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PET-Determination of Robalzotan (NAD-299) Induced 5-HT_{1A} Receptor Occupancy in the Monkey Brain

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The serotonin 5-hydroxytryptamine-1A (5-HT_{1A}) receptor subtype is of central interest in research, particularly in the area of pathophysiology and pharmacological treatment of psychiatric disorders. Robalzotan (generic name for NAD-299) is a new putative drug that binds with high selectivity and affinity to 5-HT_{1A}-receptors in the rodent brain in vitro and in vivo. The aim of this positron emission tomography study was to determine 5-HT_{1A} receptor occupancy in the cynomolgus monkey brain in vivo after IV injection of robalzotan. Two healthy monkeys were examined with Positron Emission Tomography (PET) and the radioligand [carbonyl-^{11}C]WAY-100635, the first after IV administration of 2 μg/kg and 20 μg/kg, and the second after 10 μg/kg and 100 μg/kg IV. 5-HT_{1A} receptor occupancy was calculated using an equilibrium-ratio analysis. Robalzotan occupied 5-HT_{1A} receptors in a dose-dependent and saturable manner. The highest 5-HT_{1A} receptor occupancy (70–80%) was attained after 100 μg/kg. The relationship between robalzotan drug concentration and 5-HT_{1A} receptor occupancy could be described by a hyperbolic function, which can be used to guide the selection of appropriate doses for the initial studies in man. The study further corroborates that quantitative neuroimaging of receptor binding has potentials for the evaluation and dose finding of new CNS drugs.
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KEY WORDS: *5-HT_{1A} receptors; Robalzotan; NAD-299; Antidepressive & Anxiolytic drugs; Monkey brain; [carbonyl-^{11}C]WAY-100635; Positron Emission Tomography*

Serotonergic neurotransmission includes distinct 5-hydroxytryptamine (5-HT) receptor subtypes, classified within at least seven separate families (Hoyer et al. 1994; Peroutka and Howell 1994). The 5-HT_{1A} receptor belongs to the family of G-protein coupled 5-HT_1-receptors and is of central interest as a target for the treatment of psychiatric disorders (Saxena 1995).

Presynaptic 5-HT_{1A} receptors mediate inhibition of 5-HT release and are highly concentrated on the cell bodies in the raphe nuclei (Romero et al. 1994). This receptor has been given a key role in current hypotheses on the treatment of anxiety and depression (Artigas et al. 1994; Baldwin and Rudge 1995; Berendsen 1995). An important observation is that (−)pindolol, a compound with affinity for β-adreno-and 5-HT_{1A} receptors, antagonizes 5-HT_{1A}-mediated response in the raphe nuclei (Hjorth and Carlsson 1986; Oksenberg and Peroutka 1988). Interestingly, pindolol may facilitate improvement of depressive patients by reducing the latency and enhancing the response rate to certain selective serotonin reuptake inhibitors (SSRI) (Artigas et al. 1994; Blier and Bergeron 1995). An augmenting or accelerating ef-

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fect of pindolol has recently been confirmed in some placebo-controlled clinical studies (Perez et al. 1997; Tome et al. 1997; Zanardi et al. 1997), although negative results have also been reported (Berman et al. 1997; McAskill et al. 1998; Moreno et al. 1997; Perez et al. 1999). In another PET study, we have recently reported that pindolol binds specifically to 5-HT_{1A} receptors in the living human brain (Andrée et al. 1999). This lends further support for a specific action and possible clinical implications of pindolol treatment.

Postsynaptic 5-HT_{1A} receptors have been found in high densities in the hippocampus and in the superficial layers of the neocortex in man (Hall et al. 1997; Marazziti et al. 1994; Pazos et al. 1987). The high density of postsynaptic 5-HT_{1A} receptors in the neocortex is of interest in schizophrenia research, since several studies have reported elevated 5-HT receptor binding in the neocortex and the hippocampus of post-mortal schizophrenic brains (Burnet et al. 1996; Gurevich and Joyce 1997; Hashimoto et al. 1991,1993; Joyce et al. 1993; Sumiyoshi et al. 1996). However, the antipsychotic potential of selective 5-HT_{1A} receptor antagonists or agonists has not been explored in man.

