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Chapter 8

IDENTIFICATION OF THE OPERATIONAL MODEL OF AGONISM FOR THE EEG EFFECTS OF OPIOIDS: ESTIMATION OF THE *IN VIVO* AFFINITY AND INTRINSIC EFFICACY AT THE μ -OPIOID RECEPTOR

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ABSTRACT

The objective of the current study was to identify the operational model of agonism for the EEG effects of opioids. Unbound biophase concentration-EEG effect relationships of the opioids alfentanil, fentanyl, sufentanil, morphine, butorphanol and nalbuphine were simultaneously analysed with a) the Hill equation and b) the operational model of agonism.

Individual concentration-effect relationships were analysed with the Hill equation and showed that large differences in potency (EC_{50} range 0.22 – 1215 nM) and intrinsic efficacy (α range 0.11 – 1).

Subsequent analysis with the operational model of agonism was performed with the values of the system maximum E_m (123 μ V) and n (1.44) constrained to the values of alfentanil which displayed the highest intrinsic activity. The values of the *in vivo* affinity parameter pK_A ranged from 5.64 (morphine) to 9.15 (sufentanil) and of the efficacy parameter $\log \tau$ from 0.421 for alfentanil to -0.342 for nalbuphine. When the estimated *in vivo* pK_A values were correlated with the *in vitro* pK_i values, indications for two distinct subpopulations were obtained. In addition, a poor correlation was observed between the *in vitro* Na/GTP-shift and the *in vivo* $\log \tau$ indicating that the *in vitro* efficacy measures cannot predict the *in vivo* EEG effect. These observations might be explained by 1) the involvement of active transport processes in distribution from blood to biophase, 2) the existence of μ -opioid receptor subtypes and 3) the interaction with other types of opioid receptors.

INTRODUCTION

Mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) models contain specific expressions for processes on the causal path between drug administration and effect. This includes expressions to describe a) the pharmacokinetics in blood or plasma, b) the biophase distribution kinetics, which for CNS-active drugs includes blood-brain barrier transport (BBB) transport, c) target binding and activation and d) transduction (Danhof *et al.* 2007). Recent investigations on the PK-PD correlations of opioids (alfentanil, fentanyl, sufentanil, morphine, butorphanol and nalbuphine) have focused on the role of biophase distribution kinetics as a determinant of the time course of the EEG effect as a biomarker for μ -opioid receptor activation. A number of structurally different biophase distribution models have been proposed and these models have been successfully applied to derive the biophase concentration-EEG effect relationships of this wide range of opioids (Groenendaal *et al.*, 2007 – chapter 6, chapter 7). It has been shown that particularly for morphine the functionality of transporters at the BBB is a major determinant of the time-course of the EEG effect as a biomarker of μ -opioid receptor activation (Groenendaal *et al.*, 2007 – chapter 6).

The biophase concentration effect relationships of opioids have so far primarily been described on the basis of the sigmoidal E_{\max} pharmacodynamic model (Hill equation). Moreover, an analysis of the relationship between the *in vivo* pharmacodynamic parameters and the *in vitro* receptor binding characteristics has not been accomplished. In this respect it is important that although the Hill equation is useful for descriptive purpose, it provides only limited insight in the factors that determine the shape and the location of the concentration-effect relationships (van der Graaf *et al.* 1997). Specifically, the potency (EC_{50}) and intrinsic activity (E_{\max}) are functions of both compound (i.e. target affinity, intrinsic efficacy) and system (i.e. receptor density and signal transduction) characteristics. To fully understand the *in vivo* concentration-effect relationships, more mechanistic modelling approaches are needed to describe target binding and activation processes, including a clear distinction between drug-specific and biological system-specific properties (Danhof *et al.* 2005; 2007).

In the recent years, important progress has been made with the incorporation of receptor theory in PK-PD modelling for the prediction of *in vivo* concentration-effect relationships (van der Graaf & Danhof 1997b). Meanwhile, receptor theory has been successfully applied in the PK-PD analysis of adenosine A₁ receptor agonists (van der Graaf *et al.* 1997; 1999) benzodiazepines (Tuk *et al.* 1999; 2003; Visser *et al.* 2001), neuroactive steroids (Visser *et al.* 2002) and 5-HT_{1A} receptor agonists (Zuideveld *et al.* 2004). For the adenosine A₁ receptor agonists a good correlation was observed between the *in vivo* pK_A and the *in vitro* pK_i and also between the *in vivo* efficacy parameter τ and the *in vitro* GTP shift thus enabling the prediction of *in vivo* concentration-effect relationships. In addition, excellent *in vitro-in vivo* correlations have also been observed

for benzodiazepines and neuroactive steroids (Visser *et al.* 2002). In contrast, for the 5-HT_{1A} receptor agonists, despite a good correlation between *in vivo* efficacy parameter τ and the *in vitro* GTP shift, a rather poor correlation was found between the *in vivo* pK_A and the *in vitro* pK_i. This could in part be explained by complexities at the level of blood-brain distribution (Zuideveld *et al.* 2004).

