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# Compartmental Analysis of Diprenorphine Binding to Opiate Receptors in the Rat In Vivo and Its Comparison with Equilibrium Data In Vitro

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**Summary:** The regional binding of the opiate receptor ligand diprenorphine has been examined in rat brain both in vivo and in vitro. The time course of total label in specific brain regions was followed up to 2 h after intravenous bolus injection of [$^3$H]diprenorphine, with or without a pulse chase of unlabelled diprenorphine at 30 min. In addition, total label was measured 30 min after injection of labelled diprenorphine at nontracer concentrations over a range of specific activities. Total data sets for each region were fitted simultaneously to a compartmental model to give estimates of maximal binding capacity ($B_{max}$), the second-order apparent association rate constant, and the first-order dissociation rate constant of the receptor-ligand complex. The model incorporated the use of a reference region with low specific binding (cerebellum). The binding of diprenorphine to rat brain homogenates was measured in vitro under equilibrium conditions at 37°C, pH 7.4, in the presence and absence of naloxone, to give corresponding regional estimates of $B_{max}$ and the half-saturation constant $K_d$. The results showed a close correlation between in vitro and in vivo regional estimates of $B_{max}$ over a wide range. There were no significant interregional differences either in $K_d$ in vitro or in the $K_d$ derived from the in vivo analysis, although in vitro and in vivo estimates differed by an order of magnitude. This work was carried out as part of a validation study with a view to the application of the compartmental model to data obtained in vivo in humans using positron emission tomography, when successive studies over a range of specific activities are not feasible. Restriction of the rat data to tracer alone and pulse chase protocols showed that the compartmental model gave regional estimates of the combined forward rate constant consistent with estimates obtained using the complete data set. **Key Words:** Compartmental model—Diprenorphine—Opiate receptors.

Diprenorphine binds to μ- δ-, and κ-opiate receptor subtypes with similar affinities for each in vivo (Iwamoto and Martin, 1981; Richards and Sadée, 1985). Previous studies using [$^{11}$C]diprenorphine and positron emission tomography (PET) suggest that it is a promising tracer for quantitation of regional opioid receptor binding in humans (Jones et al., 1988; Frost et al., 1989*a,b*). The purpose of the present study in the rat was to examine the relationship between regional estimates of binding parameters derived from compartmental analysis of dynamic tracer data in vivo and direct regional measures carried out under equilibrium conditions in vitro. The compartmental analysis involves the use of a reference region displaying low specific binding relative to the region of interest (ROI).

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*Abbreviations used:* PET, positron emission tomography; ROI, region of interest.

## METHODS

### Animals

Male Sprague-Dawley rats (Halan Olac Ltd., Bicester) weighing 250–280 g were used for all procedures. They were housed on a 12-h light-dark cycle with food and water ad libitum. For experiments in vivo, a tail artery and vein were cannulated with polythene tubing (ID 0.58 mm, OD 0.96 mm; Portex Ltd., Kent) for blood sampling and intravenous administration of radioligand, respectively. Cannulation was carried out under isoflurane anaesthesia with $N_2O$ plus $O_2$. The animals were then allowed to recover for 2–3 h under light restraint prior to the experiments, which were all initiated between 1300 and 1400 h.

## Chemicals

[15,16(*N*)-$^3$H]Diprenorphine (1.147–1.284 GBq/μmol) was purchased from Amersham International. Naloxone chlorhydrate was obtained from Dupont and diprenorphine was kindly donated by Reckitt & Coleman.

## Binding assay in vitro

The in vitro binding assay is based on that of Geary and Wooten (1983) except that it was carried out at 37°C. Rats were decapitated, the brain was removed, and ROIs (thalamus, anterior cortex, posterior cortex, caudate-putamen, hippocampus, brainstem plus medulla, and cervical spinal cord) were dissected on ice. For each experiment, tissue was pooled from two rats and ~250 mg of each region was homogenised for 10 s in ice-cold phosphate-buffered saline at pH 7.4 (75 ml/g, vol/wt), using an Ultra-Turrex homogeniser at setting 0.5. Aliquots of the homogenates were taken for assay of binding (below) and for assay of protein (Lowry et al., 1951).

Binding assay incubations (1 ml) contained 3.33 mg wet wt tissue (~300 μg protein) and [$^3$H]diprenorphine in a range of concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 5.0, and 10.0 n*M*) with or without naloxone (20 μ*M*) in buffer. Incubation was at 37°C for 25 min and was terminated by rapid filtration through Whatman GF/B filters in a Brandel Cell Harvester, followed by two rapid washes of the filtrate with 5 ml ice-cold buffer. Filters were left overnight in 4 ml scintillant (Hionic-Fluor; Packard) and counted using a Beckman LS 6800. Within each experiment, triplicate measures were carried out at each concentration of diprenorphine and naloxone. The experiments for each region were repeated four times.

Binding isotherms were analysed in terms of the equations $B_{tot} = B_{max} \cdot F/(K_d + F) + K_{ns} \cdot F$ (in the absence of naloxone) and $B_{tot} = K_{ns} \cdot F$ (in the presence of naloxone), where $B_{tot}$ (pmol/g wet wt) is the observed bound diprenorphine in the presence or absence of naloxone and $F$ (n*M*) is the free concentration of diprenorphine (original total concentration with correction for observed bound fraction).

Estimates of the parameters $B_{max}$ (maximal binding capacity; pmol/g wet wt), $K_d$ (equilibrium dissociation constant; n*M*), and $K_{ns}$ (nonspecific binding constant; ml/g wet wt) were obtained from simultaneous, nonlinear, least-mean-squares fits of the data, with and without naloxone, to the above equations, using a Simplex algorithm (Nelder and Mead, 1965) with all data points being given equal weight.

