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Regional Vesicular Acetylcholine Transporter Distribution in Human Brain: A [^{18}F]Fluoroethoxybenzovesamicol Positron Emission Tomography Study

Running Title: In Vivo Human Brain VAChT Distribution

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Abstract: Prior efforts to image cholinergic projections in human brain *in vivo* had significant technical limitations. We used the vesicular acetylcholine transporter (VAChT) ligand [^{18}F]fluoroethoxybenzovesamicol ([^{18}F]FEOBV) and positron emission tomography to determine the regional distribution of VAChT binding sites in normal human brain. We studied 29 subjects (mean age 47 [range 20-81] years; 18 men; 11 women). [^{18}F]FEOBV binding was highest in striatum, intermediate in the amygdala, hippocampal formation, thalamus, rostral brainstem, some cerebellar regions, and lower in other regions. Neocortical [^{18}F]FEOBV binding was inhomogeneous with relatively high binding in insula, BA24, BA25, BA27, BA28, BA34, BA35, pericentral cortex, and lowest in BA17-19. Thalamic [^{18}F]FEOBV binding was inhomogeneous with greatest binding in the lateral geniculate nuclei and relatively high binding in medial and posterior thalamus. Cerebellar cortical [^{18}F]FEOBV binding was high in vermis and flocculus, and lower in the lateral cortices. Brainstem [^{18}F]FEOBV binding was most prominent at the mesopontine junction, likely associated with the pedunculopontine-laterodorsal tegmental complex. Significant [^{18}F]FEOBV binding was present throughout the brainstem. Some regions, including the striatum, primary sensorimotor cortex, and anterior cingulate cortex exhibited age-related decreases in [^{18}F]FEOBV binding. These results are consistent with prior studies of cholinergic projections in other species and prior post-mortem human studies. There is a distinctive pattern of human neocortical VChAT expression. The patterns of thalamic and cerebellar cortical cholinergic terminal distribution are likely unique to humans. Normal aging is associated with regionally specific reductions in [^{18}F]FEOBV binding in some cortical regions and the striatum.

Keywords: Acetylcholine, striatum, basal forebrain, pedunculopontine nucleus, cerebellum, thalamus, aging, RRID: SCR_001847, RRID: SCR_007037

Abbreviations:

Acetylcholine	ACh
Acetylcholinesterase	AChase
Alzheimer Disease	AD
Amygdala	AM
Anterior Cingulate Cortex	AC
Basal Forebrain	BF
Choline Acetyltransferase	ChAT
Flocculus	FL
Fluoroethoxybenzovesamicol	FEOBV
Hippocampal Formation	HIP
Lateral Geniculate Nucleus	LG
Medial Vestibular Complex	MVC
Methyl-4-piperidinyl propionate	PMP
N-methyl-4-piperidyl acetate	MP4A
Mesopontine	MP
Parkinson disease	PD
Positron Emission Tomography	PET
Pedunculopontine-Laterodorsal Tegmental Complex	PPN-LDT
Single Photon Emission Computed Tomography	SPECT

Sensorimotor Cortex	SMC
Standardized Uptake Value Ratio	SUVR
Thalamus	THAL
Vermis	VER
Vesicular Acetylcholine Transporter	VAChT

Introduction: Acetylcholine (ACh) is the primary neurotransmitter of several CNS neuron populations. These include neurons of the basal forebrain (BF) complex projecting to numerous cortical regions, striatal cholinergic interneurons, the medial habenula-interpeduncular nucleus projection, projections of the pedunculopontine-laterodorsal tegmental (PPN-LDT) complex, the parabigeminal nucleus, some medial vestibular complex (MVC) neurons, and brainstem motor and autonomic neurons. BF cholinergic pathways participate in the modulation of important cognitive functions such as attention and executive function (Ballinger, Anath, Talmage, & Role, 2016; Hasselmo & Sarter, 2011; Prado, Janickova, Al-Onasi, & Prado, 2017). Projections of the PPN-LDT complex may play important roles in the regulation of sleep and waking, and some data implicates these neurons in control of balance and gait (Gut & Winn, 2016; Mena-Segovia & Bolam, 2017; Pienaar, Vernon, & Winn, 2016). Striatal cholinergic interneurons are the subject of intense investigations and play important roles in the control of a variety of functions mediated by the striatal complex (Deffains & Bergmann, 2015; Gonzales & Smith, 2015; Tanimura, Pancani, Lim, Tubert, Melendez, Shen, & Surmeier, 2017).

Abnormalities of some of these pathways are implicated in neurologic and psychiatric disease, particularly Alzheimer disease (AD), Parkinson disease (PD), and tobacco abuse. Degeneration of the BF complex is documented well in AD and PD, and degeneration of cholinergic PPN-LDT neurons may drive important aspects of declining gait and balance functions in PD and Progressive Supranuclear Palsy (Bohnen et al., 2012; Gilman et al., 2010; Hirsch, Graybiel, Duyckaerts, Javoy-Agid, 1987; Mesulam, 2013; Mufson, Ginsberg, Ikonomovic, & DeKosky, 2003; Schliebs & Arendt, 2011). Augmenting cholinergic neurotransmission with acetylcholinesterase inhibitors is an accepted treatment for dementias,

including AD, Lewy body dementia, and PD associated dementia. Anti-muscarinic cholinergic agents are used for treatment of dystonias and for treatment of PD associated tremor. Manipulations of cholinergic neurotransmission are being pursued to improve gait and balance functions in PD (Chung, Lobb, Nutt, & Horak, 2010; Henderson et al., 2016; Kucinski, De Jong, & Sarter, 2017; Li et al., 2015). Nicotinic cholinergic receptors mediate the addictive effects of nicotine and drugs active at this receptor family are used to treat nicotine addiction (Athenelli et al., 2016). Improved understanding of the organization of cholinergic pathways in human brain is relevant to efforts to expand useful cholinergic pharmacology in the treatment of human neurologic and psychiatric diseases.