Robalzotan with the substance name NAD-299, (R)-3-N,N-dicyclobutylamino-8-fluoro-3,4,dihydro-2H-1-benzopyran-5-carboxamide hydrogen (2R,3R)-tartate monohydrate), is a novel compound with high selectivity and affinity (0.6 nM) to 5-HT_{1A}-receptors in the rodent brain *in vitro* and *in vivo* (Larsson et al. 1998; Stenfors et al. 1998). Functional and behavioral studies with robalzotan indicate antagonistic activity at the 5-HT_{1A}-receptor (Johansson et al. 1997). The safety and tolerability of robalzotan is currently examined in healthy subjects.

[*carbonyl*-^{11}C]WAY-100635 is a suitable radioligand for quantitative determination of 5-HT_{1A} receptors in the monkey and human brain *in vivo* using positron emission tomography (PET) (Farde et al. 1997, 1998; Pike et al. 1996). This suitability has been supported by autoradiography studies, in which the radioligand [^{3}H]WAY-100635 has been used for the characterization of the 5-HT_{1A} receptor distribution *in vitro* (Hall et al. 1997; Pazos et al. 1987) showing a consistent result for the regional 5-HT_{1A} receptor distribution as that shown in the living human brain by PET.

The aims of this PET study were to determine 5-HT_{1A} receptor occupancy in the cynomolgus monkey brain *in vivo* after IV injection of robalzotan, and to establish the hyperbolic function between plasma concentration and receptor occupancy. Two healthy monkeys were examined with PET and the radioligand [*carbonyl*-^{11}C]WAY-100635, the first after administration of 2 μg/kg and 20 μg/kg and the second after 10 μg/kg and 100 μg/kg IV. 5-HT_{1A} receptor occupancy in the neocortex and the raphe nuclei were calculated using an equilibrium-ratio analysis with the cerebellum as reference region.

MATERIALS AND METHODS

This study was performed at the Department of Clinical Neuroscience at the Karolinska Hospital after approval by the Animal Ethics Committee of Northern Stockholm. Principles of laboratory animal care (NIH publication No. 85–23, revised 1985) were followed).

Compound

Robalzotan (NAD-299) was obtained from AstraZeneca AB, Södertälje, Sweden. The solutions of NAD-299 were prepared as an intravenous stock-solution (0.4 μmol/ml) batch 2230–4–1 according to GLP routines.

Radiochemistry

[*carbonyl*-^{11}C]WAY-100635 was prepared by ^{11}C acylation of the precursor WAY-100634 with [*carbonyl*-^{11}C]-cyclobexanecarbonyl chloride, as previously described in detail (Hall et al. 1997; Pike et al. 1996). The specific radioactivity was about 2000 Ci/mmol (74 GBq/μmol) at EOS (end of synthesis). The radiochemical purity was higher than 99%.

Robalzotan (NAD-299) Concentration in Plasma

Venous blood samples (1–2 ml) were drawn from a femoral vein, before drug administration (0 minutes), at start of the PET measurement (20 minutes) and at about 40, 60, 80, and, after the time for the highest doses, 100 minutes. The exact timing was dependent on success with the venopuncture at each sampling. The mean plasma concentration values ($C_{pl.mean}$), determined for the time-interval 40–60 minutes after drug administration, were calculated and used for the determination of the hyperbolic function between plasma concentrations and 5-HT_{1A} receptor occupancy. NAD-299 was isolated from monkey plasma samples by liquid-liquid extraction and analyzed by reversed phase liquid chromatography (LC) combined with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS). Standard samples, prepared in monkey plasma, were used for the calibration curves. An automated LC-MS-MS system allowing unattended over-night operation was used. NAD-299 could be reliably measured down to 0.2 nM. See Figure 1.

