmission followed by a 60 min emission scan. PET scans were conducted under light isoflurane anesthesia (2% in medical air) delivered by a nose cone. Temperature, heart rate and blood pressure were monitored throughout the procedure using a BIO PAC system. The transmission scan was obtained using a rotating [57Co] point source. Emission scans were initiated immediately after the transmission scan with a bolus injection of 11.1 to 18.5 MBq (SA = 227.6 TBq/mmol) of [18F]FEOBV administered in the tail vein. Transmission and emission scans were obtained using list mode acquisition. Images were histogrammed into 27 sequential time frames of increasing duration (8 × 30 s, 6 × 1 min, 5 × 2 min, 8 × 5 min) over 60 min. Images were reconstructed using a maximum a posteriori (MAP) algorithm, normalized and corrected for scatter, dead time and decay.

Images processing and estimation of binding parameters

Imaging analysis was conducted using mimtools (http://www.bic.mni.mcgill.ca/ServicesSoftware/HomePage). Time-averaged tissue-radioactivity images were manually co-registered to a standard rat histological template (Rubins et al., 2003) using 7 degrees of freedom (rigid body transformation plus one scaling constant). The image outcome measure non-displaceable binding potential (BPND) was estimated using a reference-tissue based graphical method for reversible ligands (Logan et al., 1990). Equilibrium was reached at 10 min after injection. The cerebellar cortex served as a reference region due to its negligible amounts of cholinergic markers, as revealed by histological studies (De Bartolo et al., 2009; Schafer et al., 1994). [18F]FEOBV BPND was estimated for every dynamic scan. The resulting images were convolved using a Gaussian kernel (FWHM = 0.6 mm), then normalized to their global BP value to account for inter-individual differences.

Regional distribution

In order to quantify [18F]FEOBV distribution in the rat brain, the following volumes of interest (VOI) were traced on a standard stereotaxic template: 1) frontal cortex; 2) temporo-parietal cortex; 3) striatum; 4) hippocampus, and 5) thalamus. The populational regional BPND of all seven young non-lesioned rats was computed for each of these VOIs.

NBM lesion study

NBM immunocytotoxic lesions were induced in one group of seven young rats using unilateral (left hemisphere) stereotaxic injection of the cholinergic neuron-selective immunotoxin 192 IgG-saporin (Pizzo et al., 1999). Animals were first placed in a surgical stereotaxic frame, following a brief anesthetic induction with 5% isoflurane administered in an induction chamber. During the stereotaxic procedures, anesthesia was maintained with 2% isoflurane. 192 IgG-saporin (Advanced Targeting Systems, lot 64–124) was infused with a microsyringe in the NBM (0.2 μL dissolved in 0.2 μL, n = 3), or 0.25 μg in 0.5 μL (n = 4). The stereotaxic coordinates, taken from Paxinos and Watson (2009), were the following: 1 mm posterior to bregma, 2.8 mm lateral to midline, and 7.6 mm ventral to cranium surface (n = 5), or 0.5 mm, 2 mm and 8.5 mm (n = 2). During the 2 weeks following the surgery, no other experimentation was done with these rats in order to allow a full recovery.

Aging study

Aging effects on the cholinergic systems were estimated cross-sectionally in the group of 18 months-old rats (n = 7; all males; average weight of 800 g). These animals were only used for this experiment.

Statistical analysis

Regional brain differences in the non-lesioned young rats (n = 7) were estimated across the five VOIs using one-way analysis of variance (ANOVA). Differences between the lesioned (n = 7) and all non-lesioned rats (n = 14) rats were assessed using both VOIs and voxel-based analyses, via one-way ANOVAs with age as covariate.
The threshold for significance for VOI analysis was \( p = 0.05 \). The threshold for significance of the voxel level analysis was set at \( t = 3.5 \) (\( p = 0.002 \)) for clusters of \( k > 5 \) mm\(^3\). In order to evaluate group effect in a significant cluster, BP\(_{\text{ND}}\) was averaged in voxels with \( t\)-values greater than 50% of the local maxima. The variability of NBM lesions was assessed by computing the probabilistic distribution of low \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) in the lesioned rats (\( n = 7 \)). Individual binary maps representing declines of \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) (\( z\)-score > 2) were compiled into a probabilistic map. Individual \( z\)-scores were obtained based on statistical values from non-lesioned animals. Comparisons between the young non-lesioned rats (\( n = 7 \)) and the old rats (\( n = 7 \)) were conducted at the voxel level using \( t\)-statistic analysis (Rmnc, https://launchpad.net/rmnc). The threshold for significance was \( t = 3.5 \) (\( p = 0.004 \)) for clusters of at least 100 contiguous voxels.

