PET Imaging of Norepinephrine Transporters

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Abstract: The involvement of the norepinephrine transporter (NET) in the pathophysiology and treatment of attention deficit hyperactivity disorder (ADHD), substance abuse, neurodegenerative disorders (e.g., Alzheimer's disease (AD) and Parkinson's disease (PD)) and depression has long been recognized. However, many of these important findings have resulted from studies *in vitro* using postmortem tissues; as of now, these results have never been verified *via in vivo* methods because brain imaging of NET in living systems has been hampered due to the lack of suitable radioligands. The fact that all three monoamine (dopamine, norepinephrine, and serotonin) transporters (DAT, NET and SERT) are involved in various neurological and psychiatric diseases further emphasizes the need to develop suitable NET ligands so that researchers will be able to probe the contributions of each monoamine transporter system to specific CNS disorders. In this review article, the design and biological evaluation of several radioligands for imaging the brain NET system with PET are discussed. Based on these characterization studies, including C-11 labeled desipramine (DMI), 2-hydroxydesipramine (HDMI), talopram, talsupram, nisoxetine (Nis), oxaprotiline (Oxap), lortalamine (Lort) and C-11 and F-18 derivatives of reboxetine (RB), methylreboxetine (MRB) and their individual (R, R) and (S, S) enantiomers, in conjunction with studies with radiolabeled 4-iodo-tomoxetine and 2-iodo-nisoxetine, we have identified the superiority of (S, S)-[^{11}C]MRB and the suitability of the MRB analogs as potential NET ligands for PET. In contrast, Nis, Oxap and Lort displayed high uptake in striatum (higher than thalamus). The use of these ligands is further limited by high non-specific binding and relatively low specific signal, as is characteristic of many earlier NET ligands. Thus, to our knowledge, (S, S)-[^{11}C]MRB remains by far the most promising NET ligand for PET studies.

Key Words: Norepinephrine transporter, PET, depression, substance abuse, ADHD, methylreboxetine, reboxetine, nisoxetine.

I. INTRODUCTION

The norepinephrine transporter (NET) has been associated with attention deficit hyperactivity disorder (ADHD), substance abuse and depression; however, *in vivo* brain imaging studies of NET have not been possible due to the lack of suitable radioligands. Here we provide the background supporting the need to develop novel radiotracers to study the brain NET system in order to better understand its role in brain function and diseases.

NET regulates the duration of norepinephrine (NE) neurotransmission by removal of the neurotransmitter from the extracellular space [1]. As much as 80-90% of released NE may be recaptured and re-released, which suggests a crucial role for NET in synaptic activity [2]. It belongs to a super family of Na^+/Cl^- -dependent neurotransmitter transporters, including the dopamine (DA) and serotonin transporters (DAT and SERT), which share genetic, structural and functional homologies [3, 4]. NE neuron projections innervate many targets throughout the central and peripheral nervous systems (CNS and PNS) and play important modulatory roles in attention, pain perception, learning, memory, and autonomic functions [5, 6]. Changes in NET have been associated in depression [7, 8], cardiovascular disease [9, 10], and neurodegenerative disorders including Alzheimer's and Parkinson's diseases [11, 12]. NET is therefore an important target of ntidepressants such as desipramine and reboxetine a [13-15] and for drugs of abuse including cocaine (Coc) and amphetamine [16, 17], as well as for drugs used in the treatment of ADHD including methylphenidate, amphetamine, and tomoxetine.

A number of radioligands have been used to identify and locate NET in the human and animal brain *in vitro*, beginning with the use of [^{3}H]desipramine to identify and characterize the NET sites [18, 19] and to show that the level of NET sites varies with norepinephrine concentration [20]. A quantitative autoradiography study with [^{3}H]desipramine in rat brain was also reported [21]. [^{3}H]Nisoxetine ([^{3}H]Nis) has also been used for mapping of NET by quantitative autoradiography, with results indicating a pattern that was in good agreement with the distribution of NE terminals [22, 23]. The highest density was found in the locus coeruleus (LC); the anteroventral nucleus of the thalamus (TH), ventral portion of the bed nucleus of the stria terminalis (BNST) and dorso-medial nucleus of the hypothalamus were also densely labeled. Moderate binding was seen in the dorsal aspect of the BNST, median raphe nucleus and amygdala, and lowest binding was found in the CA1 layer of the hippocampus and the caudate putamen.

Norepinephrine has been labeled with C-11 ((±)-[^{11}C]NE) [24, 25], and F-18 labeled catecholamines (fluoronorepinephrine ([^{18}F]FNE), and fluorodopamine ([^{18}F]FDA) [26-30] along with false neurotransmitters such as [^{123}I]meta-iodobenzylquanidine ([^{123}I]MIBG) [31], [^{18}F]fluorometaraminol [32], [^{11}C]phenylephrine [33], [^{11}C]meta-hydroxyephedrine [34], have been developed for PET studies of sympathetic

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1381-6128/06 $50.00+.00

innervation in the heart; however, their use as tracers to study brain NET is limited due to their inability to cross the blood-brain barrier. Several potent NET reuptake inhibitors have been labeled for *in vitro* or *in vivo* mapping of brain NET; however, the results were not promising due to their high non-specific bindings. To name a few, [^{3}H]desipramine [18, 19, 21] and [^{3}H]mazindol [35] showed high nonspecific binding *in vitro*; similarly, [^{11}C]desipramine also is not a suitable *in vivo* radiotracer [89]. [^{3}H]Nis is a suitable radioligand for *in vitro* study [22]; unfortunately, the binding of racemic (R/S)-[^{11}C]Nis *in vivo* appeared to be nonspecific [36]. An imaging study of an iodinated analog of tomoxetine (4-iodo-tomoxetine) showed high nonspecific binding *in vivo* as well [37]. Thus, attempts to develop radiotracers for *in vivo* imaging of NET eluded chemists for many years until the recent development of C-11 labeled reboxetine derivatives that show specific localization and highly encouraging binding kinetics in rats and non-human primates with PET [38-40].

Clinical Relevance of Studying NET

a. Importance of NET in Depression

One in five individuals are affected by a mood disorder during their lifetime [41]. Depressed people have greater mortality and impairment in many areas of functioning compared with non-depressed persons [42, 43]. Early antidepressant medications, e.g. tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), are effective because they enhance either NE or 5-HT mechanisms, or both. Unfortunately, these compounds block cholinergic, histaminergic and alpha-1-adrenergic receptor sites, interact with a number of other medications, and bring about numerous undesirable side effects. Newer drugs, including selective serotonin reuptake inhibitors (SSRIs) or noradrenaline (NARIs, reboxetine) or both (SNRIs), reversible inhibitors of monoamine oxidase, and dopamine antagonists, are the results of rational design strategies to find drugs that were as effective as the TCAs but of higher safety and tolerability profile [44, 45].

Postmortem studies showed that in the midcaudal portion of the LC, the binding of [^{3}H]Nis from major depressive subjects was significantly lower than that from age-matched, normal control subjects. These results of decreased binding of [^{3}H]Nis to NET's in the LC in major depression, accompanied by no change in the number of noradrenergic cells, suggest a compensatory down-regulation of NET in response to an insufficient availability of NE at the synapse [46, 11]. There is evidence to indicate that the NE system is more associated with increased drive, whereas the serotonergic (5-HT) system relates more to changes in mood; however, this remains to be elucidated.