The PET System

The PET system used (i.e., Siemens ECAT Exact HR47), has a spatial resolution in the imaging plane of about 3.8 mm FWHM (Full Width at Half Maximum) (Wienhard et al. 1994). The system was used in the three-dimensional mode and images were displayed in 47 horizontal sections. Radioactivity in the brain was mea-

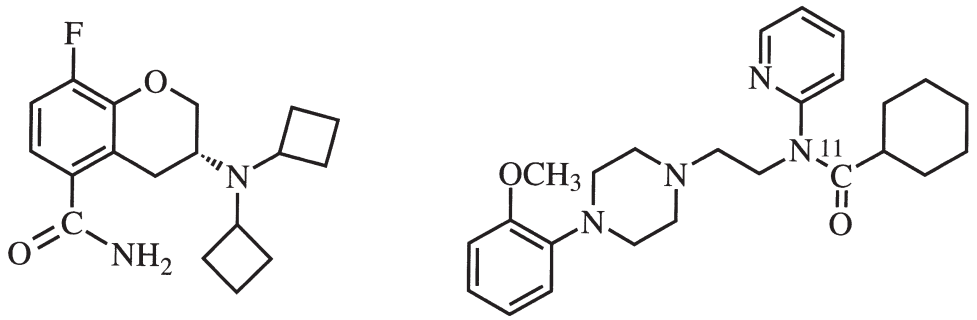

Figure 1. Structural formulas of Robalzotan (NAD-299) (left) and [*carbonyl*-^{11}C]WAY-100635 (right).

sured continuously according to a pre-programmed sequence of frames for up to 93 minutes after injection of [*carbonyl*-^{11}C]WAY-100635. See Figure 2.

PET Studies on Monkeys

Two cynomolgus monkeys with a weight of 3–4 kg were obtained from the National Laboratory of Bacteriology, Solna, Sweden. Anesthesia was induced by repeated IM injection of ketamine (Ketalar® 5–10 mg kg^{-1} h^{-1}). Blood pressure and pulse rate were recorded every 20 minutes. Temperature was kept at about 37°C using a heating pad that was regulated by the rectal temperature. A head fixation system secured a fixed position of the monkeys' head in a plane parallel to that defined by the canto-meatal line (Karlsson et al. 1993).

Each of the two monkeys participated in three PET-measurements that were performed on the same day. The first was performed at baseline conditions, the second after a low dose of robalzotan that was administered IV, and the third after a 10-times higher dose (20, 100 μg/kg). The 5-HT$_{1A}$ receptor occupancy values in the first monkey were used to select suitable doses that were administered to the second monkey. The strategy was to select doses that should cover a wide occupancy interval.

In each PET-measurement, a sterile physiological phosphate buffer (pH 7.4) solution containing about 1.3–1.5 mCi (48–56 MBq) of [*carbonyl*-^{11}C]WAY-100635 was injected as a bolus into a sural vein during 2 seconds. The specific radioactivity (SR) was 867–2039 Ci/mmol (32–75 GBq/μmol) for the measurements in the first monkey, and 192–905 Ci/mmol (7.1–33 GBq/μmol) for the second, at the time of IV administration. Each PET-measurement was performed at expected robalzotan peak plasma levels.

Regions of Interest

For analysis of regional brain radioactivity, regions of interest (ROIs) were drawn on the reconstructed PET images. ROIs were drawn for the whole brain, the neocortex, the raphe nuclei, and the cerebellum. The anatomical delineation of regions was guided by an atlas showing a series of horizontal sections of a cynomolgus monkey brain in situ obtained by serial cryomicrotomy (Karlsson et al. 1993).

Calculation of 5-HT$_{1A}$ Receptor Occupancy by the Ratio Method

Regional radioactivity was determined for each ROI measured for each sequential time-frame, corrected for ^{11}C-decay and plotted versus time. The 5-HT$_{1A}$ receptor occupancy induced by pretreatment was calculated according to a procedure that has been described previously (Farde et al. 1992, 1997). Assuming negligible specific binding, the cerebellum can be used as reference region for free and non-specific binding (C_f) in brain. Specific [*carbonyl*-^{11}C]WAY-100635 binding (C_b) to 5-HT$_{1A}$ receptors was calculated as the difference between total binding (C_t) in a ROI and cf. Receptor occupancy (%) was defined as the percent reduction of C_b/C_f. The ratio C_b/C_f was calculated for the 20–40 minutes time interval of each PET-measurement according to Equation 1:

$$\frac{C_b}{C_f} = \frac{\int_{20}^{40} C_b(t)\,dt}{\int_{20}^{40} C_f(t)\,dt} \qquad (1)$$