## Distribution of label in brain in vivo

The distribution of label in brain following intravenous bolus administration of [$^3$H]diprenorphine was measured in three experimental protocols; tracer alone, pulse chase, and varying specific radioactivity.

In the tracer alone protocol, rats received ~370 kBq of tracer in 200 μl Na citrate/acetate buffer (pH 7.4) at zero time and were killed at specified times up to 120 min after injection. In the pulse chase protocol, rats received the same dose of [$^3$H]diprenorphine at zero time, a further intravenous bolus injection of unlabelled diprenorphine (470 pmol/g body wt) at 30 min, and were killed at times up to 90 min. In the varying specific radioactivity protocol, the zero time injectate also contained additional unlabelled diprenorphine at six concentrations such that the total dose of diprenorphine ranged from 8 to 1,154 pmol/g body wt. These rats were killed at 30 min. In all studies, up to five blood samples (200 μl each) were withdrawn from each rat at various times up to death and aliquots of blood and plasma taken for radioactive counting.

Rats were killed by intravenous injection of Expiral (Ceva Ltd., Watford), the brains were rapidly removed, and ROIs were dissected as described for the in vitro experiments. Samples of brain tissue from individual rats were weighed, solubilised in Soluene-350 (Packard, Groningen; 0.5 ml), and assayed for radioactivity in scintillant (10 ml) containing 1% acetic acid to suppress chemiluminescence.

All data were normalised for precise dose and body weight before further calculation and for presentation purposes are expressed as units of uptake, defined as: (dpm per gram wet weight tissue)/(dpm injected per gram body weight).

## Kinetic analyses

Compartmental analyses of the time course of labelled diprenorphine in brain regions in vivo were based on the model illustrated in Fig. 1. The model is derived from those summarised by Huang et al. (1986) and includes a

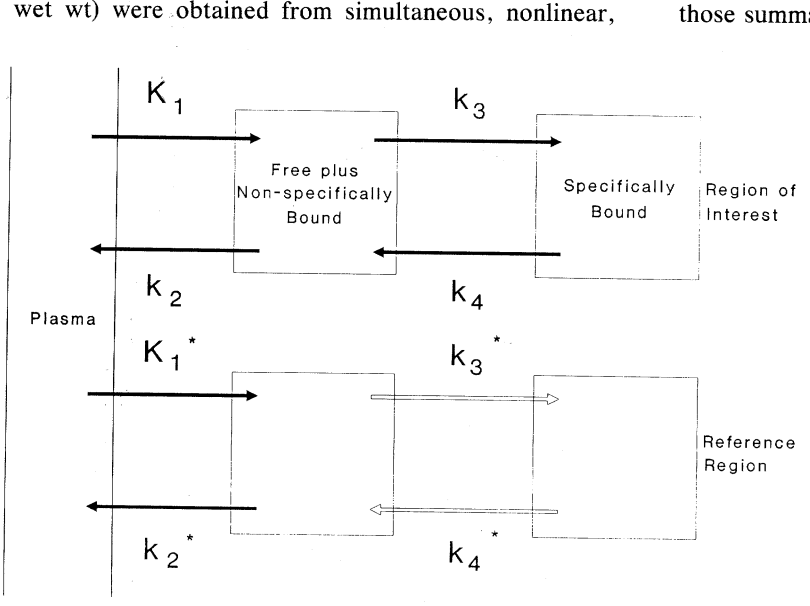


**FIG. 1.** Compartmental model incorporating reference region. The definitions of the rate constants are given in Methods under Kinetic Analyses.

modification of the reference tissue approach described by Blomqvist et al. (1989). Sadée et al. (1982) have shown that >90% of label in brain can be accounted for by the parent ligand 60 min after intravenous injection of labelled diprenorphine. It is therefore assumed that only the parent ligand, and not its labelled metabolites, can cross the blood–brain barrier. Within the ROI, two compartments are distinguished: the first comprising free and nonspecifically bound ligand, which are assumed to be in rapid equilibrium with each other, and the second deriving from specific ligand binding sites within the region. It is assumed (initially) that specific binding sites are absent from the reference region. The validity of these assumptions and the sensitivity of the analysis to them will be examined later.

Total accumulation of label in the ROI ($C_{tot}$; dpm/g) as a function of time ($t$; min) is then described by

$$c_{tot}(t) = c_f(t) + c_b(t) \quad (1)$$

$$\frac{dC_f(t)}{dt} = K_1C_p(t) - (k_2 + k_3)C_f(t) + k_4C_b(t) \quad (2)$$

$$\frac{dC_b(t)}{dt} = k_3C_f(t) - k_4C_b(t) \quad (3)$$

and that in the reference region ($c_{ref}$; dpm/g$^{-1}$) by

$$\frac{dC_{ref}(t)}{dt} = K_1{}^*C_p(t) - k_2{}^*C_{ref}(t) \quad (4)$$

where $C_p$ (dpm/ml) is the concentration of parent ligand label in plasma (the plasma input function); $C_f$ (dpm/g) is the concentration of label in the combined free plus nonspecifically bound pool of the ROI; $C_b$ (dpm/g) is the concentration of label in the specifically bound pool; $K_1$ and $K_1{}^*$ (ml/g/min) are the unidirectional clearances of ligand from plasma for ROI and reference region, respectively; $k_2$ and $k_2{}^*$ (min$^{-1}$) are the efflux rate constants of the combined free plus nonspecifically bound pool in ROI and reference region, respectively; $k_3$(min$^{-1}$) = $k_{on}(B_{max} - C_b/SA)$, where $k_{on}$(min$^{-1}$/[pmol/g]) is the apparent second-order association rate constant; $B_{max}$ (pmol/g) is the maximum specific binding capacity of the ROI; and $SA$ (dpm/pmol) is the specific activity of the labelled ligand.

