Development of Radioligands for Imaging of Brain Norepinephrine Transporters *In Vivo* with Positron Emission Tomography

Magnus Schou[1,*,+], Victor W. Pike[2] and Christer Halldin[1]

[1]*Karolinska Institutet, Department of Clinical Neuroscience, Psychiatry Section, Karolinska Hospital, S-17176 Stockholm, Sweden,* [2]*Molecular Imaging Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892-1003, USA*

Abstract: In the central nervous system (CNS) and in the periphery, specific proteins (transporters) are responsible for the regulation of the synaptic concentrations of the major monoamine neurotransmitters, noradrenaline (NE), serotonin (5-HT) and dopamine (DA). Several reports have shown that the expression of these transporters within the CNS may be altered in patients with certain neurodegenerative or neuropsychiatric disorders. Therefore, in the CNS the monoamine transporters are major targets for existing and developmental drugs. The best known drugs targeting these transporters are the selective 5-HT reuptake inhibitors (SSRIs) (e.g. citalopram, Celexa®) that are most frequently used in the treatment of clinical depression. Selective NE reuptake inhibitors (NRIs) have also found use for the treatment of depression and other conditions such as attention deficit hyperactivity (ADHD) disorder. Given that the NE transporter (NET) is also a binding site for cocaine and drugs of abuse, there is a great need for a probe to assess the densities of NET *in vivo* by brain imaging with either positron emission tomography (PET) or single photon emission tomography (SPET). PET in particular has the potential to measure NET densities quantitatively and with high resolution in the human brain *in vivo.*

The quality of a PET image depends crucially on the radioligand used in the emission measurement. Commonly used radionuclides in PET radioligands are carbon-11 ($t_{1/2}$ = 20.4 min) and fluorine-18 ($t_{1/2}$ = 109.8 min). This review specifically summarizes the present status of the development of ^{11}C- or ^{18}F-labeled ligands as tools for imaging NET in brain with PET in support of neuropsychiatric clinical research and drug development.

Keywords: NET, radioligand, PET, carbon-11, fluorine-18.

INTRODUCTION

Norepinephrine (NE, noradrenaline) is a chemical messenger that is part of a greater family of hormones and neurotransmitters commonly referred to as the catecholamines. The catecholamines, especially NE and epinephrine (E, adrenaline), play a central role in the regulation of heart rate and glucose metabolism and are vital in preparing the body for fight or flight responses [1]. Within the central nervous system (CNS), NE is involved in a number of important regulatory processes such as the regulation of sleep, mood and the degree of alertness and arousal [1].

The biological effects of NE in the synapse are primarily mediated by variations in the concentration of the neurotransmitter and of the responding receptors. Whereas receptor concentrations may be regulated by desensitization, NE concentrations are actively limited, in magnitude and duration, by a transporter protein (NET) [2]. NETs are localized pre-synaptically on noradrenergic nerve terminals, are activated upon polarization and recover about 70 to 90% of released NE from the synapse [1]. Neurons are thus largely dependent on NETs for the efficient removal of synaptic NE, which make them vulnerable to abnormalities in NET expression or pharmacological blockade of these proteins.

NET AND PSYCHOBIOLOGY

Abnormalities in the central noradrenergic system have been implicated in the pathophysiology of several neuropsychiatric and neurodegenerative disorders, such as anxiety disorder [3], attention deficit hyperactivity disorder (ADHD) [4], clinical depression [5, 6], drug abuse [7] and Alzheimer's disease [8]. More specifically, decreased levels of NET have been observed in the brains of patients with major depression [5, 6] and Alzheimer's disease [8]. Increased levels of NET have been observed in the brains of rhesus monkeys upon chronic self-administration of cocaine [7]. Given the implication of NET in the aforementioned disorders, significant efforts have been directed from the pharmaceutical industry to develop potent and selective NET inhibitors.

*Address correspondence to this author at the Karolinska Institutet, Department of Clinical Neuroscience, Psychiatry Section, Karolinska Hospital, S-17176 Stockholm, Sweden; Tel: 46-8-51775598; Fax: 46-8-51771753); E-mail: magnus.schou@astrazeneca.com

[+]Present Address: AstraZeneca, Discovery R&D, Södertälje, Sweden

NET inhibitors have been most widely used as antidepressants [6]. The treatment of depression by alterations of synaptic monoamine concentrations was introduced in the 1950s. In 1957, Kuhn presented data from clinical trials of a tricyclic antidepressant drug, which had its efficacy partly based on NET inhibition (imipramine, Tofranil®) [9]. The same year, Loomer *et al*. reported clinical evidence for iproniazid, another type of antidepressant acting through inhibition of monoamine oxidase (MAO), which is the enzyme responsible for metabolism of NE and serotonin (5-HT) [10]. Until the development of the selective serotonin transporter (SERT) inhibitors (a.k.a., SSRIs, e.g. citalopram, Celexa®; paroxetine, Prozac®; sertraline, Zoloft®) in the 1980s, tricyclics and MAO inhibitors were the predominant clinical antidepressants. More recently, a hypothesis has been developed that NET and SERT inhibitors treat different kinds of depression and may be used concomitantly with benefits to the patient [11].

Whereas the noradrenergic system appears to play a central role in depression, inhibition of NET in the treatment of ADHD has been suggested to be related primarily to the modulation of dopaminergic levels in the prefrontal cortex [12]. Such a mechanism is plausible for two reasons. Firstly, the concentration of dopamine transporters (DATs) in the prefrontal cortex is low [13]. Secondly, dopamine (DA) has higher affinity for NET than NE itself [14]. Both these facts together imply that NET may also accept DA as a substrate in brain regions devoid of DATs; this adds an extra dimension to the elucidation of the underlying mechanisms of other neuropsychiatric disorders in which NET is the target for treatment.

BRAIN IMAGING IN STUDIES OF NEUROPSYCHIATRIC DISORDERS

There are several techniques that allow non-invasive imaging of the living human brain. These can be divided into two fundamentally different classes of technique. One class, which provides anatomical information of the human brain, comprises imaging techniques such as magnetic resonance imaging (MRI) and X-ray computed tomography (CT). The other class, which provides functional information of the human brain, comprises tomographic methods such as positron emission tomography (PET) and single photon emission tomography (SPET) [15]. The latter two techniques require target or process-specific radiolabelled probes (radio-

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ligands) to be administered before the tomographic measurement is performed. When comparing PET and SPET, PET has the advantage of higher spatial resolution (~ 2−3 mm for a high resolution PET camera, vs. 8 mm for SPET) and better means of quantification (due to more accurate scatter and attenuation correction).

