THE NEONATAL PHARMACOLOGY OF MORPHINE-6-β-D-GLUCURONIDE IN THE GUINEA PIG

by

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II. Morphine-6-β-D-glucuronide respiratory pharmacodynamics in the neonatal guinea pig.
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III. Diffusion of morphine-6-β-D-glucuronide into the neonatal guinea pig brain during drug-induced respiratory depression.

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List of Abbreviations

ANOVA	analysis of variance
AUC	area under the time-action curve
BBB	blood-brain barrier
β-FNA	β-funaltrexamine
B_{max}	maximal number of binding sites
CNS	central nervous system
CSF	cerebrospinal fluid
DADLE	[D-Ala^2,D-Leu^5]enkephalin
DAMGO	[D-Ala^2,N-methylPhe^4-Glyol^5]enkephalin
DPDPE	cyclic [D-Pen^2,D-Pen^5]enkephalin
DRG	dorsal respiratory group
DSLET	[D-Ser^2,Leu^5,Thr^6]enkephalin
ECD	electrochemical detection
ECF	extracellular fluid
f	breathing frequency
F	free drug
FBM	fetal breathing movements
HPLC	high-performance liquid chromatography
HMO	hydromorphone
IC_{50}	concentration causing 50% inhibition of maximal binding
i.c.v.	intracerebroventricular

i.v.	intravenous
K_D	equilibrium dissociation constant
K_I	binding inhibition constant
k_i	isocratic capacity factor
log P	log of octanol/water partition coefficient
MOR	morphine
M3G	morphine-3-β-D-glucuronide
M6G	morphine-6-β-D-glucuronide
NA	nucleus ambiguus
NOR	normorphine
NRA	nucleus retroambigualis
NTS	nucleus tractus solitarius
Pa_{CO2}	arterial carbon dioxide tension
Pa_{O2}	arterial oxygen tension
p.o.	oral
SB	specific binding
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SIDS	sudden infant death syndrome
T_I	inspiratory time
UDPGA	uridine diphosphoglucuronic acid
UDPGT	uridine diphosphoglucuronysl transferase

UVD	ultraviolet detection
V	minute ventilation
V_I	inspiratory minute ventilation
VRG	ventral respiratory group
V_T	tidal volume
V_T/T_I	mean inspiratory flow

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Abstract

Morphine-6-β-D-glucuronide (M6G), an active morphine metabolite, is produced by many mammalian species, including human and guinea pig adults and neonates. Opioid activity of M6G has been appreciated only during the last several years. Previously, all studies on M6G utilized adult animals. Neonatal pharmacology with regards to respiratory depression, drug entry into the brain and opioid receptor binding was investigated. Reverse-phase, ion-pair, high-performance liquid chromatography microassay for morphine, M6G, morphine-3-β-D-glucuronide (M3G) and normorphine in plasma, and later brain tissue, was developed. The assay uses solid phase sample extraction, chromatography on a octadecylsilica column, a phosphate buffer and acetonitrile mobile phase with sodium dodecly sulfate as the ion-pairing agent, and ultraviolet and electrochemical detection. Limits of detection are 23 ng/ml for all compounds. Neonatal guinea pigs produce M6G and M3G from morphine *in vivo* and *in vitro*. Because of metabolite and neural development profile, and respiratory response to hypercarbia and to opioids, the neonatal guinea pig was used as a model of human neonatal pharmacology. Respiratory depressant effects of M6G in neonatal guinea pigs over the first two weeks of life were determined in a placebo-controlled, randomized study using a plethysmograph and computerized data acquisition to measure breathing. The time-action of a subcutaneous (s.c.) M6G (05. to 5.0 mg/kg) and morphine (1.5 to 15 mg/kg) over 90 min were determined. Peak respiratory depressant activity for all doses of M6G occurred 21 min later

than for morphine while breathing a 5% CO_2, 30% O_2, balance N_2 gas mixture. At peak depression, breathing the elevated CO_2 gas mixture, M6G and morphine produced dose-dependent decreases in minute ventilation on days 3, 7 and 14 after birth. This was due primarily to decreases in breathing frequency. M6G and morphine are equipotent on day 3, but M6G potency, relative to itself on day 3 and to morphine, increased 8-fold by day 7 and the change persisted through day 14. After s.c. administration, plasma M6G levels at time of average peak depression were significantly lower in day 7 animals than in day 3 animals. Brain drug levels were also lower in 7-day old than in 3-day old pups, but this was not statistically significant. Therefore, M6G distribution to brain cannot explain the observed potency increase with aging. Entry of M6G and M3G into brain is a linear, gradient-driven process in accord with their known degree of lipophilicity. This suggests entry into brain occurs by diffusion. M3G, 75 mg/kg s.c., causes mild respiratory depression, due to a 0.6% morphine impurity in the drug stock. No respiratory stimulant activity by M3G was noted in the neonatal guinea pig, contrary to published studies in adult rats and dogs. Receptor binding experiments using the *mu* opioid receptor specific radioligand, [^{3}H]DAMGO, showed a 42% increase in the number of *mu* receptors in brain stem between day 3 and 7, whereas affinity for the radioligand remains constant (1.1 nM). In competitive displacement studies, no change in 50% inhibitory concentrations of morphine, M6G or DAMGO were seen with aging. Increase in receptor number may partially explain the potency increase in M6G observed between days 3 and 7 for respiratory depression.

Alternatively, there may be neonatal development of an uncharacterized opioid receptor with which M6G, but not morphine, interacts.

Introduction

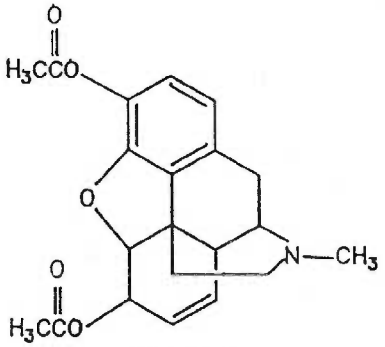
I. Pharmacology of Morphine

Opium is one of the oldest medications known to mankind, and its use predates recorded history. The first unequivocal mention of the use of poppy juice is from the third century B.C. writings of Theophrastus, and opium was included in the first century, A.D., text of Dioscorides, *De Materia Medica* (Macht, 1915; Goodman and Gilman, 1941). Derived from juice of unripe seed capsules of the poppy, *Papaver somniferium*, opium is composed of several alkaloids.

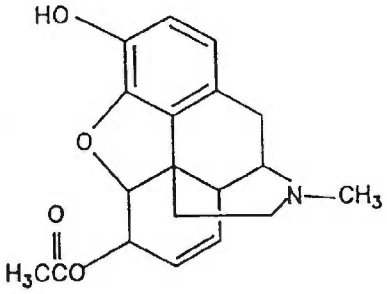
The principle phenanthrene alkaloid of opium is morphine, and it was the first to be isolated, in 1803 by Sertürner, a German pharmacist (Macht, 1915). Other, less abundant opioid alkaloids, including codeine and thebaine, as well as the non-opioid benzylisoquinoline alkaloids such as papaverine and noscapine, have also been purified (Macht, 1915; Goodman and Gilman, 1941). Morphine is a weak base with a pK_a of 8.0 (Kaufman *et al.*, 1975) and molecular weight of 285. Structures of morphine and metabolites are given in figure 1. Morphine, along with other natural, semi-synthetic and synthetic opioid alkaloids, exist as levo- and dextro-rotary isomers. Levorotary opioid alkaloids are active, whereas the dextrorotary isomers are not (Pert and Snyder, 1973; Boerner *et al.*, 1975; Jacquet *et al.*, 1977).

Morphine has diverse effects upon several physiological systems, however, its most valued therapeutic use is for analgesia. Indeed, morphine remains the

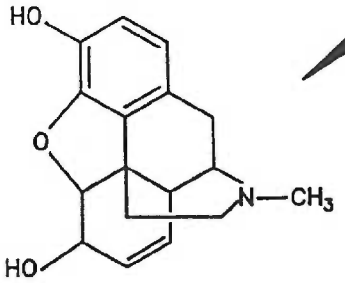
Fig. 1. Structure of heroin, morphine and major metabolites formed in human and guinea pig. Heroin is deacetylated to morphine, and morphine undergoes phase I oxidative *N*-demethylation and phase II glucuronide conjugation of the phenolic and aliphatic hydroxyl groups.



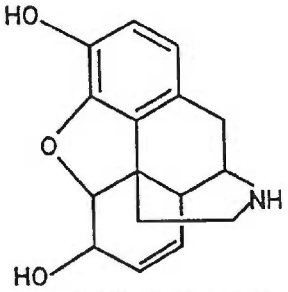
HEROIN
(DIACETYLMORPHINE)



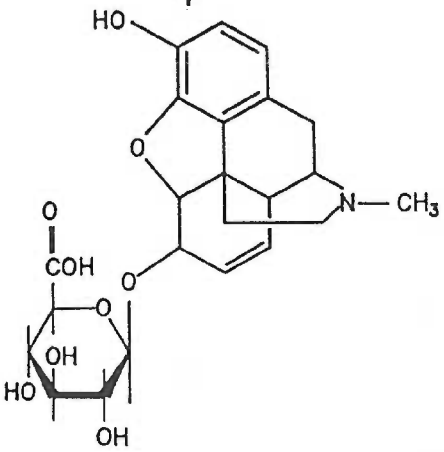
6–MONOACETYLMORPHINE
(6–MAM)



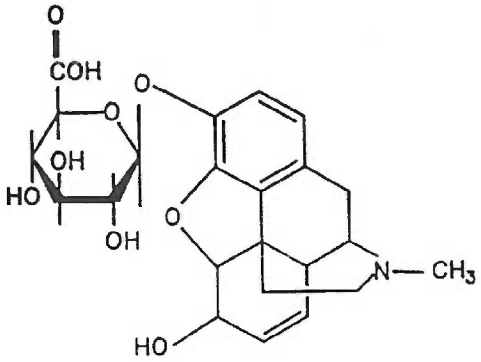
MORPHINE
(MOR)



NORMORPHINE
(NOR)



MORPHINE–6–GLUCURONIDE
(M6G)



MORPHINE–3–GLUCURONIDE
(M3G)

standard to which all other analgesics are compared (Jaffe and Martin, 1990; Glare and Walsh, 1991). Morphine interacts with opioid receptors in the brain, the spinal cord and in peripheral tissues to produce analgesia (Stein *et al.*, 1991; Ferreira *et al.*, 1991; Pasternak, 1993). The distinguishing feature of morphine, and opioid analgesics in general, is the production of dose-related increases in analgesia until a state of anesthesia is achieved (Stanski and Roizen, 1992), whereas salicylates and other non-steroidal anti-inflammatory drugs have a lower ceiling effect than opioids and cannot relieve severe pain (Stanski and Roizen, 1992).

Several side-effects can occur after morphine administration. Morphine produces somnolence and a blunting of cognition in humans (Jaffe and Martin, 1990). In the hypothalamus, morphine inhibits the secretion of gonadotropin-releasing hormone and corticotropin-releasing factor (Jaffe and Martin, 1992) and produces hypothermia (Martin *et al.*, 1976) by perturbing the body temperature set-point. Pupillary constriction is seen after administration of morphine (Martin *et al.* 1976; Jaffe and Martin, 1990) and transit through the gastrointestinal tract is delayed (Paul *et al.*, 1989). Nausea and vomiting are observed, caused by direct interaction of morphine with the chemoreceptor trigger zone in the medulla. Respiratory depression is the limiting toxicity of morphine use. All of these effects are opioid receptor mediated. A full discussion of the respiratory effects of opioids is found in Section V of the Introduction.

Central nervous system (CNS) excitation is also produced by morphine. High doses of morphine (40 μg) injected into the periaqueductal gray brain region

(Jacquet *et al.*, 1977) or 50 to 75 μg given *via* intracerebroventricular (i.c.v.) cannula (Labella *et al.*, 1979) cause excitatory behaviors and seizures in rats. By comparison, a dose of 2 μg into the periaqueductal gray causes analgesia in rats (Pasternak *et al.*, 1987). When 150 μg morphine is given by intrathecal catheter, hyperesthesia is noted (Woolf, 1981; Yaksh *et al.*, 1986), whereas 15 μg produces analgesia. Naloxone did not reverse the hyperesthesia, indicating that it is not mediated by opioid receptors.

Tolerance and dependence develop after prolonged administration of morphine. Tolerance is the phenomenon in which increasing doses of drug are required to maintain the same level of response. This may be quite pronounced in patients taking morphine for relief of chronic cancer pain. Dependence is of two types, physical and psychological. Physical dependence is a need for continued presence of drug in order to avoid withdrawal, which is the unpleasant response of an organism to the sudden absence of a drug for which it is physically dependent. Psychological dependence is the desire to continue taking a drug for its euphoric or mood altering effects and is linked to addiction, which is discussed below.

Tolerance to morphine can occur at several levels (Trujillo and Akil, 1991), including changes in drug distribution (Lange *et al.*, 1981; Lange *et al.*, 1983) or changes in clearance of drug with chronic exposure (Jaffe, 1990). Lange *et al.* (1981, 1983) reported differential tolerance to s.c. but not i.c.v. morphine after implantation of a s.c. morphine pellet in mice. They attributed the tolerance to s.c.

morphine to changes in drug distribution. There is good evidence, however, that tolerance is also pharmacodynamic at the level of the opioid receptor. Morphine acts primarily through the *mu* opioid receptor. Chronic exposure to morphine induces decreases in *mu* opioid receptors, and uncouples remaining receptors from their effectors in cell culture (Puttfarcken, *et al.*, 1988) and in guinea pig brain (Werling, *et al.*, 1989). In addition, *N*-methyl-D-aspartate pathways, which induce production of nitric oxide, may be involved in tolerance. This is based on the observed prevention of tolerance formation and the reversal of tolerance to morphine by nitric oxide synthase inhibitors (Kolesnikov *et al.*, 1993). Interestingly, this blockade was only to morphine tolerance and not to tolerance produced by *kappa* agonists.

A defining property of opioids is the ability of drugs with similar receptor selectivities to produce cross-tolerance. Chronic exposure to morphine, a *mu* agonist, produces tolerance not only to itself, but also to other *mu* agonists (Woolverton and Schuster, 1983). Similarly, the substitution of another *mu* agonist for morphine will maintain physical dependence and prevent withdrawal symptoms (Fraser *et al.*, 1958; Woolverton and Schuster, 1983).

Addiction involves a state of psychological dependence to the euphoric actions of opioids, and intake of drug in the absence of medical need. Addiction is usually concurrent with tolerance and physical dependence (Jaffe, 1990). Drug seeking behavior is also seen with addiction, which is usually not the case for patients taking opioids for medicinal reasons. The ability of a drug to produce self-

administration behaviors (Jaffe, 1990) or conditioned place preferences (Abbott and Franklin, 1991) in animal models correlates with its potential for abuse.

Morphine may be given *via* several routes, including intravenous (i.v.), subcutaneous (s.c.), intramuscular and oral (p.o.) administration. In healthy adults following i.v. administration of 10 mg per 70 kg, peak concentrations of morphine are 275 nmol/L or 78 ng/ml, and glucuronide metabolites are seen by five min after administration (Osborne *et al.*, 1990). In the same study, the elimination half-life is 1.7 hr, clearance is 27 ml/min/kg, volume of distribution is 3.9 L/kg and 8% of the dose is excreted unchanged in urine. Morphine undergoes extensive hepatic first pass metabolism, and the bioavailability after p.o. administration of 10 mg per 70 kg is 20% (Osborne *et al.*, 1990). After p.o. morphine, glucuronide metabolite levels exceed that of morphine by 15 to 20 min. The peak concentration of morphine is approximately ten-fold lower than after i.v. administration and occurs 50 min following drug administration. Only 1.6% of the p.o. dose is excreted unchanged in urine.

The pharmacokinetics of morphine in children and neonates have also been studied. In surgical patients, given a single dose of morphine, no difference in pharmacokinetic parameters is seen between ages 1 and 15 yr (Dahlström *et al.*, 1979). In another study of children aged 1 to 16 yr (mean 9.6 yr), receiving morphine infusion, clearance (26 ml/min/kg) is similar to adult values (Choonara *et al.*, 1989) In the same study, for neonates 2 to 12 days old (mean gestational age 31 weeks) clearance is significantly less (4.7 ml/min/kg).

Chay *et al.* (1992) reported the pharmacokinetics of morphine in neonates (less than 3 days old), during and after morphine infusion. The elimination half-life does not differ between preterm and term neonates (10.6 and 7.6 hr, respectively). Similarly, clearance (2.67 and 2.31 ml/min/kg, respectively) and volume of distribution (2.04 and 2.07 L/kg, respectively) do not differ between preterm and term neonates. Another study in neonates receiving a single dose of morphine (Bhat *et al.*, 1990) reported similar elimination half-lives as Chay *et al.* (1992), but higher clearance values (3.4 to 15.5 ml/kg/min). McRorie *et al.* (1992) reported that morphine pharmacokinetics reach adult values between 6 mo and 2.5 yr of age.

Morphine is metabolized by hepatic UDP-glucuronosyltransferase (UDPGT) to two primary glucuronide metabolites in many mammalian species. Morphine-6-β-D-glucuronide (M6G) and morphine-3-β-D-glucuronide (M3G) are produced in humans (Oguri *et al.*, 1970; Boerner *et al.*, 1975; Svensson *et al.*, 1982; Osborne *et al.*, 1990) and guinea pigs (Oguri *et al.*, 1970; Murphey and Olsen, 1993, 1994a). Based upon the selective inhibition of M3G formation by oxazepam in human liver microsomes, Wahlström *et al.* (1988) concluded that the two compounds are produced by separate UDPGT isozymes in humans.

M6G is produced to a lesser extent than M3G in humans and guinea pigs. After i.v. morphine administration, 8% of the dose is recovered in urine unchanged at the end of 24 hr, whereas 11% of the dose is recovered as M6G and 44% of the dose as M3G (Osborne *et al.*, 1990). The M3G to morphine and M6G to morphine ratios in urine are higher in children (1 to 16 yr), than in neonates (2 to 12 day

old), indicating that morphine metabolism is not fully developed in the human neonate (Choonara *et al.*, 1989). In the same study, the ratio of M3G to M6G was constant with age, showing that the metabolic pathways of the two glucuronides develop in parallel. Considering that glucuronidation accounts for almost all of the clearance of morphine in humans, UDPGT activity is therefore fully developed when morphine clearance in children reaches adult levels. This occurs between 6 mo and 2.5 yr of age (McRorie *et al.*, 1992). Data regarding neonatal guinea pig morphine metabolism is contained in Manuscript I and Manuscript III of this dissertation.

II. Pharmacology of Morphine Glucuronides

M6G and M3G have identical molecular weights of 461. Both drugs are weak bases due to the morphine alkaloid, and weak acids, due to the carboxyl group of glucuronic acid. The pK_a of the base is the same as for morphine (8.0). M3G is more acidic than M6G and the pK_a of the carboxyl groups are 2.8 and 3.2, respectively (Carrupt *et al.*, 1991). Further discussion of the physical chemistry of M6G and M3G is contained in Manuscript III of this dissertation.

M6G was first reported to have opioid-like activity by Shimomura *et al.* (1971). These investigators found M6G to be an analgesic in mice using the hot-plate test. M6G was 3- to 4-times more potent than morphine following s.c. administration and 45 times more potent following i.c.v. administration. These data were largely ignored for the next 15 years. In 1986, case reports appeared of

respiratory depression in patients with renal failure, who had received morphine for analgesia. These reports were remarkable for the absence of morphine in plasma of the affected patients, however, very high levels of M6G (and M3G) were present (Osborne *et al.*, 1986). The authors concluded that M6G was responsible for the observed respiratory depression in these patients.

Data of Shimomura *et al.* (1971) were confirmed shortly after the case reports of Osborne *et al.* (1986) were published. Pasternak *et al.* (1987) used injection of 0.1 to 0.5 μg M6G and morphine into the periaqueductal gray brain region of rats. In this study, M6G was 20-fold more potent than morphine in producing analgesia to thermal stimuli in the tail-flick test. This study also reported seizures in rats following higher doses of M6G (2 μg). Using thermal stimuli with the tail-immersion test in rats, M6G is 145- to 200-fold more potent than morphine, following i.c.v. administration, and 1- to 4-times more potent than morphine following s.c. administration (Abbott and Palmour, 1988). These investigators also reported a biphasic M6G dose-response curve for both i.c.v. and s.c. administration. Following i.c.v. M6G, analgesia increased between 0.01 and 0.025 μg, maintained a plateau to 0.2 μg, then sharply increased with doses to 1 μg, at which point cyanosis, catalepsy and severe seizures were seen.

Further evidence of M6G-induced analgesia have been reported for mice using the tail-flick test (Paul *et al.*, 1989). In this study, M6G was 90 times more potent than morphine with i.c.v. administration, but was 650 times more potent with intrathecal administration. Direct recording of spinal cord C-fiber neurons during

intrathecal administration of M6G and morphine in anesthetized rats were remarkable for a 13-fold greater potency of M6G (Sullivan *et al.*, 1989). M6G has also been shown to be a potent analgesic in several subsequent studies (Francés *et al.*, 1990, 1992; Smith *et al.*, 1990; Gong *et al.*, 1991, 1992).

The first clinical report of M6G analgesia in human involved a study of 6 patients given 0.5 or 1 mg per 70 kg, i.v. (Osborne *et al.*, 1988). This dose of M6G provided pain relief in 5 of 6 patients. The analgesic effect of M6G in humans was confirmed in a larger study (Osborne *et al.*, 1992). In this second study, side effects of sedation, nausea, vomiting, euphoria and respiratory depression were not present following M6G administration. M6G also produces pain relief in humans following intrathecal administration (Hanna *et al.*, 1990) and is implicated in providing a portion of the analgesia observed after administration of morphine to humans (Portenoy *et al.*, 1992).

Other aspects of opioid pharmacology have also been defined for M6G in adult animals. M6G is more potent than morphine in inhibiting gastrointestinal transit in mice (Paul *et al.*, 1989). Tolerance to M6G analgesia develops following chronic administration of the drug to mice (Shimomura *et al.*, 1971). In mice made tolerant to morphine, cross-tolerance to M6G is observed (Shimomura *et al.*, 1971; Paul *et al.*, 1989). In rats, M6G is a behavioral reinforcer, as measured by the conditioned place preference test (Abbott and Franklin, 1991). M6G was equipotent in producing a conditioned place preference with s.c. administration and 146 times more potent than morphine with i.c.v. administration.

Respiratory effects of M6G have not been as well studied as have the analgesic effects. Pelligrino *et al.* (1989) reported a dose-dependent decrease in arterial oxygen tension (Pa_{O2}) produced by fourth cerebroventricular infusions of M6G into the awake dog. In a study using i.c.v. administration of M6G to anesthetized rats, minute ventilation (V) was depressed (Gong *et al.*, 1991). In both of these studies, M6G was 5- to 10-times more potent than morphine. In one study in humans, M6G had no effect on breathing frequency (f), tidal volume (V_T) or V, while subjects breathed room air. However, M6G depressed V when subjects were given a 5.5% CO_2 challenge (Peat *et al.*, 1991). Unfortunately, only two doses of M6G and one, larger, dose of morphine were used, making potency comparisons difficult.

Unlike M6G and morphine, M3G is devoid of opioid activity. Several investigators have failed to show analgesia by M3G in rats and mice using hot-plate and tail-flick assays and direct spinal nerve recordings (Shimomura *et al.*, 1971; Schulz and Goldstein, 1972; Pasternak *et al.*, 1987; Sullivan *et al.*, 1989).

Rather than CNS depression, M3G has stimulative properties. M3G causes excitatory behaviors such as jerking, exploring and grooming when given i.c.v. to awake rats (Labella *et al.*, 1979). When given intrathecally to awake rats or anesthetized cats, M3G produces hyperesthesia (Yaksh *et al.*, 1986). In this regard, M3G is 10 times more potent than morphine, which produces hyperesthesia at very high doses. M3G also accumulates in the sheep fetus causing seizures (Olsen *et al.*, 1988). With fourth cerebroventricular infusions to the

awake dog (Pelligrino *et al.*, 1989), or with i.c.v administration to anesthetized rats (Gong *et al.*, 1991), M3G produces respiratory stimulation. The opioid antagonists naloxone and naltrexone do not reverse the excitatory effects M3G, indicating a non-opioid mechanism (Labella *et al.*, 1979; Yaksh *et al.*, 1986; Gong *et al.*, 1991).

M3G antagonizes morphine-induced analgesia (Smith *et al.*, 1990) and M6G-induced respiratory depression (Gong *et al.*, 1992). This antagonism is thought to be functional, not pharmacological. Hewett *et al.* (1993), failed to demonstrate antagonism by M3G of morphine-induced changes in recordings from spinal dorsal horn neurons. This finding is consistent with M3G functional antagonism, mediated *via* separate, excitatory pathways, seen in a whole animal model. Pharmacologic antagonism is mediated at the opioid receptor, and would not be expected in an isolated neural recording.