So far, limited progress has been made with the incorporation of receptor theory in mechanism-based PK-PD models of opioids. For the opioids alfentanil, fentanyl and sufentanil, it has been shown by simulation that the concentration-effect relationships could be explained by the operational model of agonism under the assumption of a considerable receptor reserve (Cox *et al.* 1998). Moreover, after pre-treatment with the irreversible μ -opioid receptor antagonist β -funaltrexamine, a shift in the concentration-effect relationship of alfentanil was observed, which is consistent with the 40-60% reduction in the number of specific μ -opioid binding sites as shown in an *in vitro* receptor bioassay (Garrido *et al.* 2000). However, despite these efforts, a formal incorporation of receptor theory in a mechanism-based PK-PD model of opioids has not been accomplished. Complexities at the level of biophase distribution are presumably kinetics were an important factor in this respect.

The objective of the current study was to simultaneously analyse the biophase concentration-effect relationships of six opioids (alfentanil, fentanyl, sufentanil, butorphanol and nalbuphine), as obtained in a previous investigation (chapter 7), with the operational model of agonism. The relationships between the values of the drug-specific parameters receptor affinity (K_A) and intrinsic efficacy (τ), as determined with the operational model of agonism, and the estimates of the receptor affinity and intrinsic efficacy, as determined in an *in vitro* binding assay, are also analysed.

MATERIALS AND METHODS

In vivo PK-PD experiments

The details of the PK-PD experiments have been described previously (chapter 7). Briefly, these studies were conducted in male Wistar rats (Charles River, Maastricht, The Netherlands) weighing between 250 and 300 grams. Nine days prior to the experiments, seven cortical electrodes were implanted for continuous EEG monitoring. In addition, three/four indwelling cannulas were implanted, one in the right femoral artery for collection of serial blood samples and two in the left jugular vein (interna and externa) for opioid and midazolam administration. The fourth cannula was implanted into the right femoral vein to administer vecuronium bromide which was only required for the experiments with alfentanil, fentanyl, sufentanil and morphine. At the day of the experiments, after recording of the EEG baseline, the opioids or saline were administered in a zero-order infusion. The EEG signals were recorded up to a maximum of 360 minutes after start of the opioid infusions. An overview of the experimental groups is shown in table 1. During and after the infusion of the opioids, arterial blood

Table 1: Experimental design of the studies investigating the PK-PD relationships of the EEG effects of opioids in rats.

Compound	N	Dose (mg/kg)	Infusion time (min)	Body weight (kg)
Alfentanil ^a	7	3.14	40	0.278 ± 0.005
Fentanyl ^a	8	0.15	20	0.290 ± 0.012
Sufentanil ^a	7	0.03	40	0.297 ± 0.006
Morphine ^b	24	4	10	0.297 ± 0.003
	7	10	10	0.260 ± 0.006
	18	40	10	0.298 ± 0.006
Butorphanol ^b	7	2.5	10	0.284 ± 0.006
	6	5	10	0.260 ± 0.002
	6	10	10	0.254 ± 0.004
Nalbuphine ^b	6	5	10	0.275 ± 0.009
	9	10	10	0.273 ± 0.006
Saline ^b	6	n/a	10	0.290 ± 0.007

^aExperiments described previously by Cox *et al.* 1997

^bExperiments described previously by Groenendaal *et al.* 2007c

samples were collected to monitor arterial pH, pO₂ and pCO₂ levels. During and after administration of 40 mg/kg morphine, alfentanil, fentanyl and sufentanil, artificial ventilated was required to maintain arterial blood gas values within physiological limits. For determination of drug concentrations, serial arterial blood samples were collected at predetermined time intervals and immediately haemolysed with 0.5 ml of millipore water and stored at -20 °C pending analysis with gas chromatography (Cox *et al.* 1997), radio-immunoassay (Cox *et al.* 1998) or HPLC coupled to electrochemical detection (Groenendaal *et al.* 2005 - chapter 3). Changes in the amplitudes in the δ -frequency band of the EEG (0.5-4.5 Hz) averaged over 1-minute time intervals were used as a pharmacodynamic endpoint. Further reduction of the EEG data was performed by averaging the signals over predetermined time intervals.

Protein binding

Protein binding of morphine, nalbuphine and butorphanol was determined *ex vivo*, whereas for alfentanil, fentanyl and sufentanil literature values were used (Meuldermans *et al.* 1982). For determination of the degree of plasma protein binding, blood samples were collected and incubated with morphine, nalbuphine or butorphanol for 1 hour at 37 °C. The concentrations were 100, 1000 and 5000 ng/ml for nalbuphine and butorphanol and 250, 2500 and 25000 ng/ml for morphine. Blood was centrifuged and from the remaining plasma, the free fraction was isolated using ultra filtration (Centrifree, Millipore Corporation, Belford, MA).

In vitro receptor binding assays

Brain homogenates were prepared according to the method of (Lohse *et al.*1984). Briefly, Wistar rat brains (minus cerebellum and corpus striatum) were collected in 0.32 M sucrose solution and homogenized at 25 °C. The suspension was centrifuged for 10 minutes at 1000 rpm and the supernatant was collected. Next, the supernatant was centrifuged for 30 min at 31000 rpm at 4 °C and the remaining pellet was resuspended and centrifuged (15 minutes, 31000 rpm) twice in 50 mM Tris-HCl solution. The remaining pellet was resuspended in 20 ml Tris-HCl and aliquotted. The protein concentration in the stock-homogenate was 15 mg/ml, as determined with the Pierce Micro BCA assay (Pierce, Rockford, IL). Before the experiments, the brain homogenate was diluted to 1.5 mg/ml.