Brain imaging with PET is today a recognized tool in clinical studies of neuropsychiatric disorders and in drug development. The first quantitative study of dopamine D_2 receptor (D2R) occupancy in relation to antipsychotic drug-treated patients was made 20 years ago with [^{11}C]raclopride [16]. Since then, PET has proven useful for optimization of clinical treatment [17] and proof-of-concept studies [18]. A major advantage of PET within drug development is that detailed information regarding bio-distribution and metabolism of a new drug can be obtained after administration of only a few micrograms. Thus, information regarding the success or failure of a new drug may be available before entering into large-scale clinical trials, which are costly and time-consuming [19-21].

Although the scope of PET is great, there are some limitations with the technique, which need to be addressed for PET imaging facilities. PET cameras measure pairs of γ-rays emitted from positron-electron annihilation events and thus require ligands labelled with a positron-emitting nuclide, usually ^{11}C ($t_{1/2}$ = 20.4 min) or ^{18}F ($t_{1/2}$ = 109.8 min). These radionuclides are preferably produced in a cyclotron adjacent to the PET camera, due to their short half-lives. Large investments are required in equipment such as the cyclotron, PET camera, 'hot-cells' and other radiochemistry laboratory equipment, which so far has hampered the widespread use of PET. In contrast, the commonly used SPET radionuclide, ^{99m}Tc, can be obtained more inexpensively from commercially available generators.

The biological specificity of a PET imaging study depends wholly on the radioligand used in the emission measurement. Generally speaking, a useful PET radioligand is specific for a certain protein or biological process. Proteins that may be visualized and measured include enzymes, transporters and receptors. Examples of biological processes that may be visualized by PET are metabolism and blood flow.

RADIOLIGAND DEVELOPMENT – GENERAL CONSIDERATIONS

Many factors that determine the success of a candidate PET radioligand are similar to those that govern the success of a candidate drug. For example, suitable *pharmacodynamic* properties, such as high *affinity* and *selectivity* for the target protein, are of great importance in both cases and thus often render existing drugs to be useful leads to candidate PET radioligands. Nevertheless, experience has shown that mere radiolabelling of a useful drug seldom provides a useful radioligand, since the *pharmacokinetic* properties are often required to be different; successful drugs are usually required to be long-acting while PET radioligands are ideally required to show rapidly reversible kinetics to facilitate bio-mathematical data analysis.

In PET radioligand development, the density of target proteins (B_{max}) is a key issue that needs to be considered beforehand, as this will dictate the degrees of affinity (as indexed by the reciprocal of IC_{50}, K_i or K_d) and selectivity that will be required from a successful candidate. The target protein density (B_{max}) must well exceed the ligand K_d value for good image contrast. In Table **1**, the ratio between these two parameters (B_{max}/K_d), which equates to binding potential (BP), is listed for some useful PET radioligands.

It should be noted that the BP values from Table **1** are not strictly comparable because of the different methods by which they have been obtained. Also, several ligands have probably been forgotten in this table, which is included to give a general idea of what is required from a successful candidate radioligand in terms of

Table 1. Some Useful PET Radioligands, their Dissociation Constants and *cLogP* Values, Concentrations of Target Proteins in Various Brain Regions with Theoretical (B_{max}/K_d) and *In Vivo* Measures of BP. See List for Explanation of Abbreviations

Target protein	Radioligand	Tissue	B_{max} (nM)	K_d (nM)	B_{max}/K_d	*cLogP*[a]	BP[b]	Refs.
Amyloid	[^{11}C]PIB	Cortex	1407	2.5	563	2.98	2	[22]
CBR	[^{11}C]Flumazenil	Cortex	72	1.7	42	1.84	1.8	[23, 24]
DAT	[^{11}C]PE2I	Striatum	300	0.9	333	4.16	11	[25, 26]
D1R	[^{11}C]SCH23390	Caudate	103	0.4	257	4.10	1.5	[27, 28]
D1R	[^{11}C]NNC112	Caudate	103	0.18	572	4.75	3	[27, 29]
D2/D3R	[^{11}C]Raclopride	Striatum	33	1	33	3.71	2	[30, 31]
D2/D3R	[^{11}C]FLB457	Thalamus	1	0.02	50	2.98	2.6	[30−32]
D2/D3R	[^{18}F]Fallypride	Caudate	17	0.03	567	3.30	21.7	[30, 33, 34]
D2/D3R	[^{18}F]Fallypride	Thalamus	1	0.03	33	3.30	2.2	[30]
D2/D3R	[^{11}C]PHNO	Caudate	17	0.35	49	2.14	2	[30, 35, 36]
nAChR	[^{18}F]FA-85380	Thalamus	8	0.046	174	1.14	2	[37-39]
PBR	[^{11}C]PK11195	Cerebellum	35	3	12	5.28	0.2	[40, 41]
PBR	[^{11}C]DAA1106	Cerebellum	35	0.9	39	4.28	5	[41−43]
SERT	[^{11}C]DASB	Striatum	43	3.5	12	2.76	1.4	[44−46]
SERT	[^{11}C]MADAM	Striatum	43	0.9	48	3.12	1	[44, 47, 48]
5HT1A	[^{11}C]WAY100635	Hippocampus	187	1.0	187.0	3.28	7.4	[49, 50]

[a]Calculated with Pallas 3.0 (Compudrug; S. San Francisco, CA) for the neutral microspecies of the ligand.
[b]Measured *in vivo* with PET.

affinity versus target density. The lowest ratio between target protein density and radioligand affinity from Table **1** is 12 for [^{11}C]PK11195, a radioligand that gives low signal or BP *in vivo*. Measures of BP *in vivo* are generally much lower than those obtained by simple division of B_{max} by the ligand K_d *in vitro*. The B_{max} of NET in human insular cortex is about 4.4 nM [22]. By consideration of the data in Table **1** a radioligand suitable for visualizing NET in this region should probably have a K_d below 0.4 nM. Other regions of the primate brain that are more dense in NET, like the locus coeruleus (LC) which contains 4−8 times more NET [23, 24], might however be visualized with radioligands of lower affinity. In this manner, approximate thresholds may be set with regards to the minimal affinity required for imaging a target protein region *in vivo* with PET. However, species differences in the expression of NET exist and these estimates should not be extrapolated across species [24, 25]. In addition, because affinity values may vary greatly in the literature depending on the employed assay conditions, it may be wise not to use these thresholds strictly.