Thus, $k_3$ is a time-dependent pseudo-first-order forward rate constant when nontracer doses of the labelled ligand with finite specific activity are present in the system. $k_4$(min$^{-1}$) is the first-order dissociation rate constant of the receptor-ligand complex in the specifically bound pool.

The principal advantage of this model is that it allows the removal of the plasma input function term in the differential equations (see below) and hence avoids the need to separate and quantify the labelled parent ligand in the plasma over the time course of the experiments, when significant peripheral metabolism is occurring. Perry et al. (1980) report up to 50% of label in metabolites in plasma 60 min after intravenous injection of labelled diprenorphine in rats, although at this time >90% of the label in brain tissue was accounted for by the parent ligand (Sadée et al., 1982).

Rearrangement of Eq. 4 gives the unknown $C_p(t)$ as a function of the observed time course of label in the reference region:

$$C_p(t) = \frac{dC_{ref}(t)/dt}{K_1{}^*} + \frac{C_{ref}(t) \cdot k_2{}^*}{K_1{}^*} \quad (5)$$

Blomqvist et al. (1989) suggest supplementary measures of $K_1{}^*$ and $k_2{}^*$ as a means of deriving the unknown $C_p(t)$. A simpler implementation of the reference tissue technique may however be applied with the additional assumption that

$$K_1{}^*/k_2{}^* = K_1/k_2 \quad (6)$$

i.e., that the ratio of influx to efflux rate constants (but not their absolute values) is the same in the ROI as in the reference region (see Discussion).

Substitution of Eq. 6 into Eq. 5 with rearrangement gives

$$K_1C_p(t) = R_{influx}\frac{dC_{ref}(t)}{dt} + k_2C_{ref}(t) \quad (7)$$

where $R_{influx}$ is the influx ratio $K_1/K_1{}^*$, which expression may in turn be substituted into Eq. 2. Thus, if the time course of label in a reference region is known, then that in the ROI may be described by five parameters: $R_{influx}$, $k_2$, $k_{on}$, $B_{max}$, and $k_4$.

## Fitting of data

The total data set for each region consisted of the time courses of label in tissue in the tracer alone and pulse chase protocols, plus label in tissue 30 min after intravenous administration of tracer over a range of specific activities, as described above. This total data set was used to obtain simultaneous estimates of the five model parameters ($R_{influx}$, $k_2$, $k_{on}$, $B_{max}$, and $k_4$) on the basis of a nonlinear least-mean-squares fit of the model to data from each region, using the tracer alone time course of label in the cerebellum as the reference function in each case. It was assumed that the same reference function applied for all protocols and that there was no specific binding in cerebellum. (The sensitivity of the analysis to specific binding in the reference region will be considered in the next section.) Furthermore, the same set of five parameters was assumed to apply for all protocols. This allows for data from all three protocols to contribute simultaneously to the estimates of the parameters. Any given set of parameter estimates was compared with data from the three protocols on the basis of the above basic model as follows:

The time course of label for tracer alone in cerebellum was first fitted to an arbitrary expression involving a sum of exponential terms that gave a good description of its shape (see Results). This expression and its derivative were then used in Eq. 7 and substituted into Eq. 2. Equations 2 and 3 are the two differential equations comprising the model. These equations were integrated numerically using a fourth-order Runge–Kutta scheme with the five parameters as variables (unless otherwise specified). For the varying specific activity protocol, the equation set was as given above. When comparing fits with data from the tracer alone protocol, $C_b/SA$ was set to zero during integration so that $k_3 = k_{on} \cdot B_{max}$. This was also done during comparison with data from the pulse chase protocol. In addition, the delivery of the displacing dose was modelled by including a delay from the time of injection of the pulse chase after which $k_3$ was set to zero (see below). In this way a residual sum of squares for the total

regional data set was obtained for any given set of parameters. Iterative minimisation of the residual sum of squares was obtained by varying the parameter set using the Simplex method (Nelder and Mead, 1965) or a derivative-free nonlinear regression technique (Ralston, 1983). The inclusion of a delay in the model for the pulse chase protocol is an approximation to the time-dependent process of saturation of specific binding sites. In supplementary work the effect of delays between 0 and 6 min on the residual sum of squares was investigated. In all regions, a minimum residual was obtained with delays between 2.5 and 3.5 min. The delay was therefore fixed at 3 min for all regions in all the fits presented in this article. The dose of cold diprenorphine used (470 pmol/g body wt) was sufficient to cause maximal displacement of the label (see Fig. 5).

### Correction for specific binding in the reference region

The compartmental model described above involves the assumption of negligible specific binding in the reference region. The sensitivity of the analysis to this assumption was examined as follows:

When specific reference binding occurs and the labelled ligand is present in trace amounts, then

$$C_{ref}(t) = C_f^*(t) + C_b^*(t) \quad (8)$$

$$\frac{dC_b^*(t)}{dt} = k_3^* C_f^*(t) - k_4^* C_b^*(t) \quad (9)$$

where $C_f^*$ is the concentration of the combined free plus nonspecifically bound tracer in the reference region and $k_3^*$ and $k_4$ are turnover rate constants of specifically bound tracer in the reference region. Equations 8 and 9 may be solved to give

$$C_b^*(t) = k_3^* C_{ref}(t) \otimes \exp[-(k_3^* + k_4^*)t] \quad (10)$$

where "$\otimes$" denotes convolution. The time course of the total tracer in the reference tissue is given by

$$\frac{dC_{ref}(t)}{dt} = K_1^* C_p(t) - k_2 C_f^*(t) \quad (11)$$

Equations 8–11, together with the assumption expressed in Eq. 6, may then be algebraically rearranged to give an expression analogous to Eq. 7, in which the unknown plasma input function term for the ROI may be replaced by a function of the observed time course of tracer in the reference region:

$$K_1 C_p(t) = R_{influx}\left[\frac{dC_{ref}(t)}{dt}\right] + k_2[C_{ref}(t) - C_b^*(t)] \quad (12)$$

Equation 12 reduces to Eq. 7 when $k_3^* = 0$.