First, the μ -opioid receptor binding characteristics of the radioligand ^3H -naloxone (Amersham, specific activity 63 Ci/mmol), K_d and B_{max} , were determined in a saturation experiment. Brain aliquots of 100 μl were incubated with various concentrations (0.5 – 12 nM) of ^3H -naloxone at 25 °C. After 30 minutes, the incubation was stopped by adding 1 ml 50 mM Tris-HCl buffer of 4 °C and the samples were filtered through a presoaked glass fiber filter (Whatman GF/B) and eluted six times using 3 ml 50 mM Tris-HCl buffer of 4 °C. The filters were submerged in 3.5 ml Packard Ultima Gold scintillation fluid and radioactivity was measured for 5 minutes by a Hewlett Packard Tri-Carb 1500 liquid scintillation counter. Non-specific binding was determined by calculating the binding of ^3H -naloxone in the presence of 10^{-4} M fentanyl. Free radioligand concentrations were calculated by subtracting the non-specific binding from the total concentrations. In the displacement studies, the concentration of ^3H -naloxone was equivalent to the K_d value as determined in the saturation experiments in the presence of buffer.

Secondly, the μ -opioid receptor binding was determined by displacement of ^3H -naloxone. Brain homogenate aliquots of 100 μl were incubated with 2.5 nM ^3H -naloxone at various concentrations of the opioids (10^{-10} – 10^{-5} M). The experimental conditions were similar as described above with the exception of the number of elutions, which was three times in the displacement studies.

To investigate the agonistic character of the opioids, the receptor affinity of the opioids and ^3H -naloxone was determined in the presence of buffer, 100 mM NaCl or 1 mM GTP. Previously, it has been shown that the shift in K_i observed after incubation with a high concentration of sodium (100 mM) is a reflection of the agonist efficacy of the ligand (Pert & Snyder 1974). The sodium shift is expressed as the ratio between the K_i in the presence and absence of 100 mM NaCl. Another measure of efficacy is the GTP shift, which is ratio between the K_i in the presence and absence of 1 mM GTP (see (Kenakin 1996)). In each experiment, the binding characteristics were determined in buffer, 100 mM NaCl and 1 mM GTP to minimise inter-assay variability. All experiments were repeated three times and within an experiment duplicates were obtained.

Data analysis

Both the blood pharmacokinetics and the EEG effects of the opioids were analysed using non-linear mixed effect modelling as implemented in the NONMEM software version V, level 1.1 (Beal & Sheiner 1999). Population analysis was undertaken using the first-order conditional estimation method (FOCE interaction). All fitting procedures were performed on an IBM-compatible computer (Pentium IV, 1500 MHz) running under Windows XP with the Fortran Compiler Compaq Visual Fortran version 6.1.

Blood pharmacokinetics and biophase distribution analysis

The population analyses of the blood pharmacokinetics and the biophase distribution kinetics of the various opioids have been described previously (chapter 7). Briefly, the pharmacokinetics of alfentanil, morphine and nalbuphine were best described with a three-compartment model whereas a two-compartment model best described the pharmacokinetics of fentanyl, sufentanil and butorphanol. The biophase distribution kinetics of morphine was best described with the extended-catenary biophase distribution model, while for the other opioids the one-compartment distribution was preferred.

PK-PD analysis

The derived biophase concentrations were converted from ng/ml to nM and corrected for protein binding. The concentration effect relationships were then simultaneously analysed with the sigmoid E_{\max} model (Hill equation) using the following equation:

$$E = E_0 + \frac{E_{\max} \cdot \alpha \cdot C_{e,u}^{n_H}}{EC_{50,u}^{n_H} + C_{e,u}^{n_H}} \quad (1)$$

where E_{\max} is the maximum effect of the drug with highest intrinsic activity (alfentanil), while α is the fraction of the E_{\max} that can be reached by the opioid other the alfentanil; for alfentanil $\alpha=1$, $EC_{50,u}$ is the potency expressed as the unbound concentration, $C_{e,u}$ is the unbound biophase concentration and n_H is the Hill factor, describing the steepness of the concentration-effect relationships.

Inter-animal variability on E_{\max} or n_H (when applicable) was described with a additive error model according to equation:

$$P_i = P_{typ} + \eta_i \quad (2)$$

with

$$\eta_i \sim N(0, \omega^2) \quad (3)$$

where P_i is the individual value of the model parameter P , P_{typ} is the typical value (population value) of parameter P in the population, and η_i is inter-animal random variable.

The inter-animal variability on all other parameters was described with a log normal

distribution, using:

$$P_i = P_{typ} \cdot \exp(\eta_i) \quad (4)$$

with

$$\eta_i \sim N(0, \omega^2) \quad (5)$$

Inter-animal variability was investigated for each parameter and was fixed to zero when the MVOF did not improve. Correlations between the inter-animal variability of the various parameters were graphically explored. In addition, correlations between the PD parameters and dose and between the PD parameters and the co-infusion of GF120918 were also investigated graphically.