Both M6G and M3G are cleared from the body *via* renal excretion. The pharmacokinetics of M6G in healthy human volunteers have been described following doses of 2.1 and 4.2 mg per 70 kg (Hanna, *et al.*, 1991). The clearance of M6G is 1.9 to 2.8 ml/min/kg, approximately 10-fold lower than for morphine. However, volume of distribution is 0.28 to 0.43 L/kg, also approximately 10-fold lower than for morphine. The elimination half-life, therefore, is 1.8 to 2.1 hr, the same as for morphine. A second study with cancer patients reported M6G clearance (1.5 ml/min/kg), volume of distribution (0.09 L/kg) and elimination half-life (3.2 hr) in the same range (Osborne *et al.*, 1992). This study also found a strong correlation between M6G plasma clearance and calculated creatinine clearance.

In patients with renal failure, M6G and M3G quickly achieve high levels in plasma that persist for much longer than 24 hr (Osborne *et al.*, 1993; Hanna *et al.*, 1993). The elimination half-life of morphine is unchanged from normal volunteers (Osborne *et al.*, 1993). This underscores the unimportance of the kidney for morphine elimination, and the essential contribution of renal elimination to M6G and M3G clearance.

Opioid receptor binding studies of both M6G and M3G have been done. The three main classes of opioid receptors, *mu*, *delta*, and *kappa*, have been investigated. Morphine and M6G are primarily *mu* agonists. Using bovine, rat and neonatal guinea pig brain membranes, affinity of M6G for the *mu* opioid receptor, as determined by displacement of radiolabelled opioid peptides, is 3.5 to 4 times less than the affinities of morphine for the *mu* receptors (Pasternak *et al.*, 1987; Paul *et al.*, 1989; Murphey and Olsen, 1994b). Chen *et al.* (1991) reported that the affinity of M3G for the *mu* receptor in rat brain is 50 fold less than that of morphine. *Delta* receptor affinities for M6G have been reported to be almost double that of morphine in rat brains. In the same study, the affinity of M6G for the *kappa* receptor is 100-fold less than morphine *kappa* affinity (Francés *et al.*, 1990).

III. Control of Respiration

Oxygen is the common denominator of virtually all animal life on the planet. It is molecular oxygen that serves as the final electron acceptor in the process of oxidative phosphorylation. This process, in turn, produces energy-rich ATP

molecules which power cellular function. Conversely, carbon dioxide is the ubiquitous waste product of cellular respiration and oxygen utilization. The need for efficient delivery of oxygen as well as for removal of carbon dioxide has led to the evolution of a precisely regulated system of gas exchange in the mammalian lung.

While continual delivery of oxygen is paramount for the organism to function, the immediate environment of all macromolecules in the body must be maintained within a narrow range of hydrogen ion concentration, as reflected by pH. If the pH is not kept within the correct range, conformations of these macromolecules change, critical inter- and intra-molecular interactions will be disrupted, and normal cellular processes cease. To control life-threatening fluxes in pH, mammals have developed several extracellular buffering systems to absorb or release hydrogen ions as needed to maintain pH in the appropriate range. One of the most important of these buffers is the bicarbonate system, which relies upon carbon dioxide in the acute phase to regulate acidity *via* the following equilibrium: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. Carbon dioxide, therefore, serves an important physiological role in acid-base balance, in addition to being a product of cellular processes.

The consequence of the double role of carbon dioxide is that the activity of the lung is not regulated solely by the oxygen demand of the body, but also by the acid-base balance. This duality has resulted in a tightly regulated respiratory system, responsive to both the oxygen demands of the body, and to the pH of the

organism. As would be expected, part of this regulatory system is sensitive to decreases in Pa_{O2}, and low oxygen tension serves to stimulate alveolar ventilation. Because carbon dioxide is the product of cellular respiration, a process requiring oxygen, it serves as an indirect indicator of oxygen consumption and increases in Pa_{CO2} can signify an increase in oxygen demand and therefore also serve to stimulate respiration. Carbon dioxide increases may also indicate a decrease in pH independent of ventilation (metabolic acidosis) and a shift in the bicarbonate buffer system equilibrium, which would also necessitate an increase in ventilation to restore acid-base status. Because of this, carbon dioxide is a very potent stimulator of ventilation. The regulation of respiration by the tensions of oxygen and carbon dioxide in blood is accomplished by two populations of chemoreceptors: the peripheral chemoreceptors, responsive to changes in oxygen and carbon dioxide tensions, and central chemoreceptors, which mediate responses to changes in carbon dioxide. Peripheral chemoreceptors will be addressed briefly, followed by a more detailed introduction to the central carbon dioxide response, which was involved in the study of drug effects on respiration throughout the research reported in this dissertation. Last, the neural areas involved in respiratory control and a model of how the system is integrated will be discussed.

Peripheral chemoreceptors are located bilaterally in the carotid bodies at the bifurcation of the common carotid artery. There are also aortic chemoreceptors, but in man these appear to be more involved in cardiovascular rather than

respiratory control. Histologically, the carotid chemoreceptors consist of glomus cells and afferent fibers of the glossopharyngeal nerve. Efferent innervation is by parasympathetic and sympathetic fibers. The autonomic presence is presumably to regulate blood flow to the carotid body (Mines, 1992). While the structure of these bodies is well known, the exact sensing mechanisms of the chemoreceptor are unknown. Some have postulated that the afferent nerve fibers are the actual chemoreceptive elements (McDonald and Mitchell, 1975). However, it is possible that the glomus cells may be the receptors instead.

Primary function of the peripheral chemoreceptors is to sense Pa_{O2} or dissolved oxygen content (as opposed to oxygen saturation) in the arterial blood. Decreases in plasma Pa_{O2} cause an increase in afferent firing. Peripheral chemoreceptors are also responsive to changes in Pa_{CO2} and help regulate the second-to-second changes in arterial carbon dioxide due to variations in alveolar ventilation (Mines, 1992). To a lesser extent, the chemoreceptors respond to experimentally induced primary changes in pH, occurring without changes in the amount of carbon dioxide in the blood.

Chemoreception also occurs in the CNS and is mediated by the central chemoreceptors. The existence of central chemoreceptors has been accepted for decades, their existence postulated following experiments in which the peripheral chemoreceptors were denervated but where the hypercapnic response remained. Mitchell and coworkers (1963) were the first to show discrete regions of the brain that mediated stimulation of ventilation following application of acid or high partial

pressures of carbon dioxide. These chemosensitive zones are located on the ventral surface of the medulla, lateral to the pyramidal tracts between the pontomedullary border and the roots of the vagus nerve. A second area of the medullary surface with similar properties was later identified near the roots of the hypoglossal nerve (Schlaefke *et al.*, 1970). While these zones have been identified, to date, no anatomic identification has been made of the "chemoreceptor", and the medullary areas only exist as physiologically defined chemosensitive zones.

Further characterization by Mitchell *et al.* (1963) showed that the chemosensitive zones were very superficial ($\leq$ 0.2 mm). This was based on the observation that procaine, a local anesthetic, applied to the areas blocked the usual hypercapnic response in animals under anesthesia. It was concluded that the procaine blocked conduction on the neurons of the chemoreceptor, eliminating the hypercapnic response. Because procaine only superficially penetrated the medulla, it was reasoned that the chemoreceptor must be close to the ventral surface of the medulla. Application of cold, another blocker of nerve conduction, to these areas also gives similar results (Mines, 1992). From the close approximation to the cerebrospinal fluid (CSF) of the chemoreceptive zones, it was hypothesized that extracellular fluid (ECF) near chemoreceptors is rapidly equilibrated with pH and carbon dioxide tensions of CSF, allowing immediate monitoring of and rapid response to changes in physiology.

Millhorn and Eldridge (1986) have raised valid criticisms of the studies and conclusions of Martin and colleagues, which have become dogma. It should be

noted that the changes in pH or in carbon dioxide tension that were used to stimulate changes in respiration were outside the range of normal physiology and produced only 10% increases in ventilation, and that these studies were also carried out using anesthetized animals that may not demonstrate normal physiology. In addition, confirmation of the procaine experiments in unanesthetized animals have had only limited success. Cozine and Ngai (1967) could not ablate the hypercapnic response with procaine in unanesthetized cats, and only reduced the slope of the carbon dioxide response curve.

Testing of the CSF-ECF equilibration theory of chemoreceptor regulation has had equivocal results. Changing the pH of CSF perfusate has been shown to increase efferent activity of respiratory neurons by some (Trzebski *et al.*, 1975) while others (Malcolm *et al.*, 1980) have shown no effect with such maneuvers.

Attempts to anatomically define neurons in the chemosensitive zones that would serve as chemoreceptors have had equivocal results. One study found changes in the extracellular potentials of superficial neurons in these zones that corresponded with increases or decreases in CSF pH (Schlaefke *et al.*, 1975). On the other hand, Lipscomb and Boyarsky (1972) could find no change in the rate of firing of cells in the superficial chemosensitive areas, but they reported changes in the firing rates of deep medullary cells associated with the respiratory centers coincided with decreases in CSF pH. Pokorski (1976) reported some superficial medullary cells changed firing rates in agreement with pH changes, while others changed firing rates in the opposite direction than predicted by pH shifts. A third

group of cells failed to respond at all to pH changes.

From the above experiments, it appears that the chemosensitive zones of the ventrolateral medulla are involved to some extent in central chemoreception, but it would be misguided at this time to assign the definitive label of "central chemoreceptor" to these areas. One alternative interpretation of the preceding data is that neurons critical in the relaying of impulses from chemoreceptors to respiratory centers pass superficially in the medulla in the areas identified as the chemosensitive zones. Any general disturbance in neural conduction (e.g. local anesthetics, cold, extremes in pH) in these areas could elicit (or fail to elicit) a response which could be wrongly interpreted as chemoreception. Such an interpretation leads to speculation as to whether there exist other candidates within the CNS for the central chemoreceptors. This question has been explored using several approaches, with varying results.

Mitchell and Herbert (1974) reported that neurons from the nucleus ambiguus (NA) and nucleus retroambigualis (NRA), which compose the ventral respiratory group (VRG) of the medulla, actually became hyperpolarized when carbon dioxide tension increased. They concluded that neurons of the respiratory centers are not directly responsive to changes in carbon dioxide or pH. This study has drawn reasonable criticism from Millhorn and Eldridge (1986) due to the fact that the hyperpolarization was such a small change as to be almost immeasurable, while the carbon dioxide tension was increased 250% and that recordings from other known respiratory centers were not made in order to provide supporting

evidence of unresponsiveness. Several other studies refute the conclusion of Mitchell and Herbert, beginning with an early study by Comroe (1943). In this study, deep injections of bicarbonate into brain stem areas involved with respiration caused a stimulation in ventilation. A later study by Miles (1983) using brain slices showed excitation of neurons in the dorsal respiratory group (DRG) [nucleus tractus solitarius (NTS)] and VRG by decreases in pH. This is in agreement with a recent study by Rigatto *et al.* (1992) where fetal rat cells pooled from the VRG and DRG were cultured *in vitro*. Populations of cells with spontaneous "pacemaker" activity were identified. These cells responded with an increased firing frequency to increases in carbon dioxide tensions or to lowered pH. These cells also responded to hypoxia, a property that intact central chemoreceptors in adults do not possess. Autoradiography studies of opioid receptors in the brain stem of adult and neonatal rats indicate there are significant numbers of *mu* opioid receptors in the NTS of the DRG and the NA of the VRG (Xia and Haddad, 1991). *Mu* receptors are important in opioid-induced respiratory depression (See Section V).

The aforementioned studies provide evidence that the central chemoreceptor is not confined to superficial areas of the ventral medullary surface. Considering the evidence to this point, it is reasonable to hypothesize that the central chemoreceptor is not one discrete collection of cells, but a network of chemosensitive cells spread throughout the medulla, and perhaps the entire brain stem and/or cortex. From an evolutionary standpoint, it would make sense that the

controlling mechanism for CNS oxygenation and acid-base balance be more widespread than confined solely to the ventral surface of the medulla. This would allow the organism to respond better to localized CNS disturbances rather than wait until a disturbance is of great enough magnitude to trigger a single set of distant chemoreceptors. It is also of note that deep brain structures, and probably superficial brain regions as well, are much more influenced by the immediate ECF than the more distant CSF. In fact, most ECF is in closer communication with capillary flow and Pa_{CO2} than to CSF flow and gas tensions. ECF monitoring may be more meaningful for the control of respiration than chemoreceptors responsive only to CSF changes.

Up to this point carbon dioxide tensions and pH have been used interchangeably to describe the stimulus for central chemoreceptors. Traditionally, it was assumed the stimulus for central chemoreceptors was changes in pH, reflecting changes in carbon dioxide tensions *via* the bicarbonate buffer system. Carbon dioxide was not thought to be a stimulus on its own right. However, it had also been noted that a respiratory acidosis (which is due to a primary increase in carbon dioxide tensions) appears to cause a greater stimulation of respiration than a metabolic acidosis (which is due to an increase in $[H^+]$) (Borison *et al.*, 1977; Eldridge *et al.*, 1985).

Several investigators have tried to test the resulting hypothesis that carbon dioxide is a primary respiratory stimulant. Borison *et al.* (1977) reported that decreases in arterial pH secondary to carbon dioxide inhalation caused greater

increases in ventilation than decreases in pH secondary to metabolic acidosis, or primary increase in H^+. This study utilized unanesthetized animals in which the peripheral chemoreceptors were denervated, avoiding difficulties in interpreting central versus peripheral effects. Eldridge *et al.* (1985) used phrenic discharge as an endpoint, and found carbon dioxide was a more potent stimulus of phrenic activity than a pH decrease caused by infusion of HCl. It appears carbon dioxide molecules are not only primary stimulants of respiration but that this stimulation is more effective than that induced by primary changes in $[H^+]$.

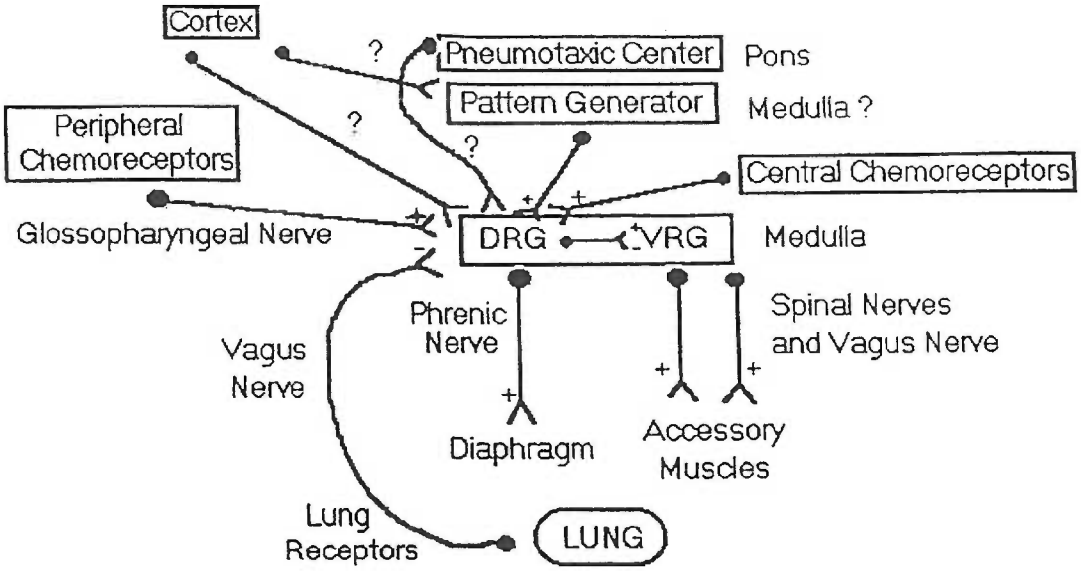
Considering the control that central chemoreceptors exert over respiration, the pathways by which this control is exercised should be examined. Spontaneous breathing is centrally mediated, as opposed to cardiac activity which is locally generated and modulated by central inputs. Identified components of the respiratory system include the DRG and VRG located in the medulla, and the pneumotaxic center in the pons. The DRG, located in the NTS, is mostly inspiratory cells that are upper motorneurons for the diaphragm and other muscles of respiration. The DRG also receives sensory input from lung receptors and the peripheral chemoreceptors. The VRG is driven by the DRG and has both inspiratory and expiratory cells that drive the accessory muscles of respiration. Cell bodies of the VRG are located in the NA and NRA. The pneumotaxic center regulates the rate of breathing and is in the nucleus parabrachialis, located between the cerebral peduncles in the pons. An apneustic center has also been postulated to serve as the inspiratory cut-off switch, but such a center has not

been identified at this time. A pattern generator must also exist to produce the unconscious, rhythmic impulses which are conveyed *via* the DRG and VRG to the respiratory muscles causing lung inflation. The pattern generator resides in the medulla. This is based upon animal experiments in which the brain stem was sectioned at various levels and it was found that respiration continues when the medulla was separated from the upper brain structures (Mines, 1992).

Figure 2 is a proposed "wiring diagram" of central respiratory control. In this suggested system, multiple central chemoreceptors project to the DRG, which modulates respiratory output from the pattern generator based upon the input of several systems. These systems include the central and peripheral chemoreceptors and lung stretch-, irritant- and J (edema)-receptors.

The need to assure an adequate supply of oxygen as well as maintain the acid-base status of the body has lead to a tightly regulated gas exchange system. This system responds to both oxygen and carbon dioxide levels via peripheral and central chemoreceptors. The central chemoreceptors are probably scattered throughout the brain, where they monitor the pH and carbon dioxide tensions of the local ECF. These chemoreceptors respond to carbon dioxide molecules to a greater extent than primary alterations in $[H^+]$, and project to the DRG where they exert their influence upon respiratory output and ultimately ventilation.

Fig. 2. Proposed neural pathways involved in the control of respiration. The plus (+) and minus (-) signs indicate stimulatory and inhibitory input, respectively. The question marks (?) indicate aspects of respiratory control which are incompletely understood or where conflicting experimental data exist. Note that the vagus nerve has afferent and efferent pathways with respect to breathing. Phrenic innervation is to inspiratory muscles only. Spinal and vagus nerve efferents are to inspiratory and expiratory muscles. The DRG, in the nucleus tractus solitarius of the medulla, consists of upper motor neurons for inspiratory muscles of respiration *via* the phrenic nerve. The VRG, located in the nucleus ambiguus and nucleus retroambigualis of the medulla, receives input from the DRG and subsequently drives accessory muscles of respiration. These accessory muscles are both inspiratory and expiratory. A pattern generator is unidentified, but is probably located in the medulla. The pneumotoxic center is in the nucleus parabrachialis of the pons and regulates the rate of breathing. Central chemoreceptive areas are on the ventral surface of the medulla, and respond to changes in pH and carbon dioxide tensions. In addition to pH and Pa_{CO2} , the peripheral chemoreceptors of the carotid and aortic bodies also respond to changes in Pa_{O2}. The lung provides feedback via stretch-, irritant- and J(edema)-receptors. Unknown cortical pathways also influence voluntary respiratory control.


Cortex
?
Pneumotaxic Center
Pons
Pattern Generator
Medulla ?
Peripheral Chemoreceptors
?
?
Central Chemoreceptors
Glossopharyngeal Nerve
DRG
VRG
Medulla
Phrenic Nerve
Spinal Nerves and Vagus Nerve
Vagus Nerve
Diaphragm
Accessory Muscles
Lung Receptors
LUNG

IV. Opioid Receptor Subtypes

Opioid receptors exist as three different types. This classification is based upon the differential binding affinities of radioligands, peripheral tissue bioassays and *in vivo* pharmacological profiles (Martin *et al.*, 1976; Jaffe and Martin, 1990; Pasternak, 1993). The types are designated *mu*, *delta* and *kappa* opioid receptors. Evidence also suggests that these classes may be divided into two or more subclasses each.

Early evidence for opioid receptor multiplicity came from studies with what came to be known as mixed agonists, which at lower doses antagonized the effects of morphine, but at higher doses produced analgesia. This led to the hypothesis of dual opioid receptors, one at which morphine was an agonist and the mixed agonist was an antagonist. The second receptor type was one at which the mixed agonist was an agonist. Further work with several series of agonists defined three distinct syndromes in the dog, caused by the prototypical agonists morphine, ketocyclazocine and SKF 10,047. Tolerance to each set of drug effects could be produced by administration of the appropriate agonist, but the lack of cross-tolerance by one of the other prototype agonists led to the full concept of opioid receptor multiplicity (Martin *et al.*, 1976; Gilbert and Martin, 1976).

Since the concept of multiple receptors was proposed, more specific opioid radioligands have been discovered and developed, which have led to better characterization of several opioid receptors. Most recently, the *delta* opioid receptor was cloned (Evans *et al.*, 1992; Kieffer *et al.*, 1992), followed by the *mu*

(Chen *et al.*, 1993) and *kappa* (Yasuda *et al.*, 1993) opioid receptors.

V. Opioids and Respiration

Since the discovery of multiple opioid receptors in the mammalian CNS, much work has been undertaken to discern which receptors mediate the clinically important adverse respiratory effects of opioids, and to develop narcotic analgesics devoid of respiratory depression. Opioid receptor subtypes are discussed in Section IV. The following is a brief introduction to the current understanding of the *mu*, *delta* and *kappa* opioid receptors and their importance in respiration. Reviews by Yeadon and Kitchen (1989) and Shook *et al.* (1990) contain additional discussion.

McGilliard and Takemori (1978) were the first to demonstrate that morphine analgesia and respiratory depression are produced by separate receptor mechanisms. This was accomplished by the determination of apparent pA_2 values for morphine-induced analgesia and respiratory depression, antagonized by the non-specific opioid antagonist naloxone. These two pA_2 values were not equal, indicating the involvement of separate receptors. This conclusion was strengthened by the analysis of the perturbation of the pA_2 for analgesia by morphine pretreatment, compared to the unchanged pA_2 for respiratory depression following such pretreatment. It was concluded that the mechanism for analgesia became tolerant while a separate mechanism for respiratory depression remained unchanged. With the development of more specific agonists and antagonists, the

receptors mediating the above observations have been more clearly defined.

Morphine was the prototype agonist for the *mu* receptor when it was described in 1976 (Martin *et al.*, 1976). Since then agonists and antagonists with greater selectivity for the *mu* receptor have been developed. These include DAMGO (also called DAGO), morphiceptin, FK 33,824, PLO17, TRIMU-4 and dermorphin among the agonists (table 1) and β-funaltrexamine (β-FNA) as an antagonist (table 2). As discussed in the review by Shook *et al.* (1990), several investigators have used these various agonists in different species in many studies to show respiratory depression, measured as decreases in f, V_T, V, P_{O2} or arterial blood pH. It was concluded from these studies that the observed effects were *mu* mediated. One needs to realize the assumptions that were made in drawing this conclusion though, and the limitations of using peptide agonists. It was assumed that the above agonists produced the observed effects by interaction solely with the *mu* receptor. While these agonists are relatively *mu* specific, all are able, to some extent, to interact with *delta* and *kappa* receptors, and the above experiments do not rule out a *delta* or *kappa* mediated effect. A scenario where the *mu* receptor has no role in respiratory depression and the agonists interacted with *delta* or *kappa* to an extent adequate to produce respiratory depression could also be deduced from the above data. In addition, only FK 33,824 and TRIMU-4 are capable of crossing the blood-brain barrier (BBB) and penetrate the CNS following peripheral administration (Morin-Surun *et al.*, 1984; Muraki *et al.*, 1988). Studies with the other agonists involve the use of i.c.v. injections of the drug in

question. It is therefore possible that high concentrations of agonist locally in the CNS following i.c.v. injection could produce the above hypothesized scenario of cross-receptor interaction and effect.

TABLE 1. **Opioid agonists, compound compositions and receptor selectivities.** Adapted from Yeadon and Kitchen (1989) and Pasternak (1993). Pen represents penicillamine.