A full sensitivity analysis would require an investigation of the behaviour of the model and the five derived ROI parameters over a range of fixed values for both reference tissue rate constants $k_3^*$ and $k_4^*$. To simplify this, we may take advantage of the observation (see Results) that the rate constants $k_{on}$ and $k_4$ show insignificant interregional variation, and therefore assume that the specific binding characteristics in the reference region are the same, except for total capacity, as those in the ROI. Thus, if, under tracer conditions,

$$k_3^* = k_{on} \cdot B_{max}^* \quad (13)$$

and

$$k_4^* = k_4 \quad (14)$$

where the asterisk denotes the reference region and $k_{on}$ and $k_4$ refer to the ROI as above, then the equation set is simplified and again involves the five variables $R_{influx}$, $k_2$, $k_{on}$, $k_4$, and $B_{max}$ for the ROI, together with one fixed parameter, $B_{max}^*$, summarising the specific binding capacity of the reference tissue. Sensitivity analyses were therefore carried out with the constraints given in Eq. 13 and 14 over a range of fixed values for $B_{max}^*$.

## RESULTS

### Binding parameters in vitro

Representative binding isotherms under equilibrium conditions in vitro in the presence and absence of naloxone, together with lines of best fit, are shown in Fig. 2 for thalamus. Parameter estimates for the eight individual regions dissected are summarised in Table 1.

There was significant regional variation in $B_{max}$, with the highest value (43 pmol/g wet wt tissue) in thalamus and with a small but significant saturable component (1.3 pmol/g wet wt tissue) in cerebellum. There was no significant regional variation in $K_d$ (overall mean, 0.198 n$M$) or in $K_{ns}$ (overall mean, 2.74 ml/g wet wt tissue). Sadée et al. (1982) have previously reported values in whole-brain homogenates for $K_d$ of 0.13–0.16 n$M$ and for $B_{max}$ of 30 pmol/g.

### Transport and binding parameters in vivo

The time course of total label in the reference region, cerebellum, after injection of tracer alone is shown in Fig. 3. Following pulse chase administration of unlabelled diprenorphine, there was a small but significant displacement of label from cerebel-

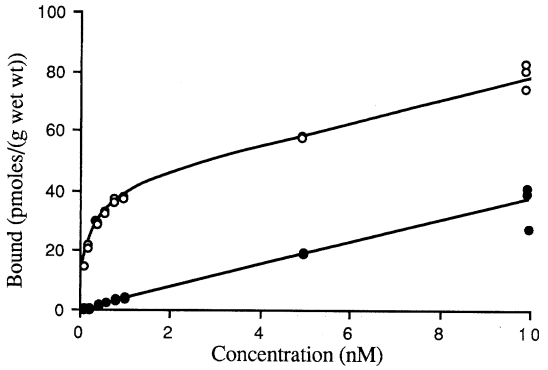


**FIG. 2.** Binding of [$^3$H]diprenorphine to a homogenate of thalamus in vitro in the presence (●) and absence (○) of naloxone at 37°C, pH 7.4. The data are from one of four replicate studies and the lines of best fit correspond to $B_{max}$ = 41.2 pmol/g wet wt, $K_d$ = 0.162 n$M$, and $K_{ns}$ = 3.78 ml/g wet wt.

TABLE 1. *Binding parameters in vitro*

| Region | $B_{max}$ (pmol/g wet wt) | $K_d$ (n*M*) | $K_{ns}$ (ml/g wet wt) |
|---|---|---|---|
| Thalamus | 43.0 ± 5.4 | 0.156 ± 0.030 | 3.45 ± 0.62 |
| Striatum | 36.3 ± 3.8 | 0.184 ± 0.060 | 3.67 ± 1.32 |
| Anterior cortex | 27.7 ± 2.4 | 0.238 ± 0.044 | 2.38 ± 0.84 |
| Posterior cortex | 22.9 ± 1.8 | 0.239 ± 0.044 | 2.26 ± 0.60 |
| Hippocampus | 19.6 ± 4.0 | 0.168 ± 0.056 | 2.08 ± 0.52 |
| Medulla | 18.5 ± 2.2 | 0.179 ± 0.026 | 3.12 ± 0.72 |
| Spinal cord | 13.3 ± 2.4 | 0.174 ± 0.058 | 2.48 ± 0.76 |
| Cerebellum | 1.3 ± 0.2 | 0.247 ± 0.138 | 2.48 ± 0.28 |

Values are means ± SD of parameters obtained from individual fits to four binding isotherms, measured at 37°C, pH 7.4, as described in Methods.

lum. The corresponding data sets for the thalamus are illustrated in Figs. 4 and 5. Figure 4 shows the retention of tracer and its marked displacement by pulse chase. Figure 5 shows the titration of label in the tissue at 30 min as a function of the coinjected dose of cold diprenorphine. There were no discernible differences in the time profiles of total $^3H$ counts in plasma between the three studies (data not shown).