The residual error, which accounts for unexplained errors (such as measurement and experimental errors) in the EEG measurements, was best described with an additive error model according to equation:

$$C_{obs,ij} = C_{pred,ij} + \varepsilon_{ij} \quad (6)$$

where $C_{obs,ij}$ is the j -th observation of the i -th individual, $C_{pred,ij}$ is the predicted concentration and ε_{ij} is a realisation from the normally distributed residual random variable with mean zero and variance σ^2 :

$$\varepsilon_i \sim N(0, \sigma^2) \quad (7)$$

Next, the *in vivo* concentration-effect relationships were analysed according to the operational model of agonism (Black & Leff 1983):

$$E = E_0 + \frac{E_m \cdot \tau^n \cdot C^n}{(K_A + C)^n + \tau^n \cdot C^n} \quad (8)$$

where E_m is the maximum effect achievable in the system, K_A is the agonist dissociation equilibrium constant, n is the slope index for the occupancy-effect relationship and τ is the efficacy parameter. This efficacy parameter is expressed according to equation:

$$\tau = \frac{R_0}{K_E} \quad (9)$$

where R_0 is the total number of available receptors in the biological system and K_E is the concentration of the drug-receptor complex required to produce the half-maximal effect for that drug.

Inter-animal variability on the parameters was described according to equations 2 and 3. The residual error was best described with a proportional error model according to

equation:

$$C_{obs,ij} = C_{pred,ij} \cdot (1 + \varepsilon_{ij}) \quad (10)$$

where $C_{obs,ij}$ is the j -th observation of the i -th individual, $C_{pred,ij}$ is the predicted concentration and ε_{ij} is a realisation from the normally distributed residual random variable with mean zero and variance σ^2 :

$$\varepsilon_i \sim N(0, \sigma^2) \quad (11)$$

In vitro receptor binding

The receptor binding characteristics of the radioligand ³H-naloxone and the opioids were analysed using the non-linear regression curve-fitting program GraphPad Prism, version IV (Graphpad Software Inc, San Diego, CA). The receptor binding characteristics of ³H-naloxone were determined by fitting the data, as obtained from the saturation experiments, to the following equation:

$$B = \frac{B_{max} \cdot C_f}{K_d + C_f} \quad (12)$$

where B is the number of receptors occupied, B_{max} is the total number of specific binding sites, K_d is the ligand concentration at which 50% of the receptors is occupied and C_f is the free ligand (³H-naloxone) concentration.

The IC_{50} values for the six opioids were determined by fitting the data, as obtained with the displacement experiments, to the following equation:

$$B = \frac{B_0 \cdot IC_{50}}{IC_{50} + C_d} \quad (13)$$

in which B_0 is the specific binding for the radioligand in the absence of the displacer (opioid) and C_d is the concentration of the displacer added and IC_{50} is the opioid concentration that displaces 50% of the radioligand ³H-naloxone. The K_i values were calculated from the IC_{50} values according to the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + (L^* / K_d^*)} \quad (14)$$

where L^* is the concentration of ³H-naloxone and K_d^* is the equilibration dissociation constant of ³H-naloxone as obtained from the saturation experiment.

RESULTS

In vivo concentration-effect relationships

After administration of the opioids, a gradual increase in the delta frequency (0.5-4.5 Hz) band of the EEG was observed (figure 1).

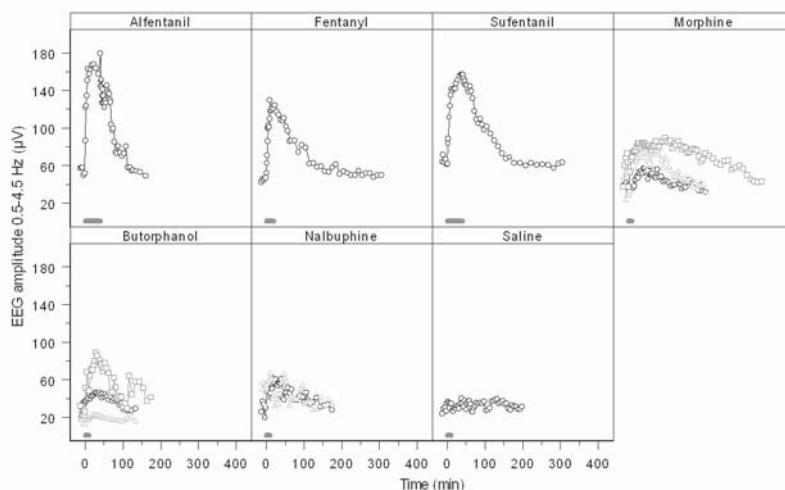


Figure 1: EEG amplitude –time profile of a typical rat after intravenous administration of alfentanil 3.14 mg/kg in 40 min, fentanyl 0.15 mg/kg in 20 min, sufentanil 0.03 mg/kg in 40 min, morphine 4 (black circles), 10 (light gray triangles) and 40 (gray squares) mg/kg in 10 min, butorphanol 2.5 (black circles), 5 (light gray triangles) and 10 (gray squares) mg/kg in 10 min, nalbuphine 5 (black circles) and 10 (light gray triangles) mg/kg in 10 min and saline.

Previously, the pharmacokinetics in blood and the biophase distribution kinetics have been investigated (chapter 7). The pharmacokinetics in blood of alfentanil, morphine and nalbuphine were best described with a three compartment model, whereas for fentanyl, sufentanil and butorphanol, a two-compartment model best described the pharmacokinetics in blood. The fraction unbound (mean \pm SD) was 0.77 ± 0.01 , 0.25 ± 0.06 and 0.097 ± 0.021 for morphine, nalbuphine and butorphanol, respectively. No differences were found between the different concentrations tested (data not shown). For alfentanil, fentanyl and sufentanil literature values of the fraction were used. These values were 0.164, 0.166 and 0.069 for alfentanil, fentanyl and sufentanil, respectively (Meuldermans *et al.* 1982). For all opioids except alfentanil and morphine, biophase distribution was best described with a one-compartment distribution model for all opioids except morphine. For morphine, the extended-catenary biophase distribution model was developed which consists of two sequential distribution compartments.