Reboxetine (RB) represents the first of a new class of antidepressants. Prior to the availability of RB, it has not been possible to elucidate the safety profile of a drug having a selective action on the NE system. It has been postulated that drugs acting selectively at NE sites will have fewer side effects, and studies with RB indicate that this is the case. The introduction of RB is welcomed as its use greatly clarifies the role of NET in depression, and may help to reveal whether patients who do not respond to antidepressants of one pharmacological class might respond to the other. Clinical studies of RB clearly demonstrate that there is a role for a selective NE drug in the treatment of depression, either alone or as an adjunct therapy [47].

b. Involvement of NET in Neurodegenerative Disorders (e.g., Alzheimer's Disease (AD) and Parkinson's Disease (PD)) and Aging

LC is affected in a number of neurological and psychiatric disorders [48]. Neuronal populations within the human LC have been found to decrease with normal aging and also as a result of neurodegeneration [22]. In normal aging, the LC shows a cell loss of ~40%, while in AD a more severe cell loss (40-60%) occurs. Since the loss of [^{3}H]Nis binding appears to parallel neuronal loss in the LC in AD and aging, it's possible that the decrease in [^{3}H]Nis binding is due to a loss of cells in LC. It has also been shown that degeneration of the NE system is due to neuronal loss and cell shrinkage in the LC, which suffers disease-specific lesions in both PD and AD [12, 49, 50].

c. The Role of NET in ADHD

Attention deficit-hyperactivity disorder (ADHD) is a complex developmental, behavioral and cognitive disorder that affects approximately 3-7% of school-aged boys [51]. For more than 50 years, the most widely prescribed pharmacological treatment for patients with ADHD has been methylphenidate (MP, Ritalin). The precise mechanisms of the therapeutic effects and side effects of MP are not yet well understood, though most studies have focused on its effects on the dopamine system and suggest that its therapeutic effects are due to its ability to increase the synaptic concentration of dopamine by blocking the DAT [52, 53, 51, 54-57]. However, MP binds to both DAT and NET, and with an even higher potency in inhibiting NE reuptake (IC_{50} 37.7 and 193 nM to NET and DAT, respectively) [58]. Neurochemical, neurophysiological and neuroimaging studies in animals also suggested that the facilitative effects of stimulants on locomotor activity, reinforcement processes, and rate-dependency are mediated by dopaminergic effects at the nucleus accumbens, whereas effects on delayed responding and working memory are mediated by NE afferents from LC to prefrontal cortex [55, 59]. The view that selective NET inhibitors are useful for the treatment of ADHD is strongly supported by successful clinical studies with desipramine, nortriptyline, and atomoxetine [59]. Thus, there is a great need for more research on the role of NET in ADHD, and it will be important for basic research to examine NE mechanisms altered by stimulants and other medications. In fact, the currently used NET inhibitors for the treatment of ADHD, including desipramine, nortriptyline, and atomoxetine (previously called tomoxetine), are not selective and they bind with high affinity to both NET and SERT. For example, atomoxetine has Kd values of 2.0 and 8.9 nM for NET and SERT, respectively. It's therefore of mechanistic importance to investigate the therapeutic properties of highly selective monoamine transporter inhibitors in order to tease out the roles of individual transporters underlying this specific CNS disorder. It's equally important to develop selective NET radioligands to examine whether NET is abnormal in ADHD subjects.

d. The Role of NET in Substance Abuse

Many psychostimulants are useful medications (such as MP) while others are highly addictive substances (such as cocaine (Coc)) with considerable morbidity and mortality. In some cases, the same drug can possess therapeutic and reinforcing properties (e.g., MP and amphetamine), depending upon the route of administration, as we have shown previously [60-64].

It's believed that the broad spectrum of effects produced by psychostimulants results from their interactions with monoamine transporters in the CNS [65]. So far, a generalization has been tentatively made from studies with animal models and from clinical and behavioral effects of abused drugs that blockade of DAT seems to be associated with stimulant activity. The interaction of Coc with dopamine systems has been considered critical to its reinforcing effects [66-68]; therefore, much attention has been paid to alterations in the regulation of dopamine transporters and receptors as a result of long-term exposure to Coc [69, 70]. Although it has been shown that amphetamine-type CNS stimulants release norepinephrine more potently than they release dopamine and serotonin [71], and that Coc accumulates in high concentrations in NE-rich brain regions [72], the role of NE systems as mediators of the acute or chronic actions of Coc has been far less studied.

Important evidence supporting a prominent role of the NET in substance abuse has come from *in vitro* binding studies and behavioral studies. In one study, a 'binge' schedule of cocaine administration (repeated doses in one session) in rats was employed along with in situ hybridization to quantify the abundance of the mRNA of three monoamine transporters in three brain regions. The results documented the difference in the effects on the three monoamine transporters' expression immediately following a binge regimen of chronic Coc, with a decrease in SERT mRNA (in raphe) and DAT mRNA (in midbrain) and an increase in NET mRNA (in LC) [73]. A profound upregulation (up to 52% relative to controls) in NET binding site density assessed by [^{3}H]Nis was also found in the bed nucleus of the stria terminalis (BNST) of rhesus monkeys after chronic Coc exposure [74]. In an extended study, the same group found that the effects extended to the nucleus prepositus, as well as forebrain regions such as hypothalamic nuclei, basolateral amygdala, parasubiculum, and entorhinal cortex [75].

NE involvement in the discriminative stimulus (DS) effects of Coc was investigated in squirrel monkeys by using a two-level drug discrimination task [76]. The selective NET inhibitors talsupram, tomoxetine, nisoxetine and desipramine substituted for Coc in the majority of monkeys under the low-dose (0.30 or 0.18 mg/kg i.m.) condition, whereas the selective DAT inhibitor GBR 12909 substituted for Coc under both low- and high-dose (1.0 mg/kg i.m.) conditions, and the selective SERT inhibitor citalopram failed to substitute for Coc under either condition. These results support a role for NET in the DS effects of Coc.

Another interesting study investigated dopaminergic and NE inhibition of lateral hypothalamic self-stimulation in a signaled, discrete-trials shuttle-box paradigm [77]. The results from the pimozide (D_2 antagonist) and clonidine (a_2 adrenoceptor antagonist) studies do not support a role for DA nor for NE in mediating brain-stimulation reward; instead, they suggest that both pimozide and clonidine specifically inhibit the initiation of motor response. However, LU 5-003, which selectively inhibits NET, inhibited self-stimulation in almost exactly the same way as did reducing reward by reducing stimulation frequency. These data support a primary role for NET in mediating brain-stimulation reward.

In a classical test for antidepressant drugs, the NET-deficient (NET-/-) animals behaved like antidepressant-treated wild-type mice [78]. Mutants were hyper-responsive to locomotor stimulation by Coc or amphetamine. These responses were accompanied by dopamine D_2/D_3 receptor supersensitivity. This suggests that altering NET expression significantly modulates midbrain dopaminergic function; an effect that may be an important component of the actions of antidepressants and psychostimulants.

NET may be the major cellular target in the human placenta for the abused drugs amphetamine and methamphetamine. Ramamoorthy *et al*. hypothesized that the two monoamine transporters, SERT and NET, that are expressed in the human placenta are direct targets for these drugs. The results from their study showed that the sensitivity of the NET to inhibition by these drugs is at least two orders of magnitude greater than that of the SERT [79].

Taken together, the data from behavioral and *in vitro* binding studies indicate a significant functional role of NET in ADHD, substance abuse, depression and neurodegenerative disorders, and support the development of suitable NET radioligands to carry out PET studies to better understand the role of NET in living human brain.

II. CHARACTERISTICS OF AN IDEAL PET TRACER FOR STUDYING THE BRAIN

Several potent NE reuptake inhibitors have been well characterized *in vitro*; examples of such compounds with high affinity and high selectivity for NET are listed in Table **1**. Some of these compounds are established drugs, such as desipramine, tomoxetine and reboxetine, and their toxicity profiles have been examined. Prior to 2003, progress in developing a tracer for *in vivo* imaging of brain NET was disappointing. In this paper, we describe efforts aimed at developing radiopharmaceuticals for PET imaging of brain NET system. The evaluation of several NET radioligands are presented and compared in this review (Fig. **1**). Although high lipophilicity (high log P) may be a reasonable explanation for the high non-specific binding *in vivo* in many cases, there are multiple other requirements for an appropriate PET tracer. General parameters that define an acceptable tracer for brain studies with PET are listed below. However, we will present a detailed discussion and examples how calculated log P values (clog P) may be misleading and how the poor ability to predict the behavior of chemical compounds *in vivo* based on their log P's and affinities cries out for more knowledge in this area.

1. Affinity, Specificity and Selectivity

The molecule must be specific and selective for a molecular target, with an appropriate Kd, in the nanomolar or subnanomolar range. However, this also depends on the den-

sity of the molecular target in the brain; lower density targets require higher affinity ligands. Lower affinity is usually associated with poor signal to noise ratio, while higher affinity may result in irreversible binding that may render poor quantification of the target sites.