The affinity of a radioligand will also influence its *in vivo* binding kinetics. For example, a high affinity radioligand will require a longer time to equilibrate than a low affinity radioligand in a high-density target region (e.g. [^{11}C]raclopride vs. higher affinity [^{11}C]FLB-457) [26]. Radioligands showing slow kinetics (i.e. those that do not reach a peak equilibrium of specific binding within 90 min) may be advantageously labelled with fluorine-18 due to the longer half-life of this radionuclide. Attainment of a peak equilibrium of specific binding is an asset in the biomathematical modelling of PET data [16].

The *selectivity* of a candidate radioligand *in vivo* is related not only to affinity for non-target protein but also to the densities of these proteins. In the case of NET radioligands, the selectivity versus DAT is of central interest, since DAT protein is highly abundant in brain. [^{11}C]Cocaine for example, despite having a reported affinity of 68.5-640 nM for DAT [27, 28], has been used to image DAT in human striatum [27]. The selectivity of a candidate radioligand vs. SERT is not nearly as crucial, since the density of SERT in rat hypothalamus (a high density SERT region) is about three times that of NET in the LC of the rat brain [25, 29, 30]. The data from the rat is given due to lack of suitable comparable data from humans.

Whereas the *in vitro* pharmacodynamic properties, affinity and selectivity, are the most important guides in the selection of a candidate radioligand for PET evaluation, the pharmacokinetic properties of a candidate radioligand may only be determined by *in vivo* experiments. However, there are some guidelines that have been shown to be useful in drug development that may also be successfully applied in the development of PET radioligands.

PET radioligands are invariably administered intravenously. Thus, the *absorption* of a radioligand is achieved directly into the blood stream for efficient delivery to the target organ, which in this case is the brain. The *bio-distribution* of a CNS radioligand should be such that it allows sufficient amounts of radioactivity to be accumulated in brain after administration, to ensure adequate counting statistics with the PET camera. This varies between targets depending on the concentration and distribution of target sites. Several factors may govern the extent of accumulation of candidate radioligands in brain, including binding to plasma proteins, metabolism, clearance from plasma or exclusion from brain by efflux pumps [31-34]. These factors are hard to predict, but some guidelines based on the physicochemical properties of a candidate drug have been developed, such as the Lipinski 'rule of five' for good drug permeability and tissue absorption [35]. For CNS drugs, it has been argued by Waterhouse [36] that more stringent rules need to be applied, namely molecular weight should not exceed 450 g/mol and *LogP* not exceed four [37]. Furthermore, a parabolic relationship has been shown between *LogP* and brain extraction in a series of small non-ionized molecules, where the maximum brain accumulation was observed with *LogP* values between three and four [38]. Although increased *LogP* value might be expected to lead to increased non-specific binding the latter involves heterogeneous interactions of ligand with fats and proteins and no firm evidence for such a direct relationship has been established. Even so, given the importance of lipophilicity in brain extraction and its at least tenuous involvement in non-specific interactions, calculated *LogP* values (*cLogP*s) are still quite valuable in guiding the selection of candidate radioligands alongside the previously mentioned pharmacodynamic parameters, affinity and selectivity. Utilization of measured *LogP* values rather than *cLogP* values is preferred because of the inaccuracies that might attend the latter. It is emphasized that these values should only be used as a guide in radioligand development and not as a strict discriminator; the useful PET radioligands listed in Table **1** have *cLogP* values that range widely (from 1.14 to 5.28).

The *metabolism* of a suitable PET radioligand must be such that no BBB-permeable radiometabolites are formed that may contribute to brain radioactivity, since the PET camera merely measures radioactivity and does not distinguish between radiochemical entities. If a radiometabolite enters brain, it may have detrimental effects on the quantification of the PET data, especially if the radiometabolite has some degree of target selectivity [33]. An estimate of the relative lipophilicity of a formed radiometabolite, and thus its BBB permeability, as well as quantitative data on the rate of metabolism of a radioligand, can be obtained by radio-chromatography techniques such as thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC) [31]. The latter, coupled with mass spectrometry, may be especially useful in the identification of radiometabolites [39]. To test whether radiometabolites are present in brain during PET measurement, *ex vivo* analyses of brain tissue extracts can be performed. Such experiments require the animal to be sacrificed after the radioligand is injected and are mostly limited to rodents, which may diminish the utility of the obtained results, as species differences in metabolism (especially in the rate of metabolism) may be significant.

RADIOCHEMISTRY WITH POSITRON EMITTERS

Due to the short half-lives of ^{18}F and ^{11}C (110 and 20 min, respectively), these radionuclides need to be introduced at the latest possible stage of a radiosynthetic procedure to maximize the radiochemical yield (RCY). Mild conditions used in conventional organic chemistry (stirring over night at room temperature etc.) are not applicable to radiochemistry with positron emitters because of high losses due to decay of the radionuclide. High temperatures or more reactive labelling agents are often applied to accelerate reactions. Microwave reactors have also been used with success in PET radiochemistry [40].

Most radiosyntheses with ^{11}C originate from in-target produced ^{11}C-carbon dioxide, which is formed from proton bombardment of nitrogen gas containing traces of oxygen. ^{11}C-carbon dioxide is most often used to carboxylate organometallic compounds, which in turn may be converted into various alkyl or acyl halides. In some cases the ^{11}C-acid derivative obtained from carboxylation is used as such for the PET study (e.g. ^{11}C-acetate) but most often it is further functionalized (to amides etc.).