The results of the simultaneous analysis of the unbound biophase concentration-effect relationships of all six opioids are shown in figure 2. Analysis with the Hill equation was performed on all individual concentration-effect data to provide estimates

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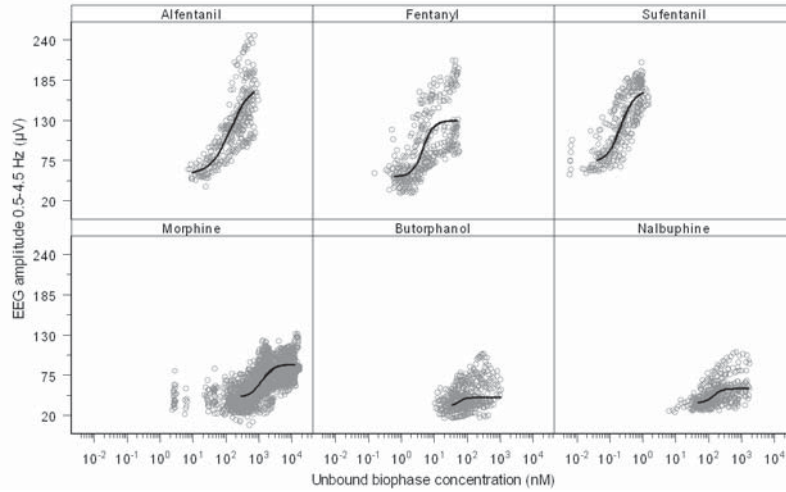


Figure 2: Unbound biophase concentration-effect relationships for the effect on the delta-frequency (0.5-4.5 Hz) band of the EEG after intravenous infusion of the opioids alfentanil, fentanyl, sufentanil, morphine, butorphanol and nalbuphine. The grey dots represent the individual observations and the solid and dashed lines were obtained by simultaneous fitting of the data to the Hill equation.

(mean \pm SEM and ω^2 for inter-animal variation) of the PD parameters including maximum achievable effect (E_{max}), the fraction of the E_{max} that can be reached by the opioid (α), the potency expressed as the unbound concentration ($EC_{50,u}$) and the Hill factor (n_H). The derived parameters are shown in table 2.

Table 2: Population pharmacodynamic estimates and standard error of estimate (mean \pm SE) for, E_{max} , fraction of E_{max} (α), potency (EC_{50}) and Hill slope (n_H). The variances (w^2) describing the inter-individual variability are shown in parentheses.

Compound	E_{max} (μV)	F	$EC_{50,u}$ (nM)	n_H
Alfentanil	123 \pm 13	1 FIXED	136 \pm 29	1.44 \pm 0.16
	-878		-0.2	(-)
Sufentanil		0.81 \pm 0.10	0.21 \pm 0.03	2.06 \pm 0.26
		(-)	-0.21	(-)
Fentanyl		0.62 \pm 0.11	4.48 \pm 0.40	2.74 \pm 0.22
		-0.06	-0.06	(-)
Morphine		0.36 \pm 0.05	1223 \pm 42	2.51 \pm 0.14
		-0.09	(-)	(-)
Nalbuphine		0.16 \pm 0.03	141 \pm 4	3.34 \pm 0.43
		-0.13	-0.13	(-)
Butorphanol		0.10 \pm 0.03	54 \pm 10	3.97 \pm 0.59
		-0.74	-0.33	(-)

Between the opioids, large differences in intrinsic activity and potency were observed with values of α ranging from 1 (alfentanil) to 0.10 (butorphanol) and of $EC_{50,\mu}$ ranging from 0.21 nM (sufentanil) to 1223 nM (morphine).

Mechanism-based analysis: estimation of in vivo affinity and intrinsic efficacy at the μ -opioid receptor

Individual unbound biophase concentration-effect relationships for all agonists, as obtained by the analysis with the Hill equation, were simultaneously analysed on the basis of the operational model of agonism according to the comparative method with $n = 1.44$ and $E_{\max} = 123 \pm 13 \mu V$. The *in vivo* pK_A of sufentanil was fixed to the *in vitro* pK_i value in the presence of 100 mM NaCl or in the presence of 1 mM GTP. Only small differences were observed between the analyses, but the parameter estimation was more precise when the *in vitro* pK_i of sufentanil in the presence of 100 mM NaCl was constrained. The estimates of *in vivo* affinity (pK_A) and efficacy ($\log \tau$) are shown in table 3. The pK_A ranged from 5.64 (morphine) to 9.15 (sufentanil) and of the $\log \tau$ from 0.421 (alfentanil) to -0.342 (nalbuphine).