Prior investigations in other species, including non-human primates, and work with post-mortem human tissues provided considerable information about the organization of human brain cholinergic pathways (Albin, Howland, Higgins, & Frey, 1994; Barmack, Baughman, & Eckenstein, 1992; Barmack, Baughman, Eckenstein, & Shojaku, 1992; De Lacalle, Hersh, & Saper, 1993; De Lacalle, Lim, Sobreviela, Mufson, Hersh, & Saper, 1994Emre, Heckers, Mash, Geula, & Mesulam, 1993; Fitzpatrick, Diamond, & Rackowski, 1989; Fukushima, Kitahara, Takeda, Saika, & Kubo, 2001; Gilmor, et al., 1999; Gilmor, et al., 1996; Heckers, Gelua, & Mesulam, 1992; Ichikawa, Ajiki, Matsuura, & Misawa, 1997; Jaarsma, et al., 1997; Kus, et al., 2003; Levey, Hallanger, & Wainer, 1987; Mahady, Periz, Emerich, Wahlberg, & Mufson, 2016; Manaye, Zweig, Wu, Hersh, De Lacalle, Saper, German, 1999; Mesulam, 2004; Mesulam & Geula, 1988; Mesulam, Geula, Bothwell, & Hesh, 1989; Mesulam, Hersh, Mash, & Geula, 1992; Mesulam, Mash, Hersh, Bothwell, & Geula, 1992; Roghani, Shirzadi, Butcher, & Edwards, 1998; Schafer, Weihe, Erickson, & Eiden, 1996; Schafer, Eiden, & Weihe, 1998; Woolf, 1991; Woolf,

Eckenstein, & Butcher, 1984; Woolf & Butcher, 1985, 1986, 1989; Zhang, Zhou, & Yuan, 2016). Working with post-mortem tissues has intrinsic limitations and inferences from studies of other species may be confounded by inter-species differences.

To advance our understanding of the organization of cholinergic pathways in humans, we assessed the regional distribution of human brain cholinergic neuron terminals with [^{18}F]fluoroethoxybenzovesmicol ([^{18}F]FEOBV) positron emission tomography (PET; Aghourian et al., 2017; Petrou et al., 2014; Xiao et al., 2011). FEOBV is a high affinity ligand for the vesicular acetylcholine transporter (VAChT), the protein responsible for pumping ACh from the cytosol into synaptic vesicles (Prado, Roy, Kolisnyk, Gros, & Prado, 2013; Prado, Janickova, Al-Onasi, & Prado, 2017). VAChT is expressed at uniquely high levels by cholinergic neurons (Arvidsson, Riedl, Elde, & Meister, 1997). Prior human imaging studies of brain VAChT expression used less resolute single photon emission computed tomography (SPECT) methods (Lamare et al. 2013; Kuhl et al., 1994; Kuhl et al., 1996). Prior PET studies of regional cholinergic terminal distribution used acetylcholinesterase (AChase) substrates ([^{11}C]methyl-4-piperidinyl propionate [PMP]; N-[^{11}C]-methyl-4-piperidyl acetate [MP4A]) to identify cholinergic synapses (Iyo et al., 1997; Kuhl et al., 1999). These methods are less specific. AChase, for example, is produced by midbrain dopaminergic neurons and high regional AChase activity was detected in regions, the lateral cerebellar cortices, not suspected to have substantial cholinergic terminal innervation (Greenfield, 1991; Kuhl et al., 1999). AChase enzyme activity may be regulated by synaptic activity and disease states, a potential obstacle to quantifying regional cholinergic terminal density (DeKosky et al., 1991). The very high AChase expression characteristic of the striatum limits quantification of striatal AChase as PMP and MP4A metabolite accumulation may be flow,

rather than AChase activity, limited. PMP and MP4A are also, though to a considerably lesser extent, butyrylcholinesterase substrates and butyrylcholinesterase activity changes might influence results of PET studies with these tracers (Snyder et al., 2001).

We describe the regional distribution of [^{18}F]FEOBV binding in human volunteers with no evidence of neurologic or psychiatric disease. Our results largely confirm inferences from post-mortem human studies and non-human mammal studies but indicate distinctive features of human cholinergic pathways and indicate effects of aging.

Methods:

Subjects: We studied 29 subjects without histories of neurologic or psychiatric disease. We studied 18 men and 11 women. The mean age was 47 years (SD 21 years); age range 20-81 years. No subject was using a medication that might affect cholinergic neurotransmission and none were using tobacco or nicotine products. Informed consent was obtained from all subjects prior to study entry and this study was approved by the University of Michigan Medical School IRB. Limited results were reported previously from subsets of these subjects (Albin, Minderovic, & Koeppe, 2017; Petrou et al., 2014).

[^{18}F]FEOBV PET: [^{18}F]FEOBV was prepared as described previously (Petrou et al., 2014; Shao et al., 2011). [^{18}F]FEOBV (288-318 MBq) was administered via intravenous bolus. Twenty eight subjects were scanned on an ECAT Exact HR+ PET tomograph (Siemens Molecular Imaging). One subject was scanned on a Biograph TruePoint scanner (Model 1094; Siemens Molecular Imaging). Brain imaging was conducted in three imaging periods. The first period began at injection and continued for 90 min. The subjects were then given a 30-min break, followed by a

second imaging period from 120–150 min, an additional break, and then a third imaging period from 180–210 min. Dynamic PET scans were reconstructed using Fourier rebinning (FORE) and the iterative 2D-OSEM algorithm, with four iterations, 16 subsets, and no post-reconstruction smoothing. The entire dynamic sequence was coregistered to the final form of the first 90-min scanning period. The first seven studies were acquired with a slightly different protocol, where the first scanning period last for 120 min, and the second and third periods lasted from 150-180, and 210-240 min respectively.