Compound	**Structure**	**Selectivity**
DAMGO (DAGO)	[D-Ala^2,N-methylPhe^4-Glyol^5]enkephalin	*mu*
Morphiceptin	Tyr-Pro-Phe-Pro-NH_2	*mu*
FK 33,824	[D-Ala^2,N-methylPhe^4-Metol^5]enkephalin	*mu*
PLO17	Tyr-Pro-N-methylPhe-D-Pro-NH_2	*mu*
TRIMU-4	Tyr-D-Ala-Gly-NH-$CH(CH_3)CH_2$-$CH(CH_3)_2$	*mu*
Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	*mu*
Enkephalin	Tyr-Gly-Gly-Phe-(Leu or Met)	*delta*
DPDPE	[D-Pen^2-D-Pen^5]enkephalin	*delta*
DPLPE	[D-Pen^2-L-Pen^5]enkephalin	*delta*
DSLET	[D-Ser^2-Leu^5-Thr^6]enkephalin	*delta*
DADLE	[D-Ala^2-D-Leu^5]enkephalin	*delta*
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	*kappa*
Ethylketocyclazocine	Alkaloid	*kappa*

TABLE 2. **Opioid antagonists used in respiratory pharmacology, type of antagonism demonstrated and receptor selectivity.**

Compound	**Antagonism Type**	**Selectivity**
β-funaltrexamine	Irreversible	*mu*
Naloxonazine	Irreversible	mu_1
ICI 174,864	Competitive	*delta*
ICI 154,129	Competitive	*delta*
Naltrindole	Competitive	*delta*

To try and control for cross-receptor interactions, *mu*-specific antagonists such as β-FNA were used. Treatment with β-FNA has no effect on f when given alone. When it is given in conjunction with morphine, some investigators have shown it to antagonize morphine-induced decreases in f (Hayes *et al.*, 1985), while Ward and Takemori (1983) have shown it to either antagonize morphine or to have no effect, depending upon the method used to quantitate a decrease in f. These contradictory results again raise questions of selectivity of either the agonists or of β-FNA. One could also hypothesize a dual receptor mechanism of respiratory depression with β-FNA antagonizing a *mu*-mediated mechanism, and depending upon the concentration of agonist used, a lower affinity mechanism being activated to produce respiratory depression. This dual mechanism gains some support from experiments carried out with *delta*-specific agonists and antagonists (Pazos and Floréz, 1983; Morin-Surun *et al.*, 1984; Haddad *et al.*, 1984; Hurlé *et al.*, 1985; Kiritsy-Roy *et al.*, 1989), to be discussed later.

Pasternak and colleagues (Pasternak *et al.*, 1980; Wolozin and Pasternak,

1981; Hahn and Pasternak, 1982) have shed further light on the role of *mu* receptors in respiratory depression with the possible identification of high affinity mu_1 and low affinity mu_2 receptor sub-types. This was accomplished using the irreversible mu_1 antagonist naloxazone which is activated to naloxonazine (NLZ) *in vivo* (Hahn and Pasternak, 1982). A considerable body of literature has been produced utilizing this tool, including a 1985 paper by Ling *et al.* in which NLZ was used to selectively inactivate mu_1 receptors and antagonize morphine analgesia, but not respiratory depression. This showed a direct separation of these effects, as McGilliard and Takemori (1978) had predicted seven years earlier. These results were later confirmed with the more *mu* selective agonist dermorphin (Paakkari *et al.*, 1990). From these it was concluded morphine acts via mu_2 receptors to produce respiratory depression. In addition, as discussed in Shook *et al.* (1990) metkephamid, a mu_2 preferring agonist, has been shown to produce respiratory depression, supporting the role of mu_2 receptors as the mediator of opioid induced respiratory depression.

Several investigators have observed that low doses of morphine and other *mu* agonists cause respiratory stimulation in adult animals (Paakkari *et al.*, 1990). Morphine infusion has a biphasic effect on fetal breathing movements (FBM) in sheep (Olsen and Dawes, 1983, 1985; Szeto *et al.*, 1991) causing stimulation followed by respiratory depression. NLZ has been used in adult rats (Paakkari *et al.*, 1990) to antagonize this low dose respiratory stimulation while having no effect on high dose respiratory depression. In the fetal lamb (Szeto *et al.*, 1991), NLZ

ablates the stimulation of FBM caused by low doses of morphine. Both of these studies conclude that mu_1 receptors not only have no part in mediating respiratory depression, but cause respiratory stimulation. Additionally, in the FBM study, administration of the muscarinic agonist pilocarpine produced a dose dependent increase in FBM similar to morphine, and a link between muscarinic pathways and the mu_1 receptor proposed. In studies in adult dogs and rabbits, anticholinesterases have been shown to antagonize morphine-induced respiratory depression (Weinstock *et al.*, 1980), implicating a cholinergic mechanism. However, more investigation into this needs to be done before any firm conclusions can be made.

These studies using NLZ are not without critics. Several investigators have questioned both the *in vivo* and *in vitro* studies done to characterize the mu_1 and mu_2 receptor sub-types, leading many to doubt the existence of *mu* sub-types. In reviewing experiments carried out using NLZ, one needs to be aware of the limitations of the technique of selective inactivation. The ability of NLZ to irreversibly antagonize mu_1 receptors directly depends upon its affinity for the receptor, and there is not total acceptance of the early studies using NLZ to characterize mu_1 and mu_2. The results observed after selective inactivation of a receptor depend also on the relative amount of receptor present, the intrinsic activity of the agonist tested (these two factors, in combination, determine the receptor reserve for a given agonist in a given tissue), as well as the fact that it is unlikely an irreversible antagonist will inactivate 100% of its target receptor population. Therefore, an

agonist with low intrinsic activity and a small receptor reserve in the target tissue may lose its ability to elicit a response following (partial) receptor inactivation while an agonist with the same affinity but a high intrinsic activity and large receptor reserve in the target tissue will still elicit a response following the same level of inactivation. This response could incorrectly be interpreted as occurring *via* a different receptor mechanism than the effect of the low intrinsic activity compound, which was abolished.

High affinity selective agonists and antagonists for *delta* receptors have only been developed recently, and less is known about what role the *delta* receptor plays in opioid-induced respiratory depression. The most selective *delta* agonists currently available at this time include DPDPE and DPLPE (table 1). Earlier work used the less selective DSLET and DADLE (table 1) while ICI 174,864, ICI 154,129 and naltrindole are *delta* selective antagonists (table 2).

Failure of β-FNA, the *mu* selective irreversible antagonist, to block the respiratory depression induced by the poorly-selective *delta* agonist DADLE (Ward and Takemori, 1983) led investigators to conclude that there is a *delta* component to opioid respiratory depression and that DADLE wasn't acting through *mu* receptors. However, the same criticisms regarding irreversible antagonists apply to this study as well. Other studies using DADLE (Haddad *et al.*, 1984; Hurlé *et al.*, 1985) have also demonstrated respiratory depression, but the possibility of a *mu*-mediated mechanism cannot be ruled out. One study by Pazos and Floréz (1983) did provide evidence that a non-*mu* receptor (presumably *delta*) mediates the

effects of DADLE by the determination of the apparent pA_2 for DADLE as compared to FK 33,824. In this experiment the pA_2 for DADLE was significantly different than that for the *mu* specific agonist, suggesting different receptors were responsible for each of the two compounds' effect. DSLET, the other earlier generation *delta* agonist, has also been shown to cause respiratory depression (Morin-Surun *et al.*, 1984).

Fewer studies on respiration have been done using the newer, more *delta* selective agonist DPDPE. In one study, it was shown to depress respiration in adult rats, as measured by a decrease in arterial blood pH (Kiritsy-Roy *et al.*, 1989), and it was 250 times less potent than DAMGO, the *mu* selective agonist. These results are inconclusive, as the high dose of DPDPE used brings into question the specificity of the compound, and whether or not DPDPE was possibly acting through a *mu* mechanism. Similarly, while ICI 174,864 antagonized the DPDPE-induced acidosis, ICI 174,864 also partially antagonized the DAMGO response leaving open the conclusion that DPDPE acted through a lower affinity *mu* interaction that was completely antagonized by ICI 174,864. This study is further limited by its lack of use of a specific *mu* antagonist, using instead the non-specific antagonist naloxone as a *mu* antagonist. A study in rabbits (May *et al.*, 1989) found even less response than above and failed to show any effect by DPDPE on respiration. Contrary to the above two reports, preliminary work by another group involving DPDPE in adult rats has shown stimulation of respiration by this peptide (Kramer *et al.*, 1992). In this last study the response was antagonized by the *delta*

specific antagonist naltrindole.

Recent studies have suggested that there may be *delta* receptor sub-types (Jiang *et al.*, 1991). What role, if any, that these suggested *delta* receptor sub-types have on opioid-induced respiratory depression and the seemingly paradoxical findings described above for *delta* agonists remains to be elucidated.

Studies by several investigators using relative *kappa* selective agonists have failed to show involvement of this receptor in respiratory depression. (Hayes *et al.*, 1985; Howell *et al.*, 1988; May *et al.*, 1989; Butelman *et al.*, 1993).

Respiratory depressant effects of opioids are mediated via a *mu* receptor mechanism. Debate persists as to whether there are mu_1 and mu_2 sub-types, with mu_2 mediating respiratory depression and mu_1 mediating analgesia and perhaps respiratory stimulation. There is also evidence that *mu* mediated respiratory effects involve the muscarinic and cholinergic systems. Less clear is what role *delta* receptors play in opioid respiratory effects, with the evidence contradictory at this point. More work is needed, with more specific agonists to resolve this issue. At this time, after several studies, it appears that *kappa* receptors play no role in opioid respiratory effects.

VI. Aims of Thesis

The work described in this dissertation was undertaken for several reasons. As reviewed above, respiratory depression can be a fatal toxicity of the use of opioids. In addition, as a public health concern, neonates of mothers who have

abused opioids during pregnancy have a five- to ten- fold increased risk of dying from the sudden infant death syndrome (SIDS) (Kandall and Gaines, 1991; Kandall *et al.*, 1993). Any data regarding the neonatal respiratory effects of opioids will serve to better define the action of these drugs on breathing, and may lead to further insight of neonatal respiratory physiology and control of breathing. M6G is an active opioid compound with greater potency than the parent compound, morphine. Morphine is used increasingly for analgesia in neonatal surgery and procedures (Bhat *et al.*, 1990; Chay *et al.*, 1992) as the ability of newborns to feel pain is better appreciated, and as a sedative during mechanical ventilation (Chay *et al.*, 1992). Newborns metabolize morphine to M6G (Choonara *et al.*, 1989; Chay *et al.*, 1992), and, therefore, information on the pharmacology of this compound in the neonate is needed in order to make informed therapeutic decisions.

With these considerations in mind, this body of work commenced with the development of a high performance liquid chromatography (HPLC) microassay with ultraviolet detection (UVD) for the quantitation of morphine and metabolites in small volumes of plasma which are available in neonatal pharmacology. The hypothesis was that the neonatal guinea pig would produce both M6G and M3G after morphine administration, which was shown. This assay was later modified to quantitate morphine and metabolites in brain tissue with electrochemical detection (ECD) added. The acute respiratory pharmacodynamics of M6G in the neonatal guinea pig were determined and compared to morphine. It was hypothesized that M6G was a respiratory depressant in the neonatal guinea pig after s.c.

administration and would be more potent than morphine. This was the first report of the respiratory effects of M6G in any neonatal animal. Analysis of this study revealed that M6G was more potent than morphine, but only on days 7 and 14 after birth, not on day 3, when the drugs were equipotent. Several hypothesis regarding this potency change were developed. M6G may become more potent with aging due to differential systemic or brain distribution of the drug (pharmacokinetic explanation), or due to changes in affinity or density of opioid receptors in the brain stem (pharmacodynamic explanation). To explore the pharmacokinetic hypothesis for the potency change, the relationship of dose, plasma levels, and brain levels of M6G with respect to age was determined, but this did not explain the potency difference. In the course of investigation, data suggested that M6G and M3G enter the brain by diffusion. The acute respiratory effects of M3G, hypothesized to stimulate respiration, were also determined, but stimulation was not observed. The work concluded by examining the pharmacodynamic hypothesis of M6G potency change with the determination of the density and affinity of *mu* opioid receptors in the neonatal guinea pig brain stem. There was no change in affinity but there was evidence for a change in receptor density which may assist in partially explaining the observed potency change. The receptor study was the first report on development of *mu* receptors in the guinea pig, and the first report of neonatal receptor binding of M6G in any species.

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Manuscript I

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Title: A STEREOSPECIFIC MICROASSAY FOR THE DETERMINATION OF MORPHINE-6-β-D-GLUCURONIDE AND OTHER ACTIVE MORPHINE METABOLITES IN THE NEONATAL GUINEA PIG.

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ABSTRACT

A stereospecific microassay for the simultaneous determination of morphine and active metabolites morphine-6-β-D-glucuronide, morphine-3-β-D-glucuronide and normorphine using reversed-phase high-performance liquid-chromatography with ultraviolet detection is described. Solid-phase extraction was followed by chromatography on an octadecylsilica column eluted by 16.5% acetonitrile in phosphate buffer, pH 2.1 with sodium dodecyl sulfate as an ion-pairing agent. Using an 80 μl sample size the limits of quantitation were 94 ng/ml for all drugs. Coefficients of variation ranged from 2.0% to 6.3%. The assay is specific and separates the stereoisomers morphine-6-α-D-glucuronide and morphine-6-β-D-glucuronide. This microassay has been applied to samples of guinea pig plasma, following subcutaneous injections of morphine, and to hepatic microsomal enzyme preparations from a pregnant guinea pig. Neonatal guinea pigs produce substantial amounts of morphine-3-glucuronide and morphine-6-glucuronide from morphine *in vivo*, but normorphine was not detectable, whereas the liver microsomal enzyme preparations from a pregnant dam rapidly produced normorphine.

INTRODUCTION

Morphine is metabolized in man *via* at least two routes that produce active metabolites: conjugation with glucuronic acid at the three or six carbon, and N-demethylation at the 17 position[1-3]. Heroin (3,6-diacetylmorphine), which undergoes *in vivo* deacetylation to morphine[4,5], is also transformed to these active metabolites. Morphine-3-β-D-glucuronide's (M3G) activity is stimulatory in nature and M3G has been shown to causes seizures[13,14]. Normorphine (NOR), the N-demethylated metabolite of morphine, has activities similar to morphine[11,12], but at a reduced potency. Recently, considerable interest has arisen in the opioid activity of morphine-6-β-D-glucuronide (M6G) which has analgesic[6-8], respiratory depressant[9] and reinforcing properties[10] similar to morphine, but in these respects M6G is more potent than the parent compound. Very few data are currently available on the developmental pharmacology of these active compounds. However, morphine is used as an analgesic in neonatal and pediatric medicine[15] and *in utero* exposure to these metabolites may occur following the use of obstetrical analgesia or maternal heroin or morphine abuse.

Several assays for morphine and at least one metabolite, using high-performance liquid-chromatography (HPLC) with ultra-violet (UVD), electro-chemical (ECD) or fluorescence detection have been reported[16-23]. Assays using ECD, however, are unable to detect the M3G molecule[17-19,23] as it lacks an oxidizable phenolic hydroxyl group. To counter this, either UVD or fluorescence detection has been used in line with ECD to detect M3G[17-19]. A limit to the

usefulness of these assays still exists, though, due to the large sample sizes required (500-1000 μl of plasma) which are incompatible with the sample sizes available from small animals such as the guinea pig used in developmental pharmacology studies. The use of fluorescence detection or UVD allows the simultaneous determination of morphine and the active metabolites[16,20-22], but the assays available are again limited by the large sample volumes required in these methods (400-1000 μl). Many of these methods also use a time consuming extraction procedure processing only one sample at a time[16-18,22,23] and some require a double extraction process[16-18].

A microassay (an assay utilizing small sample volumes) for the determination of morphine and its active metabolites, M6G, M3G and NOR in guinea pig plasma has been developed. The procedure uses hydromorphone as the internal standard, an 80 μl sample size and a convenient, single-step detection with the readily available UVD technology. A rapid solid phase extraction allows simultaneous preparation of up to ten samples. The method is sensitive and specific for M6G, M3G, NOR and morphine, and is even able to separate the isomers morphine-6-β-D-glucuronide (referred to as M6G) and morphine-6-α-D-glucuronide. While the beta configuration is produced *in vivo* in mammals, the alpha isomer has been reported as a contaminant of standards synthesized for HPLC[14]. The stereospecificity of the assay therefore adds a potential quality control application which has not been addressed by other methods[16-23]. Plasma samples from neonatal guinea pigs treated with morphine subcutaneously

(s.c.) and the *in vitro* production of NOR from morphine in hepatic microsomal incubations have been examined using this microassay.

MATERIALS

Morphine•SO_4, morphine-6-α-D-glucuronide, morphine-6-β-D-glucuronide, M3G, NOR•HCl, nalorphine•HCl, dihydromorphine, morphine-3-ethereal sulfate, amphetamine, benzoylecgonine, benzoylnorecgonine and norcocaine were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the National Institute on Drug Abuse (Bethesda, MD, U.S.A.). Hydromorphone, naloxone•HCl, nalorphine•HCl, apomorphine and cocaine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nalbuphine•HCl, oxymorphone•HCl, oxycodone•HCl and hydrocodone bitartrate were a gift from DuPont Pharmaceuticals (Wilmington, DE, U.S.A.). Codeine was a gift from Merck & Co. (Rahway, NJ, U.S.A.). l-Methadone•HCl was a gift from Eli Lilly and Company (Indianapolis, IN, U.S.A.). Stock solutions of 100 ng/μl were prepared by dissolving morphine, M6G, M3G, NOR and hydromorphone in water and were stored in 1.5 ml aliquots at -15°C until use. Acetonitrile and methanol (Malinckrodt, Paris, KY, U.S.A.) were HPLC grade. Water was purified with a Milli-Q Plus Water System for analytical applications (Millipore Corp., Bedford, MA, U.S.A.). Sodium dihydrogen phosphate (monohydrate) (J.T. Baker Co., Phillipsburg, NJ, U.S.A.) and sodium dodecyl sulfate (SDS, Sigma Chemical Co.) were chromatography grade. All other chemicals were reagent grade.

METHODS

Liquid Chromatography

A Model 334 liquid chromatography system (Beckman Instruments, Berkeley, CA, U.S.A.) in line with a Model AS-100 HRLC automatic sampling system (Bio-Rad, Richmond, CA, U.S.A.), a 100 mm X 3.2 mm I.D., 3 μm particle size, octadecylsilica coated reversed-phase cartridge column (Velosep RP-18, Applied Biosystems, San Jose, CA, U.S.A.) and a Model 783A programmable absorbance detector (Applied Biosystems, Ramsey, NJ, U.S.A.) set at 214 nm and 0.002 absorbance units full scale was used to analyze extracted samples reconstituted in mobile phase. The detector response was integrated by a Model 3396 II integrator (Hewlett-Packard, Los Angeles, CA, U.S.A.) and stored on a computer using PEAK-96 software (Hewlett-Packard). Mobile phase was a modification of that reported by Svensson *et al.*[16], and consisted of 10 mM NaH_2PO_4, pH 2.1 with 1 mM SDS as an ion pairing agent and 16.5% (v/v) acetonitrile. The flow rate was 1.5 ml/min and the injection volume was 100 μl.

Solid Phase Extraction

Sample purification was accomplished using a C-18 Bond Elut extraction column with a 50 mg sorbent bed attached to a Vac Elut manifold (Varian Associates, Harbor City, CA, U.S.A.). After pretreating the column with 2 ml methanol and 2 ml water, an 80 μl sample was added to 720 μl of 0.5 M ammonium sulfate, pH 9.3, containing 675 ng of hydromorphone as the internal

standard, in the reservoir of the Bond Elut. Vacuum was applied, and the column was washed with 3 ml of 10 mM ammonium sulfate, pH 9.3 and 1 ml of dichloromethane at an average flow of 1.5 ml/min. Elution was with 300 μl of 90:10 methanol:dichloromethane acidified with 2% (v/v) 1.0 N HCl. The eluate was dried at 45°C under nitrogen and samples were reconstituted in 300 μl of mobile phase. Standard curves were constructed from the analysis of plasma spiked with 94 to 3000 ng/ml of morphine, M6G, M3G and NOR. The ratio of morphine or metabolite peak area to the internal standard peak area was plotted against the concentration of drug in plasma to produce standard curves.

Animals

Dunkin-Hartley guinea pigs were purchased pregnant (Simonsen Labs, Gilroy, CA, U.S.A.), and parturition occurred approximately two weeks after arrival (neonatal studies) or were time-bred on site (microsomal studies). Animals were housed indoors with controlled light cycles, continuous *ad libitum* food and water, and daily health checks. The Animal Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The Department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health. Experiments were carried out on 15-17 day old neonates. Three pups of two dams were dosed with 15 mg/kg morphine s.c. and blood samples were drawn into heparinized syringes *via* cardiac puncture following ether anesthesia 35-40 min after injection. Whole blood was centrifuged

at 4°C and plasma was stored in polypropylene microfuge tubes at -15°C until analysis.

Microsomal Preparation and Incubation

Hepatic microsomes were prepared from a pregnant Dunkin-Hartley guinea pig on day 60 of gestation (term is 69 days) by the method of Williams *et al.*[24] and protein was determined by the method of Lowry *et al.*[25]. Morphine (1 mM) was incubated with 0.5 mg microsomal protein according to the method of Williams *et al.*[24].

Statistical Analysis

Least-squares linear regression analysis was used to determine standard curves for the assay, and to determine the initial rate of NOR production by hepatic microsomes.

RESULTS

Resolution and Linearity

Well resolved peaks for morphine, M6G, M3G, NOR and hydromorphone were obtained using this method, and no interfering peaks were encountered in plasma (Figure 1) or microsomal preparations. Standard curves for all compounds were linear. Figure 2 shows typical standard curves for each compound. The r^2 values of the curves for morphine and M6G were 0.999, M3G was 0.997 and NOR was 0.996.

Sensitivity, Specificity, Recovery, Precision and Accuracy

Considering the 80 μl sample size and the dilutions inherent to the assay, the sensitivity of the assay was 23 ng/ml for M6G, M3G and NOR and 47 ng/ml for morphine for an injection volume of 100 μl. The lowest quantifiable amount for all compounds was 94 ng/ml. Other assays using single wavelength UVD have reported lower limits of quantitation[16,22], however, the most sensitive of these assays requires a prohibitively large sample volume (1.0 ml of plasma)[16] for use in small animals. The microassay presented here has the sensitivity required for developmental pharmacology studies in small (Figure 3) and large[14] animal models.

The assay is specific for morphine and its metabolites and is able to distinguish the natural morphine-6-β-D-glucuronide from the stereoisomer morphine-6-α-D-glucuronide. Relative retention times of related compounds are

listed in Table 1.

TABLE 1
Retention Times of Various Compounds Relative to Morphine[a,b].

Compound	*Relative retention time*
Morphine-3-β-D-glucuronide	0.28
Apomorphine	0.39
Morphine-6-α-D-glucuronide	0.40
Morphine-6-β-D-glucuronide	0.48
Normorphine	0.83
Dihydromorphine	0.90
Morphine-3-ethereal sulfate	0.99
Morphine	1.00
Oxymorphone	1.02
Hydromorphone[c]	1.50
Naloxone	2.29
Nalorphine	2.78

[a]20 ng of unextracted compound was injected and the chromatogram recorded for 30 min.

[b]No peaks were seen during the 30 min for amphetamine, benzoylecgonine, benzoylnorecgonine, cocaine, codeine, l-methadone, nalbuphine, norcocaine, or oxycodone.

[c]225 ng was injected.

Data on recovery, precision and accuracy were determined at a drug concentrations of 750 ng/ml, the midpoint of the standard curve. These data are presented in Table 2.

TABLE 2
Recovery, Coefficient of Variation and Accuracy for Morphine, Morphine-6-β-Glucuronide, Morphine-3-β-Glucuronide, Normorphine and Hydromorphone.

Drug	*Recovery*[a] *(%)*	*Coefficient of Variation (%)*[a,b]	*Accuracy*[a] *(%)*
Morphine	100	4.9	95.6
Morphine-6-β-D-Glucuronide	92	2.0	97.0
Morphine-3-β-D-Glucuronide	85	6.3	97.2
Normorphine	84	5.4	90.0
Hydromorphone	95	5.7	-

[a]Plasma concentration of 750 ng/ml. Accuracy was defined as observed divided by expected values, multiplied by 100.

[b]n=6.

In Vivo and In Vitro Metabolite Formation

The production of active morphine metabolites was investigated in three neonatal guinea pigs. Blood was sampled 35-40 min after a s.c. injection of 15 mg/kg morphine and reported as nmol/ml to correct for differences in the molecular weights of the compounds (Figure 3). The molecular weights of morphine and the two glucuronides are 285 and 461 ng/nmol, respectively. This dose is the upper range used in respiratory studies in this laboratory and the time point was chosen to correspond to peak respiratory depression. Morphine represented (mean ± S.D.) 28.3 ± 7.7% of total morphine equivalents (conjugated + unconjugated drug) in plasma at the time of sampling. M6G and M3G accounted for 21.6 ± 8.1% and 50.1 ± 3.3% of total morphine equivalents, respectively. NOR was not detected in any samples.