Table 3: Estimates of pK_A , $\log \tau$ and $EC_{50,\mu}$ as derived from simultaneous analysis with the operational model of agonism. Results are presented as mean \pm SE

Compound	pK_A	Log τ
Alfentanil	6.42 \pm 0.23	0.421 \pm 0.215
Sufentanil	9.15 *	0.393 \pm 0.060
Fentanyl	7.81 \pm 0.14	0.296 \pm 0.177
Morphine	5.64 \pm 0.06	-0.064 \pm 0.058
Nalbuphine	6.73 \pm 0.09	-0.342 \pm 0.073
Butorphanol	7.25 \pm 0.22	-0.326 \pm 0.165

* pK_A of sufentanil has been fixed to the pK_i value in the presence of 100 mM NaCl as obtained in *in vitro* receptor bindings assay

In vitro receptor binding assays

The results of the binding assays are shown in table 4. In buffer, the receptor affinity for the μ -opioid receptor ranged from 0.09 nM for sufentanil to 5.84 nM for alfentanil. In the presence of either 100 mM NaCl or 1 mM GTP, the receptor affinity of the opioids decreased substantially. As a measure of intrinsic efficacy, both the Na-shift and the GTP-shift were calculated. In the presence of Na, alfentanil showed the highest efficacy, whereas in the presence of GTP, fentanyl had the highest agonist character. The sodium-shift ranged from 22 (alfentanil) to 3.8 (morphine) and the GTP-shift ranged from 11. (fentanyl) to 2.4 (butorphanol).

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Table 4: *In vitro* receptor binding characteristics as obtained in the displacement studies. Results are presented as mean \pm SEM

Compound	K _i (nM)	K _i +Na (nM)	K _i +GTP (nM)	Na-shift	GTP-shift
Alfentanil	5.84 \pm 1.69	129.27 \pm 32.59	59.88 \pm 12.30	22	10
Sufentanil	0.09 \pm 0.01	0.70 \pm 0.22	0.43 \pm 0.22	7.7	4.8
Fentanyl	1.28 \pm 0.46	15.13 \pm 5.57	14.36 \pm 4.54	12	11
Morphine	5.55 \pm 1.38	21.22 \pm 6.89	25.14 \pm 10.33	3.8	4.5
Nalbuphine	0.67 \pm 0.20	5.33 \pm 0.74	7.44 \pm 1.04	7.9	11
Butorphanol	0.45 \pm 0.12	2.12 \pm 0.78	1.07 \pm 0.44	4.7	2.4

In vitro – *in vivo* correlations

When the estimated *in vivo* pK_A values were correlated with the *in vitro* pK_i values, evidence for two distinct subpopulations was obtained. Figure 3 shows the correlation between the apparent *in vivo* pK_A estimates with the pK_i values found *in vitro* in the presence of either 1 mM GTP (left panel) or 100 mM NaCl (right panel). When taking all data together, no statistically significant correlation between the *in vivo* pK_A and the *in vitro* pK_i values was obtained. On contrast, for the subset containing alfentanil, fentanyl and sufentanil highly significant correlations were obtained. These correlations could be best described by pK_A = 1.3096*pK_i - 2.7991 (R² = 0.9584, P = 0.131) and by pK_A = 1.1911*pK_i - 1.681 (R² = 0.9873, P = 0.072) for 1 mM GTP and 100 mM NaCl, respectively. In addition, for the second set containing morphine, butorphanol and nalbuphine the correlation for 1 mM GTP could be best described by pK_A = 1.1231*pK_i - 2.7064 (R² = 0.8932, P = 0.211), whereas for 100 mM NaCl the correlation was best described by pK_A = 1.6264*pK_i - 6.8076 (R² = 0.9925, P = 0.055). For alfentanil, fentanyl and sufentanil the correlation between the *in vivo* pK_A and the *in vitro* pK_i in the presence of 100 mM NaCl was closest to the line of unity. In general, for morphine, butorphanol and nalbuphine a rightward shift was observed compared to the line of unity.

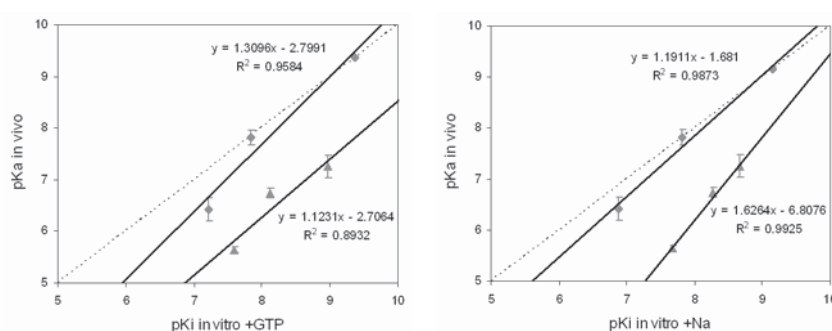


Figure 3: Relationship between the apparent *in vivo* pK_A estimates for the EEG effect of opioids and the *in vitro* pK_i values for the opioids in the presence of 1mM GTP (left panel) or 100 mM NaCl (right panel). The dashed line represents the line of identity. The solid lines were obtained by linear regression. The compounds are depicted with the first letter of the opioid name

Figure 4 depicts the relationship between *in vivo* measure for efficacy $\log \tau$ and the *in vitro* efficacy parameter GTP-shift (left panel) and Na-shift (right panel). No statistically significant relationship between the *in vivo* efficacy parameter $\log \tau$ and the *in vitro* efficacy measures was observed ($P > 0.1$). When describing the correlations between the two parameters with the equation $pK_A = a \cdot pK_i + b$, the correlation between $\log \tau$ and Na-shift was $R^2 = 0.4096$ ($P = 0.171$), while the correlation was $R^2 = 0.1332$ between $\log \tau$ and GTP-shift ($P = 0.478$).