Following an IV bolus injection, the relation between receptor binding and the concentration of ligand is given by a hyperbolic (curvilinear) function, derived from the law of mass action (Farde et al. 1986, 1989; Ito et al. 1998), according to Equation 2:

$$B = \frac{B_{max} * F}{K_D + F} \qquad (2)$$

where B is the concentration of ligand (i.e., robalzotan), bound to receptors, B_{max} is the total number of available receptors, K_D is the apparent equilibrium dissociation constant, and F is the concentration of free (unbound)

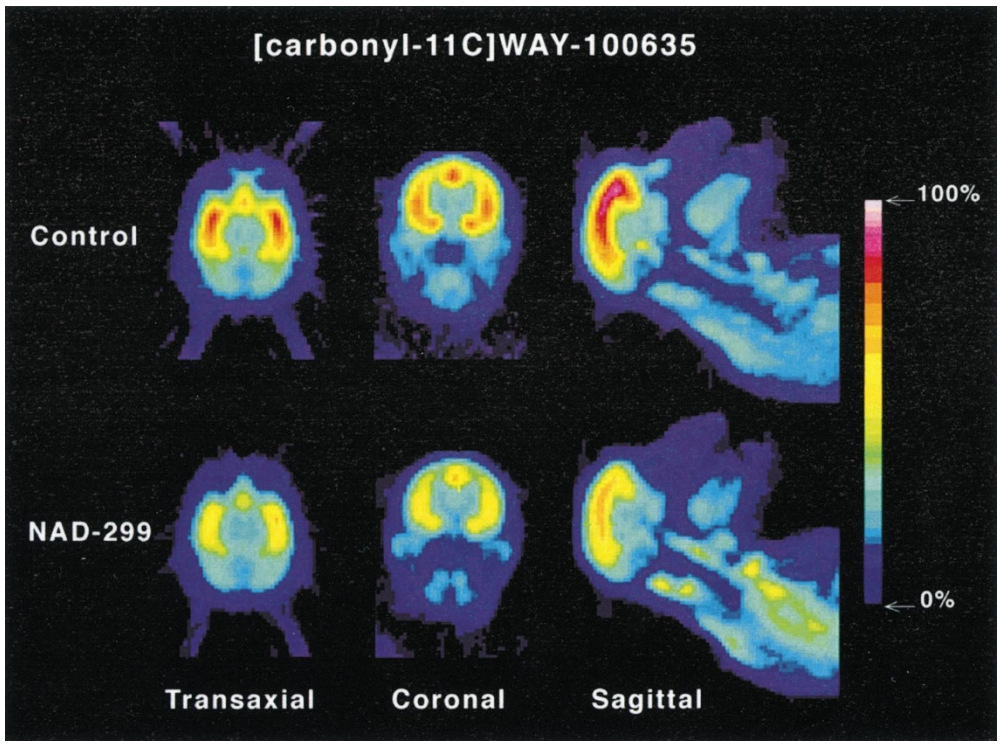

Figure 2. Color-coded PET-image of [*carbonyl*-^{11}C]WAY-100635 in three-dimension of the cynomolgus brain. Pretreatment with 20 μg/kg Robalzotan (NAD-299) IV.

ligand. Assuming that the relationship between robalzotan in brain and plasma and the free fraction are not concentration dependent, *F* may be substituted with the robalzotan plasma concentration (Farde et al. 1989), that, thus, represents an estimate of the free concentrate in brain (Karlsson et al. 1995b). All receptors (B_{max}) are occupied at 100% occupancy. Replacing *F* with $robalzotan_{pl.}$ and B_{max} with 100%, Equation 1 can be rewritten as Equation 3:

$$5\text{-HT}_{1A}\text{receptor occupancy (\%)} = \frac{100*\text{robalzotan}_{pl.}}{K_D + \text{robalzotan}_{pl.}} \quad (3)$$

5-HT_{1A} receptor occupancy was plotted versus the mean plasma concentrations of robalzotan 40–60 minutes after robalzotan administration. This time corresponds to 20–40 minutes after injection of the radioligand [*carbonyl*-^{11}C]WAY-100635. Each data point was treated as an independent observation, and Equation 1 was fitted in a least square sense through an iterative procedure to the experimental data points, using Kaleidagraph® 3.0 (Abelbeck Software, USA).

