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Paroxetine as an in vivo indicator of 3,4-methylenedioxymethamphetamine neurotoxicity: a presynaptic serotonergic positron emission tomography ligand?

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The present study sought to determine whether [^{3}H]paroxetine, a potent and selective inhibitor of serotonin uptake in vitro, could be used to label the serotonin transporter in the rat brain in vivo such that it might be employed to develop a presynaptic serotonergic positron emission tomography ligand. Tritium labeled paroxetine was administered intravenously to rats by means of tail vein injection. Four hours later, specific [^{3}H]paroxetine binding was determined by subtracting non-specific binding in the cerebellum from total binding in other brain regions of interest. The distribution of specific [^{3}H]paroxetine binding paralleled the distribution of serotonin uptake sites in all brain regions examined. Pretreatment with serotonin re-uptake inhibitors (citalopram or sertraline) reduced in vivo specific [^{3}H]paroxetine binding by as much as 99%. Specific in vivo [^{3}H]paroxetine binding was further characterized through the use of 5,7-dihydroxytryptamine (5,7-DHT), a known serotonergic neurotoxin. 5,7-DHT (200 μg, i.c.v.) caused a marked reduction in specific [^{3}H]paroxetine binding, and induced a prolonged depletion of regional brain serotonin. In a final study, the feasibility of using in vivo [^{3}H]paroxetine binding as an indicator of serotonergic damage induced by another neurotoxin (3,4-methylenedioxymethamphetamine, MDMA) was tested. MDMA-treated rats showed a profound reduction in in vivo [^{3}H]paroxetine binding, along with a lasting depletion of regional brain serotonin. These results demonstrate that [^{3}H]paroxetine can be used to label serotonin uptake sites in the rat brain in vivo, and that the damage induced by serotonergic neurotoxins can be detected using in vivo [^{3}H]paroxetine binding as an indicator. Paroxetine (or one of its derivatives) therefore holds promise as a PET ligand for studying serotonergic neurons in the living human brain in health as well as after neurotoxic injury.

INTRODUCTION

With the advent of positron emission tomography (PET) and the development of receptor-specific ligands, it is now possible to study some neural circuits in the living human brain in great detail. For example, the nigrostriatal dopamine system can be visualized both pre- and postsynaptically through the use of 6-[^{18}F]fluoro-L-DOPA[15] and 3-*N*-[^{11}C]methylspiperone[7,35], respectively. Recently, PET techniques have also made it feasible to detect clinically silent neural damage in man during life. This has been accomplished in heroin addicts who unwittingly took low doses of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Although these individuals appear normal on detailed neurological examination, PET studies have revealed clear reductions in striatal 6-[^{18}F]fluoro-L-DOPA uptake[8]. Given this precedent, it seems likely that PET will emerge as a major modality for detecting subclinical neural damage in other human populations at risk. One such population consists of individuals using 3,4-methylenedioxymethamphetamine (MDMA); this is a psychoactive recreational drug that produces brain serotonergic damage in a variety of experimental animals[2,9,29–31], including non-human primates[17,25,26].

While great strides have been made in the study of human dopamine systems with PET, little progress has thus far been made with serotonin systems. Lack of progress in this area is largely attributable to a paucity of suitable PET ligands. 3-*N*-([^{11}C]methyl)-spiperone (NMSP) was introduced some years ago[22,35], however, it binds to both serotonin and dopamine receptors[12]. Therefore, its use in the study of the serotonin system is limited by problems of selectivity. More recently, 1-*N*-([^{11}C]methyl)-2-bromo-LSD (MBL) was developed[34]. This serotonergic PET ligand has the advantage of being more selective than NMSP. However, its utility is limited by the fact that it only labels postsynaptic 5-HT$_2$ receptors, and these often fail to change even after marked and persistent disruption of serotonergic neurotransmission[10,11]. Given these precedents, a more fruitful approach may be to develop a label for the presynaptic element of serotonergic neurons.