MRI: MRI imaging was performed on a 3 Tesla Philips Ingenia System (Philips) using a 15-channel head coil. A standard T1 weighted series of a 3D inversion recovery prepared turbo-field echo was performed in the sagittal plane using TR/TE/TI = 9.8/4.6/1041 ms; turbo factor = 200; single average; FOV = 240 × 200 × 160 mm; acquired matrix = 240 × 200. One hundred and sixty slices were reconstructed to 1-mm isotropic resolution.

Analysis of [^{18}F]FEOBV binding: The primary analysis was performed using the average of the coregistered 180-210 min scan period (termed “late static”). For the seven scans that had a slightly different acquisition sequence, we used the average of both the 150-180 and 210-240 time windows, thus matching the median of the scan data used of 195 min. FreeSurfer (freesurfer-i386-apple-darwin11.4.2-stable6-20170119; https://surfer.nmr.mgh.harvard.edu/fswiki/FreeSurferMethodsCitation; RRID: SCR_001847) was used on each individual’s T1-weighted MR to define gray matter and white matter voxels. The entire co-registered PET sequence was registered to the MR. The segmented white matter mask from FreeSurfer (1 for WM; 0 elsewhere) was smoothed to PET resolution, and then a threshold of 0.90 applied to the mask, yielding a volume of interest where the partial volume

contribution from non-white matter regions to the mask was <10% for every voxel. This white matter volume-of-interest was then applied to the PET data and used as a normalizing (intensity scaling) factor for the late static scan. Accordingly, [^{18}F]FEOBV binding in each volume of interest was expressed as standardized uptake value ratios (SUVRs). We showed previously that this approach is equivalent to kinetic modeling approaches for quantifying regional brain [^{18}F]FEOBV binding (Albin, Minderovic, & Koeppe, 2017).

We used NeuroStat (https://neurostat.neuro.utah.edu/) and Statistical Parametric Mapping (SPM12; http://www.fil.ion.ucl.ac.uk/spm/; RRID: SCR_007037) to non-linearly warp all PET scans into the Talairach and International Consortium for Brain Mapping atlas spaces, respectively. This was done to allow creation of mean images of all subjects, plus mean images for young and elderly cohorts, and to allow better visualization of effects of aging. We also used our standard VOI set developed for Talairach space, which is based entirely on the PET scans (no MR), as described previously (Bohnen et al., 2006). Brainstem parcellation was performed as described previously (Albin, Koeppe, Wernette, Bohnen, Kilbourn, & Frey, 2008). To complement this regional analysis, cortical and subcortical segmentation was performed with FreeSurfer. The programs were run in the fully automated mode. Volumes for all FreeSurfer-defined brain structures were obtained as part of the standard FreeSurfer ouptut. The VOIs were then applied in native MR-space to the co-registered PET scans. Results were essentially identical to those of the Neurostat-SPM based analysis and are not shown.

To analyze thalamic [^{18}F]FEOBV binding, we used the FreeSurfer-defined thalami, assumed an ellipsoid volume, and estimated the long axis of the ellipsoid volume. We then

divided each hemisphere of the thalamus into octants, based on voxels medial vs. lateral, anterior vs. posterior, and superior vs. inferior to the axis.

Aging Effects: To assess potential aging effects, we took advantage of the fact that our study population was divided into 2 groups; a younger group (N= 13; Mean 25 years; range 20-38) and an older group (N=16; Mean 66 years; range 52-81). Inspection of averaged images for these groups suggested regionally specific age-related declines in [^{18}F]FEOBV binding accompanying aging. Selected regions exhibiting possible age-related declines were compared with regions that appeared unchanged with aging. Examined regions included the striatal complex, thalamus, primary sensorimotor cortex (BAs 1-3,5), anterior cingulate cortex (BAs 24,32), hippocampal formation, amydala, posterior cingulate cortex (BAs 23,31), cerebellar vermis, occipital cortex (BAs 17-19), lateral frontal cortex (BAs 44-47), and medial frontal cortex (BAs 8-10).

Statistical Analysis: [^{18}F]FEOBV binding was compared within some regions using standard parametric methods. Analyses are described in each relevant Results section.

Results:

Overall: Brain [^{18}F]FEOBV binding was distributed inhomogeneously (**Figure 1**; **Table 1**). Binding was highest in the striatal complex, followed by portions of the thalamus, amygdala and hippocampal formation, some neocortical regions, rostral brainstem, and some regions of the cerebellar cortex. The overall distribution and regional density of [^{18}F]FEOBV binding is largely consistent with the distribution of cholinergic terminals described in prior human post-mortem and non-human mammal studies (Albin, Howland, Higgins, & Frey, 1994; Barmack, Baughman,

& Eckenstein, 1992; Barmack, Baughman, Eckenstein, & Shojaku, 1992; De Lacalle, Hersh, & Saper, 1993; De Lacalle, Lim, Sobreviela, Mufson, Hersh, & Saper, 1994; Emre, Heckers, Mash, Geula, & Mesulam, 1993; Fitzpatrick, Diamond, & Rackowski, 1989; Fukushima, Kitahara, Takeda, Saika, & Kubo, 2001; Gilmor, et al., 1999; Gilmor, et al., 1996; Heckers, Gelua, & Mesulam, 1992; Ichikawa, Ajiki, Matsuura, & Misawa, 1997; Jaarsma, et al., 1997; Levey, Hallanger, & Wainer, 1987; Mahady, Periz, Emerich, Wahlberg, & Mufson, 2016; Manaye, Zweig, Wu, Hersh, De Lacalle, Saper, German, 1999; Mesulam, 2004; Mesulam & Geula, 1988; Mesulam, Geula, Bothwell, & Hesh, 1989; Mesulam, Hersh, Mash, & Geula, 1992; Mesulam, Mash, Hersh, Bothwell, & Geula, 1992; Roghani, Shirzadi, Butcher, & Edwards, 1998; Schafer, Weihe, Erickson, & Eiden, 1996; Schafer, Eiden, & Weihe, 1998; Woolf, 1991; Woolf, Eckenstein, & Butcher, 1984; Woolf & Butcher, 1985, 1986, 1989; Zhang, Zhou, & Yuan, 2016). Our results are consistent as well with prior VAChT ligand human SPECT imaging studies (Lamare et al. 2013; Kuhl et al., 1994; Kuhl et al., 1996).