**Results**

Time-activity curves display a clear and fast \([^{18}\text{F}]\)FE0BV penetration and washout from various brain regions (see Fig. 1 for example). This reversibility was confirmed by multiple-time point analysis for irreversible ligands (Logan et al., 1990) using the cerebellum as reference tissue, as shown in Fig. 2. Regional distribution of \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) in the young normal rats (see Fig. 3) shows a significant effect of VOI; \( F(4, 13) = 14.44, p < 0.0005 \). Highest activity was found in the striatum (\( p < 0.001 \)), followed by frontal cortex or thalamus (\( p < 0.001 \)) and lastly by the hippocampus or temporo-parietal cortex (\( p < 0.01 \)). Fig. 4 shows this BP\(_{\text{ND}}\) distribution on the brain surface, together with selected slice examples.

In NBM lesioned rats, \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) declines occurred within a limited range of cortical areas and were diluted by large VOIs encompassing the whole frontal areas. Therefore group differences were not evident using VOIs, as seen in Fig. 3. In contrast, voxel-based analysis showed a large cluster in the left ventral portion of the frontal cortex (from the anterior commissure: \( x = -3.56 \) mm, \( y = 1.24 \) mm, \( z = -0.18 \) mm, \( k = 41.88 \) mm\(^3\)), with a mean BP\(_{\text{ND}}\) of 1.33 ± 0.04 in controls, and 1.14 ± 0.05 in the lesioned rats (\( t = 6.5, p < 0.0005, d = 4.2 \); Fig. 5). In the homologous region of the contralateral (right) hemisphere, a similar but smaller cluster was found (\( x = 4.3 \) mm, \( y = 0.89 \) mm, \( z = -1.1 \) mm, \( k = 23.34 \) mm\(^3\)). The BP\(_{\text{ND}}\) mean value in the contralateral cluster was 1.36 ± 0.03 in controls, and 1.18 ± 0.05 in the lesioned rats (\( t = 5.6, p < 0.0005, d = 4.37 \); Fig. 5). The probabilistic distribution of abnormal \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) revealed a large variability of NBM lesions effects (see Fig. 6).

Comparisons between young and aged rats showed significant \([^{18}\text{F}]\) FE0BV BP\(_{\text{ND}}\) declines in the hippocampi of the old rats, for both the left hemisphere (right) hemisphere, a similar but smaller cluster was found (\( x = 5.58 \) mm, \( y = -5.01 \) mm, \( z = -0.98 \) mm, \( k = 11.2 \) mm\(^3\)) and right (\( x = 5.58 \) mm, \( y = -6.13 \) mm, \( z = 1.31 \) mm, \( k = 13.44 \) mm\(^3\)). The average BP\(_{\text{ND}}\) in these clusters was 1.13 ± 0.04 and 1.03 ± 0.03 (\( t = 7.2, p = 0.0005, d = 2.83 \)) in young and old rats, respectively.

**Discussion**

The present study has established the quantitative regional distribution of \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) in the rat telencephalon using PET with a bolus injection and reference tissue method. In addition, a \([^{18}\text{F}]\) FE0BV BP\(_{\text{ND}}\) decline was described for the first time following both a unilateral cholinergic lesion of the NBM and as a result of aging.