Total data sets for the three experimental protocols were fitted simultaneously to the compartmental model described in Methods. It was assumed initially that the reference region contained no specific binding sites. Thus, the reference input function was as shown in Fig. 3, i.e., the fit to the time course of tracer alone in cerebellum. The algebraic form of this function was chosen to give a good description of the reference tracer time course and its component parameters have no physiological interpretation. This approach allowed the estimation of $R_{influx}$ ($K_1/K_1^*$), $k_2$, $B_{max}$, $k_{on}$, and $k_4$ in the seven

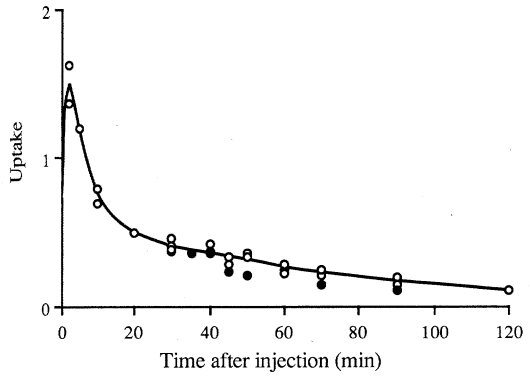


**FIG. 3.** Time course of total label in cerebellum following intravenous injection of trace amounts of [$^3$H]diprenorphine (○). At 30 min, seven rats were given a bolus intravenous injection of cold diprenorphine (200 μg/kg body wt; ●). The line of best fit is of the form $p_1(e^{-p_2t} - e^{-p_3t}) + p_4(e^{-p_5t} - e^{-p_6t})$ with $p_1 = 0.600$, $p_2 = 0.014$, $p_3 = 1.82$, $p_4 = 1.59$, $p_5 = 0.199$, and $p_6 = 1.22$. Each datum point represents a separate animal.

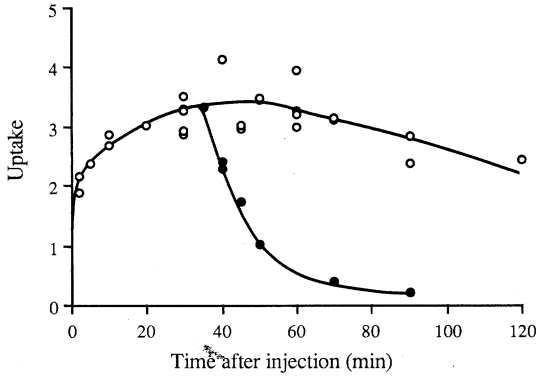


**FIG. 4.** Time course of uptake in the thalamus after intravenous bolus injection of trace amounts of [$^3$H]diprenorphine, with (●) or without (○) a bolus pulse chase of cold diprenorphine at 30 min. The lines of best fit were derived from the compartmental model assuming negligible specific binding in the reference region, cerebellum. The parameters of the fit are given in Table 2.

discrete brain regions. The fits to the data for thalamus are shown in Figs. 4 and 5. Similar fits were obtained for all regions dissected and parameter estimates are summarised in Table 2.

Regional estimates of $B_{max}$ obtained on the basis of this model in vivo (Table 2) varied over a wide range and may be compared with those obtained from the equilibrium binding study in vitro described above (Table 1). In general, estimates of $B_{max}$ obtained in vivo were higher than those obtained in vitro, and a bias was apparent in the relationship with the in vivo/in vitro ratio increasing with increasing specific regional binding capacity.

Estimates of both the second-order apparent association rate constant, $k_{on}$, and the first-order dissociation rate constant, $k_4$, showed no significant interregional variation, with overall mean values of

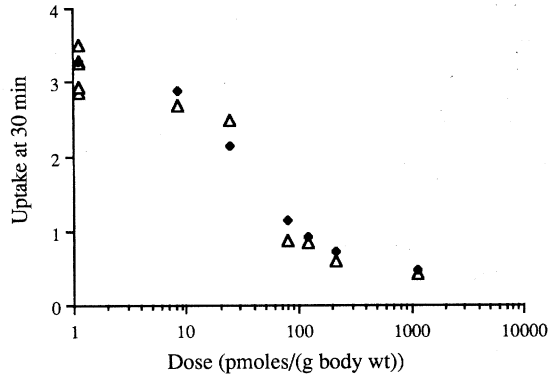


**FIG. 5.** Uptake in thalamus 30 min after intravenous bolus injection of [$^3$H]diprenorphine in a range of specific activities (△) (see the third protocol in Methods). The corresponding best-fit values (◆) were obtained from the compartmental model assuming negligible specific binding in the reference region, cerebellum. The parameters of the fit are given in Table 2.

TABLE 2. *Transport and binding parameters in vivo*

| Region | $R_{influx}$ | $k_2$ ($min^{-1}$) | $k_{on}$ ($min^{-1}$/[pmol/g]) | $k_4$ ($min^{-1}$) | $B_{max}$ (pmol/g) | CBF (ml/min/g) |
|---|---|---|---|---|---|---|
| Thalamus | 1.25 ± 0.14 | 0.247 ± 0.023 | 0.0155 ± 0.0045 | 0.112 ± 0.020 | 72.1 ± 15.0 | 1.02 |
| Striatum | 1.13 ± 0.10 | 0.228 ± 0.018 | 0.0153 ± 0.0037 | 0.124 ± 0.019 | 61.4 ± 10.1 | 0.97 |
| Anterior cortex | 1.27 ± 0.11 | 0.313 ± 0.040 | 0.0173 ± 0.0047 | 0.111 ± 0.019 | 37.0 ± 7.4 | 1.15 |
| Posterior cortex | 1.30 ± 0.11 | 0.308 ± 0.050 | 0.0226 ± 0.0073 | 0.119 ± 0.025 | 24.7 ± 5.6 | 1.08 |
| Hippocampus | 0.93 ± 0.07 | 0.198 ± 0.014 | 0.0275 ± 0.0063 | 0.154 ± 0.024 | 31.0 ± 4.8 | 0.82 |
| Medulla | 1.15 ± 0.09 | 0.247 ± 0.031 | 0.0203 ± 0.0014 | 0.120 ± 0.023 | 28.6 ± 5.8 | 0.83 |
| Spinal cord | 0.80 ± 0.09 | 0.209 ± 0.042 | 0.0184 ± 0.0078 | 0.090 ± 0.027 | 18.0 ± 5.1 | — |

Estimates (± SEM) were derived from compartmental analysis (see Fig. 1) assuming negligible specific binding in the reference region, cerebellum. CBF values were taken from Hargreaves et al. (1986) with haematocrit of 40% (CBF cerebellum, 0.80 ml/min/g).