[^{3}H]Paroxetine (α(–)-*trans*-4-(*p*-fluorophenyl)-3-[3,4-

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methylenedioxyphenoxymethyl]piperidine) (FG 7051) is a potent and selective inhibitor of serotonin uptake in vitro[16,21,32]. Recently it has been used to successfully label the serotonin transporter in the mouse brain in vivo[28]. The fact that this is feasible suggests that, once rendered positron-emitting, paroxetine may be of value as a presynaptic serotonergic PET ligand, which could be used to screen humans for MDMA neurotoxicity. Unfortunately, the mouse is not sensitive to MDMA neurotoxicity[30]. As such, it cannot be used to test the usefulness of paroxetine as an in vivo indicator of MDMA neurotoxicity. For this reason, the present study was undertaken with the following specific aims: (1) to extend previous observations in the mouse[28] to an experimental animal (the rat) which is highly sensitive to the toxic effects of MDMA[2,3,9,29,31]; and (2) to determine if, in the rat, in vivo [^{3}H]paroxetine binding can serve as an indicator of MDMA neurotoxicity such that it might ultimately be used for similar purposes in man.

MATERIALS AND METHODS

Chemicals

[^{3}H]Paroxetine was obtained from DuPont de Nemours, N.E.N. Research Products, Boston, MA. Serotonin uptake inhibitors were kindly provided by the following sources: sertraline hydrochloride: Pfizer, Inc., Groton, CT; citalopram hydrobromide: H. Lundbeck and Co., Copenhagen, Denmark. Racemic MDMA hydrochloride was obtained from the National Institute on Drug Abuse, Rockville, MD and 5,7-dihydroxytryptamine (5,7-DHT) creatinine sulfate was purchased from the Sigma Chemical Co., St. Louis, MO.

Animals

Male Sprague–Dawley rats, weighing between 180 and 200 g, were used in all experiments. Rats were obtained from the Charles River Laboratories, Wilmington, MA. Animals were housed singly in wire mesh cages with free access to food and water.

In vivo [^{3}H]paroxetine binding

Specific [^{3}H]paroxetine binding in vivo was studied by adapting the method of Scheffel and Hartig[28] from the mouse to the rat. Rats were injected with 7 μCi (approx. 80 ng) of [^{3}H]paroxetine (NEN) via a tail vein. Four hours later, the animals were killed by decapitation, the brain was removed and dissected over ice. Tissue samples were weighed, placed in glass counting vials containing 1 ml of tissue solubilizer (Protosol, NEN) and solubilized by keeping them in Protosol at 50 °C for 2 days. After cooling to room temperature, the samples were diluted with 10 ml Econofluor (NEN) and counted in a β scintillation counter with an error of 3% or less. Quench corrections were made using a precalibrated curve. Aliquots of the injectate were prepared as standards and counted along with the tissue samples. 'Specific binding' was calculated by subtracting 'non-specific' [^{3}H]paroxetine binding in the cerebellum from that in the other brain regions. [^{3}H]Paroxetine binding in the cerebellum was designated as 'non-specific' because it was not blocked by selective inhibitors of serotonin uptake (citalopram and sertraline), and because it was not reduced after 5,7-DHT/DMI treatment (see Results).

Brain dissection

The brain was removed and placed over ice on its dorsal surface. The olfactory tubercles and hypothalamus were removed first. The brain was then placed on its ventral surface and the cerebellum was removed. Samples of prefrontal cortex were obtained from the rostral aspect of both hemispheres, with each sample weighing 10–15 mg. A mid-sagittal cut was then made along the corpus callosum, separating the two cerebral hemispheres, but leaving the underlying subcortical structures attached along the midline. The resulting cortical mantles were folded back laterally with care being taken to separate the hippocampi from the cortex. Subsequently, the striata were removed with full-curved microdissecting forceps. Cortical tissue was pooled into a single cortical sample. This sample contained all cortical areas except the prefrontal regions removed previously and portions of the cortex overlying the corpus striatum which were excluded to avoid possible contamination from striatal remnants. After removal of all cortical tissue, the superior colliculi were isolated, followed by the thalamus.