Our results likely identify the distribution of and quantify the regional density of major cholinergic terminal systems in human brain; cholinergic projections originating in the BF, striatal cholinergic interneurons, ascending and descending projections from the PPN/LDT complex, and cholinergic MVC projection neurons. It was not possible to clearly identify cholinergic nuclei or terminals associate with the medial habenular-interpeduncular nucleus projection or the parabigeminal nucleus to collicular projection, though the parabigeminal nucleus may be included in a region of relatively high [^{18}F]FEOBV binding at the mesopontine junction (see below). It was not possible to identify brainstem motor or autonomic cholinergic nuclei. More detailed description of the identifiable projections-regions follows.

Striatal Complex: Consistent with the known very high expression of cholinergic markers in the striatal complex, striatal complex [^{18}F]FEOBV binding was the highest of all brain regions, well above levels expressed in any other region. Striatal complex [^{18}F]FEOBV binding was mildly heterogeneous. [^{18}F]FEOBV binding was highest in the putamen and lower in the caudate nucleus (**Table 1**; paired t-test, $p<0.0001$). The striatal complex was further subdivided in dorsal caudate, ventral striatum (nucleus accumbens and ventral caudate), anterior putamen, and posterior putamen (**Table 1**). [^{18}F]FEOBV binding was modestly different in all these subregions (one-way ANOVA for correlated samples, $p<0.0001$; all subregions different from each other, Tukey's HSD, $p<0.01$). The rank order of striatal complex [^{18}F]FEOBV binding was Anterior Putamen > Posterior Putamen > Dorsal Caudate > Ventral Striatum.

Cortex: [^{18}F]FEOBV binding was distributed widely and somewhat inhomogeneously throughout the cortex. Most neocortical regions (BA1-23, 26, 29-32, 36-47; **Figure 1, Figure 2**) exhibited modest [^{18}F]FEOBV binding (mean SUVRs = 1.5 – 2.0 range; **Table 1**) with some regions (BA24, 25, 27, 28, 34, 35) exhibiting higher levels of [^{18}F]FEOBV binding (mean SUVRs = 2.0-2.5 range; **Table 1**). Consistent with the results of the Brodmann areas based analysis, primary sensorimotor and anterior cingulate cortices were readily distinguished from surrounding cortices (**Figure 1, Figure 2**). Similarly, there was a modest anterior to posterior gradient with frontal cortices exhibiting modestly higher levels of [^{18}F]FEOBV binding than occipital cortices (**Figure 1, Figure 2**). Insular cortex was slightly higher (mean SUVR = 2.57; **Table 1**) As expected, the hippocampal formation (mean SUVR = 2.76) and amygdala (mean SUVR= 3.14) exhibited the highest [^{18}F]FEOBV binding of any cortical region (**Table 1**, **Figure 3**).

Thalamus: The thalamus exhibited relatively high but likely inhomogeneous [^{18}F]FEOBV binding (**Table 1, Figure 1, Figure 4**). Grossly, thalamic [^{18}F]FEOBV binding distribution did not exhibit the expected ovoid shape (**Figure 1, Figure 4**). In particular, the anterolateral border of thalamic [^{18}F]FEOBV binding had a concave shape, suggesting less [^{18}F]FEOBV binding in the anterolateral thalamus. In addition, the lateral geniculate nuclei exhibited high [^{18}F]FEOBV binding and could be easily distinguished from the rest of the thalamus (**Table 1, Figure 4**). This suggests that portions of the thalamus more rostral and dorsal to the lateral geniculate nucleus, e.g., portions of the pulvinar complex, had less [^{18}F]FEOBV binding. In the absence of an established parcellation of the thalamus, we divided the thalamus into 8 subregions by assuming that the thalamus has ovoid shape with a major rostral-caudal axis, excluding the lateral geniculate nuclei (see **Methods**). In general, consistent with the impression that anterolateral regions exhibited less [^{18}F]FEOBV binding, the lateral subregions defined by this analysis had less [^{18}F]FEOBV binding than medial subregions and anterior subregions had less [^{18}F]FEOBV binding than posterior regions (**Table 2**). This was confirmed by statistical comparisons of cognate octants (e.g., superior anterolateral octant vs superior anteromedial octant; superior anterolateral octant vs superior posterolateral octant, etc.). All medial octants had significantly higher [^{18}F]FEOBV binding than cognate lateral octants and all posterior octants had significantly higher [^{18}F]FEOBV binding than cognate anterior octants ($p < 0.05$; paired t-tests with Bonferroni correction for multiple comparisons). This approach may not sample the pulvinar complex adequately. As seen in **Figure 4B**, the lateral geniculate nuclei appear relatively isolated, suggesting that the pulvinar complex, rostral and dorsal to the lateral geniculates, exhibits relatively low [^{18}F]FEOBV binding.

Cerebellum: All regions of cerebellar cortex exhibited [^{18}F]FEOBV binding with some regions exhibiting relatively high [^{18}F]FEOBV binding levels (**Table 1**, **Figure 1, Figure 5**). Highest cerebellar cortical [^{18}F]FEOBV binding was found in the vermis (mean SUVR = 3.1) with the cerebellar hemisphere cortices at significantly lower levels (mean SUVR = 1.8). The floccular region exhibited intermediate levels of [^{18}F]FEOBV binding (mean SUVR = 2.2).