0.0196 $min^{-1}$ pmol/g and 0.119 $min^{-1}$, respectively (Table 2). The relationship between these parameters in vivo and the equilibrium half-saturation constant, $K_d$, as measured in vitro will be considered in Discussion. The regional variation in both $R_{influx}$ and $k_2$ most probably reflects relative differences in blood flow between regions. Independent estimates of regional CBF, taken from Hargreaves et al. (1986), are also shown in Table 2. There was significant interregional correlation of both $R_{influx}$ ($r$ = 0.83, $p < 0.05$) and $k_2$ ($r$ = 0.84, $p < 0.05$) with CBF.

### Correction for specific binding in the reference region

Evidence in vitro (Table 1) and in vivo (Fig. 3) indicated a small but significant specific binding component in cerebellum. It was therefore of interest to examine the sensitivity of the present analysis to this factor and, if necessary, to apply a suitable correction to the regional transport and binding parameters for diprenorphine. For given values of specific binding in the reference region ($B_{max}$*), the total data sets for the ROIs were reanalysed as described in Methods. Since there was a tendency for estimates of $B_{max}$ obtained in vivo to be higher than those obtained in vitro, the in vivo ROI data were reanalysed with values for $B_{max}$* equal to, and double, the in vitro value of 1.3 pmol/g (Table 1). There were no significant changes in goodness of fit of the model to the data in either case.

The resulting estimates of the binding parameters $k_{on}$, $B_{max}$, and $k_4$ are given in Table 3. These results show that failure to take into account specific binding in the reference region, if present, would lead to an underestimation of $B_{max}$ in the ROI, and that this underestimation is approximately constant over the range of specific binding capacities throughout the regions (~7 and 15 pmol/g in the two cases considered).

For $k_{on}$, the effect was negligible in the high binding thalamus, but more marked as specific binding decreased. The mean ± SD regional values for $k_{on}$ were 0.0196 ± 0.0044, 0.0176 ± 0.0032, and 0.0162 ± 0.0027 $min^{-1}$ pmol/g for $B_{max}$* values of 0, 1.3, and 2.6 pmol/g, respectively. For $k_4$, the effect was again approximately constant across regions, with mean ± SD values of 0.119 ± 0.019, 0.100 ± 0.015, and 0.090 ± 0.013 $min^{-1}$ for $B_{max}$* values of 0, 1.3, and 2.6, respectively.

As noted above, a bias was apparent in the relationship between in vivo/in vitro $B_{max}$ ratios when no correction for specific binding in the reference tissue was made. The relationship between $B_{max}$ values obtained in vitro and those obtained in vivo, after correction for specific binding in the reference region of 1.3 pmol/g, is illustrated in Fig. 6. Regres-

TABLE 3. *Sensitivity of estimates of binding parameters in vivo to specific binding in reference tissue*

| | $B_{max}$* (pmol/g wet wt) | | |
|---|---|---|---|
| | 0 | 1.3 | 2.6 |
| $B_{max}$ (pmol/g wet wt) | | | |
| Thalamus | 72.1 | 79.6 | 90.0 |
| Striatum | 61.4 | 67.2 | 75.3 |
| Anterior cortex | 37.0 | 43.1 | 51.3 |
| Posterior cortex | 24.7 | 31.7 | 39.7 |
| Hippocampus | 31.0 | 38.3 | 47.2 |
| Medulla | 28.6 | 36.5 | 45.4 |
| Spinal cord | 18.0 | 24.9 | 33.1 |
| $k_{on}$ ($min^{-1}$/pmol/g) | | | |
| Thalamus | 0.0155 | 0.0156 | 0.0158 |
| Striatum | 0.0153 | 0.0150 | 0.0146 |
| Anterior cortex | 0.0173 | 0.0168 | 0.0162 |
| Posterior cortex | 0.0226 | 0.0203 | 0.0185 |
| Hippocampus | 0.0275 | 0.0236 | 0.0209 |
| Medulla | 0.0203 | 0.0174 | 0.0154 |
| Spinal cord | 0.0184 | 0.0148 | 0.0124 |
| $k_4$ ($min^{-1}$) | | | |
| Thalamus | 0.112 | 0.099 | 0.089 |
| Striatum | 0.124 | 0.108 | 0.096 |
| Anterior cortex | 0.111 | 0.097 | 0.088 |
| Posterior cortex | 0.119 | 0.101 | 0.092 |
| Hippocampus | 0.154 | 0.123 | 0.107 |
| Medulla | 0.120 | 0.101 | 0.090 |
| Spinal cord | 0.090 | 0.072 | 0.065 |

Estimates were derived from compartmental analysis of data in vivo (see Fig. 1) assuming specific binding in the reference region, cerebellum, of $B_{max}$*. The standard errors of the estimates are all close to the equivalent data in Table 2.