RESULTS

The plasma concentrations of robalzotan increased with dose and was maximal (C_{max}) at time of start of the PET-measurements, 20 minutes after IV drug administration (T_{max}) (Table 1). During the first PET-measurement, performed at baseline conditions, there was a high uptake of radioactivity in the neocortical regions and the raphe nuclei, whereas the radioactivity in the cerebellum was low (Figure 3). After pretreatment with robalzotan, the radioactivity in the neocortex and the raphe nuclei was reduced in a dose-dependent manner (Figure 3). The mean plasma concentration values ($C_{pl.mean}$), determined for the time-interval 40–60 minutes after drug administration, was 0.5, 4, 7.2, and 31.4 nmol/l, respectively after robalzotan administration of 2, 10, 20, and

Table 1. Robalzotan (NAD-299) Plasma Concentrations (nmol/L) in Cynomolgus Monkey Brain after IV Administration in Four Separate Doses (2–100 μg/kg)

Time (minutes)	2 μg/kg	10 μg/kg	20 μg/kg	100 μg/kg
0	nd	<1	nd	<1
20	1.8	10	17.2	84
40	0.5	4	10.6	40
60	0.5	4	5.2	23
80	<0.5	2	4	22
100		2		19
Occupancy neocortex (%)	8	33	46	68
Occupancy raphe (%)	10	42	55	75

nd, not determinable.