**Normal brain distribution of \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\)**

Our results indicate high \([^{18}\text{F}]\)FE0BV BP\(_{\text{ND}}\) in the striatum, frontal cortex and thalamus, with lower values in the hippocampus and temporo-parietal cortex. This distribution pattern is in agreement with published values for concentrations of VACHT, known to be associated with cholinergic terminals (Gilmor et al., 1996; Ichikawa et al., 1997; Weihe et al., 1996). That distribution pattern is also consistent with the one found in other immunohistochemistry studies targeting presynaptic cholinergic markers such as ChAT and VACHT (Fuhrmann et al., 1985), as well as with previous PET imaging studies with \([^{18}\text{F}]\)
FEOBV (Landry St-Pierre et al., 2008a; Mulholland et al., 1998). The time-activity profiles of cortical and striatal uptakes are also mostly concordant with those reported by Kilbourn et al. (2009), with the exception of a lower uptake toward the end of the scanning period, possibly explained by the different rat strain used. Such a good agreement between our measurements of $[^{18}F]$FEOBV BPND and the literature supports the view that PET imaging with $[^{18}F]$FEOBV is a valid technique to measure cholinergic presynaptic terminal density in living individuals. The high striatal $[^{18}F]$FEOBV BPND most likely corresponds to the numerous terminals of the ACh containing mid spiny interneurons. In the thalamus, it likely depicts the well known cholinergic terminals arising from the pedunculopontine and latero-dorsal tegmental nuclei. In the cortex the greatest $[^{18}F]$FEOBV BPND was observed in the medial prefrontal area, which receives the densest cholinergic afferents from the NBM.

It should be noted however that while cholinergic selectivity of $[^{18}F]$FEOBV has been demonstrated in mice (using haloperidol, 3-PPP and donepezil pre-treatments to exclude the possibility of interactions with dopamine D2 or $\sigma$ receptors (Mulholland et al., 1998)), the same has yet to be reproduced in other species. This constitutes an important concern regarding the validity of the present findings in other species.

**NBM lesion effects on the brain $[^{18}F]$FEOBV BP$_{ND}$**

The BP$_{ND}$ outcome used in this study expresses the ratio between tissue receptor density ($B_{max}$), and $[^{18}F]$FEOBV dissociation constant ($K_D$) (Innis et al., 2007). Therefore, we believe that low BP$_{ND}$ values observed in the rats with unilateral NBM lesions reflect a reduced tissue concentration of VACt. Although a low BP$_{ND}$ could also result from an increased $[^{18}F]$FEOBV $K_D$, such an explanation remains unlikely if we consider that very little cholinergic markers can usually be observed in tissue after lesions with 192-IgG-saporin (Pizzo et al., 1999). Alternatively, a decline in regional cerebral blood flow may have been induced in our rats by the cholinergic lesions (Kovalenko and Matsievskii, 2005). However, it is difficult to estimate the impact of these changes using reference tissue analyses.

The statistical clusters representing declines of $[^{18}F]$FEOBV BP$_{ND}$ following the immunolesions were located in the ventral part of the frontal cortex. These areas were diffuse and heterogeneous enough...
that they were not statistically detected using a VOI method. This may be explained, at least in part, by variability in the 192-IgG saporin doses and infusion sites, resulting in variable cortical denervation. The inability of the VOI method to detect group differences may also be caused by the relatively small VOIs that were analyzed, in relation to the MicroPET resolution.

The clusters, relatively limited compared to the observed deafferentation following similar lesions (e.g. Pizzo et al., 2002) probably represent the intersection of individual heterogeneous lesions rather than a single lesion homogeneous across all subjects. Such an explanation is reinforced by the large variance of presynaptic cholinergic depletion depicted by the probabilistic lesion map shown in Fig. 6.

Bilateral infusion of 192-IgG saporin in the NBM has previously shown clear although variable ChAT reductions in the cortex, with decreases ranging from 42 to 70% (Risbrough et al., 2002). Unilateral lesions with similar 192-IgG saporin doses also produce a ChAT reduction, with depletions ranging from 25 to 31% (Wenk et al., 1994). Such a discrepancy might be present because the effects of a

Fig. 4. Average normal [18F]FEOBV BPND (n = 7) cortical distribution projected on the rat brain surface (top) and on the transaxial and coronal rat brain volumes (bottom). Highest BPND can be seen in the striatum (*), frontal cortex (**) and thalamus (***)..