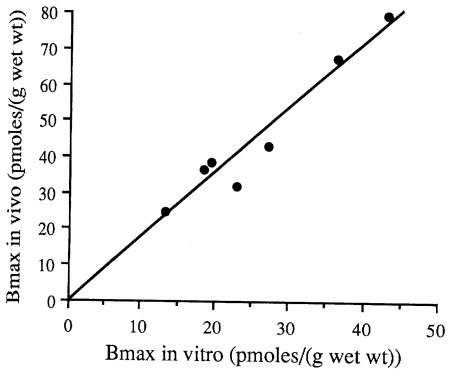


**FIG. 6.** Regression of estimates of regional $B_{max}$ values obtained in vivo assuming a specific binding capacity of 1.3 pmol/g wet wt in the reference region on estimates obtained in vitro.

sion of in vivo estimates on in vitro estimates showed a highly significant correlation ($r = 0.969$, $p < 0.001$), with the regression intercept passing close to the origin; i.e., the apparent bias has disappeared.

### Omission of varying specific activity protocol in the rat

The total data from the rat were obtained from a combination of three protocols. The third protocol, namely, varying the specific activity of the injected labelled diprenorphine, serves to distinguish the $k_{on}$ and $B_{max}$ components of the forward rate constant [$k_{on}$ ($B_{max} - C_b/SA$); see Kinetic Analyses] and hence the apparent in vivo equilibrium dissociation constant, $K_d$ (see Discussion). In the absence of these data, when only tracer alone and pulse chase data are available, only the product $k_{on} \times B_{max}$ may be obtained, with the number of variable parameters in the fit being reduced to four. Since our current studies in humans involve only tracer alone and pulse chase protocols, it was of interest to reexamine the data from the rat in vivo, omitting data from the third protocol. This also excludes the application of the correction for specific binding in the reference region. The regional estimates of binding potential so obtained are summarised in Table 4, where they are compared with those calculated from the analysis of the complete data set with a correction for specific binding in the reference region of 1.3 pmol/g wet wt. Omission of the varying specific activity data led to lower estimates of $k_{on} \cdot B_{max}$ of between 6 and 14%.

We were unable to obtain satisfactory fits for analysis of tracer alone data. Analysis of the second protocol alone (i.e., pulse chase) gave unacceptably large errors in the parameter estimates.

**TABLE 4.** *Effect of omitting the varying specific activity protocol on regional estimates of* $k_{on} \cdot B_{max}$

| Region | Tracer alone and pulse chase only | All data |
|---|---|---|
| Thalamus | 1.11 ± 0.21 | 1.24 |
| Striatum | 0.93 ± 0.18 | 1.10 |
| Anterior cortex | 0.65 ± 0.11 | 0.72 |
| Posterior cortex | 0.56 ± 0.14 | 0.64 |
| Hippocampus | 0.85 ± 0.12 | 0.90 |
| Medulla | 0.58 ± 0.12 | 0.64 |
| Spinal cord | 0.34 ± 0.12 | 0.37 |

The trace alone and pulse chase data sets were fitted in terms of four parameters including the composite rate constant $k_{on} \cdot B_{max}$ as described in Results. The corresponding estimates when all available data are taken into account were derived from the values given in Table 3 with a correction for specific binding in the reference region of 1.3 pmol/g wet wt. Data are means ± SEM.

## DISCUSSION

This work in the rat was carried out as part of a validation study, with a view to the application of compartmental analyses to dynamic PET data obtainable in humans (Jones et al., 1988). The saturable uptake of diprenorphine, its marked displacement by pulse chase in the rat in vivo, and the relationship of these phenomena to receptor binding in vitro have previously been described by Perry et al. (1980) and Sadée et al. (1982). Emphasis here has been placed on experimental protocols that are more directly comparable with those feasible in PET, i.e., those that involve intravenous administration of the label and the examination of the uptake and binding over relatively short times, when it is important to distinguish between tracer delivery to the tissue and binding in the tissue. The compartmental model is based on the use of a reference tissue to avoid the metabolite correction necessary when a plasma time-activity curve is used as the input function. We have therefore analysed the data regionally and made direct comparisons with estimates of binding parameters obtained from in vitro studies.

The use of full time-activity curves in dynamic studies represents the most efficient use of data available from PET, but the estimates so obtained, compared with equilibrium studies, are more sensitive to the simplifying assumptions on which the model is based (Huang et al., 1986). The present reference tissue model does not assume that the rate of specific binding is small relative to the transport rates across the blood–brain barrier and furthermore can allow for, and hence examine the sensitivity to, assumptions regarding the degree of specific binding in the reference region. It is, however, assumed that equilibrium between free and nonspecifically bound ligand is sufficiently rapid to allow them to be treated as a single compartment. Identity of transport rates in the ROIs and in the reference region is not required, although it is assumed that

the ratio of influx and efflux rate constants is the same in all regions. This assumption leads to a major simplification of the equations relating the behaviour of the tracer in the reference region to that in the ROI (see Eq. 7), giving rise to the parameter $R_{influx}$. Whilst this assumption has not been experimentally validated, it is justifiable since both $K_1$ and $k_2$ are dependent on regional blood flow and on the blood–brain barrier permeability–surface area product. Interregional differences in either or both of these parameters might therefore be expected to affect both $K_1$ and $k_2$ and maintain the ratio. The observed correlation of influx relative to the reference ($R_{influx}$) and $k_2$ with independent measures of blood flow lends support to this assumption.

Estimates of the regional maximum specific binding capacities in the rat in vivo correlated well with those obtained under equilibrium conditions in vitro, but estimates in vivo were higher than those in vitro and a bias was apparent. Loss of binding sites may have occurred during the preparation of the tissue homogenates in vitro. A decrease in the number of diprenorphine binding sites on increasing dilution of untreated brain homogenates was reported by Sadée et al. (1982), although this was apparent at much greater dilutions than used here.