In a separate study, the *in vitro* oxidative metabolism of morphine by hepatic microsomal enzymes from a pregnant guinea pig was investigated using the assay reported here. NOR production was linear over the first 20 min (r^2 = .945) and the initial rate was 0.439 nmol/min/mg of protein.

DISCUSSION

Previous assays for morphine and at least one metabolite have been reported, however the sample sizes required by these methods are too large for use with small animals. The 80 μl sample size in this microassay is one-fifth the requirement of the next smallest assay's sample volume[21]. The limit of quantitation of any assay is determined in part by the sample volume. Lower sensitivities have been reported[16,22], but with much larger sample sizes. The limit of quantitation of 94 ng/ml for morphine and metabolites reported here is the optimum obtainable with a small sample size, and represents adequate sensitivity for the drug levels seen in developmental work with small animals. The use of C-18 Bond Elut columns in conjunction with the Vac Elut manifold as reported here allows for a rapid extraction process and for the simultaneous preparation of up to ten samples. In both respects, this is an improvement over the previously reported use of Chromprep PRP-1[14] or Sep-Pak C_{18} cartridges[16,17,22,23]. Some assays[16-18] have also reported the use of a double extraction, using two solid phase cartridges in order to remove interfering peaks. The addition of a 1 ml dichloromethane wash to the current extraction removes such peaks and allows a single extraction per sample. Various volumes of the ammonium sulfate and dichloromethane washes were tested, and the method described produced minimal interfering peaks with no loss of drug. A 10 mM NaH_2PO_4 buffer, pH 2.1 with acetonitrile has been used to elute drugs from the extraction cartridges[14,16,17,22,23], however, this resulted in low and inadequate recoveries

when applied to a small sample size. The acidified 90:10 methanol:dichloromethane mixture presented here was found to improve recovery by 30-40%. A range of mixtures of methanol:dichloromethane from 100:0 to 50:50 were examined, and 90:10 gave the best recovery, with the acidification further improving upon the recovery obtained by use of solvents alone. Hydromorphone was chosen for the internal standard due to its retention time and good recovery (95%).

Related opioid compounds naloxone and nalorphine chromatograph under the conditions of this assay, however, they are lost in extraction due to the dichloromethane wash. The relative retention times of all compounds could be increased with decreasing amounts of acetonitrile in the mobile phase. Increase in the concentration of the ion pairing agent, SDS, also increased the retention time of morphine and its metabolites.

Both active glucuronide metabolites were produced in neonatal guinea pigs following morphine exposure. Further investigation of any role these compounds may play in morphine pharmacodynamics is being conducted. While NOR was not seen in the neonate, hepatic microsomes from the pregnant guinea pig are capable of rapid NOR production, and this is being characterized. Phase II (conjugation) metabolism of morphine has also been studied *in vitro* with this assay (data not shown).

In conclusion, an HPLC microassay for the simultaneous determination of morphine and its active metabolites is described. The assay is stereospecific and

separates the isomers morphine-6-α-D-glucuronide and morphine-6-β-D-glucuronide. The small sample size and sensitivity are suitable for use with *in vivo* and *in vitro* developmental pharmacology studies. The unique stereospecific aspect of this assay would also allow for a quality control application following the chemical synthesis of M6G. The method is reproducible and provides rapid extraction of multiple samples with good recovery of all compounds. The neonatal guinea pig produces both glucuronides *in vivo* while hepatic microsomes from the dam produce NOR *in vitro*.

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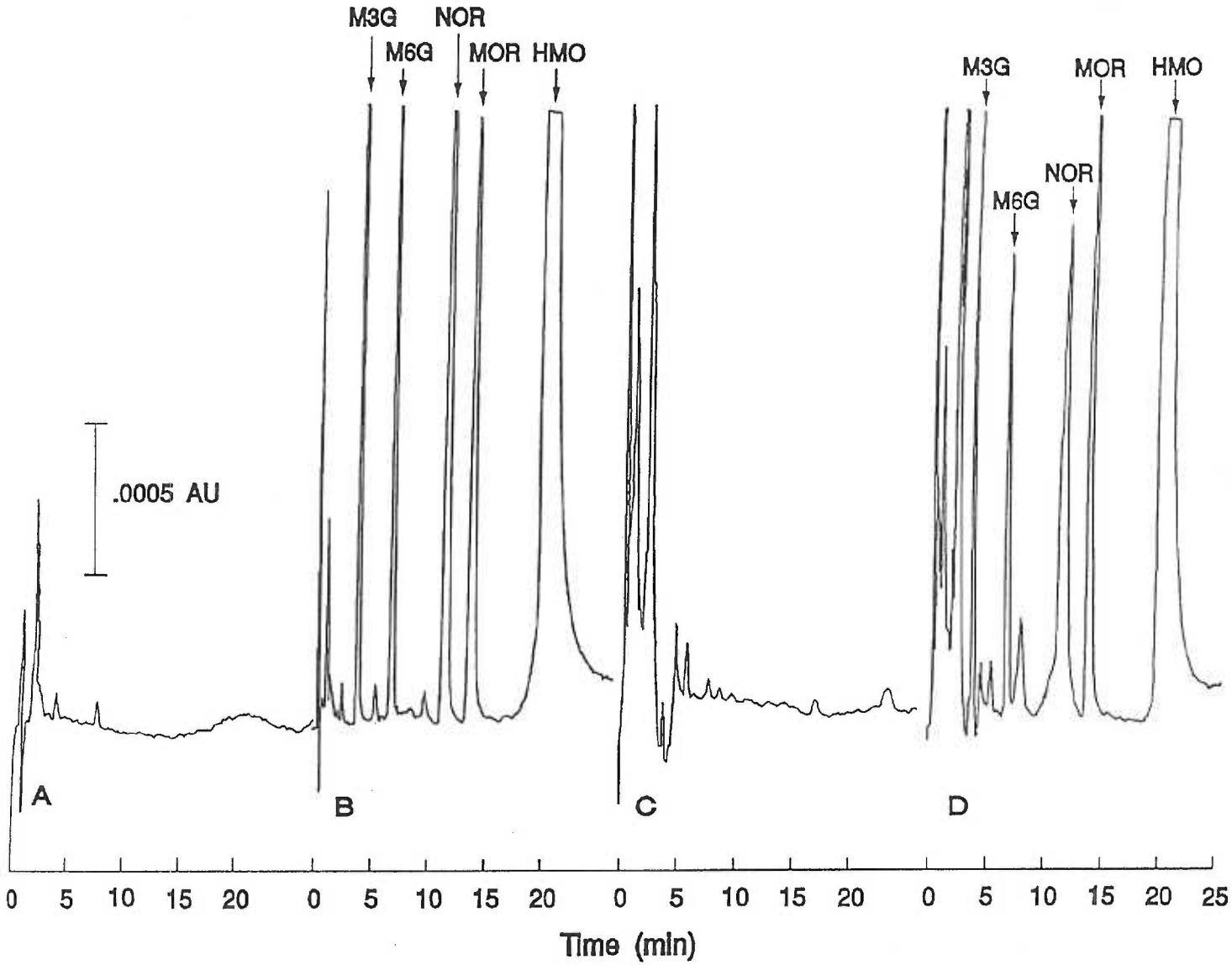
FIGURE 1. Chromatograms of (A) mobile phase; (B) mobile phase with 20 ng/100 μl injection of M6G, M3G, NOR, morphine and 225 ng/100 μl injection of hydromorphone (HMO); (C) extracted blank guinea pig plasma; and (D) extracted guinea pig plasma with 20 ng/100 μl injection of M6G, M3G, NOR and morphine, and 225 ng/100 μl injection of hydromorphone.


M3G
NOR
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A
B
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0 5 10 15 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20 25
Time (min)

FIGURE 2. Standard curves for M6G (●), M3G (○), NOR (■) and morphine (□). An 80 μl sample of guinea pig plasma was extracted on a C-18 solid phase column, the extract dried under nitrogen and reconstituted in mobile phase. The ratios of the peak area for each drug to the internal standard area, multiplied by 100 are plotted as functions of the amount of drug in unextracted plasma. Curves are produced from least squares regression analysis. Pearson's correlation coefficients (r^2) were 0.999 for morphine and M6G, 0.997 for M3G and 0.996 for NOR.

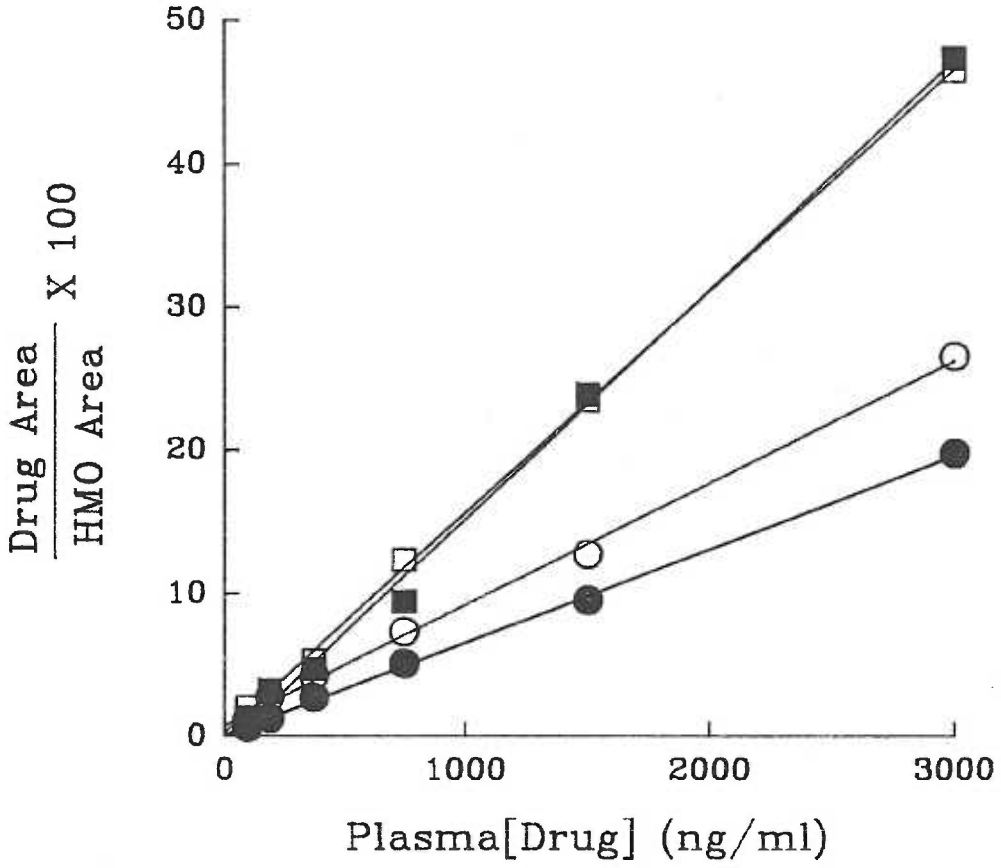
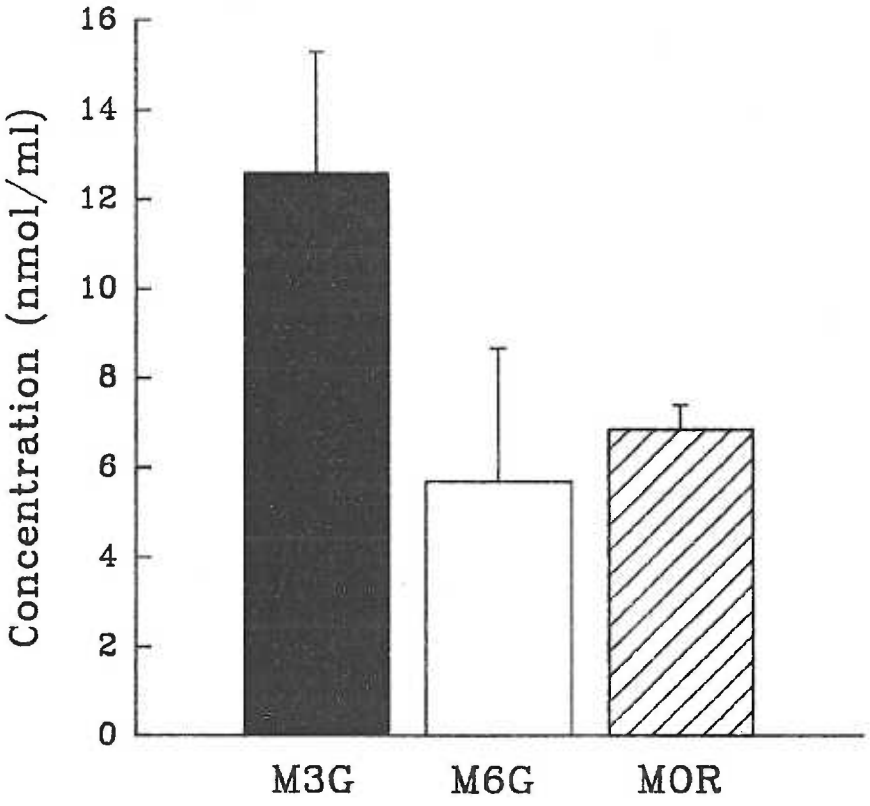

50
40
30
20
10
0
0
1000
2000
3000
Drug Area / HMO Area X 100
Plasma[Drug] (ng/ml)

FIGURE 3. Production of active morphine metabolites in neonatal guinea pigs. Blood was collected 35-40 min after three 15-17 day old pups were injected subcutaneously with 15 mg/kg morphine. Error bars represent standard deviation.


Concentration (nmol/ml)
16
14
12
10
8
6
4
2
0
M3G
M6G
MOR

Manuscript II

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Title: Morphine-6-β-D-Glucuronide Respiratory Pharmacodynamics in the Neonatal Guinea Pig[1, 2].

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Running title: M6G and Neonatal Breathing

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Abbreviations:

M6G	morphine-6-β-D-glucuronide
V_T	tidal volume
V_I	inspiratory minute volume
f	breathing frequency
T_I	inspiratory time
V_T/T_I	mean inspiratory flow
AUC	area under the time-action curve
CNS	central nervous system

ABSTRACT

Morphine-6-β-D-glucuronide (M6G) is a metabolite of morphine with opioid activity in adults. No data are available, however, on the developmental pharmacology of M6G including investigation of the respiratory effects of M6G in the neonate. A randomized, placebo-controlled study comparing the time-action, dose-response and potency of the respiratory effects of M6G to morphine was done using a nonanesthetized neonatal guinea pig model and a noninvasive computerized plethysmograph technique. Respiration was measured while the neonate breathed room air followed by 5% CO_2 in air. M6G (0.5 - 5.0 mg/kg) and morphine (1.5 - 15 mg/kg) administered subcutaneously decreased ventilation in 3-, 7- and 14-day old neonatal guinea pigs given a 5% CO_2 challenge. During CO_2 inhalation, time-to-peak action for M6G occurred 21 min later than for morphine. At maximal ventilatory depression on day 3, a dose of 1.5 mg/kg of morphine or M6G decreased minute ventilation while breathing 5% CO_2 by 30% compared to placebo. Ventilation also decreased as a function of age in both placebo and drug-treated animals. The percent respiratory depression relative to placebo remained constant for a given dose of morphine as the neonate aged, but not for M6G, which increased in potency. M6G was equipotent to morphine on day 3 after birth, but was 8-fold more potent by day 7. This increase in potency persisted through

The increased potency of M6G which accompanies aging may be due to
ange in M6G disposition or a change in opioid receptors during
nt of the neonatal guinea pig.

Introduction

Morphine-6-β-D-glucuronide (M6G) is a metabolite of morphine with opioid activity. Shimomura *et al.*, (1971) reported M6G to be analgesic in mice with potencies of 3 to 4 and 45 times that of morphine after s.c and i.c.v. administration, respectively. Analgesic activity and greater M6G potency were later confirmed by others (Pasternak *et al.*, 1987; Abbott and Palmour, 1988; Paul *et al.*, 1989; Gong *et al.*, 1991). It has been shown to be analgesic in man after systemic (Osborne *et al.*, 1988) and intrathecal (Hanna *et al.*, 1990) administration and was 2 times more potent than morphine in the latter study. M6G has reinforcing properties in the conditioned place preference test (Abbott and Franklin, 1991) and increases gastrointestinal transit time (Paul *et al.*, 1989) in rats, where it is 146 and 50 times more potent than morphine, respectively, after i.c.v. administration.

Respiratory depression often limits the clinical use of morphine. Case reports have implicated M6G in respiratory depression (Osborne *et al.*, 1986). Pelligrino et al. (1989) reported on the respiratory depressant properties of M6G, after 4th cerebroventricular administration to dogs, which was confirmed in rats using lateral cerebroventricular administration (Gong *et al.*, 1991). In both reports, M6G was 5 to 10 times more potent than morphine. M6G also depresses ventilation in humans after i.v. administration (Peat *et al.*, 1991).

M6G is produced from morphine in many mammalian species (Oguri *et al.*, 1970), by hepatic uridine diphosphoglucuronosyl transferase, including the 15- to 27-week human fetus (Pacifici *et al.*, 1982; Gawronska-Szklarz *et al.*, 1985) and

guinea pig neonate (Murphey and Olsen, 1993). There are no animal or human data on the respiratory effects of M6G during the neonatal period. This is of concern because the use of morphine in neonatal medicine has increased recently (Bhat *et al.*, 1990; Choonara *et al.*, 1989). Perinatal exposure to M6G may result after obstetrical analgesia with morphine, because morphine glucuronide crosses the hemomonochorial placenta (Olsen *et al.*, 1989). M6G is also a metabolite of heroin (diacetylmorphine). Because heroin is metabolized first to morphine (Inturrisi *et al.*, 1984), perinatal exposure may result from late gestational maternal heroin abuse.

This study was undertaken to compare the neonatal respiratory pharmacodynamics of M6G to that of morphine using a non-invasive, placebo-controlled, randomized design. Time-action, dose-response and potency comparisons were made using the neonatal guinea pig, a good model for the study of perinatal and neonatal drug effects because of its similar placental structure and other features (Olsen and Weil, 1992a).

Methods

Animals. Female Dunkin-Hartley guinea pigs (Simonsen Labs, Gilroy, CA) were time-bred on site. 126 pups from 35 litters were studied in the three dose comparison of morphine and M6G and 18 neonates from six litters were used in the extended dose study of morphine and M6G. Animals were housed indoors in cages lined with CareFRESH bedding (Absorption Corp., Bellingham, WA) with controlled light cycles, continuous *ad libitum* food and water, and daily health checks. The Animal Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The Department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health.

Drugs and dosing. M6G (Research Triangle Institute, Research Triangle Park, NC), morphine (Mallinckrodt, St. Louis, MO) and naloxone (Sigma Chemical Co., St. Louis, MO) were dissolved in saline and frozen at -20°C until use. Concentration of drug solutions were confirmed by high performance liquid chromatography analysis. Preliminary studies in this laboratory on 2- to 3-week-old guinea pig neonates and reports in the literature on adults of other species suggested a M6G: morphine mg/kg potency ratio of 3:1; therefore, a dose-range for M6G one-third of that of morphine was used. For the three-dose comparison of morphine and M6G, pups were randomized to one of seven drug treatment groups: placebo (saline); 0.5, 1.5 or 5.0 mg/kg (1.1 - 11 μmol/kg) M6G; or 1.5, 4.5 or 15.0 mg/kg

(5.3 - 53 μmol/kg) morphine for each age (day 3, 7 or 14 after birth). Additional doses of 15.0 mg/kg M6G, 30 mg/kg M6G and 30 mg/kg morphine were added to an extended dose study on day 3. Six pups were examined on each treatment at each age. Pups from a single litter were distributed across study groups and ages. Drugs and placebo were administered by s.c. injection into the shaved nape of the neck at a volume of 1 ml/kg body wt. Each animal was studied only on one day.

Noninvasive plethysmography. Neonates were studied between 8:00 a.m. and 4:00 p.m. Diurnal variations were analyzed over this time in a separate study and were not significant (data not shown). A two-chamber plethysmograph (Buxco Electronics, Sharon, CT) with a pressure transducer (Validyne Engineering, Northridge, CA) attached directly to the body chamber was used to measure breathing. Characteristics of the apparatus have been described previously (Olsen and Weil, 1992a,b). The apparatus is calibrated routinely with an air flow of 2.5 ml/sec and known volumes of 0.5 and 1.0 ml. The system is accurate, linear, reproducible and has a fast response time (Olsen and Weil, 1992a,b). The head chamber is used to provide a space for introduction of known gas mixtures for inhalation and can be completely purged with any desired gas within about 2 min.

Room air and CO_2 measurements. The animal was placed in the plethysmograph. After a 10-min acclimation period, the animal was injected s.c.

with the treatment solution while in the apparatus. Ten min after injection, the first room air data were recorded with a steady flow of compressed air (21% O_2, balance N_2, referred to as room air) through the front chamber. Pressure changes in the rear chamber were recorded *via* the transducer, converted to air flow and integrated by computer to give tidal volume (V_T) and inspiratory minute ventilation (V_I). Both V_T and V_I are expressed per 100 g of body weight. Also derived are breathing frequency (f), inspiratory time (T_I) and mean inspiratory flow (V_T/T_I). Mean inspiratory flow, a measure of central respiratory effort, is not weight corrected. Data are sampled every 10 msec (100 Hz) and averaged over 5-sec periods for 1 min.

After the first room air study is completed, the head chamber is purged with a 5% CO_2 mixture (30% O_2, 5% CO_2, balance N_2). The O_2 content is increased to 30% to ensure a purely hypercapnic response without a hypoxic component. Steady state, defined as a stable 5% CO_2 in inspired gas and a stable, elevated V_I, is reached by 3 min, and the hypercapnic data are recorded between min 4 and 5 after introduction of CO_2 (the 15th min post-injection). The same variables are recorded over this period. Data are recorded while breathing room air at 10, 25, 40, 55, 70 and 85 min and elevated CO_2 at 15, 30, 45, 60, 75 and 90 min after injection of saline or opioid drug.

Data analysis. Time-action curves were determined for morphine and M6G for 90 min after injection of drug. Area under the time-action curve (AUC) was calculated

by the linear trapezoidal rule using the HOTSHOT program (Assist/One, Portland, OR). The time of maximum, or peak, depression was analyzed for all doses of morphine and M6G. Peak depression was defined as the lowest V_I recorded during the 90 min. The time of peak depression was determined for breathing both room air and the 5% CO_2 mixture. The absolute change in a parameter (the *delta* response) was calculated by subtracting the corresponding room air value from the peak depression value while breathing CO_2.

Statistical analysis. Significance was defined as $P<.05$. Neonatal weights were analyzed by one-way ANOVA (NWA Statpak, Portland, OR). Sex ratio comparison was by chi square analysis (NWA Statpak). Variation in placebo pups' base-line respiratory parameters was analyzed with one-way repeated measures ANOVA (NWA Statpak) for each day of study. Time-action curves for placebo-, morphine- and M6G-treated animals were compared by 3-way ANOVA for drug, day and time with repeated measures for time (NWA Statpak). *Post-hoc* testing of significant effects was done by one-way ANOVA and Newman-Keuls multiple range test. Time-to-peak action was analyzed by 3-way ANOVA (NWA Statpak).

Peak drug effects and AUC data were transformed by calculating a *Z*-score, which is a comparison of the morphine and M6G data to the age-matched placebo treatment group. In the case of V_I for any given morphine- or M6G-treated pup, the *Z*-score was calculated by subtracting the average placebo value at the same time point from the individual value for the treated pup and dividing the difference by

the standard deviation of the placebo group. Because there were no statistical differences between male and female animals with respect to drug-, dose- or day-effects by ANOVA, subsequent analysis was done on pooled data. Student's *t* test was used to determine whether *Z*-scores for AUC and peak drug effects for the room air, CO_2 and *delta* data were significantly different from placebo. Further analysis of *Z*-scores was by 3-way ANOVA using SAS statistical software (SAS Institute Inc., Cary, NC) on a VAX/VMS system (Digital Equipment Corporation, Maynard, MA). Actual data are presented in figures 1 to 5, and table 1. Statistical results are given in table 2. *Post-hoc* testing was done using the Student-Newman-Keuls procedure. Extended dose data were analyzed by 2-way ANOVA for drug and CO_2 using non-*Z*-scored data.

Regression analysis used least square nonlinear and linear regression of individual pup data points (NWA Statpak). Log dose-responses were analyzed for responses between 20 and 80% of placebo. Comparison of morphine and M6G dose-response regressions was by the method of Neter and Wasserman (1974), using the *F* ratio test. For significant differences, slopes were compared using the *t* statistic. Potency was calculated on a molar basis for responses that were 50% of placebo for a given parameter. When slopes differed, potency was calculated for the upper and lower limits of responses observed.