Fig. 5. Statistical images projected on the brain surface and transaxional volumes. t-Statistical maps [non-lesioned > NBM lesioned] representing cortical declines of the [18F]FEOBV BPND following NBM lesions. Note the maximum effect on the ventral portion of the frontal cortex ipsilateral to the NBM lesions, with a similar but smaller effect on the contralateral side.
unilateral lesion are typically measured by comparing the lesioned hemisphere with the non-lesioned one. Our results suggest that the hemisphere contralateral to the lesion may not be an adequate control, as significant $^{18}$F-FEOBV BP$_{ND}$ decreases were observed on both sides. The smaller contralateral cluster of $^{18}$F-FEOBV BP$_{ND}$ decrease indicates a possible fiber crossover in NBM projections to the cortex.

Our results with the lesioned rats suggest that $^{18}$F-FEOBV may be useful for detecting cortical cholinergic denervations such as those known to be present in AD or dementia with Lewy Bodies (DLB) (Dournaud et al., 1995; Prohovnik et al., 2006). However, further studies are needed to evaluate the reliability of this detection when compared to post-mortem immunohistochemistry standards. In addition, it remains to be seen whether $^{18}$F-FEOBV can detect lesions in other areas, such as those of the pontine cholinergic projections to the thalamus, known to occur in DLB as well as in PSP (Henderson et al., 2000; Hirano et al., 2010).

**Aging effects on brain $^{18}$F-FEOBV BP$_{ND}$**

Aging effects on cholinergic neurotransmission have been extensively investigated in human brain tissue as well as in animal models with diverse results and interpretations. While postsynaptic cholinergic receptors are often shown to be sensitive to aging (Podruchny et al., 2003), there is no consensus regarding the effect on presynaptic markers; several studies have found aging-related changes in either hippocampus or cerebral cortex (or both), while others have investigated the same regions and came out with negative results (Sherman and Friedman, 1990).

Our results supporting a bilateral and symmetric aging-related depletion of cholinergic innervation in the hippocampus are consistent with previous studies showing fiber length reduction (Ypsilanti et al., 2008) or decrease of VACHT in the hippocampus of 18 and 24 months old rats (Canas et al., 2009). The age-related $^{18}$F-FEOBV BP$_{ND}$ decrease limited to the hippocampus suggests that contrary to pathological processes, normal aging primarily affects septohippocampal projections instead of those arising from the NBM or pontine nuclei.

Overall, this aging-related alteration supports specific cholinergic vulnerability linked to the aging process. Possibly, such vulnerability represents a neurodegenerative phenomenon amenable to neuroprotective treatments. However, much remains to be determined regarding the exact nature of this observation.

In conclusion, our results indicate that PET $^{18}$F-FEOBV is a sensitive method to quantify neocortical and allocortical presynaptic cholinergic terminals in the living brain. $^{18}$F-FEOBV demonstrates losses of

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**Fig. 6.** $^{18}$F-FEOBV BP$_{ND}$ declines following lesion of the NBM. Average BP$_{ND}$ images overlay on histological template (left) shows decline of binding following a NBM lesion in the left hemisphere (arrows). The probabilistic map (right) shows a large variability of NBM lesion across animals, likely caused by the variation in toxin doses and injection sites. The greatest effect can be observed in the ipsilateral frontal area, induced by most (60%) of the NBM lesions.

**Fig. 7.** $t$-Statistical maps [3 months > 18 months old rats] representing declines of $^{18}$F-FEOBV BP$_{ND}$ in the old rats projected on the brain surface and volumes. Note that the largest differences were observed in both hippocampi.
cholinergic terminals induced by lesions or physiological processes like aging. PET imaging of VACHT with \(^{[18}F]EFOBV\) represents a promising method for the quantification of presynaptic cholinergic deficits in human diseases.

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