2. Appropriate Lipophilicity

Normally, values of log P between 1 and 4 can be used as a rough initial guide; however, keeping in mind that calculated log P values do not always match measured values (see discussion below). Very low or very high log P may prevent adequate crossing of the blood-brain barrier (BBB); that is, molecules with log P <1 are not lipophilic enough to cross BBB [80], while molecules with log P >4 are generally associated with high nonspecific binding as well as slow penetration of the BBB [81-85]. The size of the molecule also affects brain penetration; typically, MW should be <500.

3. High Stability in Plasma and Low Affinity for Plasma Proteins

Fast metabolism in plasma and/or peripheral organs and high *irreversible* plasma protein binding can also result in low brain uptake.

4. Lack of Labeled Metabolites in the Brain

The presence of radiolabeled metabolites capable of crossing BBB is also undesirable, as it complicates image interpretation and kinetic analysis.

5. Appropriate Kinetics for Quantification

A suitable kinetic profile that allows proper quantification of the target sites is also essential.

6. High Specific Activity to Avoid Mass Effects

The specific activity of the radiotracer must be sufficiently high to avoid significant receptor or transporter occupancy.

7. Safety Profile

The molecule should be relatively nontoxic with a wide safety margin.

III. KINETIC MODELING

PET provides a quantitative *in vivo* measurement of the local concentration of radioactive compounds. In order to use

Table 1. NET Inhibitors: Affinity (IC50, ***IC50, the rest are *Kd (nM))) and Selectivity for Dopamine, Serotonin and Norepinephrine Transporters (DAT, SERT, NET)**

NET Inhibitors	DAT	SERT	NET	DAT/NET	SERT/NET	*CS* log P	Clog P_g
Amoxapine	4310 ±10	58 ± 2	16 ± 0.3	269	3.6	2.49	
3-Cl-methyl-reboxetine**	704	558	3.3	213	169	1.85	
Desipramine	3190 ± 40	17.6 ± 0.7	0.83 ± 0.05	3800	21	2.16	3.6
Lofepramine	18000 ± 1000	70 ± 4	5.4 ± 0.4	3400	13	4.85	
Lortalamine[$]	>10,000	>100,000	~0.2 [$]	>10,000	>100,000	2.08	1.9
Maprotiline	1000 ±20	5800 ± 200	11.1 ± 0.3	90	520	4.75	
Mazindol	8.1 ± 0.4	39 ± 1	0.45 ± 0.03	18	87	1.82	4.3
Methyl-reboxetine**	>10,000	310	2.48	4032	125	1.13	2.7
Nisoxetine	360	1000	1	360	1000	1.74	3.4
Nomifensine	56 ± 3	1010 ± 30	15.6 ± 0.4	3.6	65	2.42	
Nortriptyline	1140 ± 30	18 ± 1	4.37 ± 0.07	260	4.1	3.31	4.2
Oxaprotiline	4340 ± 30	3900 ± 100	4.9 ± 0.2	890	800	3.50	3.6
Protriptyline	2100 ± 60	19.6 ± 0.5	1.41 ± 0.02	1500	14	3.24	
Reboxetine**	>10,000	1070	8.2	>1220	130	1.01	3.0
Talopram***	44,000	1400	2.9	15172	482	3.68	3.8
Talsupram***	9300	850	0.79	11772	1075	4.46	4.6
Tomoxetine	1080 ± 50	8.9 ± 0.3	2.03 ± 0.06	530	4.4	2.11	4.0

*Kd, [45]; **IC50, [113]; ***IC50, [114]. [$]Lortalamine is a potent NET inhibitor with a potency higher than imipramine (13 fold) and desipramine (5 fold) [115]. Lipophilicity was calculated as *CS* log P using the ChemSilico LLC (Tewksbury, MA) family of property prediction software (*CSPredict*); Lipophilicity was also calculated as cLogP g using the Spartan molecular modeling software, the Ghose-Crippen model.

this data effectively it is necessary to be able to separate binding to the target receptor/transporter/enzyme from other processes. Up to now, no suitable kinetic modeling method has been developed for the NET system. The main reasons for this appear to be the widespread localization and the relatively low density of NET, and the high nonspecific (non-NET) binding exhibited by many NET tracers, which makes the identification of a reference region difficult. With other PET ligands the use of a reference region increases reproducibility of the outcome measure in test/retest studies. How best to normalize data from PET studies with NET ligands to obtain a measure related to NET availability was the subject of recent work by our group [86].

In principle, binding characteristics of successful NET ligands must necessarily be somewhat different than requirements of DAT ligands due to differences in density between NET and DAT. In the case of the DAT, the distribution is not as widespread and the density in basal ganglia is higher than NET density in most regions besides the locus coeruleus. To compensate for the lower *Bmax* of the NET compared to the DAT, the desirable kinetic constraints would be a higher k'_{on} ($k_3 = k'_{on}\ Bmax$) and/or a somewhat smaller $k_{off} = k_4$ (but high enough that the ligand is sufficiently reversible in the time course of the [^{11}C] (or [^{18}F] for the fluorinated derivatives) PET experiment to estimate a distribution volume.

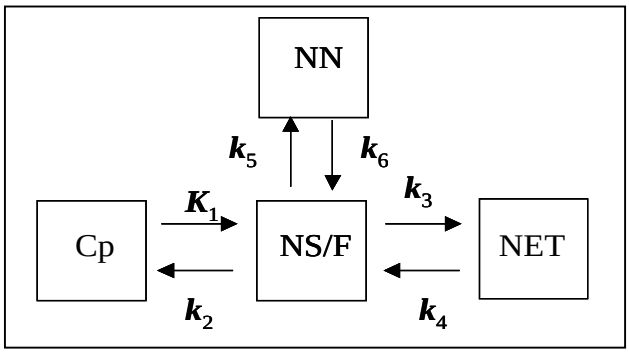
Most PET data is analyzed in terms of a reference region, which is assumed to have the same properties as the target region but without the specific binding component. The ratios of the distribution volumes (DV) of these regions is related to the target binding potential. The DV for the three-compartment model can be expressed as

$$DV = \frac{K_1}{k_2}(1 + BP^S)$$

where S refers to a specific binding. The rapid nonspecific binding component is incorporated into k_2. The DV for the reference region is assumed to be K_1 / k_2 and the distribution volume ratio (DVR) is $1 + BP^S$ so that DVR –1 is the effective binding potential, BP^S.

The binding of NET ligands can be represented by the four-compartment model shown below, and the total distribution volume for a tissue with both NET and non NET (NN) binding is given by

$$(1)\ DV = \frac{K_1}{k_2}(1 + BP^{NET} + BP^{NN})$$



where binding to the NET is characterized by the binding potential BP^{NET} which in terms of model parameters is given by

$$k_3 / k_4 = B^{NET}_{max} / K^{NET}_d$$

The non-NET binding is described by BP^{NN}, which is a summation over all types of (non-NET) binding contributing to the signal. Unlike other PET ligands both binding potential terms can vary from tissue to tissue. K_1 and k_2 are the tissue /plasma transport constants. The ratio K_1 / k_2 is also a function of plasma protein binding as well as the rapid nonspecific binding. It would be desirable to be able to evaluate all these kinetic parameters and therefore separate the individual binding components, however, the data from the NET ligands discussed here could be well described by a single compartment so that it was not possible to uniquely identify values for all these parameters. In this case the data can be described by K_1 and k_2' with DV = K_1 / k_2' where k_2' is the apparent k_2 given by $\frac{k_2}{(1 + BP^{NN} + BP^{NET})}$. Since the reference tissue may have a different amount of NN binding, the DVR (DV_T/DV_{REF}) is given by

$$(2)\ \mathrm{DVR} = \frac{1 + BP^{NN}_T}{1 + BP^{NN}_{REF}}\left(1 + \frac{BP^{NET}_T}{1 + BP^{NN}_T}\right) = \frac{f^{NN}_{REF}}{f^{NN}_T}\left(1 + f^{NN}_T BP^{NET}_T\right)$$

where *T* refers to the target region, *REF* to the reference region and we have used the notation $f^{NN}_i = \frac{1}{1 + BP^{NN}_i}$ where i refers to either *T* or *REF*.