Brainstem: Significant [^{18}F]FEOBV binding (mean SUVRs in the 2-2.5 range) was present in all regions quantified (**Table 1**). Precise parcellation of the brainstem is very limited due to the small size of brainstem structures such as PPN-LDT complex and cranial nerve nuclei. [^{18}F]FEOBV binding was particularly concentrated in dorsal brainstem from the caudal midbrain to the mid pons (**Figure 1, Figure 6**). While difficult to quantify, there also appeared to be modestly increased [^{18}F]FEOBV binding in the dorsal regions of the caudal brainstem, possibly within the periaqueductal tegmentum (**Figure 1, Figure 6**).

Aging Effects: Potential aging effects were assessed initially by comparing younger (22-38 years) and older (52-81 years) subjects. These comparisons revealed regionally specific changes accompanying aging (**Table 3, Figures 7, 8**). Statistical significance of age-related changes was assessed with one-way independent t-tests using the Holm-Bonferroni method to correct for multiple comparisons at a threshold of $p < 0.05$. Significant age-related changes were found in the striatum, primary sensorimotor cortex, and anterior cingulate cortex with trends towards age-related declines in the thalamus, hippocampal formation, amygdala, and posterior cingulate (**Table 3**). There was no evidence of age-related decline in the cerebellar vermis and other cortical regions (**Table 3**). To further explore the relationship between aging and regional [^{18}F]FEOBV binding, age was regressed against [^{18}F]FEOBV binding in selected

regions (Pearson product moment correlations; **Figure 8**). Strong negative correlations were present in striatum ($r = 0.68$; $p < 0.00003$; decline of ~4% per decade), primary sensorimotor cortex ($r = 0.52$; $p < 0.003$; decline of ~2.5% per decade), and anterior cingulate cortex ($r = 0.55$; $p < 0.001$; decline of ~2.5% per decade), and thalamus ($r = 0.42$; $p < 0.0125$; decline of ~2.5% per decade). All correlations significant after Bonferroni correction at a threshold of 0.05. An interesting example of regionally specific decrease in [^{18}F]FEOBV binding was found in primary sensorimotor cortex. In younger subjects, this region was easily distinguished from surrounding cortices by higher [^{18}F]FEOBV binding (**Figure 7**). In older subjects, primary sensorimotor cortex [^{18}F]FEOBV binding appeared more similar to surrounding cortices (**Figure 7**).

Discussion:

VAChT is thought to be uniquely expressed by cholinergic neurons. The VAChT gene is part of a distinctive cholinergic "operon," encoded within the first exon of the Choline Acetyltransferase gene (Eiden, 1998). FEOBV is a high affinity and specific ligand for VAChT (Mulholland et al., 1998). In general, our findings correlate well with prior studies of brain cholinergic systems in humans and other mammals based on ChAT and high affinity choline transporter immunohistochemistry (Kus, et al., 2003; and see below). Our results also correlate well with non-human animal and limited human studies of the distribution of brain VAChT immunoreactivity (Arvidsson, Reidel, Elde, & Meister, 1997; Ichikawa, Ajiki, Matsuura, & Misawa, 1997; Gilmor et al., 1996, 1998; Roghani, Shirzadi, Butcher, & Edwards, 1998; Schafer, Weihe, Erickson, & Eiden, 1996; Schafer, Eiden, & Weihe, 1998). Our results also

correspond very well with prior limited human PET imaging with [^{18}F]FEOBV (Aghourian et al., 2017; Albin, Minderovic, & Koeppe, 2017; Petrou et al., 2014).

Striatal Complex: The striatal complex exhibited the highest regional [^{18}F]FEOBV binding, indicating a very high density of cholinergic terminals within the striatal complex. This result is consistent with a large body of data from studies in many species indicating uniquely high expression of cholinergic system markers and terminals in the striatal complex. There are at least two, and possibly three, sources of striatal cholinergic terminals. While striatal cholinergic interneurons comprise only a small fraction (~2-3%) of the total striatal neuron population (Bernacer, Prensa, & Gimenez Amaya, 2007; Gonzales & Smith, 2015; Lecumberri, Lopez-Janeiro, Corral-Domenge, & Bernacer, 2017) they give rise to dense axonal-terminal arborizations and play an important role in integration of cortico- and thalamostriate inputs with dopaminergic functions. The striatal complex also receives innervation from mesopontine (PPN-LDT) cholinergic neurons (Dautan et al., 2014; Dautan, Hajioglu Bay, Bolam, Gerdjikov, & Mena-Segovia, 2016; Woolf, 1991), presumably collaterals of neurons innervating the thalamus and other targets of the PPN-LDT complex. Mesulam et al. (1992) suggested that the human striatal complex receives some cholinergic afferents from the basal forebrain complex, a projection that is absent in rodents (Dautan, Hajioglu Bay, Bolam, Gerdjikov, & Mena-Segovia, 2016). The relative proportion of striatal cholinergic interneuron terminals and extrinsic afferent terminals is unknown. It is generally assumed that striatal cholinergic interneuron terminals are much more abundant than extrinsic cholinergic afferent terminals. Janickova et al. (2017) genetically ablated VAChT in murine PPN-LDT neurons. As measured by western immunoblots, VAChT protein expression diminished markedly in the brainstem and thalamus,

and was unchanged in hippocampal formation, neocortex, and striatum. Genetic or toxic ablation of striatal cholinergic interneurons results in marked reductions in striatal cholinergic terminal markers (Aoki, Liu, Zucca, Zucca, & Wickens, 2015; Laplante, Lappi, & Sullivan, 2011; Martos, Braz, Beccaria, Murer, & Belforte, 2017; Pappas et al., 2015). These results are consistent with the traditional view that striatal extrinsic cholinergic afferents contribute only modestly to the total density of striatal cholinergic terminals. This is not to say that extrinsic cholinergic striatal afferents are functionally unimportant.