Pharmacological blocking studies

Inhibition studies in the rat were performed with sertraline (n = 4) and citalopram (n = 5). Each serotonin uptake blocker was injected intravenously at a dose of 3.2 mg/kg 5 min before administration of [^{3}H]paroxetine. A dose of 3.2 mg/kg was selected on the basis of pilot data indicating that this dose inhibits greater than 80% of specific [^{3}H]paroxetine binding in the hypothalamus without significantly influencing non-specific binding of [^{3}H]-paroxetine in the cerebellum. Controls (n = 6) were preinjected with saline. In all cases, rats were sacrificed 4 h after [^{3}H]paroxetine injection and radioactivity in tissue samples was measured as described above.

5,7-DHT lesions

5,7-DHT was injected i.c.v. as follows. Rats (n = 11) were anesthetized with 10% chloral hydrate (5 ml/kg, i.p.). The skull was exposed by shaving the overlying skin and making a midline incision along the calvarium. The bregma was identified, and a small hole was made in the skull using a 25 gauge needle. Intracerebroventricular injections were made 1.5 mm lateral to the mid-sagittal suture and 0.5 mm caudal to the coronal suture using a Hamilton syringe attached to a cuffed 26-gauge needle such that only 4 mm penetrated the brain parenchyma (level of lateral ventricle). Ten 10 μl of sterile saline containing 200 μg of 5,7-DHT and 0.1 mg/ml ascorbic acid were injected into the right lateral ventricle over a 30-s interval. After completing the injection, the needle was removed and the skin overlying the skull was apposed using a suture clip. All animals were pretreated with desmethylimipramine (DMI, 25 mg/kg, i.p.) 30 min prior to 5,7-DHT administration.

In vivo [^{3}H]paroxetine binding (n = 5) or serotonin levels (n = 6) were measured 1 week after 5,7-DHT injection.

FIGURE 1

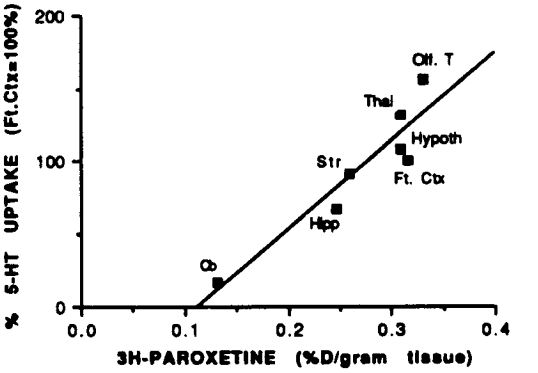

Fig. 1. Comparison between specific in vivo [^{3}H]paroxetine binding and in vitro [^{3}H]serotonin uptake. Synaptosomal serotonin uptake data is from Leysen[20]. The coefficient of correlation between these two parameters was 0.93 (P < 0.002). Cb, cerebellum; Hipp, hippocampus; Str, striatum; Ft. Ctx, frontal cortex; Hypoth, hypothalamus; Thal, thalamus; Olf. T, olfactory tubercles.

TABLE I

Pharmacological blockade of specific in vivo [^{3}H]paroxetine binding by selective inhibitors of 5-HT uptake

	Specific binding (mean % dose/g tissue ± S.D.) of [^{3}H]paroxetine 4 h after injection		
	Controls (n = 6)	*Sertraline (n = 4)*	*Citalopram (n = 5)*
Olfactory t.	0.200 ± 0.027	0.020 ± 0.004 (–90%)*	0.062 ± 0.010 (–69%)*
Hypothalamus	0.172 ± 0.068	0.017 ± 0.007 (–90%)*	0.052 ± 0.014 (–69%)*
Hippocampus	0.124 ± 0.020	0.068 ± 0.013 (–45%)*	0.093 ± 0.012 (–25%)*
Striatum	0.123 ± 0.028	0.037 ± 0.004 (–69%)*	0.071 ± 0.009 (–42%)*
Frontal cortex	0.196 ± 0.030	0.073 ± 0.010 (–62%)*	0.108 ± 0.009 (–44%)*
Thalamus	0.185 ± 0.025	0.049 ± 0.005 (–73%)*	0.084 ± 0.009 (–54%)*
Superior colliculus	0.219 ± 0.037	0.002 ± 0.004 (–99%)*	0.052 ± 0.017 (–76%)*

*% Decrease from controls, significantly different from controls.