Even though the DVR does not provide a binding potential as in the case of the three-compartment model, for example raclopride, it normalizes the data and was found to have a significantly reduced variability over that of the DV when comparing studies on the same animal [86]. This reduced variability of the DVR compared to the DV has also been observed for other PET ligands. There are potentially three sources of variation in the distribution volume. One is in the ratio K_1 / k_2 (due to, for example, plasma protein binding), the other two are the binding potential terms for NET and non-NET binding. The first (K_1 / k_2) is a global factor occurring in all regions. That the coefficient of variation is considerably smaller for the DVR than the DV indicates that a major source of this variability is global and is therefore common to both regions and cancels in the ratio. Also fluctuations between a high NET region, thalamus and a low NET region occipital cortex were found to be correlated when comparing the DV values for the same animal over a number of studies [86]. Another source of variability, the non-NET binding (the last term in Eq. (2)) would not be expected to reduce the variability in a DV ratio since these terms would not necessarily cancel.

The choice of the reference region was guided by results from experiments with (S, S)-[^{11}C]MRB in which pretreatment with pharmacological doses of cocaine preceded injection of the tracer. Averaging over all studies of a given dose of cocaine, the basal ganglia and occipital cortex consistently had the smallest % change in DV compared to baseline. The basal ganglia has been cited in many of the references listed

here as a region with very low binding, while the occipital cortex was not mentioned specifically in the same references; however from our studies it appears to have little high affinity NET binding. The use of two regions with perhaps different amounts of non-NET binding might be a better method of normalizing target regions, which may contain somewhat different amounts of non-NET binding. The results from our studies showed that the variability (as measured by the coefficient of variation, CV = standard deviation/mean) in the distribution volume ratio (DVR) of thalamus (to the reference region) was considerably reduced over that of the DV when the average DV of occipital cortex and basal ganglia was used as the composite reference region.

Since the NN binding can differ between the target and reference regions, the effective binding potential can no longer be calculated as DVR-1 and in fact the DVR could be less than 1. The ratio $f_{REF}^{NN} / f_{T}^{NN}$ represents the lowest possible value occurring when all transporters are blocked. Whether or not a particular NET tracer is useful will have to be determined by blocking studies to establish the range of DVRs, that is the sensitivity of the signal to changes in NET density. A critical examination of the kinetic properties of all new radioligands should continue to be a crucial part of NET radiotracer development strategy.

IV. RADIOLIGANDS FOR *IN VIVO* IMAGING OF NET IN BRAIN

a. ^{11}C-Desipramine

Desipramine (DMI), a well-known tricyclic antidepressant, is a highly selective and potent inhibitor of NE reuptake [87] (Table **1** & Fig. **1**). The specific, high-affinity binding sites for [^{3}H]DMI in rat have been demonstrated in tissues receiving dense noradrenergic input, such as heart, submaxillary gland, cortex and hypothalamus [88]. 2-Hydroxydesipramine (HDMI), a metabolite of DMI, retains the excellent NET selectivity profile of DMI, with a slightly lower inhibitory effect on the NE reuptake (K_i = 7.8 nM; Fig. **1**). Van Dort *et al*. [89] reported the synthesis of ^{11}C-labeled DMI and HDMI but the *in vivo* evaluation was not included. It appears that [^{11}C]DMI is not a suitable *in vivo* radiotracer with high non-specific binding, based on their unpublished results.

b. ^{11}C-Talopram and ^{11}C-Talsupram

Talopram and talsupram, developed as potential antidepressants based on benzo[*c*]furan and benzo[*c*]thiophene structures, exhibited potent and selective inhibition of [^{3}H]norepinephrine uptake in rat brain homogenates (IC_{50} 2.9, 0.79 nM for talopram and talsupram, respectively [114]). McConathy *et al.* [90] reported the synthesis, radiolabeling, and *in vivo* evaluation of [^{11}C]talopram and [^{11}C]talsupram. Their PET studies in a Rhesus monkey and the biodistribution studies in rats both showed that the brain uptake of these two C-11 labeled tracers was low, which diminished their potential application for imaging brain NET. Interestingly, the clog P for [^{11}C]talopram and [^{11}C]talsupram were 3.7 and 4.5, respectively; however, the free bases of these two compounds appeared to be very lipophilic based on their retention times on reverse-phase C18 HPLC, and the measured log $D_{7.4}$ values were 0.8 and 1.7, respectively. It was concluded that the polarity of these two ligands at physiologic pH might contribute to their low CNS availability.

c. ^{11}C-Nisoxetine

[^{3}H]Nisoxetine (Nis) has been used as a gold standard for *in vitro* mapping of NET, and Nis has a structure quite similar to reboxetine (Fig. **1**). Despite the biodistribution study of (R/S)-[N-$^{11}CH_3$]Nis in mice that showed only modest specific binding [91], its ultimate value as a PET imaging agent had never been explored. Therefore, in our lab, we labeled the more potent enantiomer of Nis in two positions (namely, (R)-[O-$^{11}CH_3$]Nis and (R)-[N-$^{11}CH_3$]Nis; IC_{50}'s for R- and S-Nis are 5.8 and 18 nM, respectively [92]) to further evaluate its potential as a PET tracer for NET using a primate model [93]. Since the profile of labeled metabolites for the labeled N- vs. O-methyl compounds may be different, we labeled Nis at different positions to probe potential differences. Comparative PET studies showed similar uptakes in three brain regions (TH, CB and ST) and relatively low uptake in frontal cortex for both tracers. Plasma metabolite assays indicated similar metabolism profile for both tracers, with a slightly slower metabolism for (R)-[O-$^{11}CH_3$]Nis as compared to (R)-[N-$^{11}CH_3$]Nis. Pretreatment with unlabeled Nis (1mg/kg iv, 10 min prior) did not reduce the tracer binding; instead, increased uptakes were observed, suggesting its high non-specific binding *in vivo*; consequently, both tracers are not suitable for PET imaging of NET.

d. ^{11}C-Methylreboxetine

Evaluation of [^{11}C]MRB, (S, S) and (R, R)-[^{11}C]MRB

Methylreboxetine (MRB, Fig. **1**) is one of the most promising ligand candidates since it is highly potent (IC_{50} =2.5 nM), more potent than RB (IC_{50} = 8.2 nM) (Table **1**), and highly selective [113], and was expected to be relatively easy to label by O-methylation with [^{11}C]methyl iodide. Two other groups also reported preclinical studies showing the potential utility of [^{11}C]MRB for imaging the NET [38, 39] at about the same time that we published our study [40]. Wilson *et al*. [38] first demonstrated its utility in rat brain; we reported the results of comparative PET studies in baboons with the active and inactive enantiomers of [^{11}C]MRB, and Schou *et al*. [39] reported similar PET evaluation of [^{11}C]MRB in monkeys. These studies demonstrate that (S, S)-[^{11}C]MRB displays much more desirable selectivity and specificity *in vivo* than any existing NET radioligand, even those with far higher affinity. Briefly, we developed an 11 step synthetic strategy for the nor precursors including the preparation and chiral separation of its enantiomers, and selective C-11 methylation at the phenolic oxygen to prepare [^{11}C]MRB [94]. Based on our baboon studies, the regional distribution of the radioactivity after injection of [^{11}C]MRB in the brain is consistent with the known distribution of NET, with the highest uptake and slower washout occurring in the thalamus, a known NET-rich region. For a NET-poor region such as ST, there were no significant changes in the striatal uptakes with the nisoxetine pretreatment. In contrast, a significant blocking effect by nisoxetine was observed in NET-rich regions such as TH and CB after injection of ra-

cemic [^{11}C]MRB, with an even greater effect after injection of (S, S)-[^{11}C]MRB (the largest DV changes occurred in TH, -48%, which were equivalent to almost complete blockade). However, no blocking effect on the uptakes of [^{11}C]MRB was observed when baboons were pretreated with GBR12909 or citalopram. These results, along with the fact that there was no regional specificity and no blocking effect by nisoxetine for (R, R)-[^{11}C]MRB, suggest the enantioselectivity and specificity of MRB *in vivo*, which is consistent with previous *in vitro* and *in vivo* studies in rodents [38, 95].

e. ^{18}F-Methylreboxetine and ^{18}F-Reboxetine

Evaluation of ^{18}F-Methylreboxetine and ^{18}F-Reboxetine, their individual Enantiomers and the Deuterated Analogues

We have shown that (S, S)-[^{11}C]MRB is a potent and highly selective NET radioligand whose regional kinetics can be quantified using a graphic kinetic modeling for reversible ligands; however, it might be beneficial to extend the PET scanning time using a F-18 labeled radioligand. The preparation of its [^{18}F]fluoromethyl analog, ((S, S)-[^{18}F]FMeNER), has been reported [96]. In spite of significant uptake of (S, S)-[^{18}F]FMeNER in the NET-rich regions of monkey brain, there was also high bone uptake due to *in vivo* defluorination. A *di*-deuterated analog, (S, S)-[^{18}F]FMeNER-D_2, was then developed with the intention of reducing its *in vivo* defluorination. Their PET studies indicated that the extent of defluorination was significantly reduced, though not totally inhibited as shown by the continuous increase of bone uptake.