Our data suggest modest inhomogeneity of striatal cholinergic terminal density. [^{18}F]FEOBV binding was higher in the putamen than the caudate. Further subdividing these regions, we found highest [^{18}F]FEOBV binding within in the anterior putamen and lowest in the ventral striatum. In a stereologic analysis of human post-mortem material, Bernacer, Prensa, & Gimenez-Amaya (2007) described inhomogeneous distribution of striatal cholinergic interneuron perikarya with higher density in the caudate nucleus than putamen. This is somewhat discordant with our results. While their definition of subregions is somewhat different from ours, their definition of caudate and putamen approximate our definition of dorsal caudate nucleus and putamen. The differences between our results and those of Bernacer, Prensa, & Gimenez-Amaya (2007) cautions against simple equation of perikaryal density and terminal density. Mesulam, Mash, Hersh, Bothwell, & Geula (1992) suggested that human cholinergic basal forebrain projections to the striatal complex more heavily innervate the putamen.

Cortex: The cortical mantle receives its cholinergic inputs from cholinergic neurons of the basal forebrain complex (Ch1 – Ch4 in the nomenclature of Mesulam, Mufson, Wainer, & Levey, [1983]). These cholinergic projections are known to participate in several important cognitive

processes including attention, executive functions, and forms of memory (Ballinger, Anath, Talmage, & Role, 2016; Hasselmo & Sarter, 2011; Prado, Janickova, Al-Onasi, & Prado, 2017). Our results are broadly consistent with data from older analyses of cholinergic terminal markers in human post-mortem specimens (De Lacalle, Lim, Sobreviela, Mufson, Hersh, & Saper, 1994; Emre, Heckers, Mash, Geula, & Mesulam, 1993; Mesulam & Geula, 1988; Mesulam, Mash, Hersh, & Geula, 1992; Mesulam, 2004; Mesulam, 2013). Consistent with these prior descriptions, highest [^{18}F]FEOBV binding was found in amygdala and hippocampal formation, followed by paralimbic cortices, including the insular cortex, perirhinal cortex (BA35), entorhinal cortex (BA34, BA28), parahippocampal gyrus (BA27), and portions of the cingulate cortex (BA25, BA24). Regional binding density is probably not the only important difference in cholinergic innervation between cortical regions. Mesulam, Mash, Hersh, & Geula (1992) also described varying laminar distributions of cholinergic terminals between neocortical regions. Detecting such differences is beyond the resolution of PET imaging. Similarly, prior studies indicate that the basolateral amygdala exhibits the highest density of cholinergic terminals with other amygdala nuclei exhibiting significantly less cholinergic innervation (Emre, Heckers, Mash, Geula, & Mesulam, 1993).

Thalamus: Analysis of the thalamus was limited by lack of an accepted parcellation method for thalamic subnuclei. Nonetheless, our data suggests significant inhomogeneity for [^{18}F]FEOBV binding within the thalamus. The lateral geniculate nuclei exhibited significant [^{18}F]FEOBV binding. Visual inspection suggests less [^{18}F]FEOBV binding in anterior, lateral and some posterior (pulvinar) thalamic subregions. These conclusions are consistent with analysis based on arbitrary division of the thalamus into 8 equivalent octants. Lateral regions exhibited less

[^{18}F]FEOBV binding than medial regions and anterior regions less [^{18}F]FEOBV binding than posterior regions. This crude parcellation approach may not adequately assess [^{18}F]FEOBV binding in the pulvinar complex, which likely also exhibits lower [^{18}F]FEOBV binding.

Our results are consistent with the work of Heckers, Geula, & Mesulam (1992) assessing cholinergic terminal density with ChAT immunohistochemistry in human post-mortem specimens. Heckers, Geula, & Mesulam (1992) documented ChAT immunoreactive fibers throughout the human thalamus with the greatest density of ChAT-immunoreactive fibers in the lateral geniculate nuclei, some intralaminar nuclei, the reunions nucleus, the anterodorsal nucleus, the reticular nucleus, and some portions of the mediodorsal nucleus. Lowest density of ChAT-immunoreactive fibers was seen in the pulvinar and medial geniculate nuclei. As Heckers, Geula, & Mesulam (1992) point out, the human subregional distribution of thalamic cholinergic terminals is likely distinct from that found in the limited number of other species studied (Levey, Hallenger, & Wainer, 1987; Fitzpatrick, Diamond, & Raczkowski, 1989).

The major cholinergic innervation of thalamus originates in the PPN-LDT complex with some contribution from the basal forebrain. PPN-LDT cholinergic efferent projections generally ramify widely and innervate several target regions (Mena-Segovia & Bolam, 2017), though Holmstrand & Sesack (2011) suggest some specialization of cholinergic PPN-LDT afferents targeting the anterior thalamus. In humans, basal forebrain cholinergic afferents probably innervate more anterior and medial thalamic nuclei, including the central medial and central lateral nuclei, and portions of the mediodorsal nucleus (Heckers, Geula, & Mesulam; 1992).

Cholinergic afferents to the thalamus are implicated in several important phenomena, notably the regulation of consciousness and sleep, though the abundance of cholinergic

terminals and receptors within the thalamus suggests cholinergic neurotransmission may modulate multiple aspects of thalamic function. Recent work, for example, from our group suggests that thalamic cholinergic neurotransmission contributes to “bottom-up” detection of salient sensory cues (Kim, Muller, Bohnen, Sarter, & Lustig, 2017).