MDMA treatment

Rats (n = 9) received 8 s.c. 20 mg/kg injections of MDMA hydrochloride at 08.30 and 17.00 h on each of 4 consecutive days. Controls were injected with an equal volume of saline at the same intervals. Seven days after the last MDMA or saline injection, in vivo [^{3}H]paroxetine binding (n = 5) or serotonin levels (n = 4) were determined.

Serotonin determinations

Serotonin was measured by means of high-performance liquid chromatography coupled with electrochemical detection using the method of Kotake et al.[18] with minor modifications detailed elsewhere[26].

Statistics

The significance of differences between groups was assessed using a two-tailed Student's t-test. Differences were considered significant when P was < 0.05.

RESULTS

In vivo distribution of specific [^{3}H]paroxetine binding

As in the mouse, the highest levels of specific [^{3}H]paroxetine binding in the rat brain were found in the superior colliculus, olfactory tubercle, frontal cortex, thalamus and hypothalamus (Fig. 1 and Table I). The striatum and hippocampus contained lesser but still considerable amounts of specific [^{3}H]paroxetine binding. The cerebellum contained the least amount of [^{3}H]paroxetine binding, but here it was 'non-specific' as evidenced by the fact that it was not blocked by serotonin uptake blockers or reduced by 5,7-DHT treatment (see below). To explore the relationship between serotonin uptake sites and specific [^{3}H]paroxetine binding in vivo, the distribution of these two parameters in the rat brain was compared. An excellent correlation between these two parameters was observed (r = 0.93, $P < 0.002$) (Fig. 1).

Inhibition of in vivo [^{3}H]paroxetine binding by 5-HT uptake blockers

To further characterize the in vivo [^{3}H]paroxetine binding, inhibition studies were carried out with two drugs known to block 5-HT uptake sites selectively,

Figure 2a

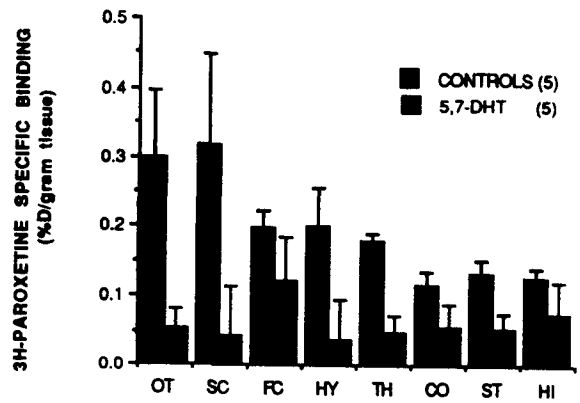

Figure 2b

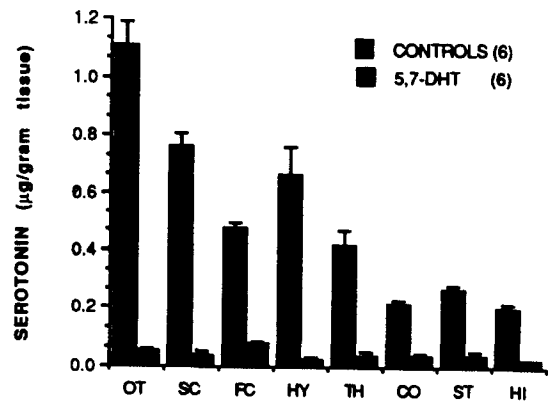

Fig. 2. Effect of 5,7-DHT on: (a) specific in vivo [^{3}H]paroxetine binding (% injected dose/g tissue – % dose/g cerebellum); and (b) 5-HT concentrations (µg/g tissue) in different regions of the rat brain. Rats were pretreated with desmethylimipramine (25 mg/kg). 5,7-DHT was given i.v.; 200 µg in 10 µl saline 7 days before determination of [^{3}H]paroxetine binding and 5-HT levels. Data are means ± S.D. Number in parentheses = number of animals. OT, olfactory tubercles; SC, upper colluculi; FC, frontal cortex; HY, hypothalamus; TH, thalamus; CO, cortex; ST, striatum; HI, hippocampus.