Results

Neonatal weights and sex ratio of 3-dose study. There was no difference in neonatal weights among the seven treatment groups at birth or on day of study. The 126 pups on day 1 weighed (mean ± S.E.) 97.3 ± 1.4 g. The 42 pups studied on day 3, 7 or 14, weighed 94.3 ± 2.3, 126 ± 3.1 and 188 ± 4.5 g, respectively. Although there were significantly more female (n = 75) than male (n = 51) animals (χ^2 = 4.57, df = 1; P<.05), no differences were present in the male: female ratio among treatment groups or in the responses of male and female pups (data not shown).

Placebo-treated animals. Respiratory parameters for placebo-treated animals are presented in table 1. V_I decreased during the 90 min study while breathing room air on days 3 and 14 ($F_{5,25}$ = 4.15, P<.01 and $F_{5,25}$ = 2.77, P<.05, respectively). Day 3 placebo V_I decreased from 106 to 74 $ml \cdot min^{-1} \cdot 100g^{-1}$ over the study period, while day 14 V_I decreased from 76 to 59 $ml \cdot min^{-1} \cdot 100g^{-1}$. While breathing room air on day 14, f decreased ($F_{5,25}$ = 4.63, P<.01) from 122 to 93. On day 3, breathing 5% CO_2, f changed ($F_{5,25}$ = 3.28, P<.05) from 188 at 15 min, to 174 to 178 $breaths \cdot min^{-1}$ for the remainder of study. V_T/T_I of placebo pups breathing 5% CO_2 on day 14 decreased ($F_{5,25}$ = 3.54, P<.05) from 17.1 $ml \cdot sec^{-1}$ at 15 min to a stable range of 13.4 to 13.9 $ml \cdot sec^{-1}$ over the last hr of study. During CO_2 inhalation, V_I had a linear age-dependent decrease during the first 2 weeks of life [V_I = 382 - 12.7•(age), $F_{1,16}$ = 21.85, P<.01] as has been described previously

in the neonatal guinea pig (Olsen and Weil, 1992a,b).

Time-action for V_I. Comparison of time-action curves for V_I breathing 5% CO_2 was significant for drug-, day- and time-effects and drug-time and drug-day-time interactions (F ranged from 1.45 - 95.0, $P<.05$) (fig. 1). There was no difference between morphine and M6G in time-to-onset and duration of action. The drug-time interaction was due to differences in time-to-peak action and the drug-day-time interaction reflects different time-action profiles as animals aged (fig. 1). Peak V_I depression was significantly later for M6G than for morphine while breathing room air ($F_{1,60} = 9.94$, $P<.01$) or 5% CO_2 ($F_{1,90} = 33.2$, $P<.0001$). Peak depression on morphine occurred at (mean ± S.E.) 40 ± 4 and 46 ± 3 min on room air and 5% CO_2, respectively, and on M6G at 55 ± 3 and 67 ± 3 min, respectively. Dose affected time-to-peak V_I depression breathing room air ($F_{2,60} = 4.29$, $P<.05$), but not CO_2. Time-to-peak for room air occurred 35% later for the low dose of the two drugs than mid or high doses (data not shown). There was no effect of age on time to peak V_I depression.

Area under V_I, f, and V_T time-action curves. The AUC of V_I, f and V_T time-action curves was calculated to analyze all features of the time-action curve: onset of action, time to peak, intensity of peak and duration of action. However, there was no difference between AUC for morphine and M6G while breathing room air or 5% CO_2 (data not shown).

Peak effect for V_I. Treatment with morphine and M6G decreased ventilation compared to placebo in neonatal guinea pigs breathing room air (t_{107} = 10.0, P<.0001) or 5% CO_2 (t_{107} = 21.7, P<.0001). Further analysis of the room air data was significant for reduced V_I on days 7 and 14 of life, but not on day 3 (data not shown). For the CO_2 data, V_I decreased compared to placebo in drug-treated animals for all doses at all ages (fig. 2A). The dose-response regressions were not significant on day 3, but were on days 7 and 14 with the M6G curve shifted to the left (fig. 3). There was a day-effect with V_I decreasing with increasing age (fig. 3). The *delta* response from room air to 5% CO_2 for V_I was depressed for all doses on all days (t_{107} = 20.1, P<.0001) (fig. 4). *Delta* V_I decreased with dose (table 2, and fig. 4). M6G: morphine potency ratios were 1:1 on day 3 and 9.9:1 on day 14, measured at 50% of placebo V_I *delta* response (data not shown). Dose-response relationship for M6G on day 7 was not significant.

Peak effect for f. Depression of V_I was caused by decreases in f for room air and both f and V_T for 5% CO_2 data. Breathing room air, morphine and M6G decreased f compared to placebo (t_{107} = 11.6, P<.0001) (data not shown). Aging enhanced the morphine and M6G depression of f. Drug treatment decreased f while breathing 5% CO_2 (t_{107} = 14.5, P<.0001) except low dose morphine on day 14 (fig. 2B). Log-linear decreases in f were significant for morphine, but not for M6G. Aging augmented the drug effect on depression of f (fig. 2B). The *delta* response for f was decreased after morphine and M6G treatment (t_{107} = 8.8, P<.0001).

Post-hoc analysis indicated a decrease only on days 3 and 7.

Peak effect for V_T. V_T was increased by drug treatment breathing room air (t_{107} = 3.7, P<.001) (data not shown). During CO_2 inhalation morphine and M6G decreased V_T (t_{107} = 8.0, P<.0001) compared to placebo response for most doses (fig. 2C). A dose-response relationship was significant only on day 14 for M6G (table 2, fig. 2). The *delta* response for V_T was decreased compared to placebo for all doses on all days (t_{107} = 12.8, P<.0001), reflecting both an increased V_T on room air and a decreased V_T on 5% CO_2 (data not shown). M6G caused a greater decrease in the *delta* V_T than did morphine and the decrease to both drugs increased with aging.

Timing at peak depression. T_I was increased by morphine and M6G while breathing room air (t_{107} = 11.6, P<.0001) and 5% CO_2 (t_{107} = 12.3, P<.0001) (data not shown). Dose-effects on room air were only significant for morphine; on CO_2 they were significant for morphine and also for M6G on day 3. The drug-induced increase of T_I was greatest on day 7.

Mean inspiratory flow at peak depression. Morphine and M6G depressed V_T/T_I while breathing room air (t_{107} = 20.4, P<.0001). Effects of both dose and day were significant (table 2), but visual inspection of data revealed no trends. For the CO_2 (t_{107} = 23.9, P<.0001) and *delta* response data (t_{107} = 16.3, P<.0001),

morphine and M6G depressed V_T/T_I compared to placebo and were affected by dose (table 2, fig. 3). On days 7 and 14, the curves of the *delta* response data differed; M6G was 5.9 and 6.1 times more potent at 50% of placebo, respectively. Slopes differed on day 14, and potency varied from 9.2 to 3.9 over the dose range (data not shown).

Extended dose study. Extended dose V_I time-action data while breathing CO_2 had a drug-effect ($F_{8,45}$ = 6.85, P<.0001) and a drug-time interaction ($F_{40,225}$ = 1.69, P<.01). The interaction was caused by a later time for the M6G peak compared to morphine (data not shown) similar to the 3 dose study. At peak effects, drug treatment decreased V_I, f ($F_{9,50}$ = 6.82 and 10.18, respectively, P<.0001) and V_T ($F_{9,50}$ = 3.07, P<.01) on 5% CO_2 for the day 3 extended dose analysis. Added doses resulted in a significant log dose-response relationship for V_I, breathing 5% CO_2 (fig. 5) and confirmed that morphine and M6G were equipotent on day-3. Morphine and M6G dose-responses on CO_2 for V_T were significant, and the drugs equipotent (fig. 5). V_T/T_I was depressed by drug treatment ($F_{9,50}$ = 11.29, P<.0001) and the day-3 dose-response trend for M6G was equipotent to morphine (fig. 5). The f regressions were significant and the slopes differed (data not shown).

Naloxone antagonism. Naloxone (0.5 mg/kg) rapidly reversed M6G (5.0 mg/kg) induced peak respiratory depression in 3- and 7-day-old animals breathing 5%

CO_2. Onset of antagonism was within 20 sec, and decreases in V_I were reversed by 65 to 100%.

Discussion

This study is the first description of the neonatal and developmental respiratory pharmacodynamics of M6G and the first after s.c. administration of M6G to unanesthetized and noninstrumented animals. Both M6G and morphine depress ventilation in neonatal guinea pigs. Time-action profiles of the two drugs differ, with M6G having a later time of peak depression. Relative potency of the two drugs change with aging, with no potency differences between the drugs on day 3 and a 8- to 9-fold greater potency for M6G on days 7 and 14. In 3- and 7-day-old animals, naloxone, at one-tenth the dose of M6G, rapidly antagonized M6G-induced respiratory depression.

Morphine is the standard drug used for analgesia in clinical medicine, and other narcotic analgesics are compared to morphine for efficacy and potency determinations. M6G contributes to the analgesia seen after administration of morphine in adults (Portenoy *et al.*, 1992). In neonatal medicine, morphine analgesia is increasingly common (Bhat *et al.*, 1990) and exposure to M6G would occur because of neonatal M6G production from administered morphine (Choonara *et al.*, 1989).

Respiratory depression is the limiting side-effect of morphine and M6G may contribute to this effect (Osborne *et al.*, 1986). Few studies have investigated the respiratory effects of M6G and no work has been done in the neonate, although neonates may be more sensitive to opioid-induced respiratory depression (Olkkola *et al.*, 1988). Exploration of M6G respiratory effects in animals has been done

using instrumented preparations and i.c.v. drug administration (Pelligrino *et al.*, 1989), often with halothane anesthesia (Gong *et al.*, 1991, 1992), raising the possibility of drug-drug interactions involving M6G as well as disruption of central nervous system (CNS) respiratory pathways. One human study (Peat *et al.*, 1991), using i.v. administration, compared two doses of M6G to one, 50% greater dose of morphine, making a potency comparison difficult.

M6G binds to opioid receptors. Pasternak et al. (1987) reported similar *mu* affinities for morphine and M6G. More recently, M6G has been reported to have 3- to 4-fold less affinity for *mu* receptors (Paul *et al.*, 1989; Francés *et al.*, 1990) but 2-fold greater affinity for *delta* receptors (Francés *et al.*, 1990). In this last study, M6G was 100 times less competitive for *kappa* sites. Paul *et al.* (1989) reported M6G to have 3 to 4 times less affinity for both *mu*-1 and *mu*-2 sites, whereas Hucks *et al.* (1992) reported similar M6G and morphine binding at *mu*-1 sites and 5-fold less affinity for M6G at *mu*-2 sites. Based upon its binding profile, M6G probably causes respiratory depression *via mu* receptors, with a possible contribution from *delta* receptors. Opioid receptor affinities for M6G do not explain adequately the reported M6G and morphine potency differences. After i.c.v. administration, M6G is 45-90 times more potent than morphine for *mu*-1-mediated analgesia (Shimomura *et al.*, 1971; Paul *et al.*, 1989) and 5-10 times more potent for *mu*-2-mediated respiratory depression (Pelligrino *et al.*, 1989; Gong *et al.*, 1991), whereas M6G affinities are equal for *mu*-1 or actually less than morphine for *mu*-2. Further research, including receptor activation and production of second

messengers is required to resolve this disparity.

Opioid-induced respiratory depression is due to decreases in central chemoreceptor CO_2 sensitivity, and is mediated by *mu* receptors (McGilliard and Takemori, 1978a,b; Ling *et al.*, 1985). Chemoreceptors in the CNS respond to changes in CO_2 and pH, the most important stimuli for breathing (Yeadon and Kitchen, 1989). Opioids may exert effects on timing, depth of breathing or on both. In the study reported here, both M6G and morphine effects were caused by changes in f and V_T and were most pronounced while breathing a 5% CO_2 gas mixture. V_T of all drug-treated animals increased while breathing room air compared to placebo, perhaps as a physiological response to drug-induced hypoventilation. The reason V_T is depressed by morphine and M6G while breathing 5% CO_2 may be the absence of hypoxic drive caused by 30% O_2 in the gas mixture.

Large, polar molecules, such as M6G generally have been thought not to cross the blood-brain barrier. However, it has been shown (Shimomura *et al.*, 1971; Yoshimura *et al.*, 1973) that M6G does cross this barrier intact. No free morphine was detected in either brain or blood in these studies, indicating that the activity of M6G is not caused by the hydrolysis of M6G and subsequent passage of morphine into the CNS. M6G also penetrates into the cerebrospinal fluid of humans (Portenoy *et al.*, 1991). Carrupt *et al.* (1991) hypothesized that M6G may be a "molecular chameleon," folding the glucuronic acid group to present a relatively hydrophobic appearance, and cross biologic membranes. Selective

increases in blood-brain barrier permeability also could explain how M6G enters the CNS after peripheral administration. Baba et al. (1988) reported that administration of *mu*-agonists in mice increases the permeability of the barrier to sodium fluorescein. Development of the blood-brain barrier is usually anatomically complete by the time of a critical period in CNS development (Bradbury, 1979), which occurs between days 41 and 45 of gestation in the guinea pig (term = 69 days) (Dawes, 1968), so it is unlikely that an anatomically immature barrier is responsible for the entry of M6G into the CNS causing the respiratory depression reported here. The possibility that M6G crosses into the brain by way of a carrier exists. Candidate carriers could include the glucose carrier (Pardridge and Oldendorf, 1977), the enkephalin transporter (Zloković *et al.*, 1987), or the monocarboxylic acid carrier (Pardridge and Oldendorf, 1977).

The current study reports a significant difference in the times-to-peak for M6G- and morphine-induced ventilatory depression while breathing room air and 5% CO_2. M6G had a later time-of-peak action in neonatal guinea pig pups, which is consistent with differential M6G and morphine distribution. Takemori and Stenwick, (1966) and Hug, (1967) reported that morphine is transported actively out of the cerebrospinal fluid by the choroid plexus in the rabbit and dog. Morphine-3-glucuronide and, presumably M6G do not share this same mechanism (Muraki, 1971). Preferential removal of morphine could explain the differences in time-to-peak action.

The most striking finding in this study was a change in potency of M6G with

development of the neonate. This is the first such report of a developmental alteration in the control of respiration by M6G. In decreasing ventilation, M6G was equipotent to morphine on a molar basis on day 3 of life, but had become 8 times more potent on day 7. The potency of morphine relative to placebo did not change with age, but M6G became more potent. Due to differences in molecular weight (M6G = 461, morphine = 285), the day-7 potency ratio is 4.7, on a mg/kg basis.

Development of opioid receptors has not been studied extensively in the guinea pig. One investigation reported late fetal guinea pigs to have the same number of naltrexone binding sites as adult animals (Clendeninn *et al.*, 1976). In the newborn rat, naltrexone binding capacity (Clendeninn *et al.*, 1976) and the ratio of *mu* to *kappa* and *delta* receptors change during the first 3 postnatal weeks (Leslie *et al.*, 1982). This time frame, however, corresponds to a period of CNS development that probably occurs *in utero* in the guinea pig. It is unknown if the relative population of opioid receptors changes after birth, and whether this plays any role in the changing potency of M6G.

Developmental pharmacokinetic changes usually result in decreased drug action and probably do not contribute to observed potency changes. Morphine is eliminated primarily by liver metabolism and has a much longer elimination half-life in the human neonate than the older child or adult. This is due to a low hepatic clearance value, which increases with age (Olkkola *et al.*, 1988). M6G is eliminated by renal excretion. If the clearance of M6G changes with age in the guinea pig, it

should increase because of increased renal function, thereby decreasing potency. Access of M6G to the CNS may change with development, but anatomic maturation of the blood-brain barrier decreases uptake of large molecules in the human (Misra *et al.*, 1987), and presumably in the guinea pig, which has a similar CNS development profile. Maturation or induction of a carrier for M6G would also explain potency changes with aging. Carriers on the blood-brain barrier are inducible (Cremer *et al.*, 1976; Pardridge and Oldendorf, 1977). For instance, the monocarboxylic acid carrier is induced in the neonate by suckling (Cremer *et al.*, 1976).

This study reports on the time-action, potency and developmental profile of the M6G effect upon respiration in the newborn guinea pig. Comparison with morphine was remarkable for differences in time-to-peak action and potency. The most striking difference was an 8-fold increased potency of M6G that occurred between days 3 and 7 of life, which has not been reported previously. M6G is a potent respiratory depressant in the neonate, and sensitivity to this active metabolite increases with aging. The change in potency of M6G may be caused by changes in drug disposition or in opioid receptors.

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Footnotes

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2. Performed as partial requirement for a Ph.D. degree in Pharmacology for L.J. Murphey.

TABLE 1. **Respiratory parameters for saline-treated animals.** Values are the average of six measurements for each of six pups at each age during the 90-min time-action study after saline injection. One-way repeated measures ANOVA was used to analyze for change over the 90 min for each parameter on each day, and significant variation within a day is noted below. Data presented as means ± S.E.

Day	f min^{-1}	V_T $ml \cdot 100g^{-1}$	V_I $ml \cdot min^{-1} \cdot 100g^{-1}$	T_I sec	V_T / T_I $ml \cdot sec^{-1}$
Room air					
3	122±5	0.74±0.04	87±3 [a]	0.19±0.006	4.0±0.2
7	143±6	0.61±0.02	84±3	0.15±0.007	5.7±0.3
14	106±4 [a]	0.69±0.03	69±2 [b]	0.23±0.010	5.9±0.3
5% CO_2					
3	178±3 [b]	1.99±0.07	348±12	0.15±0.004	13.1±0.4
7	171±3	1.70±0.06	289±8	0.14±0.002	15.0±0.4
14	126±6	1.66±0.01	207±9	0.22±0.008 [b]	14.6±0.5 [b]

[a] Significant variation over the 90 min time-action study for a given day ($P<.01$).

[b] Significant variation over the 90 min time-action study for a given day ($P<.05$).

TABLE 2. ***F* ratio and significance level for 3-way ANOVA of *Z*-scored respiratory data at peak depression while breathing room air or 5% CO_2 and for the absolute change (*delta* response) between the two gas mixtures.** There was no overall difference in the response between the morphine and M6G treatments breathing room air or 5% CO_2; no 3-way interactions among drug, dose and day; and no 2-way interactions occurred except for drug by day interactions for V_T / T_I on CO_2 and *delta* f, and drug by dose for T_I on CO_2 and *delta* V_T. Degrees of freedom are indicated in *F* subscripts. NS, not statistically significant.

Effect	f	V_T	V_I	T_I	V_T / T_I
Room air					
Dose: $F_{2,90}$	5.00	1.63	1.04	6.33	5.40
P	<.01	NS	NS	<.01	<.01
Day: $F_{2,90}$	16.02	2.52	22.58	9.99	4.47
P	<.0001	NS	<.0001	<.0001	<.05
5% CO_2					
Dose: $F_{2,90}$	12.64	6.35	20.35	16.32	15.09
P	<.0001	<.01	<.0001	<.0001	<.0001
Day: $F_{2,90}$	32.80	6.70	7.50	45.93	15.36
P	<.0001	<.01	<.001	<.0001	<.0001
Delta					
Dose: $F_{2,90}$	0.22	16.80	17.11	4.60	11.83
P	NS	<.0001	<.0001	<.05	<.0001
Day: $F_{2,90}$	45.46	10.92	15.68	12.41	17.37
P	<.0001	<.0001	<.0001	<.0001	<.0001

Fig. 1. Time-action curves for V_I while breathing a 5% CO_2, 30% O_2, balance N_2 gas mixture. On day 3 (Panel A), 7 (Panel B) or 14 (Panel C) neonates (n = 6 per treatment) received s.c. injection of either saline placebo (●), 4.5 mg/kg morphine (▾) or 5.0 mg/kg M6G (■). Placebo value for room air breathing is shown (♦). On these doses, the average peak depression occurred at 28 and 73 min for 3-day-old animals, at 48 and 78 min for 7-day-old animals, and at 45 and 63 min for 14-day-old animals after morphine and M6G treatment, respectively. These doses of morphine and M6G were chosen for figure 1 to illustrate the time-action differences, the similar potency of the two drugs on day 3 and the increased potency of M6G with respect to morphine on days 7 and 14. Other doses of the two drugs were omitted for the sake of clarity. Vertical bars represent S.E. $^{*}P<.05$, $^{**}P<.01$ compared to placebo and $^{\dagger}P<.05$ compared to 4.5 mg/kg morphine by ANOVA and Newman-Keuls test.

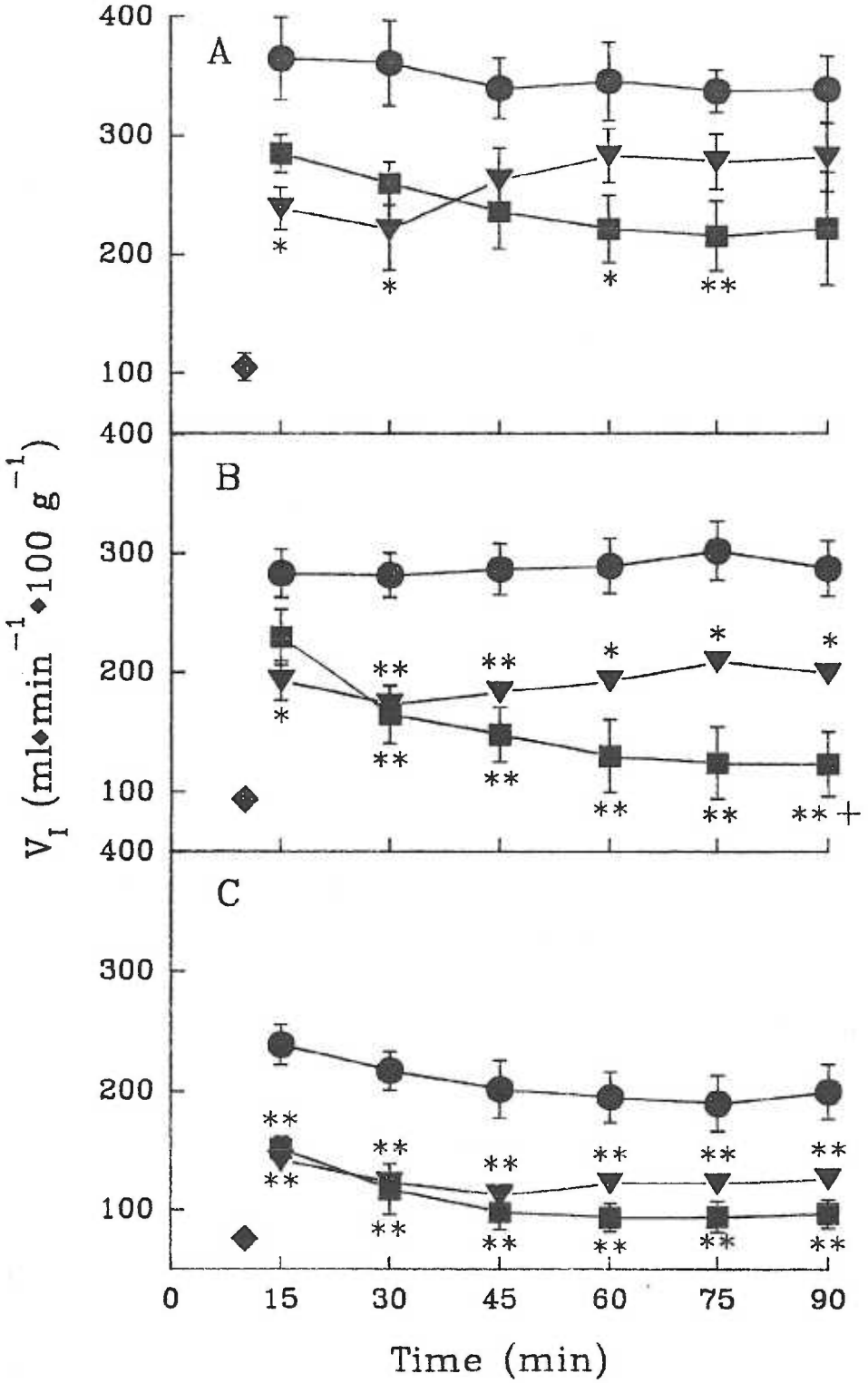

A
B
C
400
300
200
100
0
15
30
45
60
75
90
Time (min)
V_I (ml•min^{-1}•100 g^{-1})

Fig. 2. V_I, f and V_T at peak depression for morphine- and M6G-treated pups breathing 5% CO_2 compared to placebo. Data for V_I (Panel A), f (Panel B) and V_T (Panel C) are expressed as percent of average placebo response. Vertical bars represent S.E. (n=6 per treatment). Parameters were significantly decreased from placebo ($P<.001$) except as noted (+). M6G had a greater potency in decreasing V_I, relative to placebo, with aging. Morphine produced the same degree of depression in V_I at all ages for a given dose (Panel A).