Cerebellum: Prior work in several species suggests significant cholinergic innervation of some cerebellar regions (Barmack, Baughman, & Eckenstein, 1992; Barmack, Baughman, Eckenstein, & Shojaku, 1992; De Lacalle, Hersh, & Saper, 1993; Fukushima, Kitahara, Takeda, Saika, & Kubo, 2001; Jaarsma et al., 1997; Manaye, Zweig, Wu, Hersh, De Lacalle, Saper, German, 1999; Zhang, Zhou, & Yuan, 2016). There is no evidence of cholinergic neurons within the cerebellum in most species studied. Cholinergic terminals were detected throughout the cerebellar cortex, though most studies describe these as relatively sparse in most cerebellar cortical regions. [^{18}F]FEOBV PET is likely to convey a more faithful representation of cerebellar cortical innervation than AChase PET methods, which show uniform and high cerebellar cortical tracer retention. Barmack and colleagues (Barmack, Baughman, & Eckenstein, 1992; Barmack, Baughman, Eckenstein, & Shojaku, 1992) described relatively high levels of cholinergic innervation of some cerebellar cortical regions in rat, rabbit, car, and non-human primate brain. These included the uvula-nodulus (lobules 9 & 10), the floccular region, and the anterior vermis (lobules 1 & 2) (Barmack, Baughman, & Eckenstein, 1992; Barmack, Baughman, Eckenstein, & Shojaku, 1992). The predominant source of these terminals appears to be cholinergic neurons of the vestibular complex. Jaarsma et al. (1997) also described cholinergic afferents to the cerebellar cortex from numerous brainstem populations, though MVC cholinergic neurons were felt to be the predominant source of cerebellar cortical afferents. De Lacalle, Hersh, & Saper

(1993) described a ChAT-immunoreactive subpopulation of cerebellar cortical Golgi interneurons, particularly in the vermis, uvula-nodulus, and flocculus, in human post-mortem specimens.

We find uniformly high [^{18}F]FEOBV binding throughout the vermis, different from the patterns described in other mammals. The originating neurons of this extensive cerebellar vermis cholinergic innervation are unknown, though it is possible that it is an extension of the vestibulocerebellar cholinergic innervation documented in other mammals. Alternatively, the pattern of cerebellar [^{18}F]FEOBV binding may reflect the distribution of cholinergic Golgi interneurons described by De Lacalle, Hersh, & Saper (1993). The vestibular system plays a critical role in maintenance of normal upright posture and gait, as does the cerebellar vermis. A plausible speculation is that extended vestibular cholinergic innervation of the cerebellar vermis is a correlate of bipedal locomotion in humans. Seidel et al. (2015) document α-synuclein aggregate pathology in the MVC nuclei of post-mortem specimens from individuals with Parkinson disease and Lewy body dementia. It is possible that vestibular dysfunction contributes to the dopamine replacement therapy refractory gait and balance deficits of advanced Parkinson disease.

Another previously documented site of cholinergic innervation of the cerebellum is the deep cerebellar nuclei. Prior tract-tracing and functional studies in rodents indicate projections from the PPN-LDT complex to the deep cerebellar nuclei with MRI tractography studies supporting the existence of this projection in humans (Aravamuthan, Muthaswamy, Stein, Aziz, & Johansen-Berg, 2007; Vitale, Mattei, Capozzo, Pietrantoni, Mazzone, & Scarnati, 2016). These terminals are presumably too sparse to be detected by [^{18}F]FEOBV PET.

Brainstem: Cholinergic neurons within the brainstem include oculomotor (CrN III, IV, and VI nuclei), motor (motor nucleus of CrN V, and CrN VII, CrN X, XI, and CrN XII nuclei), and autonomic nuclei (Vagal Nucleus), and some MVC neurons. Most of these nuclei are beyond the resolution of PET imaging. The largest collection of brainstem cholinergic neurons lie within the PPN-LDT complex (Mesulam, Mufson, Wainer, & Levey [1983] Ch5 & 6). In immunohistochemical studies of post-mortem human specimens, Mesulam, Geula, Bothwell, & Hersh (1989) described the PPN-LDT complex as stretching from the dorsal mid-mesencephalon to dorsal mid-pons. The distribution of cholinergic perikarya did not observe specific nuclear borders nor was it delimited by fiber tracts. Cholinergic perikarya were distributed at varying densities throughout this volume of the brainstem. Similar results were obtained by Manaye et al. (1999). In rodent PPN-LDT, there is a rostrocaudal gradient of cholinergic perikarya density with highest density of these neurons in the caudal PPN-LDT complex (Mena-Segovia & Bolam, 2017). The distribution of cholinergic perikarya described in humans by Mesulam, Geula, Bothwell, & Hersh (1989) coincides well with the distribution of relatively high [^{18}F]FEOBV binding we find spanning the dorsal mesopontine junction. This area of relatively high brainstem [^{18}F]FEOBV binding may also include the cholinergic parabigeminal nucleus (Mesulam, Mufson, Wainer, & Levey [1983] Ch8), adjacent and lateral to the PPN-LDT complex in the upper pons.

We found significant [^{18}F]FEOBV binding throughout the brainstem, most likely the binding to the terminals of PPN-LDT neurons that innervate multiple targets throughout the brainstem (Woolf & Butcher, 1989). While our analysis is limited by the resolution of PET, prior studies in other species do not describe marked inhomogeneity of brainstem cholinergic

terminals. It was possible to tentatively identify what might be a column of relatively increased [^{18}F]FEOBV binding in the dorsal caudal brainstem surrounding the aqueduct (**Figure 1, Figure 5**).