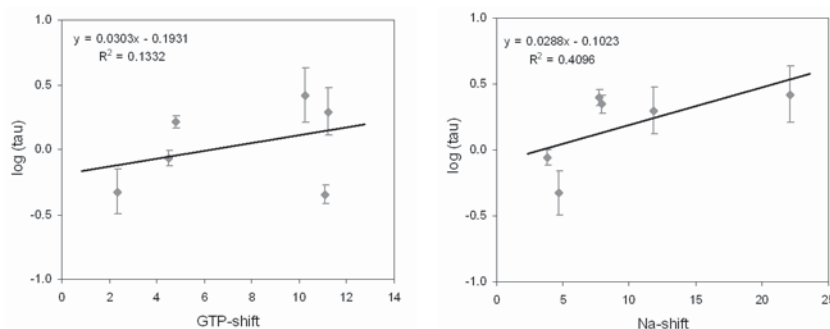


Figure 4: Relationship between the *in vivo* $\log \tau$ estimates for the EEG effect of opioids and the *in vitro* GTP-shift (left panel) and Na-shift (right panel). The solid line was obtained by linear regression. The compounds are depicted with the first letter of the opioid name.

DISCUSSION

The objective of the current study was to develop a mechanism-based pharmacodynamic model for the characterisation of the biophase concentration-EEG effect relationships of opioids. To this end, the unbound biophase concentration-effect relationships of a series of opioids which consisted of alfentanil, fentanyl, sufentanil, morphine, butorphanol and nalbuphine were simultaneously analysed with both the empirical sigmoid E_{\max} model and the mechanistic operational model of agonism. The values of the parameters obtained with the operational model of agonism, characterizing the *in vivo* affinity (pK_A) and intrinsic efficacy ($\log \tau$) were compared with the estimates of the receptor affinity and intrinsic efficacy as determined in *in vitro* binding assays.

An important feature of this investigation was that the *in vivo* biophase concentration-EEG effect relationships have been determined using previously developed biophase distribution models (chapter 7). For morphine it has been demonstrated that the biophase distribution kinetics is best described with an extended-catenary biophase distribution model consisting of a transfer and an effect compartment model. In contrast, the biophase distribution kinetics of fentanyl, sufentanil, butorphanol and nalbuphine were best described with the effect-compartment model, whereas for alfentanil a direct relationship was observed between blood concentrations and EEG-effect.

In the current investigations, EEG monitoring was used as a pharmacodynamic endpoint.

Quantitative analysis of drug effects on the electroencephalogram (EEG) yields an attractive biomarker, which is continuous, sensitive and reproducible (Dingemans *et al.* 1988). It has been shown that the synthetic opioid alfentanil, which is frequently used in anesthesia produces a progressive slowing of the EEG with a pre-dominant increase in the delta frequency band (0.5-4.5 Hz) of the EEG power spectrum in both animals (Cox *et al.* 1997; Mandema & Wada 1995; Wauquier *et al.* 1988; Young & Khazan 1984) and humans (Scott *et al.* 1985; Wauquier *et al.* 1984; Young & Khazan 1984). Meanwhile the increase in the delta frequency band of the EEG has been widely used as a biomarker in numerous studies on the PK-PD correlations of synthetic opioids. In preclinical studies evidence has been obtained that the increase in the delta frequency band of the EEG reflect μ -opioid receptor activation (Cox *et al.* 1997; 1998; 1999). However, it remains to be elucidated whether changes in the delta frequency band are solely caused by μ -opioid receptor activation.

Simultaneous PK-PD analysis with the Hill equation showed that alfentanil had the highest intrinsic activity ($123 \pm 13 \mu\text{V}$). This analysis was performed in order to enable a ranking in intrinsic activity for the set of opioids. This fraction (α) ranged from 0.81 to 0.10 for sufentanil and butorphanol, respectively which corresponds to an E_{max} value of $100 \mu\text{V}$ for sufentanil and $12 \mu\text{V}$ for butorphanol. Previously, the biophase concentration-effect relationships have been investigated with the Hill equation for each opioid separately (chapter 7). The parameters derived from the simultaneous analysis are not distinctly different compared to the separate analysis except for the E_{max} of nalbuphine. In the separate analysis an E_{max} of $56 \mu\text{V}$ was found, whereas in the simultaneous analysis an E_{max} fraction of 0.16 was obtained, which corresponds to an E_{max} of $20 \mu\text{V}$. A possible explanation for this difference is that with the simultaneous analysis one residual error is estimated whereas with the separate analysis a residual error is estimated for each compound.

A limitation of the Hill equation is that, although very useful for descriptive purposes, it is only of limited value to understand factors which determine the shape and location of the concentration-effect relationship. Specifically, the pharmacodynamic parameters of the Hill equation are mixed parameters which depend on both the properties of the drug and the biological system (van der Graaf *et al.* 1997; 1997a). To fully understand the *in vivo* concentration-effect relationship, more mechanistic modeling approaches are needed to describe target binding and activation processes, including a clear distinction between drug-specific and biological system specific properties (Danhof *et al.* 2005; 2007). Recently, the operational model of agonism has been successfully applied for explaining and predicting the effects of differential expression of agonism *in vivo* (Black & Leff 1983; van der Graaf *et al.* 1997; Zuideveld *et al.* 2004).

Previously, simulation on the basis of the operational model of agonism indicated that the μ -opioid receptor functions with high efficiency. As a result, the synthetic opioids

alfentanil, fentanyl and sufentanil were all found to behave as full agonists, which complicated identification of the operational model of agonism (van der Graaf *et al.* 1997; 1997b). The simultaneous analysis of the six opioids with the Hill equation has shown that these compounds display a wide range in intrinsic efficacy and were therefore particularly useful for identification of the operational model of agonism.