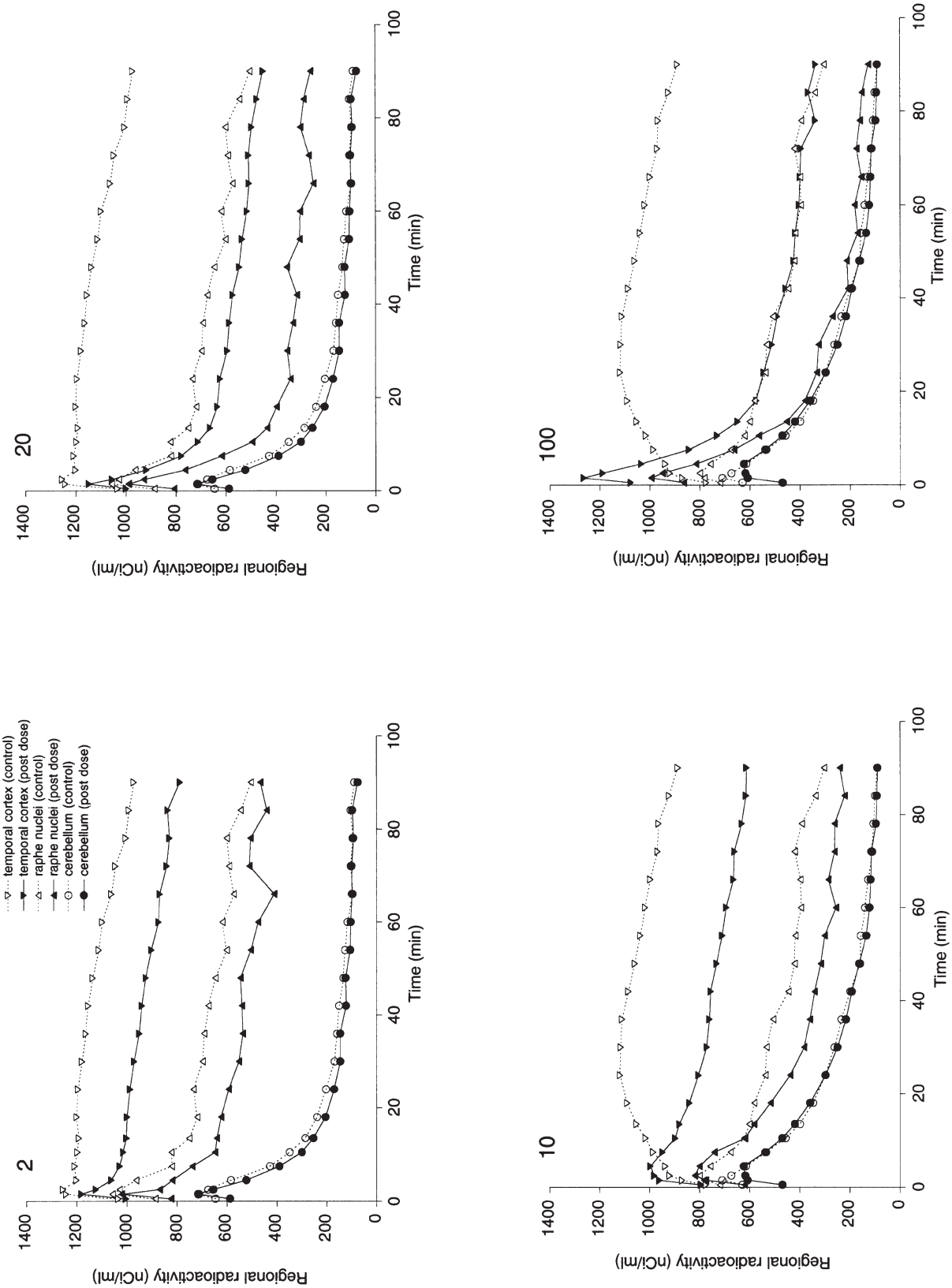

Figure 3. Time course for regional activity (nCi/ml) in the Cynomolgus brain after intravenous injection of [*carbonyl*-^{11}C]WAY-100635. Control and pretreatment with 2 μg/kg (upper left), 20 μg/kg (upper right), 10 μg/kg (lower left), and 100 μg/kg robalzotan (lower right), respectively.

100 μg/kg as an IV injection (Table 1). The highest occupancy (70–80%) was calculated for the measurement after the 100 μg/kg dose (Table 1).

The curvilinear function for a saturation hyperbola was fitted to the experimental data points (Karlsson et al. 1995a). The results indicate that 50% of the 5-HT_{1A} receptors in the raphe nuclei are occupied at a robalzotan plasma concentration of 3.9 nmol/L. The corresponding value for the neocortex was 5.4 nmol/L (Figure 4).

DISCUSSION

After IV injection of robalzotan in the two cynomolgus monkeys, there was a binding of [*carbonyl*-^{11}C]WAY-100635 to 5-HT_{1A} receptors (Figure 3). The results indicate that robalzotan (generic name NAD-299) rapidly passes the blood-brain barrier and binds to central 5-HT_{1A} receptors in the cynomolgus brain *in vivo.*

The relationship between robalzotan plasma concentration and 5-HT_{1A} receptor occupancy after IV administration of robalzotan could be described by a hyperbolic function, both for the neocortex and the raphe nuclei (Figure 4), which can be used to guide the selection of appropriate doses for initial studies in man. The study demonstrates that quantitative neuroimaging of receptor binding has potential for the evaluation and dose finding of new CNS drugs.

The 5-HT_{1A} receptor occupancy values were numerically higher in the raphe nuclei in all four measurements compared to the slightly lower receptor occupancy calculated for the neocortical region. However, it must be taken into account that this difference is based on pooled data from four measurements in two monkeys only, therefore, these values should be taken with caution. Considering the small volume of the monkey brain (65 ml) and the raphe nuclei in relation to the resolution of the PET-system (3.8 mm FWHM), it cannot be concluded that robalzotan binds preferentially to 5-HT_{1A} receptors in the raphe nucleus.

The predominant metabolite of the robalzotan in vitro in all species tested including rat, dog, and man, is the mono-N-dealkylated derivative (Gagner Milchert et al. 1998). This metabolite has more than thousand fold less affinity for the 5-HT_{1A} receptor than the parent compound (personal communications, Seth-Olov Thornberg, AstraZeneca). Hydroxylated robalzotan and its dealkylated derivative are other labeled metabolites which have been suggested in a PET-study with [^{11}C]robalzotan in cynomolgus monkeys (Sandell et al. 1999a). The hydroxylated metabolites are polar and not likely to pass the blood-brain barrier and contribute to the 5-HT_{1A} receptor occupancy.