It has been shown in the case of ^{18}F-labeled benzodiazepine receptor radioligands [97] that the extent of bone uptake from an aryl [^{18}F]fluoromethyl ether was reduced by over 98 % when a [^{18}F]fluoromethyl moiety was replaced by a [^{18}F]fluoroethyl group. In addition, the reduction of bone uptake by the replacement of a [^{18}F]fluoromethyl moiety of a serotonin transporter radioligand with a [^{18}F]fluoroethyl group has also been reported [98]. Therefore, it is conceivable that replacing the [^{18}F]fluoromethyl moiety of [^{18}F]FMeNER with a [^{18}F]fluoroethyl group would further reduce the extent of *in vivo* defluorination, while the resulting [^{18}F]FRB, which is an analog of RB, would retain the high affinity and selectivity toward NET. With the hope of minimizing defluorination, we prepared two new ^{18}F-labeled reboxetine derivatives [99], (S, S)-2-[α-(2-(2-[^{18}F]fluoroethoxy)phenoxy)benzyl]-morpholine ((S, S)-[^{18}F]FRB-H_4) and its tetradeuterated analog (S, S)-[^{18}F]-FRB-D_4, and evaluated their potential as PET tracers [93].

The racemate and (S, S) & (R, R) enantiomers of [^{18}F]FRB and [^{18}F]FRB-D_4 were obtained in 11-27 % (EOB) in 120 min with a radiochemical purity of > 98 % and specific activities of 0.57-1.29 Ci/μmole (EOB). The racemate and (S, S) enantiomer of each tracer displayed regional specificities that were consistent with the known NET distribution, and their uptakes could be blocked by NET inhibitors. In contrast, no regional specificity or blocking effect were observed for the (R, R)-enantiomers. Similar signal to noise ratios were obtained for both (S, S) enantiomers of [^{18}F]FRB and [^{18}F]FRB-D_4. The bone uptake of these two tracers was low and did not increase with time, while the clearance rate was faster for (S, S)-[^{18}F]FRB-D_4 than for (S, S)-[^{18}F]FRB-H_4. These findings were also supported by a biodistribution study in mice [99], and are quite different from previous studies with (S, S)-[^{18}F]FMeNER and (S, S)-[^{18}F]FMeNER-D_2, in that the bone uptake increased with time. In terms of signal to noise ratio, (S, S)-[^{18}F]FRB is not as good as (S, S)-[^{11}C]MRB (Table **2**)**.** However, in addition to the advantage of the longer half life of F-18 ($t_{1/2}$ = 110 min) as compared to C-11 ($t_{1/2}$ = 20 min), (S, S)-[^{18}F]FRB displayed a faster kinetics in NET-rich region, which may facilitate its kinetic analysis. Thus, (S, S)-[^{18}F]FRB or (S, S)-[^{18}F]FRB-D_4 may have potential as ligands for NET studies [93].

f. ^{11}C-Oxaprotiline

Due to the fact that our lead compound (S, S)-[^{11}C]MRB showed many desired *in vivo* properties that have not been seen with any other NET ligands, our strategy was to see if we could optimize this ligand by, for example, decreasing the high, non-specific striatal uptake that characterizes *in vivo* binding of C-11-MRB as well as many of the NET tracers in the literature. Oxaprotiline (Oxap) was chosen as a candidate because it has a quite different structure from MRB, but it has high affinity and selectivity towards NET (Fig. **1** & Table **1**). Racemic [^{11}C]Oxap was then synthesized and subjected to initial evaluation in baboons with PET [93]. The tracer uptake in baboon brain, in general, was high; however, the distribution did not match with the known NET distribution (a bio-distribution study in mice reported previously as an abstract [100] also showed that the tracer displayed relatively uniform uptake in all brain regions). The fact that Oxap has the highest uptake in striatum and low S/N ratio suggested strongly to us that an analogue with this type of molecular structure is probably not desirable for *in vivo* imaging of NET, despite the fact that its measured log P is ideal (2.1).

g. ^{11}C-Lortalamine

Lortalamine is a potent NET inhibitor with a potency higher than imipramine (13 fold) and desipramine (5 fold) [115]. Racemic [^{11}C]Lort was synthesized and subjected to initial evaluation with PET in baboon [93]. In terms of regional specificity and signal to noise ratio, [^{11}C]Lort is slightly better than [^{11}C]Oxap with similar uptakes in both thalamus and striatum in the baseline study. Pretreatment with unlabeled nisoxetine did reduce the tracer binding in TH and CB, but less blocking effect was observed in striatum, as expected. However, these positive characteristics, which indicate the specific binding of the tracer to NET, were diminished by the fact that [^{11}C]Lort still suffers high non-specific uptake in striatum (striatum has higher uptake than TH).

h. ^{11}C-3-Chloromethylreboxetine

Based on our preliminary studies, we have identified a number of reboxetine (RB) analogues that are suitable radioligands for PET studies of brain NET, including (S, S)-[^{11}C]MRB and (S, S)-[^{18}F]FERB-D_4. (R)-[^{11}C]Nisoxetine, [^{11}C]oxaprotiline and [^{11}C]lortalamine all suffer high non-specific striatal uptake (higher than TH) and low S/N ratio,

in spite of their high affinity and high selectivity *in vitro*. These results, along with many other previously reported disappointing ligands, suggest that analogues of MRB are still the most promising structures for further development of potential NET ligands. We therefore prepared another analogue of MRB. 3-ChloroMethylreboxetine (3-Cl-MRB), a compound structurally similar to MRB (Fig. **1**), which is more potent though less selective than RB (Table **1**), was also chosen because it may also provide important structure-affinity relationship (SAR) information in terms of binding and kinetics.

We used the same chiral resolution strategy followed by radiosynthesis to obtain pure (S, S) & (R, R)-3-Cl-[^{11}C]MRB [94]. Comparative PET studies were then carried out in baboons, and results showed that (S, S)-3-Cl-[^{11}C]MRB displayed appropriate regional specificities and signal to noise ratio, with TH uptake significantly higher than striatum; in contrast, high uptakes in both striatum and TH were observed for the (R, R)-enantiomer. Comparative studies of (S, S)-3-Cl-[^{11}C]MRB vs. (S, S)-[^{11}C]MRB in the same baboon indicated that both tracers have similar high uptakes in TH; however, (S, S)-[^{11}C]MRB appears to be superior since it has a faster clearance from striatum. As a result, the signal to noise ratio (calculated based on the DVR of TH to that of the composite reference region [avg. DV of ST and Occi] as described in the *Kinetic Analysis* section, above) of (S, S)-[^{11}C]MRB is significantly better than that of (S, S)-3-Cl-[^{11}C]MRB [93].

i. ^{125}I-Nisoxetine

As indicated earlier, [^{3}H]nisoxetine has been used as a gold standard for *in vitro* mapping of NET. In fact, both tomoxetine and nisoxetine are phenoxyphenyl propylamines that have high affinity for the NET. Although an iodinated analog of tomoxetine showed high nonspecific binding *in vivo* [101], tomoxetine itself displayed high *in vitro* affinity [45] (Kd = 2 nM). However, our intention to develop [^{11}C]tomoxetine as a selective NET tracer for PET studies was discouraged by our characterization studies (unpublished results) in baboon using [^{11}C]DASB, a selective SERT ligand [102]. In these studies, we demonstrated that tomoxetine exhibited the same blocking effect on [^{11}C]DASB binding as fluoxetine (a selective SERT inhibitor). A thorough investigation indicated that tomoxetine is an equally