The most common labelling agent for ^{11}C is probably ^{11}C-methyl iodide [41], which can be readily prepared from $^{11}CO_2$ or $^{11}CH_4$ using commercial available systems. An obvious limitation with ^{11}C-methyl iodide is that its use is restricted to labelling methyl groups, which are not always present in the molecule of interest. A more versatile labelling agent is ^{11}C-carbon monoxide, which has been used to label the carbonyl group of amides, esters, ketones and acids by the use of transition metal catalysts [42, 43].

Radiosyntheses with ^{18}F usually originate from in-target produced ^{18}F-fluoride ion, which is formed from bombardment of

^{18}O-water with protons. Larger cyclotrons usually also have the option to produce ^{18}F-fluorine gas, but due to practical reasons this labelling agent is isotopically diluted with ^{19}F. This isotopic dilution is usually so severe that ^{18}F-fluorine is unsuitable for PET studies of neuroreceptor systems because of receptor saturation effects.

^{18}F-fluoride ion on the other hand can be readily applied in the synthesis of radioligands for use in neuroreceptor studies. Because of its nucleophilic nature, it may be introduced by aromatic or aliphatic substitution reactions. In some cases the ^{18}F-labelled radioligand can be obtained in one step from the proper non-labelled precursor, but protecting groups are often required for basic nitrogens in the molecule, which may hamper reactivity. ^{18}F-fluoroalkyl halides are also popularly used for the labelling of fluoroalkyl chains [44, 45]. Although not so abundant in biologically active molecules, fluoroalkyl chains may in some cases be used to replace their corresponding alkyl chains with retained biological activity.

NET INHIBITORS

In this review, inhibitors of NE reuptake are divided mainly into subclasses depending on the number of fused rings in their molecular structure (Fig. (**1**)). The tricyclic antidepressants form a group that comprises several potent and selective NET inhibitors [46, 47]. Other groups, in which potent NET inhibitors may be found are monocyclics [48-51], bicyclics [52] and tropane derivatives [53, 54].

The mutual structural elements of most potent NET inhibitors (except tropanes) are two aryl rings (A and B, Fig. **1**) and a secondary amine. It has been suggested that the aryl rings should have an anti-periplanar orientation to the secondary amine nitrogen for optimal activity [52]. If an aliphatic H-bond acceptor (e.g. ether linkage or hydroxyl group) is present in the ligand, the stereochemical orientation of this moiety is of importance for affinity [49, 50, 52, 55]. For example, the eutomers (*R*)-thionisoxetine and (*S*)-oxaprotiline (Figs. **2** and 4) inhibit NE reuptake with eudismic ratios of about 155 and 1,000, respectively [47, 50]. Regarding aromatic substitution, 3´-chloro substitution of phenyl ring B seems to be optimal [49, 52, 56], whereas the optimal substitution of phenyl ring A is unclear. The rank order of pharmacological activity for substitution of ring A on the nisoxetine platform is R = 2-I > 2-SMe > 2-Me > 2-OMe [50, 57]. It is likely that the reboxetine platform will show structure-affinity relationships (SARs) similar to those of the nisoxetine platform, given the high structural similarity between the two scaffolds.

The candidate radioligands discussed within this review, alongside their affinities and *cLogP*-values, are listed in Table **2**.

MONOCYCLIC NET INHIBITORS AS PET RADIOLIGANDS

Candidate radioligands from this structural class include various analogs of nisoxetine and reboxetine (Fig. **2**). Haka and Kilbourn were the first to attempt development of a NET radioligand in 1989, when they labelled racemic nisoxetine in its *N*-methyl position with ^{11}C [69]. Although showing a heterogeneous uptake, its distribution was considered to be mainly non-specific. An iodinated analogue of nisoxetine (2-INXT) was prepared by Kung in 1999 [50], but not much more was reported until 2003, when Wilson *et al*. published on the synthesis and *ex vivo* evaluation of an *O*-^{11}C-methyl analog of reboxetine, (*S*,*S*)-[^{11}C]MeNER, in rats [70]. The binding of (*S*,*S*)-[^{11}C]MeNER was shown to be sensitive to NET inhibition and insensitive to several other pharmacological challenges, including DAT and SERT inhibition [70]. In addition, autoradiography experiments with (*S*,*S*)-[^{3}H]MeNER in normal and NET knock-out mice showed negligible amounts of specific binding in the knock-outs, whereas high binding was found in NET-rich regions of normal mice [71]. This provided further evidence for the specific binding of radiolabeled (*S*,*S*)-MeNER to NET *in vivo*, which prompted measurements in non-human primates. In monkeys and baboons a lower specific to non-specific binding ratio was observed for (*S*,*S*)-[^{11}C]MeNER (about 1.6 compared to 2.5 in rats) [70, 72, 73]. Although the binding ratio was lower than that reported by Wilson,