The expected rapid metabolism was, nevertheless, a reason for starting the PET-measurements as early as about 20 minutes after robalozotan IV administration. According to a PET study with [^{11}C]robalzotan in cyno-

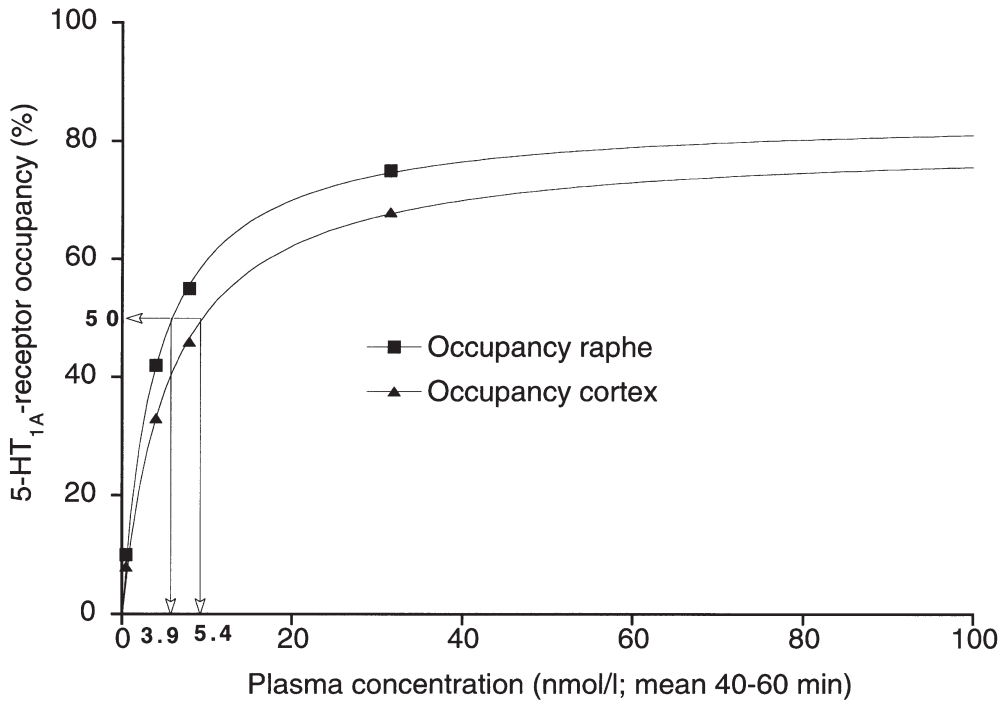

Figure 4. Robalzotan (NAD-299) plasma concentrations (nmol/l) versus 5-HT_{1A} receptor occupancy (%) in two cynomolgus monkeys after i.v. administration of robalzotan. The K_i-values for the neocortex and the raphe nucleus corresponding to 50% occupancy are indicated.

molgus monkey, this time is sufficient to allow robalzotan to be distributed to brain and to reach binding equilibrium (Sandell et al. 1999a). The plasma curves confirm that the PET-measurements started at peak plasma levels (C_{max}) in all four measurements (Figure 3A–D).

Recently, we have labeled robalzotan (NAD-299) with carbon-11 for visualization of the 5-HT_{1A} receptor (Sandell et al. 1999b). Autoradiographic examination of [^{11}C]NAD-299 binding in the post mortem human brain demonstrated high specific binding in the hippocampus, the raphe nuclei and the neocortex. The results indicate that ^{11}C-labeled NAD-299 has potential as radioligand for PET examination of 5-HT_{1A} receptors *in vivo.*

CONCLUSION

This PET-study confirms that robalzotan (NAD-299) passes the blood-brain barrier and binds to central 5-HT_{1A} receptors in a saturable and dose-dependent manner, in the primate brain *in vivo.* The relationship between robalzotan drug concentrations and 5-HT_{1A} receptor occupancy could be described by a hyperbolic function that can be used to guide the selection of appropriate doses for the initial studies in man.

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