Figure 3a

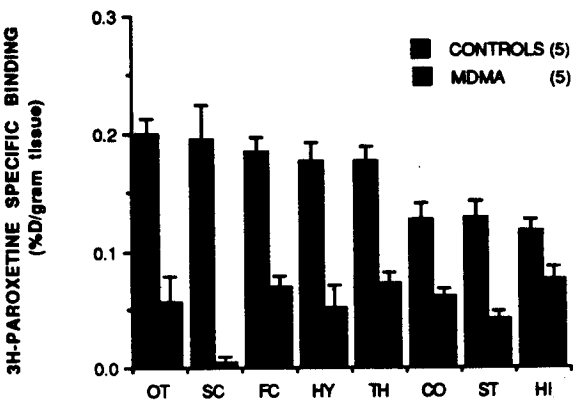

Figure 3b

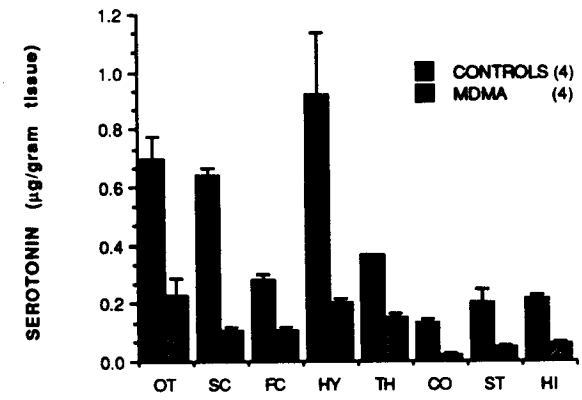

Fig. 3. Effect of MDMA on: (a) specific in vivo [^{3}H]paroxetine binding (% dose/g tissue – % dose/g cerebellum); and (b) serotonin concentrations (μg/g tissue) in different regions of the rat brain. MDMA was administered s.c. (8 × 20 mg/kg) over a 4-day period. [^{3}H]Paroxetine and 5-HT levels were determined 7 days after the last MDMA dose.

sertraline and citalopram. Both antagonists caused marked reductions in specific [^{3}H]paroxetine binding in all brain regions examined (Table I). The greatest decreases occurred in the superior colliculi, olfactory tubercles, thalamus and hypothalamus. Inhibition was less pronounced in the hippocampus, striatum and frontal cortex. No reductions occurred in the cerebellum (data not shown).

Effect of 5,7-DHT on in vivo [^{3}H]paroxetine binding

Having established that the distribution of specific [^{3}H]paroxetine binding in the rat is comparable to that in the mouse, and that selective inhibitors of serotonin uptake interfere with the in vivo [^{3}H]paroxetine binding in the rat brain, the effect of 5,7-DHT was investigated. 5,7-DHT (200 μg, injected i.c.v. 7 days before the study) markedly reduced in vivo [^{3}H]paroxetine binding in all rat brain regions examined (Fig. 2a). Greatest reductions were observed in the superior colliculi (–86%), olfactory tubercles (–82%), hypothalamus (–80%), and thalamus (–72%). Smaller reductions occurred in the hippocampus (–41%), striatum (–59%) and frontal cortex (–38%). [^{3}H]Paroxetine binding in the cerebellum was not changed by 5,7-DHT/DMI treatment.

In accord with previous findings[14], 5,7-DHT produced large depletions of serotonin in all areas of the rat brain examined (Fig. 2b). In the hippocampus, striatum, and frontal cortex, depletions of serotonin were larger than reductions of in vivo [^{3}H]paroxetine binding (compare Fig. 2a with b).

Effect of MDMA on in vivo [^{3}H]paroxetine binding

To determine if in vivo [^{3}H]paroxetine binding could be used to detect serotonergic damage induced by MDMA, rats were treated with toxic doses of MDMA (20 mg/kg s.c. twice daily for 4 days). One week later, rats were divided into two groups. In the first group (n = 5), in vivo [^{3}H]paroxetine binding was measured; in the other (n = 4), serotonin levels were determined. MDMA produced large reductions in both in vivo [^{3}H]paroxetine binding and serotonin levels (Fig. 3a,b). As after 5,7-DHT, the depletion of serotonin in the striatum and hippocampus was larger than the decrement in in vivo [^{3}H]paroxetine binding (Fig. 3a,b).