Aging Effects: Our results indicate significant and regionally specific declines in [^{18}F]FEOBV binding. Striatal complex changes are consistent with age-related declines in striatal cholinergic interneuron terminal density. There was a suggestion of reduced thalamic [^{18}F]FEOBV binding, consistent with age-related decrease in PPN-LDT complex cholinergic terminals. Ransmayr et al. (2000) described age-related loss of PPN neurons in humans. Zhang, Sampogna, Morales, & Chase (2005) et al. studied PPN-LDT neurons in aged cats. There was no evidence of PPN-LDT cholinergic perikarya loss but there was evidence of neuronal atrophy and dendritic simplification. Prior work suggests age-related changes in BF neurons. MRI morphometry studies indicate age-related loss of BF nuclear volumes (Grothe, Heinson, & Tempel, 2012; Hanyu, Asano, Sakurai, Tanaka, Takasaki, & Abe, 2002). These results are consistent with our data suggesting age-related loss of BF corticopetal terminals. Our data suggests that this is not a uniform process as we found cortical regions (primary sensorimotor cortex, anterior cingulate cortices) with significant declines in [^{18}F]FEOBV binding and others with little or no age-related change. As subopulations of BF neurons are thought to project to relatively restricted cortical regions (Gielow & Zaborszky, 2017), our results suggest inhomogeneous age-related changes within the BF complex. Prior SPECT studies of human VAChT distribution also suggested age-related decline in BF corticopetal terminals but lacked the precision to resolve changes in individual neocortical regions (Kuhl et al., 1996).

General Conclusion: [^{18}F]FEOBV PET delineates regional cholinergic terminals well in human brains. The general pattern of [^{18}F]FEOBV binding is highly consistent with the known organization of major cholinergic systems in mammalian brain. Our results indicate distinctive organization of cholinergic terminals in the human neocortex, cerebellar cortex, and the human thalamus. Our results indicate the presence of age-related and region specific changes in cholinergic terminal density. [^{18}F]FEOBV PET is likely to be a useful method for studying disease states, including several neurodegenerative disorders and psychiatric disorders.

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Figure Legends:

Figure 1: [^{18}F]FEOBV binding in human brain. (A) Dorsal to ventral survey of averaged transaxial images for all 29 subjects. Images scaled to peak SUVR of 3.0. Regions with notable [^{18}F]FEOBV binding include the striatal complex, thalamus, amygdala, hippocampal formation, neocortical mantle, mesonpontine junction, and portions of the cerebellum (see text for details). (B) Pseudocolor images of dorsal transaxial levels (identical to top row in [A] above) to illustrate differing [^{18}F]FEOBV binding in different cortical regions. Scaled to peak SUVR of 2.5. These images demonstrate relatively higher [^{18}F]FEOBV binding in primary sensorimotor and anterior cingulate cortices.

Figure 2: Neocortical [^{18}F]FEOBV binding. Transaxial images from near the vertex to the level of the striatum. Images scaled to emphasize differences in cortical regions. Consistent with Brodmann area based analysis (**Table 1**; see text), higher [^{18}F]FEOBV binding in primary sensorimotor (SMC) and anterior cingulate (AC) cortices. There is a modest anterior to posterior gradient with higher frontal cortical than occipital cortical [^{18}F]FEOBV binding.

Figure 3: [^{18}F]FEOBV binding in amygdala and hippocampal formation. (A) & (B); [^{18}F]FEOBV binding**.** (A) Transaxial averaged image of all 29 subjects at level of anterior temporal lobe. (B) Parasagittal averaged image of all 29 subjects at level of amygdala (AM) and hippocampal formation (HIP). (C) & (D); early phase averaged images, representing the relative regional blood-brain ligand transport rate – regional anatomy. (A) & (B) show significant [^{18}F]FEOBV binding and easily distinguishable amygdala and hippocampal formation.

Figure 4: Thalamic [^{18}F]FEOBV binding. Pseudocolor images of [^{18}F]FEOBV binding (top row) and relative regional blood-brain ligand transport rate – regional anatomy (bottom row). (A) Transaxial and parasagittal images at levels of dorsal thalamus (THAL). Note the inverted comma shape of high density of [^{18}F]FEOBV binding in thalamus in the transaxial image compared with the ovoid shape of the thalamus in the transaxial flow image. (B) Transaxial and parasagittal images at levels of the lateral geniculate nuclei (LG). Not the relative isolation of high [^{18}F]FEOBV binding in the lateral geniculate nuclei compared with surrounding regions, particularly the more anterior and dorsal pulvinar complex.

Figure 5: Cerebellar [^{18}F]FEOBV binding. Pseudocolor images of [^{18}F]FEOBV binding (top row) and relative regional blood-brain ligand transport rate – regional anatomy (bottom row). (A) Transaxial images at the level of flocculi (FL). Note relatively high [^{18}F]FEOBV binding in the floccular region, anterior vermis (VER), and posterior vermis (VER) with less [^{18}F]FEOBV binding in the cerebellar hemispheres. (B) Parasagittal images at the level of the flocculus. (C) Coronal images at the level of the vermis. Again, note relatively high [^{18}F]FEOBV binding in the vermis compared with the cerebellar hemispheres.

Figure 6: Brainstem [^{18}F]FEOBV binding. Pseudocolor parasagittal images of [^{18}F]FEOBV binding (top) and relative regional blood-brain ligand transport rate – regional anatomy (bottom) in the midline plane. Relatively high [^{18}F]FEOBV binding in the dorsal midbrain and spanning the mesopontine junction into rostral pons (MP). Increased [^{18}F]FEOBV binding within the more dorsal caudal brainstem, possibly around the aqueduct.

Figure 7: Effects of Aging on [^{18}F]FEOBV binding. (A) Averaged transaxial pseudocolor images scaled at a peak of 10.0. A1 & A3 – Young normal subjects. A2 & A4 – Older normal subjects. Note declines in [^{18}F]FEOBV binding in primary sensorimotor and anterior cingulate cortices in older normal subjects. (B) Averaged transaxial pseudocolor images scaled at a peak of 3.0 and at the levels of the striatal complex. B1 – Young normal subejcts. B2 – Older normal subjects. Note decline in striatal complex [^{18}F]FEOBV binding in older subjects.

Figure 8: Correlations Between Age and Regional [^{18}F]FEOBV binding. Pearson product-moment correlations between subject age and regional [^{18}F]FEOBV binding in striatum, primary sensorimotor cortex, anterior cingulate cortex, and thalamus. All correlations significant after Bonferroni correction at a threshold of 0.05.