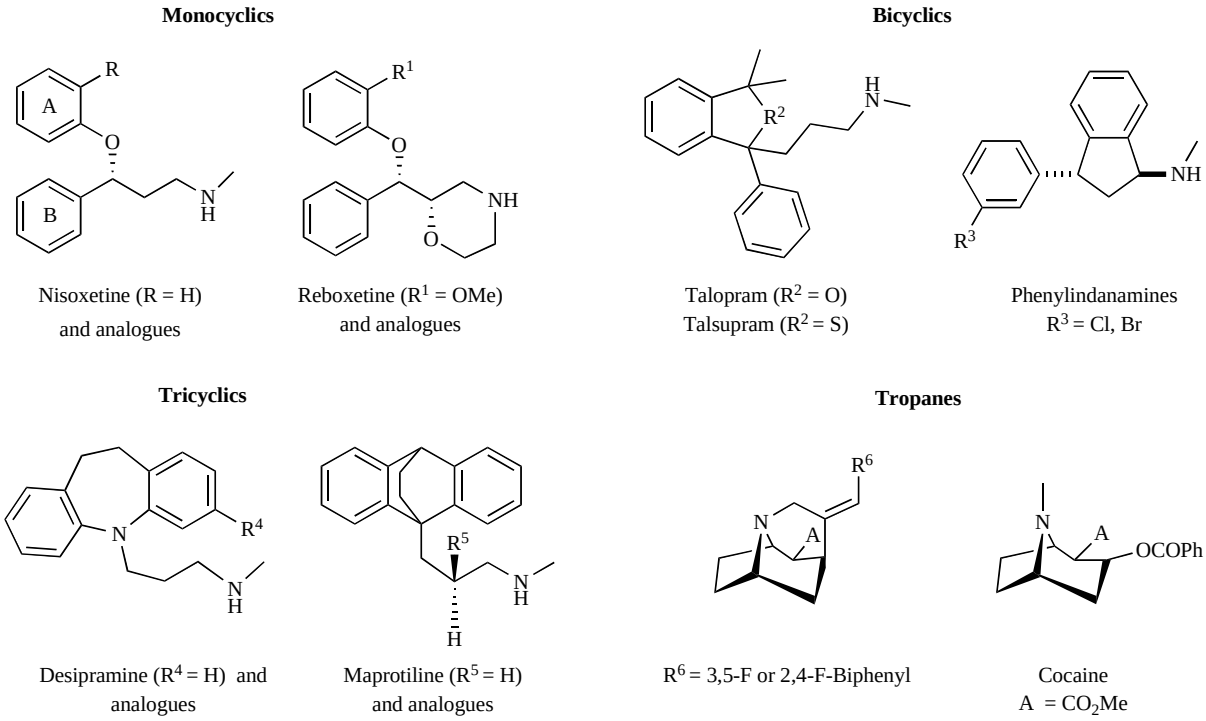


Fig. (1). NET inhibitors divided into subclasses dependent on the number of fused rings in their chemical structure, plus those based on tropanes.

Table 2. Affinities and Physicochemical Parameters of Candidate NET Radioligands Discussed within this Review Article

Structural class	Ligand		K_d (nM)		*CLogP*[a]	Refs.
		NET	SERT	DAT		
Monocyclics	(*R*)-Nisoxetine	0.4	1,000	360	3.52	[51, 58]
	(*R*)-Thionisoxetine	0.2	>200	~360	3.94	[57]
	(*R*)-2-iodonisoxetine	0.03	n.a.	n.a.	4.68	[59, 60]
	Reboxetine	11	440	>10,000	3.98	[12]
	MeNER (aka MRB)	2.5[b]	310[b]	>10,000[c]	3.52	[49]
	3-Cl-MRB	3.3[b]	558[b]	704[b]	4.3	[49]
	FMeNER-D2	3.1	75	>10,000	3.51	[61]
	FRB-D4	n.a.	n.a.	n.a.	3.57	[62]
	CFMME	10.8	59.4	>10,000	2.37	[63]
	Compound **1**	0.94	158	16.1	2.81	[64]
	Compound **2**	0.68	4.5	83.3	3.80	[64]
Bicyclics	Talopram	2.6	430	3,900	3.97	[65]
	Talsupram	6.4	240	>20,000	4.58	[65]
	trans-3-BrPA	4.1	51	8.1	4.46	[66]
Tricyclics	Desipramine	3.8	179	>10,000	4.00	[12]
	R-OHDMI	8.3	335	>10,000	2.97	[63]
	Oxaprotiline	4.9	3,400	4,340	3.49	[67]
	Lortalamine	0.2[c]	>10,000[c]	>100,000[c]	2.10	[62]
	Mazindol	4.9	94	43	3.44	[68]
	6-OCH3-DCM	1.7	3,600	60	2.89	[68]
	6-OCH3-t-OCH3-DCM	n.a.	n.a.	n.a.	3.52	n.a.

[a]Calculated with Pallas 3.0 (Compudrug; S. San Francisco, CA) for the neutral microspecies of the ligand.

[b]IC_{50}-values.

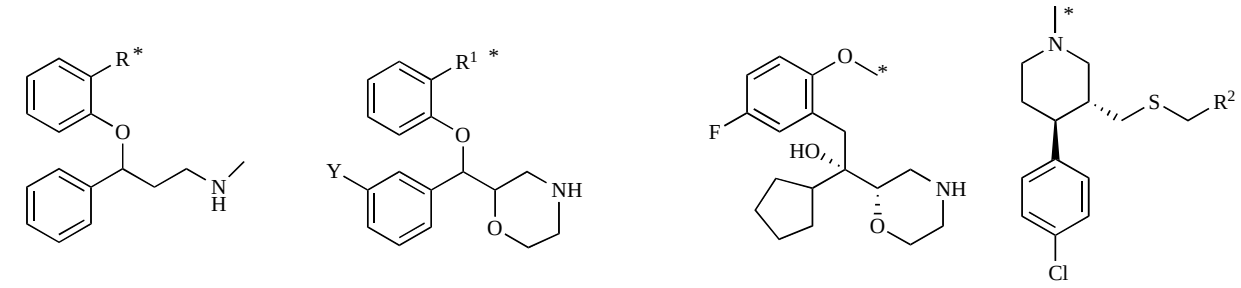
[c]Based on comparison to desipramine

the binding was confirmed to be specific to NET. In addition, the PET group in Århus demonstrated specific binding in a pig model [74]. Therefore, the ligand was tested in clinical studies, in which a high test-retest variability in binding potential (BP) was observed (range 17−31%), depending on the examined ROI [75]. This variability is too large for measurements of discrete changes of NET inhibition in brain. The large test-retest variability is primarily associated with the slow binding kinetics of the radioligand, which necessitates BPs to be estimated from emission data obtained between 63 and 93 min after injection of (*S*,*S*)-[^{11}C]MeNER. At these time-points, the data is very vulnerable to noise due to the short half-life of ^{11}C.