DISCUSSION

The present findings extend our previous results[28] and demonstrate that [^{3}H]paroxetine can also be used to label the serotonin transporter complex in the rat brain in vivo. Three lines of evidence suggest that in vivo [^{3}H]paroxetine binding labels the serotonin uptake site in the rat brain. First, the distribution of in vivo [^{3}H]paroxetine binding closely parallels the known distribution of serotonin uptake sites (Fig. 1)[20]. Second, selective inhibitors of serotonin re-uptake (sertraline and citalopram) interfere with the specific [^{3}H]paroxetine binding (Table I). Third, 5,7-DHT, which is known to destroy serotonergic nerve fibers[14], produces marked decrements in specific in vivo [^{3}H]paroxetine binding (Fig. 2). Taken together, these anatomical, pharmacological and toxicological findings strongly suggest that [^{3}H]paroxetine labels the serotonin transporter complex in the rat brain in vivo.

The present results also show that specific [^{3}H]paroxetine binding can serve as an in vivo indicator of MDMA-induced serotonergic neurotoxicity. Rats treated with toxic doses of MDMA display clear reductions in in vivo [^{3}H]paroxetine binding (Fig. 3a). Moreover, they exhibit large depletions of serotonin (Fig. 3b). Since a lasting depletion of serotonin generally provides a reliable index of serotonergic axonal damage[9,19,26,27], in vivo [^{3}H]paroxetine binding appears to provide another

means by which MDMA neurotoxicity can be detected and quantified. The fact that this can be accomplished in vivo suggests that in the future, it may be possible to use positron-emitting paroxetine (or one of its analogs) to screen for possible subclinical MDMA neurotoxicity in man during life.

In using specific [^{3}H]paroxetine binding as an in vivo indicator of serotonergic neurotoxicity, it is important to note that in certain brain regions [^{3}H]paroxetine binding may underestimate the extent of serotonergic damage. For example, this appears to be the case in the hippocampus, striatum and possibly the frontal cortex. In these regions, specific [^{3}H]paroxetine binding is reduced by only 30–40% in animals in which serotonin content is reduced by as much as 70–90% (compare Fig. 2a with b and Fig. 3a with b). Although the basis for this discrepancy is unknown, it is possible that in these brain regions [^{3}H]paroxetine binds not only to the serotonin uptake site, but also to a site which is not destroyed by 5,7-DHT or MDMA. Preservation of this second site would explain why serotonin content is reduced to a greater extent than specific [^{3}H]paroxetine binding after neurotoxic exposure. Little can be said regarding the precise location of this second site except that it is probably on other (non-serotonergic) neural elements or glia. Notably, the only brain regions in which this additional site seems to be present are the hippocampus and striatum (and possibly frontal cortex), since in all other brain regions examined, an excellent correlation (r = 0.98) was observed between reductions in in vivo [^{3}H]paroxetine binding and decreases in serotonin content.

Lending support to the notion that serotonin uptake blockers label a site in addition to the serotonin uptake site (at least in some brain regions) is a previous observation by Brunello and co-workers[5]. Following 5,7-DHT lesions, these investigators also found a discrepancy between reductions in [^{3}H]imipramine binding and serotonin content. As in this study, binding was reduced only half as much as serotonin content, and the discrepancy occurred in the hippocampus and striatum[5]. On the basis of these results, Briley[4] postulated that,in some brain regions, [^{3}H]imipramine bound to a site in addition to the serotonin uptake site, possibly a postsynaptic receptor site. While the exact location of this additional site remains uncertain[6], the observations of Brunnello et al.[5] are certainly reminiscent of those made in this study (Figs. 2 and 3), and lend credence to the notion that, at least in certain brain regions, serotonin uptake blockers label an additional site, a site which is not located on serotonergic axons or terminals.