For the analysis of the operational model of agonism, the comparative method (Black & Leff 1983; Leff *et al.* 1990; van der Graaf *et al.* 1997) was applied where E_{\max} (123 μ V) and n (1.44) were constraint to alfentanil which displays the highest intrinsic activity *in vivo* as proposed by Leff and co-workers. In addition, the pK_A of sufentanil was fixed to the *in vitro* pK_i (9.15), since this opioid displays the highest affinity. The constraint of the *in vivo* pK_A to the *in vitro* pK_i has been applied previously (Black & Leff 1983; Jonker *et al.* 2005; van der Graaf *et al.* 1997; Zuideveld *et al.* 2004). When analyzing the concentration-effect relationships of the opioids two subsets were seen in the efficacy parameter $\log \tau$ which ranged from 0.3 to 0.4 for alfentanil, fentanyl and sufentanil and from -0.3 to -0.06 for morphine, nalbuphine and butorphanol.

The *in vitro* K_i values of alfentanil, fentanyl and sufentanil were slightly lower compared to the results reported previously, whereas the values for the sodium shift were largely similar (Cox *et al.* 1998). The observed difference in binding affinity may be explained by differences in the method of membrane preparation and the source of the membranes. When using both the Na-shift and the GTP-shift as measures of the *in vitro* intrinsic efficacy, the opioids alfentanil, fentanyl, and nalbuphine displayed the highest efficacy. The GTP-shift ranged from 11 for fentanyl to 2.4 for butorphanol and the Na-shift ranged from 22 for alfentanil to 3.8 for morphine. Interestingly, nalbuphine showed a relatively high efficacy in both assays, whereas the effect *in vivo* is relatively small (E_{\max} fraction 0.16 compared to alfentanil).

When taking all compounds together, the correlations between the *in vivo* pK_A and the *in vitro* pK_i determined in the presence of 1 mM GTP or 100 mM NaCl were not statistically significant ($P > 0.05$). However, there were clear indications for two (sub-) populations of opioids. The estimated *in vivo* pK_A for alfentanil, fentanyl and sufentanil were similar to the values obtained *in vitro*, whereas for morphine, butorphanol and nalbuphine, the pK_A was higher. A possible explanation for this observation is the influence of complex biophase distribution processes with emphasis on interaction with transporters. Although analysis with the biophase distribution models has resulted in accurate biophase concentration-effect relationships, it could still be possible that interaction with active transporters influences the biophase concentration-time profiles and thereby the estimation of the pharmacodynamic parameters. The influence of active transport mechanisms as a confounder of the analysis of the *in vitro-in vivo* correlations of pK_A values has also been identified for 5-HT_{1A} receptor agonists in particular with regard to flesinoxan (Zuideveld *et al.* 2004). No significant correlations between *in vitro* pK_i and *in vivo* pK_A were observed for a set of 5-HT_{1A} receptor agonists

including flesinoxan. However, when flesinoxan was excluded from the analysis the correlation became statistically significant. Zuideveld and co-workers concluded that the *in vivo* pK_A determined on the basis of blood concentrations was not representative for the flesinoxan concentrations at the site of the 5-HT_{1A} receptor due to interaction with transporters at the BBB which had previously been shown by Van der Sandt and co-workers (2001).

Another possible explanation for the existence of two sub-populations is the interaction with different μ -opioid receptor subtypes. After pre-treatment with β -FNA, the Hill factor of alfentanil is increased to 2.75 (Garrido *et al.* 2000). It has been speculated that antagonist-induced curve-steepening could be indicative for receptor heterogeneity (van der Graaf *et al.* 1996) and that the EEG effect of alfentanil is mediated via multiple receptor types which differ in their sensitivity to β -FNA (Garrido *et al.* 2000). Recently, alternative spliced μ -opioid receptor isoforms have been identified, which might be involved with different aspects of the pharmacology of alfentanil (Pasternak, 2005; Zernig *et al.* 1994). In addition, for the opioids morphine, butorphanol and nalbuphine it is known that they have affinity for both the μ - and κ -opioid receptor, whereas fentanyl is a specific μ -opioid receptor agonist (Chen *et al.* 1993). In literature, little is known about the receptor affinity of alfentanil and sufentanil, but they have been specifically designed to bind exclusively to the μ -opioid receptor (Chen *et al.* 1988). Furthermore, it is known that heterodimerisation of opioid receptors can potentiate the effects of opioids (Gomes *et al.* 2000).

Finally, the correlation between the *in vitro* measures for efficacy, the GTP-shift and the Na-shift, and the *in vivo* $\log \tau$ were poor. This indicates that the EEG effects of opioids are not determined by interaction with a single receptor system. For example, nalbuphine shows a relatively high efficacy *in vitro* (Na-shift = 7.94) whereas the *in vivo* efficacy is the lowest of the six opioids tested ($\log \tau = -0.342$). As mentioned above, nalbuphine has affinity for both μ - and κ -opioid receptors suggesting that interaction with both receptors determines the *in vivo* EEG effect.

In conclusion, analysis with the operational model of agonism has provided insight into the complex process of receptor interaction in the EEG effect of opioids. Since many opioids have affinity for both the μ - and the κ -opioid receptor, the predictive value of the *in vitro* K_i at the μ -opioid receptor is of limited value.

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