In an attempt to extend data acquisition over the peak equilibrium of specific binding, the *O*-fluoromethyl analogue, (*S*,*S*)-FMeNER was developed. (*S*,*S*)-FMeNER is almost equipotent to (*S*,*S*)-MeNER and also has a selectivity profile similar to other reboxetine analogues. In PET measurements with (*S*,*S*)-[^{18}F] FMeNER, radioactivity entered brain to a similar extent as in PET measurements with (*S*,*S*)-[^{11}C]MeNER (2.8% I.D. for (*S*,*S*)-[^{18}F]FMeNER vs. 3% I.D. for (*S*,*S*)-[^{11}C]MeNER). Radioactivity also distributed similarly within brain, with DMI-sensitive binding in NET-rich regions. Furthermore, the specific binding reached peak equilibrium at 90 to 120 min after injection. Nevertheless, the ligand also showed defluorination, which confounds imaging of cortical regions [76]. By analogy with the work of Hamill *et al*. [77], the *di*-deuterated radioligand, (*S*,*S*)-[^{18}F]FMeNER-D_2, was developed as a metabolically more stable analogue of (*S*,*S*)-[^{18}F] FMeNER. This radioligand showed a regional distribution of radioactivity in monkey brain similar to that observed with (*S*,*S*)-[^{11}C]MeNER and (*S*,*S*)-[^{18}F]FMeNER. The binding was also sensitive to pharmacological challenge with NET inhibitor, but not DAT or SERT inhibitors. A clinical evaluation of (*S*,*S*)-[^{18}F]FMeNER-D_2 is currently underway.

Other radioligands derived from the reboxetine scaffold include (*S*,*S*)-[^{18}F]FRB-D_4, (*S*,*S*)-[^{11}C]3-Cl-MRB and the related compound [^{11}C]CFMME [62, 63, 78]. In a comparative evaluation of (*S*,*S*)-[^{11}C]MeNER, (*S*,*S*)-[^{18}F]FRB-D_4 and (*S*,*S*)-[^{11}C]3-Cl-MRB, (*S*,*S*)-[^{11}C]MeNER was considered the most promising radioligand [62]. Recently, a ^{11}C-thiomethyl analogue of reboxetine was reported by Goodman.* Given that the potency tends to increase with larger groups in the 2-position on the nisoxetine platform, it will be interesting to follow the development of this radioligand. Another highly interesting radioligand in this respect is the iodinated

* Abstract at the Annual Congress of the European Association of Nuclear Medicine (Athens, 2006).



Nisoxetine, R = OMe
Thionisoxetine, R = SMe
2-INXT, R = I

Reboxetine, R^1 = OEt, Y = H
MeNER, R^1 = OMe, Y = H
3-Cl-MRB, R^1 = OMe, Y = Cl
FMeNER, R^1= OCH_2F, Y = H
FMeNER-D_2, R^1 = OCD_2F, Y = H
FRB-D_4, R^1 = OC_2D_4F, Y = H
INER, R^1 = I, Y = H

CFMME

1, R^2 = CH_2OH
2, R^2 = CO-N

Fig. (2). Structures of candidate NET radioligands based on a monocyclic core. *Position of label.

analogue, [^{123}I]INER [79], which may be the most potent NET inhibitor that can be obtained from the reboxetine platform.

Further monocyclic NET radioligands that have been reported are (*R*)-[^{11}C]thionisoxetine and two ^{11}C-labeled piperidine-based analogues of cocaine (**1** and **2**, Fig. **2**) [64, 80]. Whereas the first mentioned ligand did not show improved target to non-target ratios with respect to the reboxetine analogues [90], the piperidine based radioligands predominantly showed binding to DAT [64, 80].

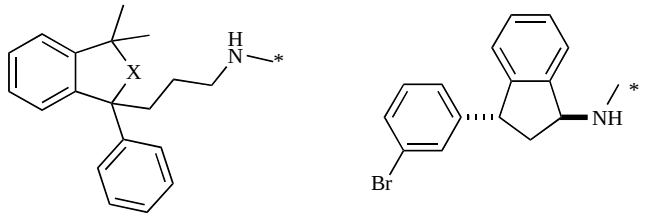
BICYCLIC NET INHIBITORS AS PET RADIOLIGANDS

A series of phenyl 1-indanamines were discovered as potent monoamine transporter inhibitors at the Danish pharmaceutical company, Lundbeck [52]. Among these compounds, two NET inhibitors, talopram and talsupram (Fig. **3**), were further evaluated in clinical trials, in which they demonstrated some antidepressant efficacy [81, 82]. Talopram and talsupram are two of the most potent NET inhibitors reported to date with K_i values of 6.4 and 2.4 nM, respectively. Furthermore, they are highly selective versus a wide range of other targets. In contrast to their clinical efficacy, the low BBB permeability of both [^{11}C]talopram or [^{11}C]talsupram hampered their development as brain imaging agents [65, 83]. A reasonable explanation for these diverse observations is that talopram and talsupram take part in saturable processes that render their availability in brain to be concentration-dependent. Possible examples of such processes are binding to plasma proteins (e.g. serum albumin) or active exclusion from brain by an efflux pump (e.g. Pg-P).

A related ligand, [^{11}C]3-BrPA (Fig. **3**), which is a mixed NET and DAT inhibitor, with inhibition constants of 4.1 and 8.1 nM vs. NET and DAT, respectively, showed modest brain uptake. The binding of this ligand was however unaffected by pharmacological challenges with either DAT or NET inhibitors [66].

TRICYCLIC NET INHIBITORS AS PET RADIOLIGANDS

Desmethylimipramine (desipramine, DMI, Fig. **4**) is the *N*-desmethyl metabolite of the tricyclic antidepressant, imipramine (IMI, Tofranil®) [9]. In contrast to IMI, which is a selective and potent SERT inhibitor, DMI is a potent and selective NET inhibitor [46], with subnanomolar affinity [56]. DMI has been widely used as an antidepressant [6]. The clinical side effects of tricyclics are usually associated with their affinity towards muscarinic acetylcholine receptors. DMI, however, has only a low affinity for these receptors (K_i = 66 nM) [84] that would be of minor importance in



Talopram, X = O
Talsupram, X = S

trans-3-BrPA

Fig. (3). Structures of candidate NET radioligands based on a bicyclic core. *Position of label.

relation to PET imaging. Before this study, [^{3}H]DMI had been used for *in vitro* autoradiography of NET in the human brain *post mortem* [85, 86]. [^{3}H]DMI was found to distribute heterogeneously in the human brain, with binding to two different sites, with that of higher affinity assumed to be the NET binding site [85]. The binding of [^{11}C]DMI was however predominantly non-specific *in vivo*. In an attempt to decrease non-specific binding, the hydroxylated analogue, (*R*)-[^{11}C]OHDMI was developed. This ligand was about equipotent to (*S*,*S*)-MeNER, but failed as a PET radioligand due to its low BBB permeability [63]. The structurally related [^{11}C]oxaproptiline and [^{11}C]lortalamine both showed high uptake of radioactivity in the striatum, which limits their utility as NET ligands [62].