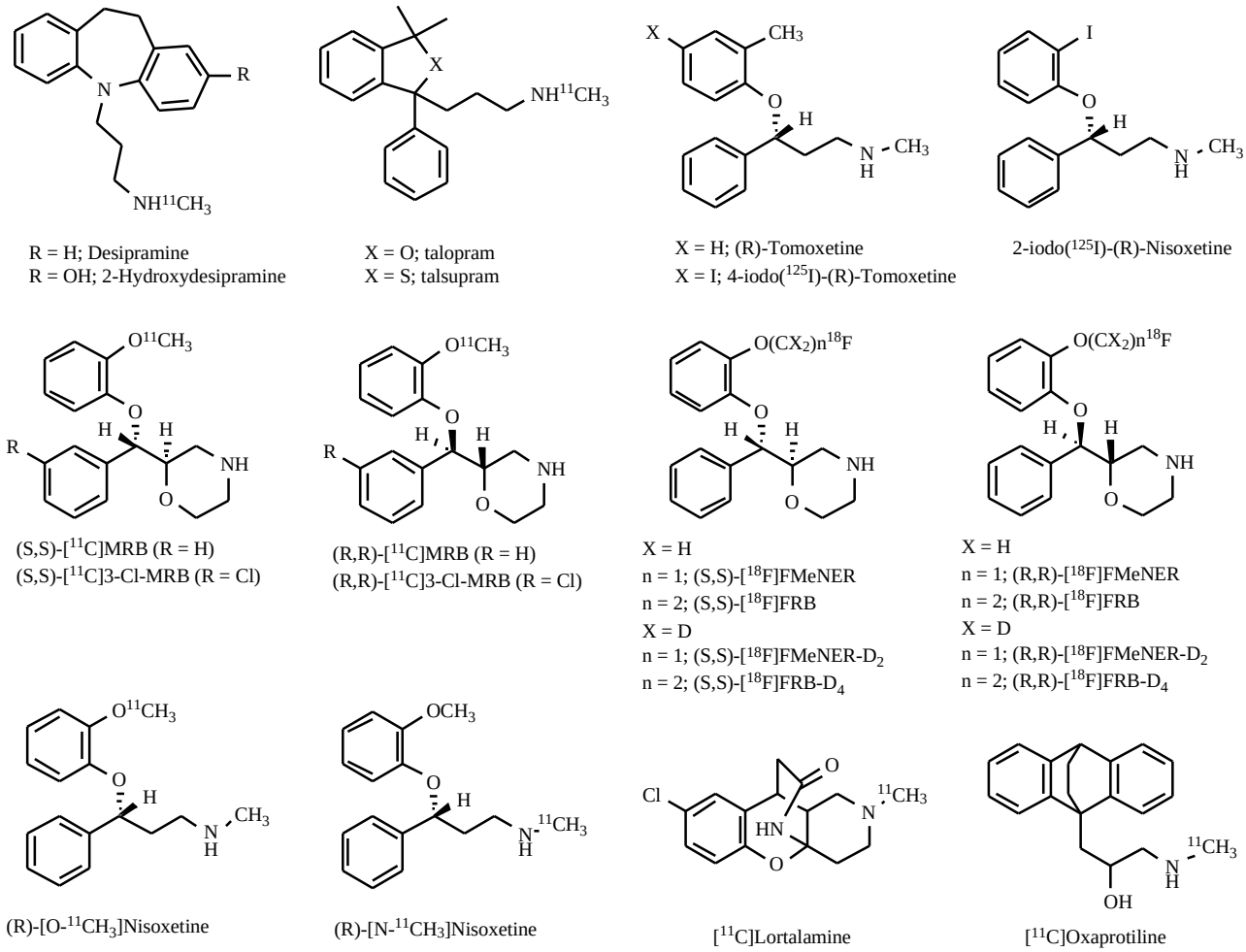


Fig. (1). Structure of NET radioligands.

potent *in vivo* inhibitor to both NET and SERT. These results were consistent with the *in vitro* data previously shown that (R)-tomoxetine bound not only to the NET but also to SERT [92]. In contrast, nisoxetine appears to be more selective; thus, analogues of nisoxetine, such as (R)-thionisoxetine and 2-iodo-nisoxetine, have been developed. Gehlert *et al*. [92] showed that thionisoxetine (thio-Nis) was more potent than (R)-Nis, and the (R)-thio-Nis was significantly more potent than its S-enantiomer, with Ki values of 0.20 and 31 nM, respectively, for inhibiting [^{3}H]Nis binding. However, it failed during the preclinical evaluation *in vivo* due to the high non-specific binding (unpublished results). 2-Iodo-nisoxetine has been labeled as [^{125}I]2-iodonisoxetine [103, 104], and it displayed extremely promising *in vitro* properties, with a very high Kd (0.06 nM) [104]. Unfortunately, this tracer also failed the *in vivo* tests; i.e., it displayed high nonspecific binding and/or binding to secondary sites resulting in a high background uptake [103]. Although evaluation of PET NET ligands is the focus of this review, the evaluation studies of [^{125}I]2-iodonisoxetine provide important insight regarding the development of new NET ligands. This will be discussed further below.

To summarize the above studies on our tracer development, the measured log P, PPB, peak brain uptake (e.g., %injected dose in global) and S/N ($DVR_{TH/Ref}$, see below for details) are tabulated in Table **2** along with their reported affinity and calculated log P. For comparison purposes, data for (R)-[^{123}I]iodoNis is also included. These results clearly indicate the superiority of (S, S)-[^{11}C]MRB and the suitability of the MRB analogs ((S, S)-[^{11}C]MRB >(S, S)-[^{11}C]3-Cl-MRB >(S, S)-[^{18}F]FRB) as NET ligands for PET. In addition to high uptake in ST (higher than TH), Nis, Oxap and Lort displayed high non-specific binding and poor S/N. According to the *in vitro* mapping of NET by quantitative autoradiography, lowest binding was found in the CA1 layer of the hippocampus and the caudate putamen [22, 23]. The mechanism for a relatively high *in vivo* uptake in the ST for all the NET radioligands that have been investigated is not known. It may reflect the presence of non-specific or low affinity non-NET binding sites in the striatum, as has been seen for other NET ligands [21, 105]. Our comparative studies indicate that although there is uptake in ST after i.v. injection of (S, S)-[^{11}C]MRB, its striatal uptake is, by far, the least significant among all the tracers.

V. OCCUPANCY STUDIES

PET studies with (S, S)-[^{11}C]MRB showed the binding to the transporter to be sufficiently reversible that it can be characterized by a distribution volume (DV). The DV is a measure of the capacity of the tissue to bind the tracer, and is therefore a function of the number of binding sites. However, there are other factors such as nonspecific binding, the presence of endogenous neurotransmitter and the presence of low affinity sites that can also alter the amount of binding and affect the DV measurement. In order to compare studies it's necessary to have some way of taking into account this variation. Normally this is done with a reference region and the DV's are reported as the ratio of the DV from the region with specific binding to that of a region with little or no specific binding. Since the NET is present in some concentration in many brain regions it is challenging to identify a reference region; also, some of the ligands may bind to sites other than the NET (see also III. Kinetic Modeling). In order to identify a potential reference region, we have used paired studies with the tracer (S, S)-[^{11}C]MRB in which a baseline scan was followed by a scan after pretreatment with a dose of cocaine [86, 106].