A further consideration is that the naloxone-sensitive binding sites as determined in vitro may not be fully representative of the total saturable diprenorphine binding sites in the tissue, the latter being defined in vivo by a diprenorphine pulse chase and by various specific activities of diprenorphine. However, the simultaneous fitting procedure applied to the in vitro data takes into account both the slope of the isotherm in the presence of naloxone and the limiting slope of the isotherm in the absence of naloxone, which were virtually identical (see, e.g., Fig. 2), which argues against this consideration.

The mean regional value for $k_4$ in the rat in vivo was 0.10 $min^{-1}$, which corresponds to a half-life of 6.9 min. This value compares with dissociation rate constants determined in vitro at 37°C by Sadée et al. (1982) who report 20–30 min in buffer alone and 10 min in the presence of 100 m*M* NaCl.

The rate constant $k_{on}$, as estimated from compartmental analysis of data in vivo, is referred to as the "apparent" second-order association rate constant for two reasons: First, it is applied to the compartment representing both free and nonspecifically bound ligand in rapid equilibrium, rather than to the free ligand alone; and second, the true physical volume (ml/g tissue) occupied by the free ligand is not defined. These considerations are necessary if the fractional rate constants and label per gram of tissue, in which the present model is formulated, are to be related to the effective concentration of free ligand at the binding site (Mintun et al., 1984). If the free fraction of the combined pool is denoted $f_2$ and physical volume $V_2$ (see notation of Mintun et al., 1984), then the association rate constant applicable to the concentration of free ligand in this volume ($k_a$; $min^{-1}/nM$) is given by

$$k_a = \frac{k_{on} \cdot V_2}{f_2}$$

Thus, given estimates of $f_2$ and $V_2$, the association and dissociation rate constants in vivo may then be related to a corresponding equilibrium dissociation constant, $K_d$ (n*M*):

$$K_d = k_4/k_a$$

The present results give a mean value for the nonspecific binding constant in tissue homogenates in vitro ($K_{ns}$) of 2.74 ml/g wet wt tissue. If a similar partition between free and nonspecifically bound ligand pertains in vivo, then an estimate of the free fraction of the combined free plus nonspecifically bound compartment (Fig. 2) may be made. Under the simplest of assumptions, in which the free ligand is contained in the tissue water space (0.76 ml/g; Mintun et al., 1984), the corresponding value of $f_2$ is 0.76/(2.74 + 0.76) = 0.22. The corresponding estimate of $K_d$ in vivo, based on the mean values for $k_{on}$ and $k_4$ (see Results), is 1.59 n*M*. This estimated value for $K_d$ in vivo is thus about an order of magnitude greater than that obtained under equilibrium binding conditions in vitro (0.198 n*M*).

Several factors may contribute to this discrepancy. Amongst those arising from a necessarily simplified compartmental analysis is the possibility of local diffusional gradients around the specific binding sites. This phenomenon, which would be manifest in dynamic studies as opposed to studies made under equilibrium conditions, could arise if reassociation of the ligand with the binding site was occurring rapidly relative to its rate of mixing with the total free pool. Perry et al. (1980) raised this possibility specifically in the case of diprenorphine binding in vivo and included an extra compartment to distinguish reassociating ligand from the bulk of the free pool. We have investigated this possibility in a preliminary analysis with data in rats collected under the tracer alone protocol (Jones et al., 1989) and with the present data from the three protocols. Briefly, an additional pool was added to the compartmental model communicating with the free plus nonspecifically bound compartment with rate constants $k_{in}$ and $k_{out}$ (see notation of Perry et al., 1980)

and communicating with the bound pool with rate constants $k_3$ and $k_4$. The total data sets were then refitted for the original five variables together with $k_{in}$ as a sixth variable and with $k_{out}$ set at fixed values. The inclusion of this local rapidly reassociating fraction of free ligand in the compartmental model affected principally the estimate of $k_{on}$. The remaining variables were little changed and there were no significant differences in the residual sum of squares ($F$ test). Thus, for example, in thalamus, where the ratio $k_4/k_{on}$ was 7.2 pmol/g in the absence of a local pool (data from Table 2), inclusion of the extra compartment with $k_{out}$ set at 10 $min^{-1}$ (Perry et al., 1980) led to a value for $k_4/k_{on}$ of 0.74. Although this value is an order of magnitude less, it cannot necessarily be interpreted as indicating the in vivo $K_d$ to be correspondingly less since account should also be taken of the physical volume occupied by the reassociating fraction. The fitted value for $k_{in}$ this particular case was 1.05 $min^{-1}$. The small apparent distribution volume of the reassociating fraction relative to the remaining free pool ($k_m/k_{out}$ = 0.105) is consistent with a correspondingly smaller physical volume. Thus, the change in the apparent second-order rate constant $k_{on}$ (applicable to concentrations expressed per gram of tissue) could equally well reflect a smaller physical distribution volume (and hence higher concentration in nanomolar terms) of the reassociating pool. Although diffusional gradients may contribute to the discrepancy between the in vitro and in vivo $K_d$ reported here, these figures based on compartmental approximations are not a sufficient explanation. Zeeberg et al. (1988) have also considered the effects of diffusional gradients and heterogeneity of receptor distribution and conclude that such effects may contribute to in vivo/in vitro discrepancies. The suitability of diffusion models for the analysis of ligand binding in vivo is currently being investigated.

In conclusion, the present study establishes experimentally a kinetic model using a reference tissue, which adequately describes the behaviour of diprenorphine in vivo. Restriction of the data to tracer alone and displacement protocols gave estimates of the forward rate constant that correlate with interregional variation of $B_{max}$, indicating that kinetic analysis of dynamic tracer studies can be used to give an adequate description of regional binding capacity. The results obtained in rat would indicate that with use of this combination of two protocols (i.e., tracer alone and pulse chase), as is feasible within the constraints of PET, the model could be applied to describe satisfactorily the regional distribution of opioid receptor sites in humans.

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