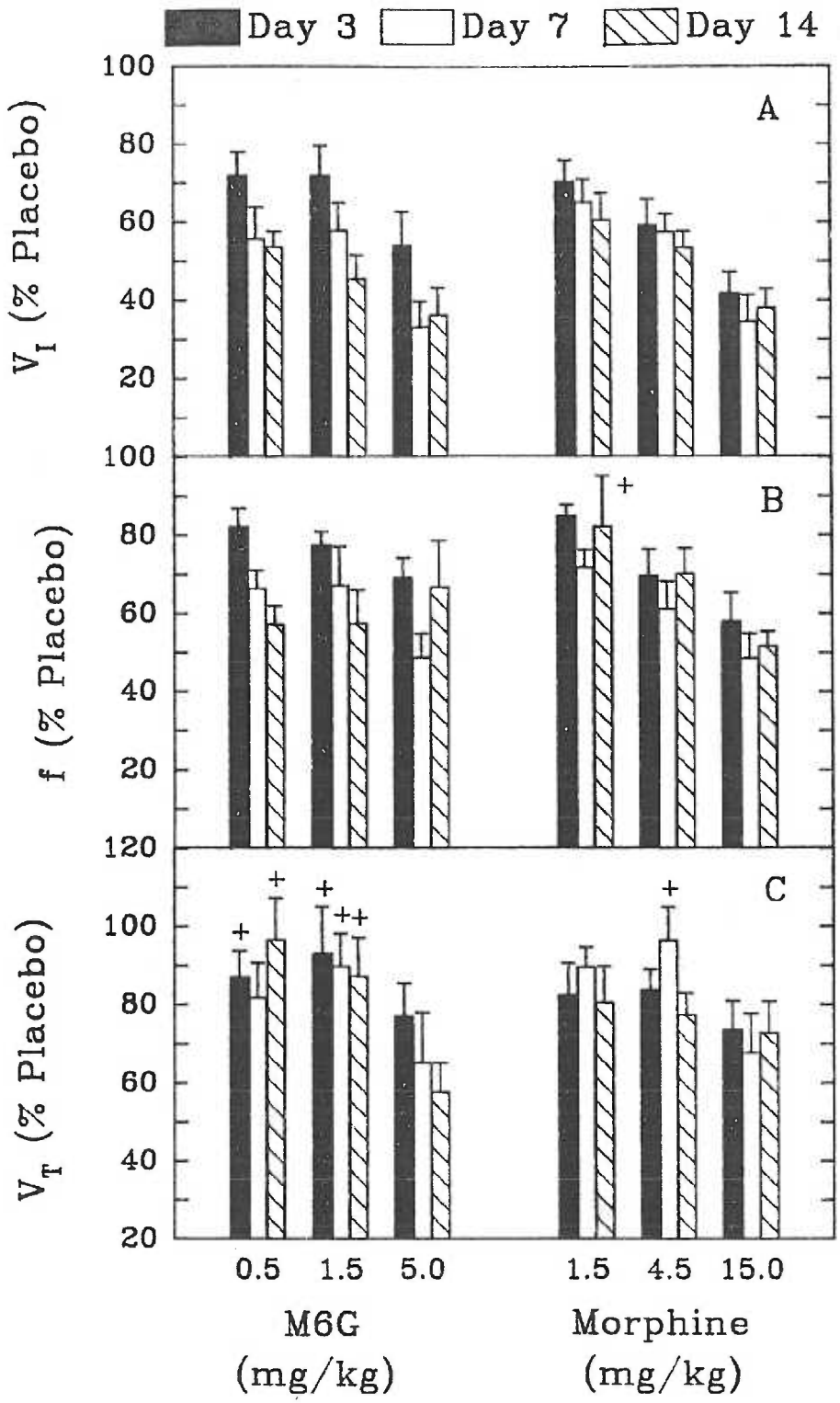

Day 3
Day 7
Day 14
A
B
C
V_I (% Placebo)
f (% Placebo)
V_T (% Placebo)
100
80
60
40
20
120
0.5
1.5
5.0
1.5
4.5
15.0
M6G
(mg/kg)
Morphine
(mg/kg)

Fig. 3. Dose-response curves for V_I and V_T/T_I at peak depression while breathing a 5% CO_2, 30% O_2, balance N_2 gas mixture. On day 3 (Panels A and D), 7 (Panels B and E) or 14 (Panels C and F) neonates (n = 6 per dose) were injected s.c. with 0.5 to 5.0 mg/kg M6G or 1.5 to 15.0 mg/kg morphine. Vertical bars represent S.E. □ = M6G, ▾ = Morphine. On day 3, regression analyses were not significant for V_I after morphine and M6G treatment or for V_T/T_I after M6G. Morphine and M6G regression lines were different on days 7 and 14 for V_I and V_T/T_I. For V_I, slopes did not differ on day 7 and M6G was 7.6 times more potent than morphine on a molar basis. On day 14, slopes differed with M6G 9.4 times more potent than morphine at 50% depression and, over the range of doses studied, the potency ratio varied from 5.9 to 10.8. For V_T/T_I, M6G and morphine slopes differed on both days 7 and 14. On day 7, the M6G potency ratio was 7.4 at 50% depression and varied over the range studied from 3.4 to 7.8. On day 14, the 50% depression and range for potency were 5.0 and 2.6 to 8.2, respectively. The equations for significant regressions are: (Panel B) V_I (□) = 155 - 66.6•log(M6G), V_I (▾) = 208 - 76.8•log(morphine); (Panel C) V_I (□) =102 - 37.6•log(M6G), V_I (▾) = 142 - 52.3•log(morphine); (Panel D) V_T/T_I (□) M6G regression not significant, V_T/T_I (▾) = 8.76 - 3.03•log(morphine); (Panel E) V_T/T_I (□) = 7.30 - 3.48•log(M6G), V_T/T_I (▾) = 10.77 - 5.43•log(morphine); (Panel F) V_T/T_I (□) = 7.85 - 2.40•log(M6G), V_T/T_I (▾) = 11.21 - 5.40•log(morphine).

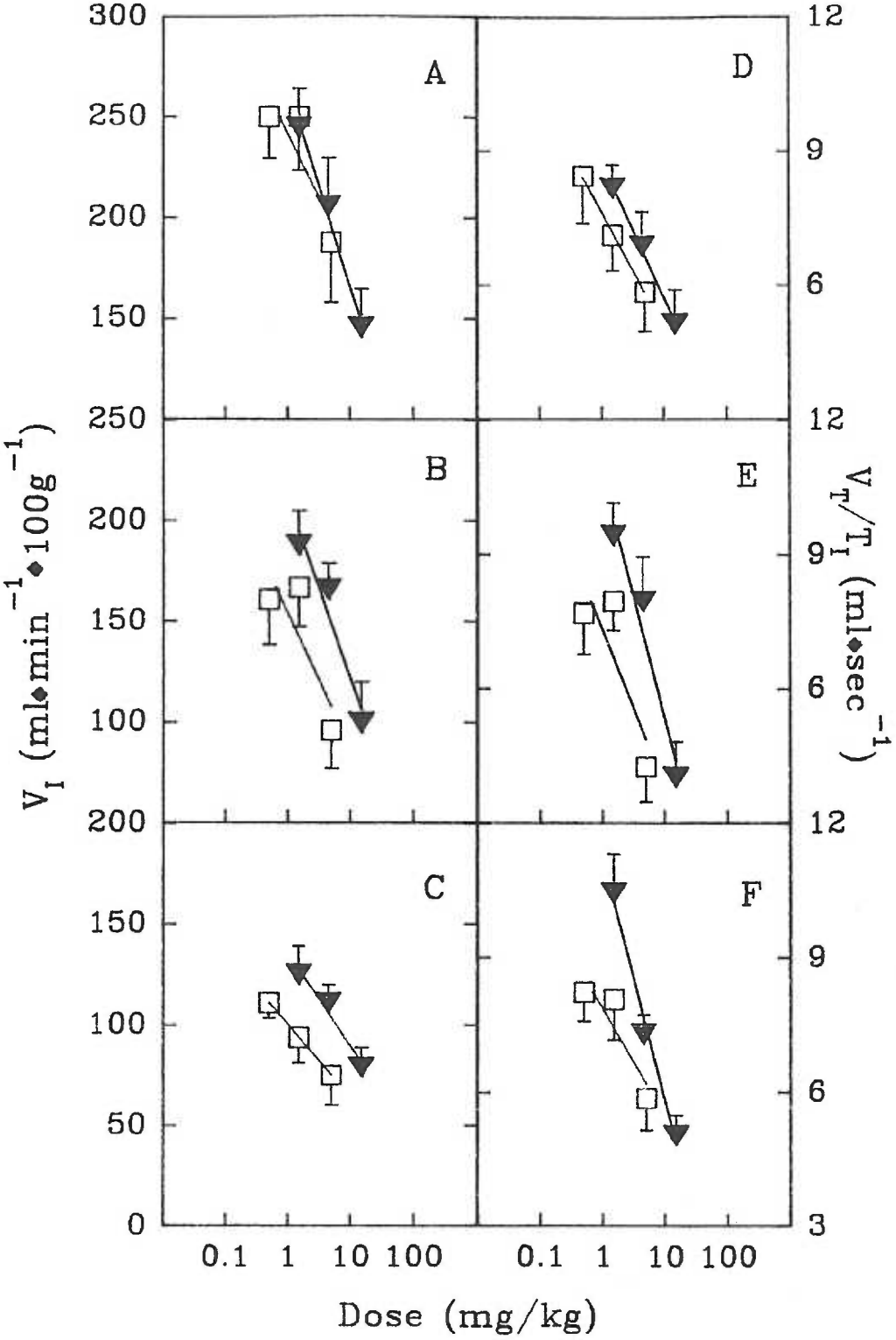

A
B
C
D
E
F
300
250
200
150
100
50
0
12
9
6
3
0.1
1
10
100
V_I (ml•min^{-1}•100g^{-1})
V_T/T_I (ml•sec^{-1})
Dose (mg/kg)

Fig. 4. *Delta* response for V_I at peak depression for morphine- and M6G-treated animals compared to placebo. The point of maximum depression following drug treatment while breathing CO_2 was determined and the difference from the preceding room air data calculated. Each pup's *delta* response is expressed as percentage of average placebo *delta*. Vertical bars represent S.E. (n=6 per treatment). *Delta* V_I was depressed significantly at all doses on all days ($P<.0001$). The potency of M6G changed with aging, producing a greater percent decrease on days 7 and 14 than on day 3, whereas the potency for a given dose of morphine did not change with aging. M6G was 9.9 times more potent than morphine on day 14 of life.

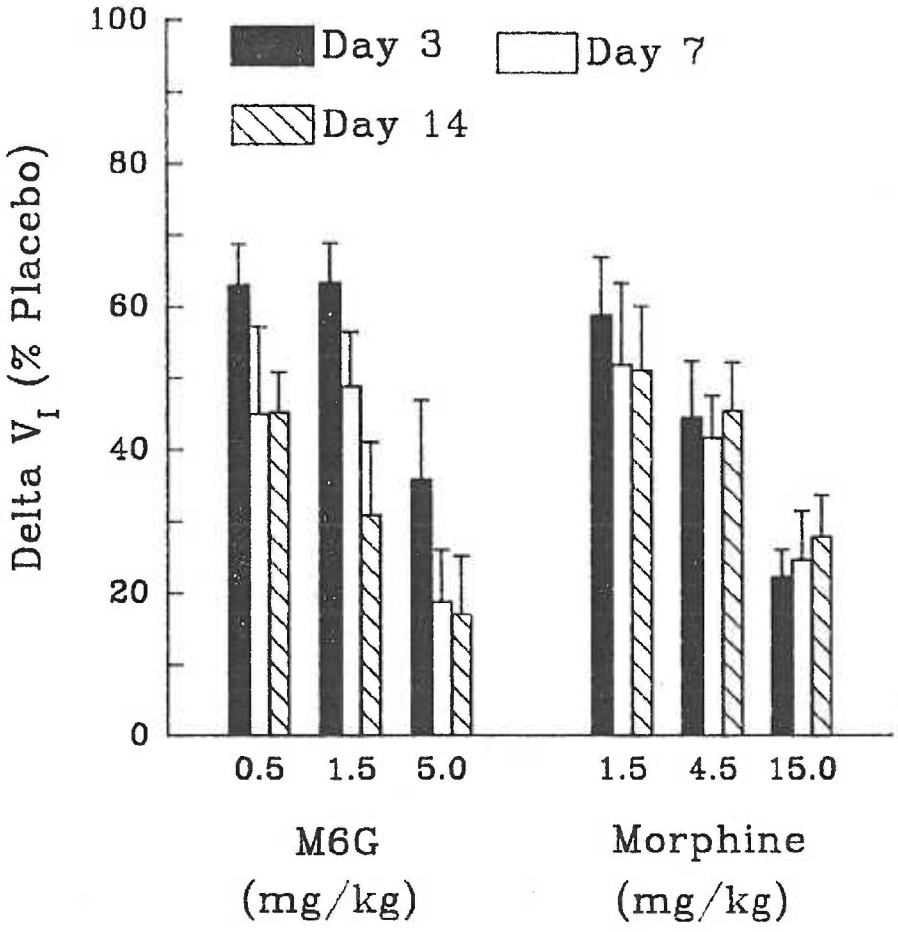
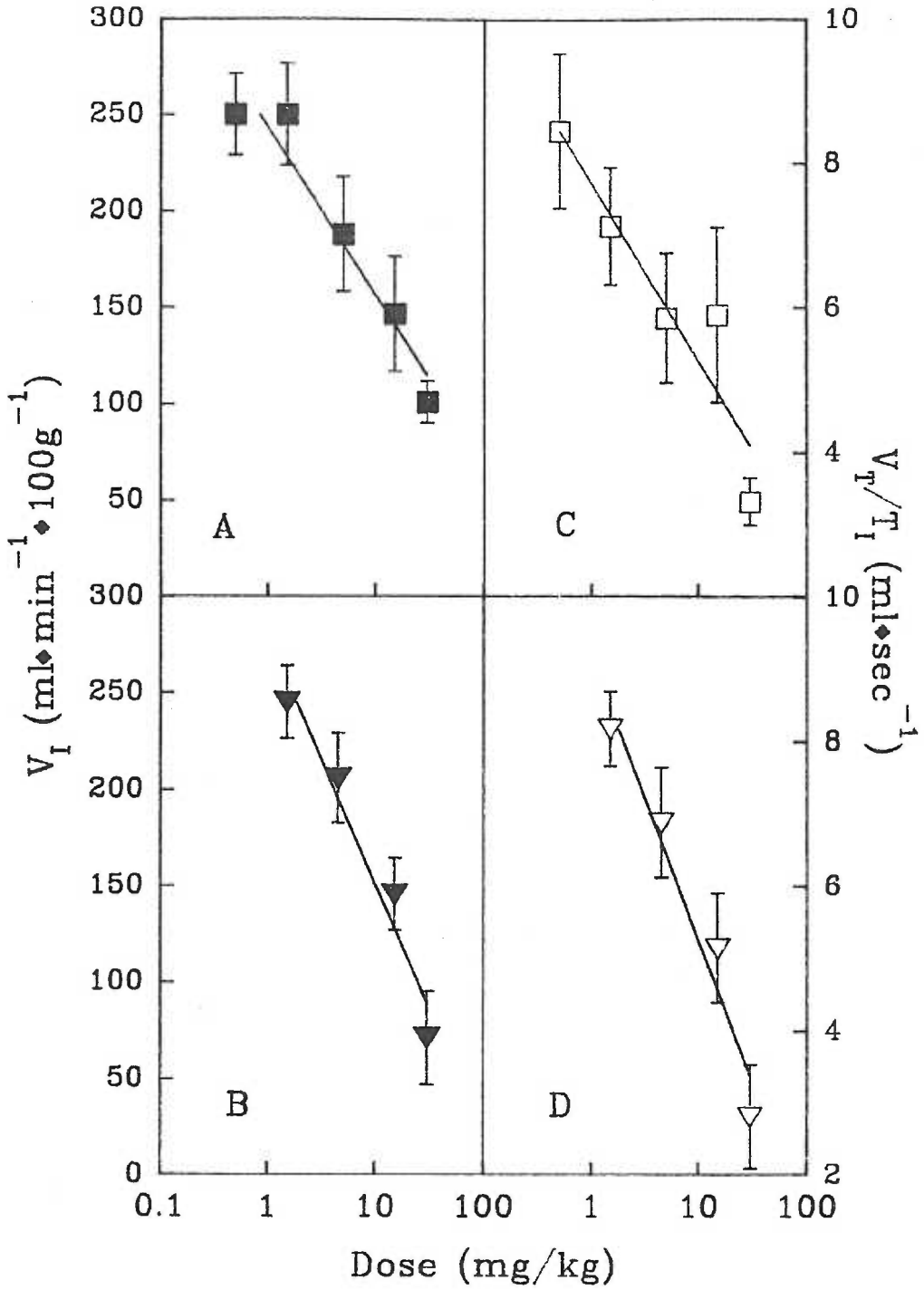

Day 3
Day 7
Day 14
Delta V_I (% Placebo)
100
80
60
40
20
0
0.5
1.5
5.0
1.5
4.5
15.0
M6G
(mg/kg)
Morphine
(mg/kg)

Fig. 5. Day 3 extended dose-response curves for V_I and V_T/T_I at peak depression while breathing 5% CO_2, 30% O_2, balance N_2. Morphine, 1.5 to 30 mg/kg (Panels B and D), and M6G, 0.5 to 30 mg/kg (Panels A and C), were administered to 3 day old neonates (n=6 per dose). Study conditions and curve fitting was described in figure 3. Vertical bars represent S.E. and are occasionally contained within the symbol. (For M6G ■=V_I and =V_I/T_I and for morphine ▾=V_I and ∇=V_I/T_I.) All log dose-responses are significant. There is no significant difference between morphine and M6G curves for a given parameter. V_I for M6G (panel A) was analyzed with and without the lowest dose with the same outcome. Results of all 5 doses are reported. Regression equations are: (Panel A) V_I (■) = 243 - 87.1•log(M6G); (Panel B) V_I (▾) = 254 - 108•log(morphine); (Panel C) V_T/T_I (□) = 7.71 - 1.06•log(M6G); (Panel D) V_T/T_I (∇) = 8.44 - 1.45•log(morphine).


300
250
200
150
100
50
0
A
B
C
D
10
8
6
4
2
0.1
1
10
100
Dose (mg/kg)
V_I (ml•min^{-1}•100g^{-1})
V_T/T_I (ml•sec^{-1})

Manuscript III

(J. Pharmacol. Exp. Ther. In Press, 1994)

Title: Diffusion of Morphine-6-β-D-Glucuronide Into the Neonatal Guinea Pig Brain During Drug-Induced Respiratory Depression[1,2].

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RUNNING TITLE PAGE

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Abbreviations:

M6G	morphine-6-β-D-glucuronide
M3G	morphine-3-β-D-glucuronide
HPLC	high-performance liquid chromatography
V_I	inspiratory minute ventilation
CNS	central nervous system
BBB	blood-brain barrier
log P	log of octanol/water partition coefficient
k_i	isocratic capacity factor

ABSTRACT

Morphine-6-β-D-glucuronide (M6G) is an active metabolite of morphine. In a previous study, M6G depressed respiration in the neonatal guinea pig, becoming more potent with aging, a finding that is confirmed in the current study. After s.c. injection, M6G is absorbed into plasma, crosses the blood-brain barrier (BBB), and is present in the central nervous system (CNS) at the time of maximal M6G-induced ventilatory depression. No hydrolysis of M6G to morphine is detected in either plasma or brain tissue by high-performance liquid chromatography (HPLC). About 30% more M6G is in plasma in 3 day old than in 7 day old pups following drug administration ($P<.05$). Mean brain concentrations of M6G are 12% higher on day 3 than day 7, but the difference is not statistically significant. Brain to plasma ratios of M6G do not differ after 5 or 15 mg/kg M6G or with age (mean ratio = 0.037). Brain drug concentration is a linear function of plasma drug levels ($r^2 = 0.84$), suggesting M6G crosses the BBB by diffusion. Differential systemic absorption or CNS distribution of M6G cannot explain enhanced respiratory depression with aging. Morphine-3-β-D-glucuronide (M3G), also crossed the BBB, but is less permeable than M6G (mean brain to plasma ratio = 0.022). Contrary to reports in the literature, M3G at a dose of 75 mg/kg, does not stimulate respiration in this study. Morphine administration to neonatal guinea pigs produces measurable plasma and brain levels of M6G and M3G. Brain to plasma metabolite ratios are 2 to 3 fold greater ($P<.01$) after s.c. morphine injection than when M6G

and M3G are administered directly, suggesting glucuronidation of morphine occurs within the CNS.

Introduction

M6G and M3G are metabolites of morphine which are produced by several mammalian species, including the human adult and neonate (Boerner *et al.*, 1975; Choonara *et al.*, 1989; Portenoy *et al.*, 1991), the 15-27 week human fetus (Pacifici *et al.*, 1982; Gawronska-Szklarz *et al.*, 1985), and the adult and neonatal guinea pig (Oguri *et al.*, 1970; Murphey and Olsen, 1993). M6G has analgesic activity in animal studies (Shimomura *et al.*, 1971; Pasternak *et al.*, 1987; Paul *et al.*, 1989; Gong *et al.*, 1991) and in human trials (Osborne *et al.*, 1988, 1992; Hanna *et al.*, 1990), with M6G more potent than morphine. In contrast, M3G is devoid of opioid activity (Shimomura *et al.*, 1971; Gong *et al.*, 1991).

Respiratory effects of M6G in adult animals have also been studied (Pelligrino *et al.*, 1989; Gong *et al.*, 1991). M6G is 5-10 times more potent than morphine in causing respiratory depression, after central administration. In these same studies, M3G stimulates ventilation. Neonatal respiratory pharmacology of M6G has recently been defined in the guinea pig (Murphey and Olsen, 1994). M6G causes equal depression compared to morphine in 3 day old pups, but is 8 to 10 fold more potent than morphine and than itself in 7 and 14 day old neonates. To explain this shift in M6G potency relative to morphine and to itself, it has been suggested that entry into the CNS might change with aging.

The current report explores whether M6G pharmacokinetic differences explain potency changes. The distribution of M6G to CNS after s.c. administration, with respect to dose and neonatal age, during M6G induced respiratory

depression is investigated. Morphine and M3G administration are also studied and compared to M6G.

Methods

Animals. Thirty-two Dunkin-Hartley guinea pig pups from 10 litters were entered into the M6G study. The morphine study used 12 pups from 4 dams. Eleven neonates from 4 litters were used in the M3G study. Dams and litters were housed in the Department of Animal Care at the Oregon Health Sciences University, and had daily care and health checks. The Department operates in compliance with the Animal Care Act and has a letter of assurance on file with the National Institutes of Health.

Drugs and dosing. M6G, M3G (Research Triangle Institute, Research Triangle Park, NC), and morphine (Mallinckrodt, St. Louis, MO) stocks were dissolved in saline and frozen at -20°C until use. For the M6G study, 8 pups received either 5 or 15 mg/kg M6G on either day 3 or 7 after birth. These doses were chosen to produce significant respiratory depression and brain drug concentrations large enough to quantitate. Litters and pups were randomized to dose and day of study. Six neonates received 15 mg/kg morphine either on day 3 or on day 7. In the M3G time-action respiration study, eleven 7 day old pups received 75 mg/kg of M3G. Five of the 11 pups also had blood and brain samples taken. Pilot studies indicated M3G had no respiratory effect at doses of 25 and 50 mg/kg. Administration was by s.c. injection into the shaved scruff of the neck in a volume of 1 ml/kg for M6G and morphine. Due to the lower solubility of M3G, 9 ml/kg of M3G stock was given. Drug concentrations in injection solutions were confirmed

by HPLC.

Sample collection. Samples were collected at times corresponding to the average time of maximal respiratory depression for M6G (70 min) and morphine (45 min) while breathing a 5% CO_2 gas mixture, as previously determined (Murphey and Olsen, 1994). After the respiratory measurement, pups were anesthetized with ether and blood was sampled by cardiac puncture followed by cervical dislocation and collection of whole brains. Blood samples were centrifuged at 1500 x *g* for 30 min at 4°C, and plasma frozen at -20°C until analyzed. Brains were homogenized in 1.2 ml/g of 0.01 M sodium phosphate, pH 2.0, (Sandberg and Olsen, 1992) and centrifuged at 10,000 x *g* for 20 min. The pellet was discarded and the supernatant was centrifuged at 13,000 x *g* for 45 min and the resulting supernatant frozen at -20°C until analyzed.