Three doses were used (0.2 mg/kg, 0.4 mg/kg and 0.8 mg/kg); each was given 5 min prior to the injection of the tracer. The results (presented in Fig. (**3**) as the % change in DV) represent the average of 4 baboons for the 0.2 and 0.8 mg/kg doses and the average of 3 for the 0.4 mg/kg dose. As expected, the greatest changes occurred in the TH, midbrain and brain stem, which are known to have high concentrations of NET. The lowest changes occurred in the occipital (OCC)

Table 2. Log P, PPB, Peak Brain Uptake* and S/N Ratio of NET Radioligands**

Radiotracer	Affinity	Log P (measured)	CSLog P (calculated)	PPB (% unbound)	Brain uptake (% inj. dose)	S/N ($DVR_{TH/Ref.}$)
(R)-[^{11}C]Nis	1	0.48 (-0.02)$^{\$}$	1.74 (3.4)		2%	poor
(S,S)-[^{11}C]MRB	2.5*	1.17	1.13	14%	3.2-4%	1.8-2.2
(S,S)-[^{18}C]FERB	----	0.91	1.85	13%	1.8%	1.3-1.6
(S,S)-[^{11}C]3-Cl-MRB	3.3*	1.91	1.85	6%	3.2%	1.4-1.6
[^{11}C]Oxaprotiline	4.9	2.1	3.50	9.1%	6.0%	poor
[^{11}C]Lortalamine	0.2	1.35	2.08	44%	2.6%	poor
(R)- [^{123}I]IodoNis	0.06	1.31	2.05	----	**	poor

All the data, except for (R)-[^{123}I]iodoNis, were generated from PET studies in baboon [93].

$^{\$}$ 0.48 is our measurement with (R)-[^{11}C]Nis; -0.02 is measured with [^{3}H]Nis by Kiyono *et al*. [103]. 1.74 and 3.4 are calculated Log P values for Nis when different software was used.

** in rats, 0.45% ID/brain for (R)-[^{123}I]iodoNis as compared to 0.53% ID/brain for (S,S)-[^{11}C]MRB [103].

***Peak brain uptake is based on % injected dose/cc x 200 g (avg. brain weight).

PPB: plasma protein binding.

and basal ganglia (BG, also called ST) regions with the standard deviation for these regions spanning 0 for all doses. Comparative studies of S, S and R, R-[^{11}C]MRB showed the smallest differences in the OCC, BG and temporal (19, 16 and 17%, respectively) and the largest differences in TH (-48%). This is consistent with lower affinity of R, R-MRB for the NET. Based on these data we have chosen to use an average of the OCC and BG as the reference region for normalization. The use of two regions should minimize the effect of fluctuations in the nonspecific binding and statistical variations. It's certainly possible that these regions contain some amount of low affinity binding. However the average DVR is on the order of 1.8, which should be sufficient as a target to non-target ratio to detect changes in NET binding. The low binding in these reference regions should not compromise our results since the tendency would be to slightly underestimate any difference due to a drug treatment.

As illustrated in Fig. (**2**) for (S, S)-[^{11}C]MRB, baseline studies (n = 18) indicated that the variation in DVR is less than the variation in DV for various brain regions (DVR calculated using an average of OCC and BG for the reference region). Fig. (**3**) shows the %change in DV for the Coc occupancy studies. Note that there is a dose-dependent occupancy by Coc at a dose lower than 0.8 mg/kg. In one baboon, the decrease in TH was 46% after 0.4 mg/kg of Coc (in terms of the binding potential, this is essentially completely blocked). It's important to point out that the estimated NET occupancies by the tracer alone were not significant, since the specific activity of the tracer was very high (avg. specific activity was ~10-15 Ci/micromol at EOB and approx. 2 Ci/micromol at the time of injection) and we would not anticipate any significant mass effects.

VI. DISCUSSION

NET has long been recognized as an important molecular target for both stimulant and therapeutic drugs to treat depression, ADHD and other CNS disorders; however, despite widespread abuse and therapeutic use, the mechanisms for addictive and therapeutic properties of stimulant drugs such as cocaine and methylphenidate (Ritalin, the most prescribed drug for treatment of ADHD) are not well understood. Cocaine binds to all three monoamine transporters (DAT, NET, SERT) with comparable affinities, and methylphenidate binds to DAT and NET with even higher affinity towards NET. Their effects on the brain DA system have been well characterized in living humans, yet our knowledge of their effects on NET has been limited to postmortem studies due to lack of suitable radiotracers. This places a sense of urgency in developing radiotracers that can characterize their binding to different molecular targets and the relationship of their behavioral and therapeutic properties in living humans. We and other researchers are making pro-

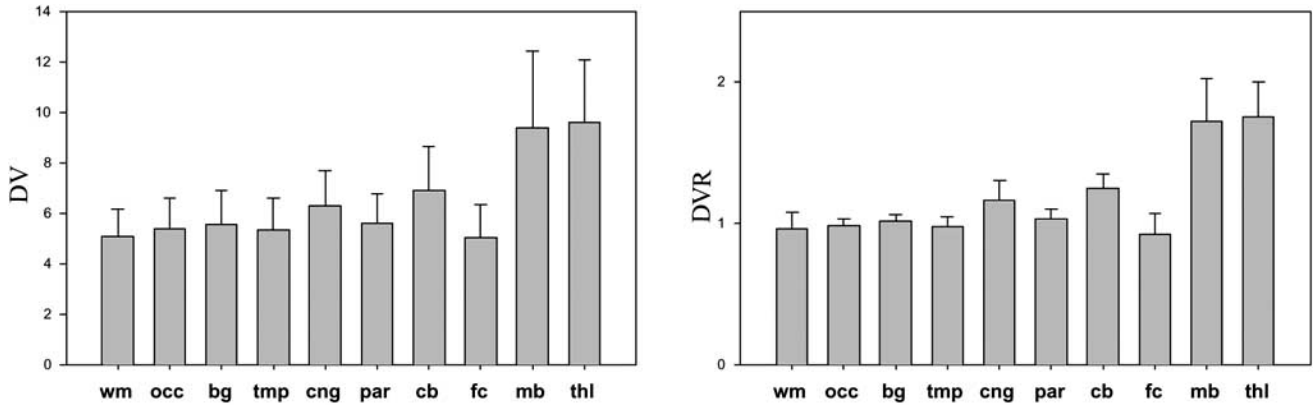


Fig. (2). DV and DVR for (S, S)-[^{11}C]MRB.

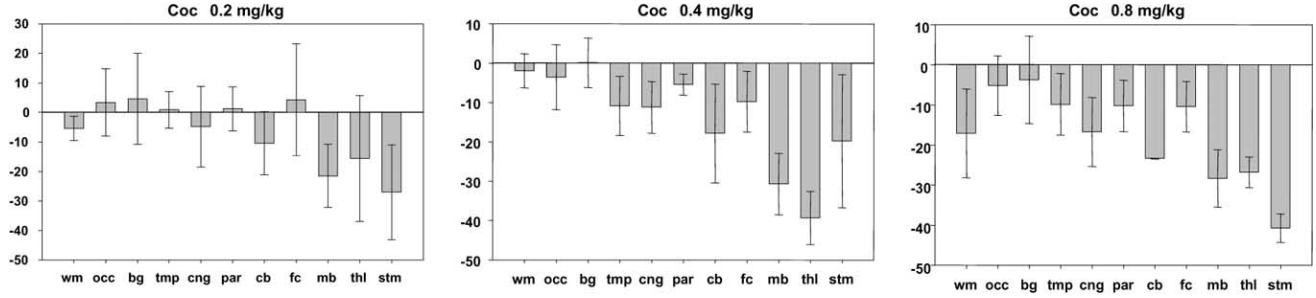


Fig. (3). % Change in DV in the occupancy studies with Coc (0.2, 0.4 and 0.8 mg/kg, IV).

gress in developing suitable ligands for mapping NET *in vivo*, in the hopes that we will soon be able to better understand the role of NET in various CNS disorders. The evaluation of several PET radioligands for the NET system are presented and compared in this review. The results indicate that reboxetine derivatives are by far the best candidates to provide specific and functional maps of NET in the human brain.