Some might argue that in areas such as the hippocampus and striatum reduced radioligand binding provides a more accurate index of serotonergic damage than serotonin depletion, particularly since serotonin can be lowered by numerous processes which do not involve neurotoxic changes (e.g. inhibition of synthetic enzymes etc.). There are several reasons why this is unlikely. First, in previous studies[3,17] reductions in in vitro [^{3}H]paroxetine binding have paralleled depletions of serotonin. Second, when serotonin uptake sites are quantified using synaptosomal preparations, decrements in the V_{max} of [^{3}H]serotonin uptake are typically of the same magnitude as reductions in serotonin content[9,27]. Third, changes in other serotonergic presynaptic markers (e.g. 5-HIAA and tryptophan hydroxylase) closely parallel changes in serotonin content[3,17,19,31]. Finally, the discrepancy between in vivo [^{3}H]paroxetine binding and serotonin content is found only in the hippocampus and striatum. These considerations suggest that, in selected brain regions, serotonin depletion is more accurate than in vivo [^{3}H]paroxetine binding as an indicator of serotonergic damage, and that in the hippocampus and striatum (and possibly the frontal cortex) in vivo [^{3}H]paroxetine binding may underestimate the extent of serotonergic damage.

Making paroxetine or one of its analogs positron-emitting promises to be a challenging task. In principle, this could be accomplished through the use of carbon-11 (^{11}C) or fluorine-18 (^{18}F). Use of ^{18}F seems preferable at first glance since it allows for an isotopic substitution (Fig. 4) with an isotope of longer half-life ($t_{1/2}$ of ^{18}F = 110 min). A longer half-life is desirable because paroxetine requires 3–4 h to attain a high ratio of total to non-specific binding. The major problem with radiofluorination of paroxetine is that appropriate synthetic methods are not yet available and their development promises to be difficult. Use of ^{11}C ($t_{1/2}$ = 20 min) is more attractive from a methodological standpoint. However, as the most convenient approach to ^{11}C-labeling necessarily involves addition of a new side chain, there is concern that this may alter the compound in an undesirable way. For example, with paroxetine, there is evidence that *N*-methylation reduces its affinity for the

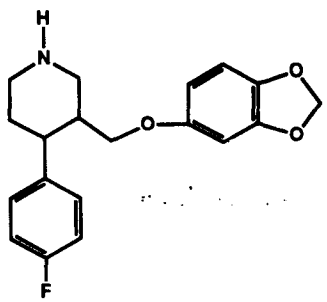

Fig. 4. Structure of paroxetine.

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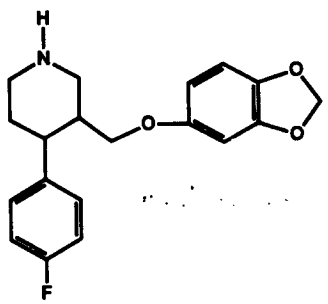

Fig. 4. Structure of paroxetine.

5-HT uptake site by a factor of ten[24]. The critical issue is whether sufficient biological activity is retained by the *N*-methylated derivative for it to still be useful as a PET ligand. With regard to *N*-methylparoxetine, there is some recent preliminary indication that there is[33]. Additional studies are needed to confirm this initial finding and to further evaluate *N*-methylparoxetine as well other paroxetine analogs as potential serotonergic PET ligands.

Once developed, a serotonergic PET ligand promises to be useful in a number of ways. First, it should greatly facilitate the study of serotonergic function in the normal living human brain. Secondly, it should permit a more critical assessment than has heretofore been possible of the role of altered serotonergic neurotransmission in the pathogenesis of depression, anxiety, and other neuropsychiatric disorders[1,13,23,36]. Finally, a serotonergic PET ligand should make it feasible to screen individuals in whom serotonergic damage is suspected but in whom there are no obvious signs or symptoms of central serotonin deficiency (e.g. recreational MDMA users).

In summary, the results of the present study demonstrate that [^{3}H]paroxetine can be used to label the serotonin transporter complex in the rat brain in vivo. In addition, they show that specific [^{3}H]paroxetine binding can serve as an in vivo indicator of MDMA-induced serotonergic neurotoxicity. Together, these findings provide a framework for developing a presynaptic serotonergic PET ligand, a ligand which should prove useful not only for evaluating humans for possible MDMA neurotoxicity, but also for enhancing our understanding of the functional role of serotonin in the living human brain in health as well as disease.

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