Other ligands from this structural class include mazindol and related analogues. Mazindol is a potent NET inhibitor with low nanomolar affinity towards NET, but not without DAT affinity. Lin has reported two *des*-chloro- and 6-methyl substituted analogues of mazindol with improved selectivity. Of these, 6-[^{11}C]O-CH_3-*t*-OCH_3-DCM showed uptake into NET-rich regions in baboons.[†] Further studies are being carried out to characterize the binding of 6-[^{11}C]O-CH_3-*t*-OCH_3-DCM.

[†] Meeting abstract. Lin, K.-S. *et al*., Synthesis and *in vivo* evaluation of C-11 labeled mazindol analogs for imaging the norepinephrine transporter with PET. *J. Label. Compd. Radiopharm*, 2005. **48**: p. S152.

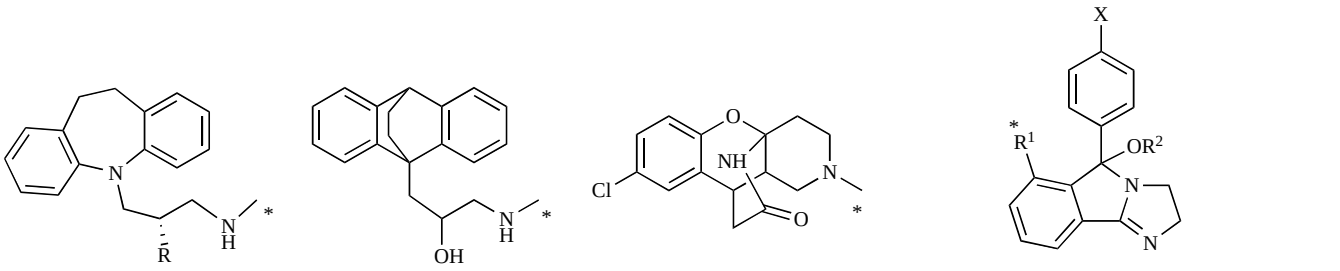


Fig. (4). Structures of candidate NET radioligands based on a tricyclic core. *Position of label.

TROPANE NET INHIBITORS AS PET RADIOLIGANDS

Although there exist several potent NET inhibitors within this subclass, the generally inherent affinity of tropanes towards DAT will probably limit the utility of radioligands derived from this scaffold. An especially illustrative example is that of the two piperidines labelled by Musachio *et al.* [64]. One of the compounds was a highly potent NET inhibitor, with a K_i of 0.94 nM and a 16-fold selectivity towards DAT. Although being selective to NET, the radioligand showed specific binding to DAT because of the high concentration of these proteins in brain. Although the piperidines are not tropane-based compounds, this example shows that great caution should be exercised with regards to DAT affinity. The demand for low affinity for DAT may be appreciated by noting that [^{11}C]cocaine can be used for DAT imaging, despite having a reportedly very low affinity (K_i = 640 nM) [27].

PRESENT STATUS OF BRAIN IMAGING OF NET WITH PET

Until just two years ago, a thorough investigation of the regional distribution of NET in the primate brain had not been performed. The scientific community was thus largely relying on extrapolating data obtained from rodents to get an idea of the regional distribution and densities of NET in the primate brain. This was accompanied with some uncertainty in radioligand development. Questions were raised about the density of NET in various regions of the primate brain [59, 70], including the absence of NET in striatum. Recently, however, *in vitro* studies have clarified the distribution and density of NET in the primate brain [22, 24].

The observed densities of NET were roughly in accordance with the relative densities of NET found in the rodent brain [25]. Thus, the highest levels of NET were found in the LC and RN, followed by intermediate levels in the hypothalamus, thalamus and other brainstem regions. The lowest levels of NET were found in the striatum and the molecular layer of cerebellum [24]. Although there was a high resemblance between rodents and primates in the expression of NET, some important species differences were observed. In primates, the expression of NET was about seven-, three- and five-fold lower in cortex, hypothalamus and thalamus relative to the LC. Furthermore, in a separate study, the B_{max} of NET in human insular cortex homogenate was found to be 4.4 pmol/g tissue (nM), which is roughly nine times less than in rodents [22]. These two studies are fundamentally very important. The regional distribution of NET in the primate brain *in vitro* can be used to verify the distribution of NET radioligands *in vivo*. The density of NET in primate brain can be applied for calculating affinity thresholds for future candidate NET radioligands.

During the past five years, about fifteen NET inhibitors have been evaluated as candidate radioligands in rodents and/or in primates [62, 65, 66, 70, 73, 87]. A number of these radioligands have shown accumulation of radioactivity in the striatum. In one case, this binding was ascribed to binding to DAT [64], but in most cases this component of the binding in brain has remained uncharacterised and been regarded as non-specific [62, 66]. Logan has reasoned that this binding, similarly to the binding of [^{3}H]DMI *in vitro*, is of low affinity and to a second non-adrenergic site [88]. However, in the *in vitro* studies with [^{3}H]DMI, this binding could be inhibited by the inclusion of a high concentration of NET inhibitor [85], whereas the binding of (*R*)-[^{11}C]nisoxetine in striatum was unaffected in a pre-treatment experiment with nisoxetine (1 mg/kg) *in vivo*. Furthermore, the binding of [^{3}H]nisoxetine to human cortical homogenate *in vitro* was found to represent a single class of binding sites [22], which contradicts Logan's hypothesis. In favour of Logan's hypothesis, the low-affinity binding of [^{3}H]DMI was shown to be sensitive to incubation conditions [89, 90], which may not be altered *in vivo*. In any case, it is important to characterize this binding site for future NET radioligand development.