Tracer Kinetics

Based on our baboon studies, (S, S)-[^{11}C]MRB exhibits high brain uptake with reasonable kinetics and suitable clearance rate from the binding sites; however, the study by the Karolinska group found that the same tracer (which they called (S, S)-[^{11}C]MeNER) has slow kinetics in the brain. The most likely explanation for the contrasting results lies in the fact that these two independent studies were carried out in two different primate species (baboons vs. cynomolgus monkeys). Different anesthesia procedures may also affect the tracer kinetics; these have been well documented [107]. In our previous paper [40], we not only reported time-activity curves but we also carried out kinetic modeling and provided distribution volume (DV) data that showed that the uptake of (S, S)-[^{11}C]MRB in striatum is lower than that in thalamus (TH) and higher than those in most cortical regions (TH/ST: 1.63, TH/Occ: 2.06, and TH/frontal cortex: 1.94). In fact, a high ratio of binding in TH to that in striatum is an important criterion for a NET tracer, considering that NET concentration in striatum is low. Almost all of the known NET ligands (*in vitro* and *in vivo*) displayed a significantly higher striatal uptake (higher than TH) than reboxetine analogues, as indicated in our studies for (R)-[^{11}C]nisoxetine, [^{11}C]oxaprotiline and [^{11}C]lortalamine.

F-18 labeled analogues of RB, such as (S, S)-[^{18}F]FRB and (S, S)-[^{18}F]FRB-D_4 that have been evaluated in our lab, as well as (S, S)-[^{18}F]FMeNER and (S, S)-[^{18}F]FMeNER-D_2 by Schou *et al.* [96], displayed relatively fast kinetics in NET-rich regions that, in principle, would facilitate the kinetic modeling. However, their characteristics, such as defluorination and a relatively poorer signal to noise ratio as compared to (S, S)-[^{11}C]MRB, make them less desirable as *in vivo* NET ligands.

(S, S)-3-Cl-[^{11}C]MRB is a promising ligand for imaging brain NET. However, comparative studies of (S, S)-3-Cl-[^{11}C]MRB vs. (S, S)-[^{11}C]MRB in the same baboon indicated that (S, S)-[^{11}C]MRB is still the best since it has a faster clearance from ST.

In addition to high uptake in striatum (higher than thalamus), non-specific binding and poor signal to noise ratio, Nis, Oxap and Lort displayed undesirable slow kinetics. Thus, the kinetics of (S, S)-[^{11}C]MRB remain by far the most promising for PET studies.

Kinetic Analysis

The cell bodies of NE neurons are located in the brain stem. Projections from these cell bodies are widespread – in thalamus, cortex, hippocampus, cerebellum. Based on *in vitro* studies, the highest binding of [^{3}H]desipramine (expressed as fmol/mg protein) was found in locus coeruleus (888), with moderately high binding in the cingulate (240) and lower (172) in cerebellar cortex, with basal ganglia (45) practically indistinguishable from nonspecific binding [21]. Autoradiographic studies in rat with [^{3}H]nisoxetine also revealed that the highest specific binding was in brain stem (locus coeruleus) and thalamus (1526 and 1444 fmol/mg protein, respectively) with 54 fmol/mg protein in caudate-putamen [22]. Our PET studies in baboon showed high uptakes of (S, S)-[^{11}C]MRB and its analogues in TH, midbrain and brain stem, which is consistent with the known NET distribution. We have also demonstrated their binding to the transporter to be sufficiently reversible that it can be characterized by a distribution volume (DV). In our previous paper [40] we reported the DV rather than the DVR since we had not yet identified a reference region. It is not unusual to see variations in absolute DV values in the same baboon on different days due to different physiological states of the animals probably related to anesthesia and other variables. That is why it is preferable to use DV ratio (DVR) instead of the absolute DV when comparing studies. Normally this is done with a reference region, and the DV's are reported as the ratio of the DV from the region with specific binding to that of a region with little or no specific binding. Since the NET is present in some concentration in many brain regions it is challenging to identify a reference region; also, some of the ligands may bind to sites other than the NET. In order to identify a potential reference region, we used paired studies with the tracer (S, S)-[^{11}C]MRB in which a baseline scan was followed by a scan after pretreatment with a dose of cocaine (see also in III. Kinetic Modeling). The advanced graphical analysis methods for quantification of NET by choosing an average of the occipital (Occ) and striatal (ST) regions (ST + Occ) for the reference region should, in principle, minimize the effect of fluctuations in the nonspecific binding and statistical variations. In fact, using the data from our *Synapse* paper [40], we calculated the DVR as the ratio of the DV from thalamus to that of (ST + Occ) for racemic, S, S and R, R compounds, and the value for the racemic compound (1.38) fell between those of the S, S (1.83) and the R, R enantiomer (1.13), which is consistent with expectations.

Lipophilicity (Log P)

The finding of a very low log P value (0.48) for (R)-[^{11}C]-nisoxetine was unexpected since it was very different from the calculated values (1.74 or 3.4). In fact, Kiyono *et al.* [103] also recently reported a very low log P value of –0.02 for [^{3}H]nisoxetine, which was similar to what we obtained for (R)-[^{11}C]nisoxetine (0.48). As we and others pointed out, the calculated log P values are not always identical with the measured log P (oxaprotiline, talopram and talsupram are other examples), and the calculated log P values are often different when different software is used. The rationalization for computational methods to generate the log P values for each structure is based on the information contained in the program library. It is believed that most estimates reflect only partitioning of the neutral species, and therefore represent more a log P value than a log D determination (which includes a partitioning value obtained by measurement of all species present in solution and therefore accounts for solubility effects associated with hydrogen

bonding and ionization) [83, 108-110]. The discrepancy in the log P measurement of nisoxetine and other ligands further supports our point that, although calculated log P values provide initial guidance for the lipophilicity of the molecule, they should be used with caution when making predictions.

Can Good Kd Guarantee the *In vivo* Suitability of a Ligand?

Measurements of the density of NET in the rat brain by various radioligands using autoradiography [22, 104] showed the Bmax ranged from 50 to 1500 fmol/mg protein in various brain regions. The DAT density in the rat striatum measured by [^{125}I]IPT, a tropane derivative, was about 2000 fmol/mg protein [111], while a Bmax value of 100 fmol/mg protein for 5-HT transporter in rat cortical homogenates was obtained. Thus, *in vivo* mapping of NET with PET, while challenging, is feasible. There are many NET inhibitors such as nisoxetine (Kd = 1 nM) and desipramine (Kd = 0.83 nM) that have higher affinity than MRB (Kd = 2.5nM); however, they did not survive the *in vivo* test. [^{125}I]2-Iodonisoxetine, which has a very high affinity (Kd = 0.06 nM), also failed the *in vivo* tests. These disappointing results were similar to those that we obtained in our studies with (R)-[^{11}C]Nis, [^{11}C]Oxap and [^{11}C]Lort. Thus, in spite of the fact that (S, S)-[^{11}C]MRB does not have as high an affinity as the above-mentioned compounds, it displays much more desirable selectivity and specificity *in vivo* than any existing NET radioligand. Similarly, [^{11}C]raclopride, which has an affinity (Ki = 1 nM) lower than many existing DA D_2 ligands, is a superior *in vivo* ligand. [^{11}C]DASB is another good example; it has an affinity (Ki = 1.77 nM) that is lower than that of [^{11}C]McN 5652 (Ki = 0.26 nM); however, [^{11}C]DASB is a much better tracer than [^{11}C]McN 5652 for *in vivo* imaging of SERT [81]. Therefore, it is not strictly an affinity issue, and high *in vitro* affinity of a ligand does not guarantee its suitability as an *in vivo* ligand.

Indeed, the poor ability to predict the *in vivo* behavior of chemical compounds based on their log P's and affinities emphasizes the need for more knowledge in this area [112]. We and others have pointed this out, and the NET system is an outstanding example of the fact that generalizations don't always work. Designing out the high non-NET uptake in the striatum is the next challenge; however, the limitations of (S, S)-[^{11}C]MRB are no more limiting than were initial studies with [^{11}C]McN 5652 for the SERT. One would speculate that human studies of this pre-clinically well-characterized ligand coupled with advances in kinetic modeling might provide extremely important information to guide us towards the development of a new generation of NET ligands.

ACKNOWLEDGEMENTS

This research was supported under contract DE-AC02-98CH10886 with the U.S. Department of Energy and Office of Biological Environmental Research and also supported by the National Institutes of Health (National Institute for Biomedical Imaging and Bioengineering EB002630 and National Institute on Drug Abuse DA-06278) and Office of National Drug Control Policy. The authors are grateful to the team of the PET group at Brookhaven National Laboratory for their assistance.

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