Analysis of plasma drug concentration. Plasma was analyzed for M6G, M3G and morphine by the method of Murphey and Olsen (1993), which uses solid phase extraction of an 80 μl sample and reverse-phase HPLC with ultraviolet detection. The limits of detection for all compounds was 47 ng/ml (1.3 ng injected onto column). Hydromorphone was the internal standard and samples were diluted to be in the range of the standard curve, when necessary.

Analysis of brain drug concentration. Brain supernatant (400 μl) was filtered with

an Ultrafree-MC 5,000 NMWL filtration unit (Millipore, Bedford, MA). 720 μl of 0.5 M ammonium sulfate, pH 9.3, containing internal standard (hydromorphone), and 80 μl of the ultrafiltrate were added to a BondElut extraction column with cyclo-hexyl packing (Varian, Harbor City, CA) and drawn through under vacuum. Drug was eluted from the BondElut with 1 ml of 0.01 M sodium phosphate, pH 2.0, with 5% (v/v) acetonitrile. One ml of 0.01 M sodium phosphate, pH 6.0, and the eluate were added to a Clean Screen extraction column (World Wide Monitoring, Horsham, PA), previously conditioned with 3 ml methanol, 3 ml water and 1 ml 0.01 M sodium phosphate, pH 6.0. The column was washed with 1 ml 0.1 N hydrochloric acid, 1 ml water and 3 ml methanol as described in Murphey *et al.* (1993). Two elution protocols were compared. In the first scheme M6G, M3G, morphine and hydromorphone were eluted with a 88:10:2 mixture of methanol:dichloromethane:ammonium hydroxide. A second elution scheme used a 78:20:2 mixture of dichloromethane:isopropyl alcohol:ammonium hydroxide (Murphey *et al.*, 1993) to elute morphine and hydromorphone, followed by the 88:10:2 mixture to elute M6G and M3G. This produced a cleaner preparation for M6G. Results for M6G using the second scheme were not significantly different from the first one ($P > .20$), therefore data using the second scheme are reported. Eluate was dried under N_2 at 40°C and reconstituted in 600 μl of mobile phase and 200 μl was chromatographed according to Murphey and Olsen (1993). In more recent experiments, a Model 5200A electrochemical detector with Model 5011 dual coulometric analytical cell set at +225 and +450 mV potential (ESA, Inc., Bedford,

MA) was added before the ultraviolet detector as a second way to monitor M6G, morphine and hydromorphone. M3G is sensed by the ultraviolet, but not the electrochemical detector. The coulometric analysis produced a less noisy baseline than the ultraviolet analysis for brain samples, and was therefore used to quantitate M6G and morphine. In samples analyzed with both ultraviolet and electrochemical detection, there was no difference between the two methods ($P>.70$). Known standards were extracted as controls. Standard curves were linear from 94 to 3000 ng/ml supernatant (113 to 3600 ng/g, or 2.5 to 80 ng onto column) for M3G and from 47 ng/ml (56 ng/g, or 1.3 ng onto column) for M6G and morphine. The limit of detection for M3G was 56 ng/g and for M6G and morphine was 28 ng/g (0.7 ng onto column). Recovery of morphine, M6G and M3G from spiked samples was greater than 95% for all 3 compounds.

Breathing studies. V_I was measured using a computerized, 2-chamber plethysmograph described previously (Murphey and Olsen, 1994; Olsen and Weil, 1992a,b). For the M6G study, pre-injection and post-injection data while animals breathed a steady flow of either atmospheric air (referred to as room air) or a 5% CO_2, 30% O_2, balance N_2 mixture were recorded to document respiratory depression. For the M3G study, animals were injected with drug immediately following collection of the baseline data and a time-action curve determined over 90 min, as in a previous study of M6G (Murphey and Olsen, 1994). V_I was normalized per 100 g body weight.

Partition coefficient determination. Octanol-buffer partitioning of morphine, M6G and M3G were determined by the shake-flask method of Hansch *et al.* (1987). Drugs (25 or 50 μM) were dissolved in phosphate buffer (0.1M, pH 7,4) saturated with HPLC grade octanol (Sigma Chemical Co., St. Louis, MO). A 2:1 ratio of mutually saturated octanol and buffer was mixed by inversion for 15 min followed by centrifugation at 1000 x *g* for 2 hr. Drug in buffer was measured by HPLC before and after equilibration with octanol. For M6G and M3G, buffer (0.01M, pH 7.4) with drug was also scanned before and after equilibration and octanol was scanned after equilibration, by spectrophotometry (Uvikon 860 Spectrophotometer, Kontron Instruments, Zurich, Switzerland).

Data analysis. To compare the penetration of M6G or M3G and morphine into brain, a new index was proposed, the relative hydrophilicity index. This index was calculated by dividing the brain to plasma ratio for morphine by the brain to plasma ratio for M6G or M3G following injection of metabolite. This index is the inverse of lipophilicity. Relative hydrophilicities from other *in vivo* studies were calculated from data in published reports (table 4). Relative hydrophilicity from *in vitro* lipophilicity data (Carrupt *et al.*, 1991) was calculated by dividing the published k_i for morphine (9.33) by the published k_i for M6G (2.09) or M3G (0.676).

Statistics. Mean ± S.E. were calculated. Neonatal weights were analyzed by one-

way ANOVA (NWA Statpak, Northwest Analytical, Portland, OR). Student's *t* test for paired data (NWA Statpak) was used to compare results of the two HPLC elution schemes, and compare results using ultraviolet and electrochemical detection. Student's *t* test for unpaired data (NWA Statpak) was used to compare drug levels for a given dose on day 3 and day 7. Mann-Whitney rank-sum test for nonparametric data was used to compare M6G ventilatory data and percentage of morphine, M6G or M3G in plasma or brain between day 3 and day 7 pups. Friedman 2-way repeated ANOVA (NWA Statpak) was used to analyze brain to plasma ratios. There were no dose or day effects for brain to plasma ratios, so data were pooled for further analysis. Least-squares linear regression (NWA Statpak) was used to analyze for significant correlations between brain and plasma drug concentrations. One-way repeated measures ANOVA (NWA Statpak) was used to analyze M3G time-action curve with post-hoc Newman-Keull's multiple comparisons test. Significance was defined as $P<.05$.

Results

Neonatal weights. Pup weights did not differ among treatment groups at birth or on day of study ($P<.05$). The 55 pups in the M6G, morphine and M3G studies weighed (mean ± S.E.) 102 ± 2 g at birth. Animals studied on day 3 weighed 96 ± 3 g (n = 22), and day 7 pups weighed 138 ± 3 g (n = 33).

M6G study. M6G was absorbed into blood and penetrated into the CNS following s.c. administration to neonatal guinea pigs (table 1). At the time of average maximal ventilatory depression (70 min), plasma levels of M6G were significantly higher in 3 day pups than in 7 day pups given either 5 or 15 mg/kg (table 1). There was no difference in brain M6G concentrations between day 3 and day 7. Morphine or M3G were not detected in plasma or in brain tissue after M6G administration. The brain to plasma concentration ratios for M6G did not differ between doses or day after birth (table 1). The average M6G brain to plasma ratio for all animals (n = 32) was 0.0374 ± 0.0018. Brain drug levels were a linear function of plasma drug levels (figure 1; [Brain drug] = 38.5 + 0.0325•[plasma drug], r^2 = 0.84, $P<.01$).

At 70 min after administration, M6G significantly depressed ventilation in 3 and 7 day old guinea pigs ($P<.0001$). Ventilatory results are presented in table 2. Animals were more depressed after a given dose on day 7 than on day 3 ($P<.05$). Depression of V_I was mainly due to decreases in breathing frequency, which was reduced to 40 to 60% of the control value in treated pups. The restricted dose

range, data variability and small sample size did not permit a dose-response determination.

Morphine study. At the time of average maximal morphine effect (45 min after injection), morphine, M6G and M3G were quantitated in plasma and brain tissue (table 3). Significantly more M3G was in plasma and brain of day 7 pups than in day 3 pups (table 3). Brain to plasma ratio for M6G was significantly higher ($P<.001$) after morphine administration than after s.c. administration of M6G (tables 1 and 3). Age did not affect ventilatory depression by morphine. All animals were depressed to 51 ± 7% of control.

The amount of metabolite and morphine, as percent of total moles of opioid present in plasma, was 30:19:51 on day 3 and 22:20:58 on day 7, for morphine, M6G and M3G, respectively. For brain tissue, percentages were 62:19:19 and 50:25:25 for day 3 and 7, respectively. These percentages did not vary with age.

M3G study. Plasma samples were obtained at the end of the 90 min respiratory time-action study for 5 animals given 75 mg/kg M3G. Plasma concentrations were 51,400 ± 3310 ng/ml for M3G, 160 ± 10 ng/ml for morphine and 221 ± 17 ng/ml for M6G. M3G brain levels in these 5 animals were 1,130 ± 78 ng/g. There was no detectable morphine or M6G in the brain samples. The brain to plasma concentration ratios 90 min after injection of M3G was 0.0221 ± 0.001. The M3G blood to plasma ratio present after morphine administration (table 3) was

significantly higher ($P<.01$) than after s.c. M3G injection. Analysis of M3G drug stocks by HPLC disclosed a 0.6% morphine impurity that was present in the manufacturer's supply. M3G, 75 mg/kg s.c., caused respiratory depression compared to pre-injection controls (figure 2). At maximal depression, 45 min after injection, V_I was 78% of the control value.

Relative hydrophilicity index. The relative hydrophilicity indices for M6G and M3G, using morphine as the standard of reference, were calculated (see Data Analysis section of Methods). There was no aging effect for the morphine blood to plasma ratio, and, therefore, the pooled value was used for the calculation of the index. This index for M6G was 5.9 (0.222 ÷ 0.0374) and for M3G was 10.0 (0.222 ÷ 0.0221). Table 4 presents relative hydrophilicities calculated from other published values for M6G, M3G and morphine concentrations across blood-brain, dural and epitheliochorial placenta barriers. Also included is the relative hydrophilicity index calculated from k_i's, which are *in vitro* determinations of M6G, M3G and morphine lipophilicity.

Partition coefficient. The apparent log P of morphine at pH 7.4 was -0.20 ± 0.02 (n = 3). By HPLC, no change was seen in buffer M6G or M3G concentrations after equilibration with octanol, suggesting that the amount of M6G or M3G entering octanol was so small that no difference in concentrations in the buffer could be detected. Therefore, concentrations of M6G and M3G in octanol after equilibration

were estimated by spectrophotometry, and the log P for M6G and M3G was approximated to be -2.4. However, because of the very small amount of M6G or M3G in the octanol, this determination represents only a rough estimate of the log P for M6G and M3G.

Discussion

Murphey and Olsen (1994) reported that the absolute potency of M6G and the potency of M6G relative to morphine-induced respiratory depression increases with age in neonatal guinea pigs. They suggested that this phenomenon may be a result of higher CNS concentrations of M6G for a given s.c. dose, as the neonate ages, due to increased systemic absorption or increased distribution of M6G to the CNS. The current report presents data rejecting these two hypotheses.

Increased absorption after s.c. administration of M6G is unlikely. There is significantly less M6G in the plasma of 7 day old pups than 3 day old pups at the time of maximal respiratory depression. This difference may be due to decreased absorption, increased clearance and/or increased volume of distribution as the neonate ages. Likewise, increased distribution of M6G to the CNS cannot explain the aging changes in M6G potency. There is not a greater amount of drug in the brains of day 7 animals compared to day 3 pups.

There may be limits to extrapolating whole brain drug levels to respiratory depression, which is caused by local opioid concentrations at the respiratory centers in the pons and medulla. However, McGilliard and Takemori (1978), showed that the opioid antagonist, naloxone, was evenly distributed throughout mouse brain after i.p. injection. Sanner and Woods (1965) reported concentrations of dihydromorphine in several brain regions, including the pons and medulla, to be similar after s.c. injection to rats.

M6G could enter the CNS by either diffusion or carrier mediated transport

across the BBB, but data from this investigation suggest that M6G crosses the BBB only by diffusion. M6G does not enter the brain against a concentration gradient and the ratio of M6G in brain relative to plasma is constant with respect to dose and age. Brain M6G concentrations are a linear function of plasma M6G concentrations over the dose range studied.

It is possible that M6G is hydrolyzed in the neonatal guinea pig to morphine which in turn enters the brain and depresses breathing, but morphine is not detected in either plasma or brain following M6G administration. Neither is M3G detected after M6G injection, which is further evidence that morphine is not produced by hydrolysis of M6G and subsequently conjugated to M3G. Entry of unhydrolyzed M6G has been reported previously for the adult rat using thin layer chromatography (Shimomura *et al.*, 1971), radiolabelled M6G (Yoshimura *et al.*, 1973) and HPLC (Francés *et al.*, 1992). M6G is also present in the cerebrospinal fluid of humans after systemic administration of morphine (Portenoy *et al.*, 1991).

In conjunction with absorption and distribution, respiration was measured at the time of maximal respiratory depression (70 min), which was determined in a previous study (Murphey and Olsen, 1994). As reported in Murphey and Olsen (1994), a potency change was noted for M6G in the current investigation. While breathing elevated CO_2, V_I in 7 day olds was more profoundly depressed than in 3 day olds, after a given dose of M6G (table 2). However, the restricted dose range, data variability and the small number of subjects does not permit a dose-response determination.

Other investigators have reported M6G-induced respiratory depression. Thirty to 3000 ng/min of M6G, infused into the fourth cerebral ventricle, depressed breathing in dogs (Pelligrino *et al.*, 1989). Gong *et al.* (1991) used single lateral cerebroventricular injections of 250 to 25,000 ng M6G and observed respiratory depression in rats. In both studies conducted in adult animals, M6G was 5-10 times more potent than morphine, which is similar to the reported potency difference in 7 day old guinea pigs (Murphey and Olsen, 1994).

Morphine absorption, distribution and respiratory effects were also examined in neonatal guinea pigs. Morphine, M6G and M3G were measured in plasma and in brain at peak morphine respiratory depression, 45 min after s.c. injection. The ratio of brain to plasma concentrations for morphine was greater than for M6G or M3G, and M6G was greater than for M3G. Both for M6G and M3G, the brain to plasma ratios were greater after morphine administration than after the glucuronides were given alone. This suggests that morphine in the CNS may be metabolized by brain. Glucuronidation of morphine by microsomes derived from both mouse and human brains has been reported (Wahlström, *et al.*, 1986, 1988).

The plasma molar amounts of the compounds, as a percentage of total opioid present, was 30:19:51 for morphine, M6G and M3G, respectively, on day 3. Similar percentages on day 7 were 22:20:58. This is comparable to the 28:22:50 reported earlier for 15 to 17 day old neonatal guinea pigs (Murphey and Olsen, 1993). In brain tissue, morphine was most prevalent, reflecting differential CNS

penetration of the 3 compounds. The molar percentages of morphine, M6G and M3G, respectively, were 62:19:19 and 50:25:25 for days 3 and 7, respectively.

In the neonatal guinea pig, M3G is also absorbed into plasma after s.c. injection and penetrates into the CNS of 7 day old pups. Brain levels of M3G (1130 ng/g) were similar to that seen with a fourth cerebroventricular infusion of 1500 ng/min in dogs (Pelligrino *et al.*, 1989), or i.c.v. injections of 5000 or 25000 ng M3G in rats (Gong *et al.*, 1991). Both morphine and M6G, in addition to M3G were quantitated in plasma. The origin of the morphine was probably the 0.6% morphine impurity in the M3G stock, and the M6G from subsequent glucuronidation of the morphine impurity. At the doses of M3G used (75 mg/kg), the injection solution contained 0.45 mg/kg morphine.

This dose of M3G decreased V_I to 78% of the control value. Lower doses of M3G (50 mg/kg) did not effect ventilation. Using the linear portion of the dose-response relationship for morphine-induced peak respiratory depression (V_I = 208-76.8•log[morphine dose]), which was previously defined in 7 day old guinea pig pups (Murphey and Olsen, 1994), 0.45 mg/kg morphine is predicted to produce a V_I of 234 $ml \cdot min^{-1} \cdot 100\ g^{-1}$ at the time of peak respiratory depression[a]. M3G administration resulted in a V_I of 231 $ml \cdot min^{-1} \cdot 100\ g^{-1}$, supporting the hypothesis that the morphine impurity was the cause of respiratory depression after M3G administration.

[a]See Appendix C for probit dose-response relationship and prediction of V_I response for 0.45 mg/kg morphine.

Several descriptions of the CNS excitatory properties of M3G have been published. M3G produces hyperalgesia when given i.c.v. to awake rats (Labella *et al.*, 1979) or intrathecally to awake rats and anesthetized cats (Yaksh *et al.*, 1986); it accumulates in the sheep fetus causing seizures (Olsen *et al.*, 1988); and M3G may induce respiratory stimulation in dogs (Pelligrino *et al.*, 1989) and rats (Gong *et al.*, 1991). The activity of M3G is *via* non-opioid mechanisms, as demonstrated by the lack of naloxone or naltrexone antagonism of excitatory effects (Labella *et al.*, 1979; Yaksh *et al.*, 1986; Gong *et al.*, 1991). M3G may also functionally antagonize morphine-induced analgesia (Smith *et al.*, 1990) and M6G-induced respiratory depression (Gong *et al.*, 1992). Hewett *et al.* (1993), however, failed to demonstrate antagonism by M3G of morphine-induced changes in recordings from spinal dorsal horn neurons. There was no evidence that M3G has any respiratory stimulative effect in the neonatal guinea pig.

Because M6G and M3G appeared to enter the CNS more readily than expected, the relative hydrophilicities of these drugs, using morphine as a reference, were calculated. The resulting ratio, the hydrophilicity index, is inversely related to lipophilicity and as the index increases, lipophilicity decreases. The values calculated in this study are in the same range and rank order as the relative hydrophilicities calculated from other published data on morphine glucuronides across several tight membranes: the blood-brain, blood-CSF and sheep placenta (table 4). The epitheliochorial placenta of sheep has very tight barriers to hydrophilic compounds (Faber and Thornburg, 1983) similar to the BBB. M6G and

M3G are more hydrophilic than morphine in these *in vivo* studies and M6G is less hydrophilic than M3G.

The above calculations suggest that the morphine glucuronides are much more lipophilic than glucuronide metabolites in general. Carrupt *et al.* (1991) have calculated and experimentally determined the k_i, an *in vitro* measure of lipophilicity. The k_i is derived from the retention of a compound on a C-18 HPLC column, compared to an unretained solute, and are superior to the shake-flask measure of lipophilicity in regards to sensitivity and precision (Braumann *et al.*, 1983; Braumann, 1986; Bechalany *et al.*, 1989; Carrupt *et al.*, 1991) The calculated k_i indicates that morphine glucuronide (isomers not distinguished) should be 1780 times more hydrophilic than for morphine. However, the experimentally determined isocratic factors for M6G are 4.5 and 4.3 times more hydrophilic than morphine at pH 7.0 and 8.0, respectively, and for M3G are 13.8 to 13.5 more hydrophilic than morphine. According to modeling studies, M6G and M3G may exist in 2 energetically favorable conformations: extended, in hydrophilic environments, and folded, in more lipophilic environments (Carrupt *et al.*, 1991). Differences in relative hydrophilicity between M6G and M3G are due to intramolecular interactions in the folded form, which mask the high polarity of the carboxylate of M6G to a greater degree than for M3G (Bernard Testa, University of Lausanne, Switzerland, personal communication). These data suggest that M6G should cross the BBB and other tight biological membranes by diffusion. The *in vivo* results reported here and the hydrophilicity indices calculated from several other studies are in the same range

and rank order as the results of Carrupt *et al.* (1991), supporting this suggestion. These data, however, do not preclude mechanisms such as facilitated transport or adsorptive endocytosis.

The disparity between the estimated log P (shake-flask method) determination of lipophilicity for M6G and the HPLC method for k_i (Carrupt *et al.*, 1991) is intriguing. Many studies have shown the two methods to be comparable (Braumann *et al.*, 1983; Braumann, 1986; Bechalany *et al.*, 1989). As mentioned, M6G and M3G exist in extended or folded conformations. Recently, similar disparities in the shake-flask and HPLC methods have also been noted for small peptides, which also undergo conformational changes *in vivo*. (Bernard Testa, personal communication). For molecules such as these, the two methods apparently differ. Braumann (1986) has argued that the stationary lipid phase of the HPLC method better models the complex regions of biologic membranes than does the liquid-liquid interface of octanol and water in the shake-flask method. The k_i may, therefore, better reflect the *in vivo* flux of molecules, such as M6G and M3G, which are capable of folding, than does the shake-flask method. The data presented here, and calculated from other studies of M6G and M3G across tight biologic membranes, are in accord with the *in vitro*, k_i based, relative hydrophilicity calculated from the known lipophilicity of the drugs.

The study reported here strongly suggests that M6G and M3G cross the BBB by diffusion. The access of the glucuronides to the CNS depends on the concentration gradient between plasma and brain and is inversely related to their

in vitro hydrophilicity index. There is no evidence that a carrier is responsible for the entry of either M6G or M3G into the CNS. Reported changes in the potency of M6G relative to morphine in depressing neonatal ventilation (Murphey and Olsen, 1994) cannot be explained by alteration in BBB permeability to M6G during the first week of life. The aging effect upon M6G potency remains unexplained, but may be due to changes in receptor density or binding affinity or a change in the efficacy of second-messenger activation once agonist binding has occurred.

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Footnotes

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2. This work was performed as partial requirement for a Ph.D. degree in Pharmacology for L.J. Murphey.

TABLE 1. **Brain and plasma M6G concentrations and brain to plasma ratios 70 min after s.c. M6G administration.** M6G was administered by s.c. injection to 3 or 7 day old guinea pigs and blood and brains were collected (Mean ± S.E.), (*n* = 8).

	Day 3			Day 7		
Dose (mg/kg)	**Brain (ng/g)**	**Plasma (ng/ml)**	**Brain to Plasma Ratio**	**Brain (ng/g)**	**Plasma (ng/ml)**	**Brain to Plasma Ratio**
5	210 ± 10	[a] 6712 ± 563	0.032 ± 0.002	173 ± 18	4250 ± 331	0.042 ± 0.005
15	628 ± 35	[b] 19,049 ± 1043	0.034 ± 0.003	591 ± 49	14,735 ± 1222	0.041 ± 0.002

[a] Day 3 different than day 7, P<.01.

[b] Day 3 different than day 7, P<.05.

TABLE 2. **Depression of V_I while breathing 5% CO_2 mixture 70 min after s.c. injection of M6G.** Baseline ventilation was recorded followed by M6G injection, and post-treatment data collected 70 min later. Data are expressed as a ratio of the pre-injection control V_I (Mean ± S.E.), (n = 8).

Dose (mg/kg)	Day 3	Day 7
5	[a] 0.664 ± 0.069	0.477 ± 0.067
15	[a] 0.619 ± 0.077	0.461 ± 0.097

[a] Day 3 different than day 7, P<.05.

TABLE 3. **Brain and plasma drug concentrations and brain to plasma ratios 45 min after morphine administration.** Morphine, 15 mg/kg, was given by s.c. injection, blood and brain tissue collected and analyzed for morphine and metabolites. Due to laboratory accident, data for 5 pups, instead of 6, are included for day 3 (Mean ± S.E.).

	Day 3 (n = 5)			Day 7 (n = 6)		
Drug	**Brain (ng/g)**	**Plasma (ng/ml)**	**Brain to Plasma Ratio**	**Brain (ng/g)**	**Plasma (ng/ml)**	**Brain to Plasma Ratio**
Morphine	170 ± 8	715 ± 49	0.24 ± 0.02	153 ± 5	755 ± 54	0.21 ± 0.01
M6G	89 ± 17	763 ± 68	0.12 ± 0.02	125 ± 10	1167 ± 155	0.12 ± 0.02
M3G	[a] 89 ± 11	[b] 2099 ± 288	0.043 ± 0.003	122 ± 5	3224 ± 179	0.038 ± 0.002

[a] Day 3 different than day 7, P<.05.

[b] Day 3 different than day 7, P<.01.

TABLE 4. **Relative hydrophilicity index for morphine-6- and morphine-3-glucuronide.** The index is calculated by dividing the brain to plasma ratio for morphine by the brain to plasma ratio for M6G or M3G. Results from this study in the neonatal guinea pig are compared to *in vivo* data from the literature involving drug concentrations across the blood-brain, dural and the epitheliochorial placental barriers. Ratios using k_i are an *in vitro* measure of hydrophilicity, based on known lipophilicity of the drugs. As the number increases, the hydrophilicity of the glucuronide metabolite increases relative to morphine.