The NET radioligands with the best properties of those evaluated so far are based on the reboxetine platform. It was long argued that (*S,S*)-[^{11}C]MeNER, although it has slow kinetics, could be suitable for PET imaging of NET in man [88]. Recently however, the test-retest variabilities in BPs when using (*S,S*)-[^{11}C]MeNER were found to be too large (17 to 31%) to reliably assess NET density. In addition, the binding of (*S,S*)-[^{11}C]MeNER was not found to be saturable at supra-therapeutic doses, a finding that was also supported by the PET group at Johns Hopkins University [91]. Although NET can be imaged with (*S,S*)-[^{11}C]MeNER, its disadvantages are such that it is not useful in clinical research. Currently, clinical studies with (*S,S*)-[^{18}F]FMeNER-D_2 are underway. Some promising *in vitro* autoradiography data have been reported [92]. In addition, a recent occupancy study with atomoxetine and (*S,S*)-[^{18}F]FMeNER-D_2 in monkeys demonstrated a saturable and dose-dependent accumulation in the LC [93]. Thus, (*S,S*)-[^{18}F]FMeNER-D_2 represents the most promising imaging agent for central NET in the human brain reported to date (Fig. (**5**)), but given its problem with low BP, it is far from an ideal PET radioligand for NET.

CHALLENGES IN FUTURE NET RADIOLIGAND DEVELOPMENT

The challenges in further NET radioligand development are similar to those faced in the development of radioligands for other receptor or transporter systems. However, there are some specific difficulties in NET radioligand development. These are listed below:

i. The expression of NET in the human brain is low; only 4.4 nM NET is present in the insular cortex. In comparison, the density of DAT in putamen is 212 nM [94] and the

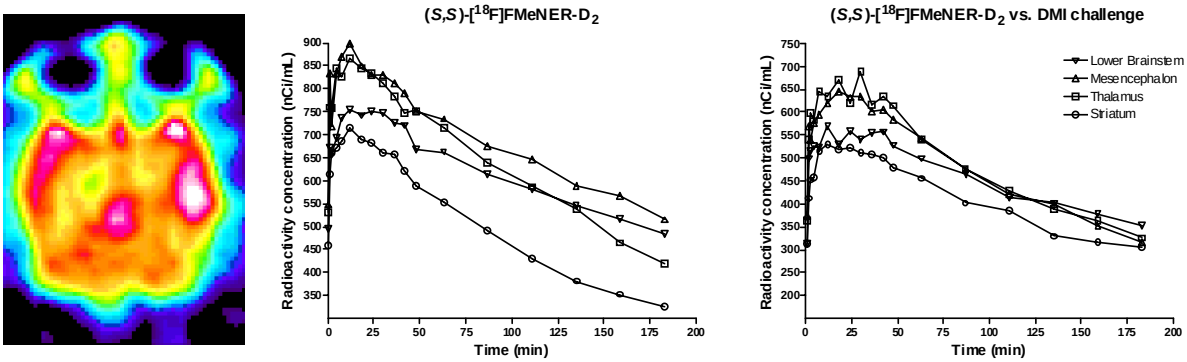


Fig. (5). Color coded PET image (left) and time activity curves (right) of brain radioactivity following injection of (*S*,*S*)-[^{18}F]FMeNER-D$_2$ into a cynomolgus monkey. The PET image is derived from a baseline experiment.

corresponding density of SERT is 14 nM in prefrontal cortex [95]. Although this comparison is not entirely fair, because the compared regions are not the regions of highest density for the given transporters (except for DAT), it gives an idea about the relationship between the monoamine transporter concentrations *in vivo*. The lower density of NET requires that a suitable radioligand would need higher affinity than those presently used for DAT [96] or SERT [97] imaging. In human insular cortex, the affinity of a sensitive radioligand should be at least 0.4 nM.

ii. Not only is the expression of NET low in brain, the highest levels of NET expression in the primate brain are localized within small brain regions, like LC and RN, which are challenging to image with PET. It is estimated that the number of neurons in the LC, which is the most dense NET-region in primate brain, is only of the order of 40,000 to 50,000 [98]. In addition, the dimensions of this region are just a few millimetres.

iii. There is a lack of potent and selective NET platforms suitable for NET imaging. Candidate radioligands from all known potent and selective NET platforms have been evaluated as candidate NET radioligands. Only one "hit" was found during this screening process, namely (*S*,*S*)-MeNER, which is based on the reboxetine platform. Further development of this scaffold resulted in (*S*,*S*)-FMeNER-D$_2$, LY 2152041 and an iodinated analogue, (*S*,*S*)-INER [79], that were about equipotent to (*S*,*S*)-MeNER. Based on SAR on the related nisoxetine platform, it is uncertain if any more potent NET inhibitors will be obtained from the reboxetine scaffold. Further NET radioligand development may require extensive synthesis and SAR analysis of new compounds.

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ABBREVIATIONS

5-HT	=	Serotonin
a.k.a.	=	Also known as
ADHD	=	Attention deficit hyperactivity disorder
B_{max}	=	Concentration of protein
BP	=	Binding potential (= B_{max}/K_d)
BBB	=	Blood-brain barrier
CBR	=	Central benzodiazepine receptor
CNS	=	Central nervous system
DMI	=	Desipramine, desmethyl-imipramine
DA	=	Dopamine
D1R	=	dopamine D_1 receptor
D2R	=	Dopamine D_2 receptor
D3R	=	Dopamine D_3 receptor
DAT	=	Dopamine transporter
E	=	Epinephrine, adrenaline
Et	=	Ethyl
$t_{1/2}$	=	Half-life
HPLC	=	High performance liquid chromatography
IC_{50}	=	Concentration of inhibitor that reduces binding of reference radioligand to protein of interest by 50%.
K_d	=	Ligand equilibrium dissociation constant
K_i	=	Inhibition constant
I.D.	=	Injected dose
LC	=	Locus coeruleus
MAO	=	Monoamine oxidase
Me	=	Methyl
MeNER	=	Methyl norethyl reboxetine
nAChR	=	Nicotinic acetylcholine receptor
NE	=	Norepinephrine, noradrenaline
NET	=	Norepinephrine transporter
P	=	Partition coefficient between octanol/water
PBR	=	Peripheral benzodiazepine receptor
PET	=	Positron emission tomography
RN	=	Raphe nuclei
ROI	=	Region of interest
SSRI	=	Selective serotonin reuptake inhibitor
SERT	=	Serotonin transporter
SPET	=	Single photon emission tomography
SAR	=	Structure-affinity relationships

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