Barrier	Species	M6G	M3G	Reference
Blood-Brain	guinea pig	5.9	10.0	Present study
Blood-Brain	rat	[a] 7.5	-	Yoshimura *et al.*, 1973
Dura	human	[b] 6.2 [c] 9.2	- -	Portenoy *et al.*, 1991
Placenta	sheep	-	[d] 14.	Olsen *et al.*, 1988; unpublished data
Isocratic capacity factors, pH 7.0	*in vitro*	4.5	13.8	Carrupt *et al.*, 1991

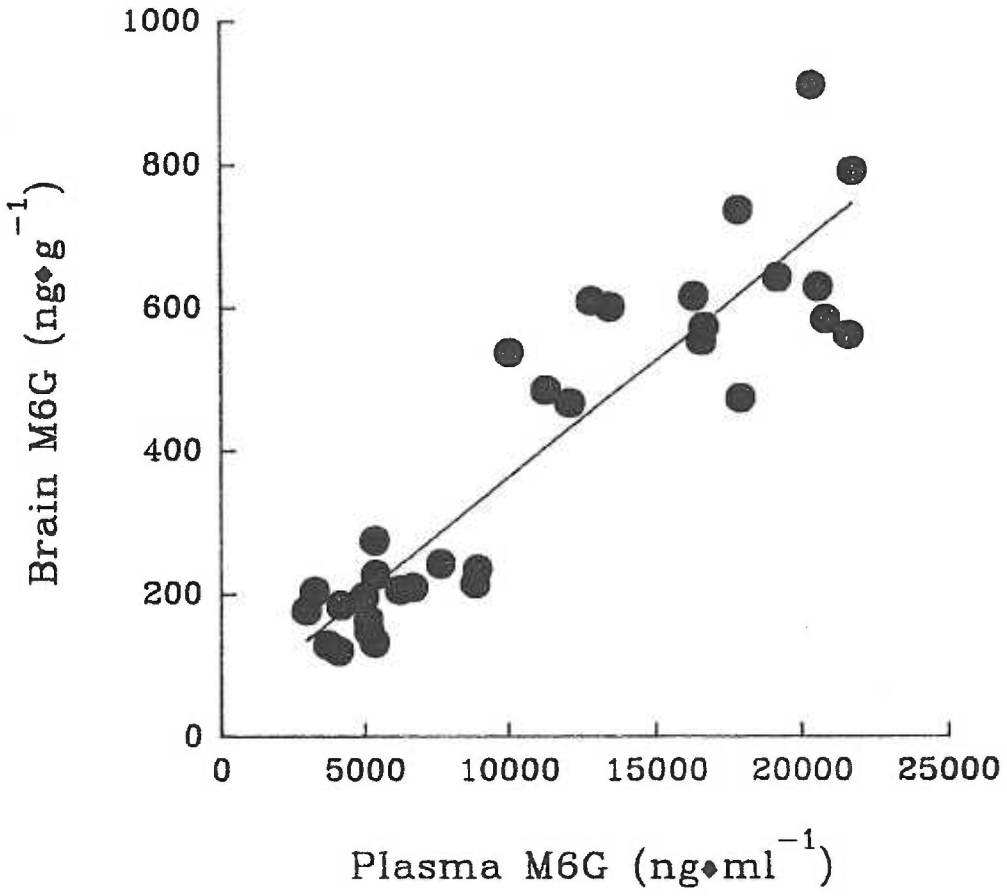
[a] Blood and brain morphine are both 0.0287% of total injected dose (ratio=1.0); blood and brain M6G are 0.043 and 0.0057%, respectively, of total injected M6G (ratio=0.133).

[b] Lumbar cerebrospinal fluid morphine and M6G ratios are 0.751 and 0.121, respectively.

[c] Ventricular cerebrospinal fluid morphine and M6G ratios are 0.71 and 0.077, respectively.

[d] Fetal to maternal plasma ratios for morphine and M3G are 0.0270 and 0.00188, respectively.

Fig. 1. Correlation between brain and plasma drug concentrations. Brain M6G concentrations were a linear function of plasma M6G levels. [Brain M6G] = 38.5 + 0.0325•[Plasma [M6G], $r^2 = 0.84$, $F_{1,30} = 153.9$, $P<.01$.


1000
800
600
400
200
0
0
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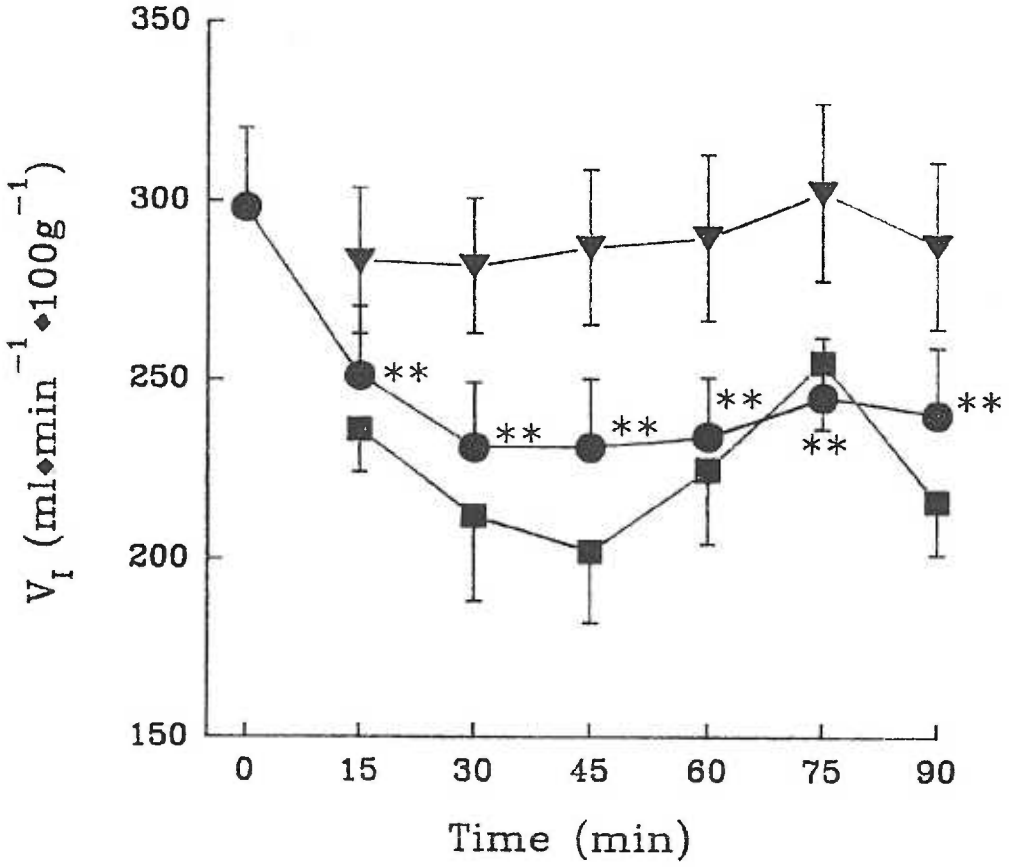
10000
15000
20000
25000
Brain M6G (ng•g^{-1})
Plasma M6G (ng•ml^{-1})

Fig. 2. Time-action curve for V_I after M3G injection to 7 day old guinea pig neonates. Following baseline recording of ventilation (time = 0 min), animals (●, n=11) received 75 mg/kg M3G by s.c. injection and breathing was studied over 90 min. Historical data (Murphey and Olsen, 1994, with permission from the American Society for Pharmacology and Experimental Therapeutics) for saline (▾, n=6) and 1.5 mg/kg morphine (■, n=6) treated pups are included for comparison. Mean ± S.E. $^{**}P<.01$ for M3G data compared to pre-injection control.


350
300
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Time (min)
V_I (ml•min^{-1}•100g^{-1})
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Manuscript IV

(J. Pharmacol. Exp. Ther. Under Review, 1994)

Title: Developmental Change of *Mu* Opioid Receptors in Neonatal Guinea Pig Brain Stem: Relationship to Morphine-6-β-D-Glucuronide Respiratory Pharmacology[1,2].

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Running Title: Neonatal M6G *Mu* Opioid Binding

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Abbreviations:

M6G	morphine-6-β-D-glucuronide
M3G	morphine-3-β-D-glucuronide
DAMGO	[D-Ala^2,N-methylPhe^4-Glyol^5]enkephalin
K_D	equilibrium dissociation constant
B_{max}	maximal number of binding sites
IC_{50}	concentration causing 50% inhibition of maximal binding
K_I	binding inhibition constant

ABSTRACT

Opioid receptor binding of morphine-6-β-D-glucuronide (M6G), morphine and [^{3}H][D-Ala^2,N-methylPhe^4-Glyol^5]enkephalin (DAMGO) was determined in neonatal guinea pigs, as a function of age. Membranes from brain stem areas important in opioid-induced respiratory depression (pons and medulla) showed specific binding of all three compounds. The concentration of competitor producing 50% inhibition (IC_{50}) of maximal [^{3}H]DAMGO binding (5 nM) did not change for M6G or morphine between day 3 and day 7 after birth. The IC_{50} (mean ± S.E.) for M6G was 84 ± 7 nM (n = 8), and for morphine it was 28 ± 3 nM (n = 7). The equilibrium dissociation constant (K_D) for [^{3}H]DAMGO binding was unchanged with aging (1.1 ± 0.1 nM), however the maximum number of binding sites (B_{max}) increased from 62.2 ± 4.8 fmol/mg protein (n = 7) to 88.3 ± 5.6 fmol/mg protein (n = 6) between days 3 and 7 ($P<.01$). This 42% increase in *mu* receptors may play a role in the increased potency of M6G respiratory effects which has been reported for guinea pigs during the first week after birth.

Introduction

M6G, the active morphine metabolite, causes analgesia (Shimomura *et al.*, 1971; Pasternak *et al.*, 1987; Paul *et al.*, 1989; Francés *et al.*, 1992) and respiratory depression (Pelligrino *et al.*, 1989; Gong *et al.*, 1991; Murphey and Olsen, 1994a,b) similar to the parent compound. In the neonatal guinea pig, M6G potency, with respect to respiratory depression, increases with aging, but morphine potency does not change (Murphey and Olsen, 1994a,b). There are no developmental changes in M6G distribution to the brain which can explain the observed increase in potency (Murphey and Olsen, 1994b).

Alteration in opioid receptor binding affinity or number of binding sites may account for the increase in M6G respiratory depressant potency with aging. Opioids cause respiratory depression by decreasing chemoreceptor sensitivity to hypercapnia. These respiratory effects are mediated *via mu* receptors located in the respiratory centers of the pons and medulla oblongata (see review by Yeadon and Kitchen, 1989). The development of opioid receptors in the guinea pig brain has not been thoroughly examined since only non-specific radiolabelled ligands and brains without the brain stem have been studied (Clendeninn *et al.*, 1976). Because the guinea pig is more neurologically mature at birth compared to the rat and mouse (Dawes, 1968), brain developmental studies in these other rodent species (Leslie *et al.*, 1982; Spain *et al.*, 1985; McDowell and Kitchen, 1987; Rius *et al.*, 1991) cannot be extrapolated to the guinea pig.

This investigation reports the *mu* opioid binding of guinea pig brain stem

tissue during the first week of life. The *mu* specific agonist, [^{3}H]DAMGO (Pasternak *et al.*, 1987), was used to define opioid binding capacity and the relative ability of M6G and morphine to inhibit this binding. This is the first report of M6G binding in any neonatal animal, and the first study of *mu* opioid binding in the neonatal guinea pig.

Methods

Animals. Neonates from female Dunkin-Hartley guinea pigs time-bred on site were used. Animals were housed on CareFresh Bedding (Absorption Corporation, Bellingham, WA) with controlled light cycles, *ad libitum* food and water, and daily health checks. The Department of Animal Care operates in compliance with the Animal Care Act, and has a letter of assurance on file with the National Institutes of Health.

Chemicals. [^{3}H]DAMGO (specific activity 50.5 Ci/mmol) was purchased from New England Nuclear-DuPont (Boston, MA). M6G, M3G (Research Triangle Institute, Research Triangle Park, NC), unlabelled DAMGO and cyclic [D-Pen^2,D-Pen^5]enkephalin (Chiron Mimotopes Peptide Systems, San Diego, CA) were obtained through the National Institute on Drug Abuse (Rockville, MD). Morphine was purchased from Mallinckrodt (St. Louis, MO) and dextrorphan was a gift from Hoffman-LaRoche (Nutley, NJ). Unlabelled drug and peptide stocks (250 μM) were made in water, stored at -20°C and diluted with 0.05 M Tris buffer before use. Tris•HCl and sucrose (Mallinckrodt, Paris, KY) were laboratory grade. Acetic acid and n-butanol (J.T. Baker, Phillipsburg, NJ) were reagent grade.

Purity of radioligand. Thin layer chromatography with silica gel and a 25:4:10 mixture of n-butanol:acetic acid:water was employed. Lanes containing unlabelled DAMGO, [^{3}H]DAMGO and radiolabel underspotted with unlabelled peptide were

chromatographed and visualized with ninhydrin reagent (Sigma Chemical Co., St. Louis, MO). Lanes containing tritiated peptide were scraped from the plate in segments and counted. Purity was 90%, and calculations were adjusted accordingly.

Tissue preparation. Brains were removed following decapitation, and the pons and medulla oblongata, which contain the respiratory centers, were dissected together. The rostral limit of dissection was the ventral fissure between the pons and the cerebral peduncles. The caudal limit of dissection was marked by the confluence of the vertebral arteries to form the basilar artery caudal to emergence of the XII cranial nerve. Cerebellar peduncles were transected and the cerebellum removed. Dorsally, any remaining portion of the cerebral peduncles were detached from the rostral limit of the fourth cerebral ventricle, and discarded.

The pons and medulla were homogenized in 20 ml/g wet weight of 0.32 M sucrose with a glass-teflon tissue grinder, and centrifuged at 2000 x *g* for 5 min. The supernatant was centrifuged at 30,000 x *g* for 10 min and the pellet resuspended in 10 ml/g original wet weight of 0.05 M Tris•HCl, pH 7.4 and frozen at -70°C for later use (Christensen and Jørgensen, 1987). Before use, membranes were thawed, brought to 30 ml/g original wet weight with 0.05 M Tris•HCl, pH 7.4, centrifuged at 30,000 x *g* for 20 min and resuspended in 10 ml/g original wet weight of 0.05 M Tris•HCl, pH 7.4. When fresh membranes were used in binding assays, the freezing step was omitted and the first 30,000 x *g* pellet was

resuspended in 30 ml/g wet weight of Tris buffer before the second 30,000 x *g* centrifugation. Protein concentrations were determined by the method of Lowry *et al.* (1951) using a bovine serum albumin standard.

Radioligand binding assays. Fresh and frozen tissue preparations were compared, and no difference was observed with respect to affinity and number of binding sites (data not shown). Subsequent binding assays, therefore, were performed using frozen tissue. Non-specific binding was defined as radioligand bound in the presence of 1 μM morphine. Assay volume was 250 μl, consisting of 50 μl of radioligand, 100 μl of membranes (200 to 300 μg protein) and 100 μl of either Tris buffer (total binding), morphine in Tris buffer (non-specific binding) or competitor in Tris buffer (displacement assays).

Saturation binding isotherms were created using 0.5 to 7 nM [^{3}H]DAMGO and binding at each concentration was determined in duplicate. [^{3}H]DAMGO binding was equilibrated at 25°C for 45 min. In preliminary studies, specific binding of [^{3}H]DAMGO was lower when equilibrated at 4°C for 60 min, however, the K_D was the same as at 25°C. Incubations at 37°C yielded the same B_{max} and K_D as observed at 25°C through 60 min, but specific binding decreased significantly over the next 24 hr. The binding assay was terminated by the addition of 4 ml of ice cold 0.05 M Tris buffer and bound and free radioligand were separated by rapid filtration over a number 30 S&S glass fiber filter (Intermountain Scientific, Kaysville, UT). Each assay tube and filter was washed twice with 4 ml aliquots of Tris buffer.

Experiments comparing 3 day old and 7 day old animals were performed concurrently. In the competitive displacement studies, a saturating concentration (5 nM) of [^{3}H]DAMGO in the presence and absence of various competitors in concentrations ranging from 100 pM to 10 μM, were incubated as described.

Filters with membranes were placed in 7 ml plastic scintillation vials and 5 ml of CytoScint liquid scintillation cocktail (ICN, Costa Mesa, CA) were added and allowed to equilibrate for 5 hours. Radioactivity was quantitated in a Model 7500 liquid scintillation system (Beckman Instruments, Inc, Fullerton, CA). Cpm were corrected to dpm using an external standard quench curve. Average counting efficiency was 47%.

Data analysis. The K_D and B_{max} for [^{3}H]DAMGO were determined by non-linear least-squares curve fit to the specific binding saturation isotherm (Graph-PAD InPlot, San Diego, CA). For displacement assays, the IC_{50} was determined following non-linear least-squares fit to the plot of fraction of total radioligand bound versus concentration of competitor (Graph-PAD InPlot). Inhibition constant (K_i) was calculated by the method of Cheng and Prusoff (1973).

Statistics. Statistical differences were determined using Students' *t*-test, and a probability level of 0.05 was considered significant. Mean ± S.E. are reported.

Results

Saturation binding studies. The *mu* opioid agonist, [^{3}H]DAMGO, showed saturable, high affinity binding to pontine and medullary membranes from both 3 and 7 day old neonatal guinea pigs (figure 1). Binding affinity did not change with aging and the K_D was (mean ± S.E.) 1.0 ± 0.1 (n = 7) and 1.1 ± 0.1 (n = 6) nM on day 3 and 7, respectively. In contrast, binding capacity increased by 42% between day 3 and day 7. B_{max} changed from 62.2 ± 4.8 (n = 7) fmol/mg protein on day 3 to 88.3 ± 5.6 (n = 6) fmol/mg protein on day 7 ($P<.01$). Specific binding of [^{3}H]DAMGO was not observed in membranes derived from liver, boiled brain, or when membranes were omitted (data not shown).

Competitive displacement studies. Morphine, M6G and unlabelled DAMGO displaced binding of 5 nM [^{3}H]DAMGO to brain membranes (figure 2). Morphine exhibited a 3-fold higher affinity for the [^{3}H]DAMGO binding site than did M6G while DAMGO had the lowest IC_{50} of the three competitors (table 1). However, there was no difference in IC_{50} between days 3 and 7 for M6G, morphine or DAMGO. M3G (which has very low affinity for the *mu* receptor), dextrorphan (an inactive opioid isomer), and cyclic [D-Pen^2,D-Pen^5]enkephalin (a *delta* opioid receptor agonist) did not displace [^{3}H]DAMGO binding, at concentrations up to 100 nM (data not shown).

Discussion

This is the first report of the neonatal opioid receptor binding of M6G in any species. The affinity of M6G and morphine for *mu* opioid receptors in the neonatal guinea pig brain stem remains constant between days 3 and 7 after birth, a time period when respiratory effects of M6G show an increase in potency (Murphey and Olsen, 1994a,b). Therefore, age related changes in M6G *mu* opioid receptor affinity do not explain the previously reported changes in M6G respiratory effect (Murphey and Olsen, 1994a,b).

The binding affinity of M6G, as reflected by its IC_{50}, was 3- to 4-fold lower for the *mu* receptor as compared to morphine at both ages which corresponds to previous reports in adult animal brains of several other species. In competition displacement studies using [^{3}H]DAMGO, M6G exhibited a 2- to 4-fold lower affinity than morphine for [^{3}H]DAMGO binding sites in mouse brain (minus cerebellum) homogenates (Paul *et al.*, 1989), and in bovine thalamic membranes (Pasternak *et al.*, 1987). However, in rat brain (minus cerebellum) homogenates, Chen *et al.* (1991) reported a 2-fold greater affinity for M6G compared to morphine. In all of these studies, the IC_{50} values for morphine and M6G ranged from 2.5 to 11 nM. In the neonatal guinea pig pons and medulla, IC_{50} values for morphine and M6G determined in the current study were higher, 25 to 30 nM and 77 to 90 nM, respectively. However, the earlier studies were conducted using 1 nM [^{3}H]DAMGO, while in this study a saturating concentration (5 nM) of [^{3}H]DAMGO was used, which would be expected to increase the IC_{50} values for M6G and morphine. The

expected IC_{50} for DAMGO competition of 5 nM [^{3}H]DAMGO is 5 nM. The observed IC_{50} of 10-13 nM is probably due to unlabelled peptide impurity.

Development of *mu* opioid receptor binding has not been previously studied in the neonatal guinea pig. The B_{max} for [^{3}H]DAMGO increased 42% between day 3 and day 7 while the K_D remained constant over this same interval. There may also be a higher affinity site which was not detected in this study due to binding of endogenous ligand. It is uncertain if the increase in the number of binding sites plays a role in the increased potency seen for M6G respiratory effects. M6G may have lower intrinsic activity and a smaller receptor reserve than morphine, which would make M6G more sensitive to changes in total receptor number. Higher numbers of *mu* receptors may preferentially augment the respiratory effects of M6G, whereas morphine achieves its respiratory depressant activity with the number of receptors available on day 3.

The brain developmental profile of *mu* receptors has been reported for the rat (Leslie *et al.*, 1982; Recht *et al.*, 1985; Spain *et al.*, 1985), mouse (Rius *et al.*, 1991) and, to a limited extent, for the human (Magnan and Tiberi, 1989) and sheep (Dunlop *et al.*, 1986). In the rat brain, newborn pups have approximately 50% lower number of *mu* receptors than does the adult. However, significant brain development in rats and mice occurs post-natally, whereas guinea pig neural function is more mature at birth, and is equivalent to a one to two week old rat pup (Dawes, 1968; Olsen and Weil, 1992). In one human study, 20-week gestation fetal brain contained *mu* opioid receptors (Magnan and Tiberi, 1989). In the

midgestation sheep fetus, the number of *mu* binding sites is about 70% of the adult level (Dunlap *et al.*, 1986). One study in the guinea pig found the same number of [^{3}H]naltrexone binding sites present at birth, as in the adult animal (Clendeninn *et al.*, 1976). However, naltrexone is not particularly selective for *mu* receptors, and these results may reflect binding of naltrexone to *delta* and *kappa* receptors as well.

Brain distribution of [^{3}H]DAMGO binding sites has been studied by saturation binding in adult rats (Bhargava *et al.*, 1989; Gulati and Bhargava, 1990) and by autoradiography in neonatal rats (Xia and Haddad, 1991). In contrast to the results in the neonatal guinea pig brain, adult rats had a much higher K_D (25 nM compared to 1.1 nM) for [^{3}H]DAMGO binding in the pons and medulla (Gulati and Bhargava, 1990). The B_{max} was lower in pons and medulla in these rat studies as well (25 to 50 fmol/ mg protein compared to 62 to 88 fmol/mg protein). *Mu* receptors are present in high levels in the nucleus tractus solitarius and nucleus ambiguus of the rat (Xia and Haddad, 1991). These areas compose the dorsal and part of the ventral respiratory centers, respectively. In these areas, newborn levels of *mu* receptors were approximately 25 to 50% of adult levels (Xia and Haddad, 1991).

Binding of M6G to membranes of the neonatal guinea pig pons and medulla has been defined for the *mu* receptor. M6G exhibits three-fold lower affinity than does morphine. The number of [^{3}H]DAMGO binding sites increases between days 3 and 7, but affinities for [^{3}H]DAMGO, M6G and morphine do not change.

Increases in the number of *mu* binding sites may play a role in the increased potency of M6G in depressing respiration during neonatal guinea pig development.

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Footnotes

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2. This work was performed as partial requirement for a Ph.D. degree in Pharmacology for L.J. Murphey.

TABLE 1. **Inhibition of [^{3}H]DAMGO binding to neonatal guinea pig pontine and medullary membranes by other opioids.** [^{3}H]DAMGO (5 nM) was incubated at 25°C for 45 min with competitor (100 pM to 10 μM), then filtered. IC_{50} (mean ± S.E., n=4) was determined following non-linear regression of the plot of fraction of total [^{3}H]DAMGO bound versus concentration of unlabelled ligand. K_I's were calculated according to the method of Cheng and Prusoff (1973). There was no difference in IC_{50} between days 3 and 7 for a given ligand. M3G, dextrorphan and cyclic [D-Pen^2,D-Pen^5]enkephalin, at concentrations up to 100 nM, did not displace [^{3}H]DAMGO binding.

	IC_{50} (nM)	
Displacing Ligand	Day 3	Day 7
Morphine	[a] 24.7 ± 6.2	29.6 ± 3.1
M6G	90.4 ± 11.3	76.9 ± 10.0
DAMGO	[a] 10.0 ± 1.9	[a] 12.6 ± 1.0
	K_I (nM)	
Displacing Ligand	Day 3	Day 7
Morphine	4.3 ± 1.1	5.6 ± 0.6
M6G	15.7 ± 2.1	14.4 ± 2.0
DAMGO	1.8 ± 0.3	2.5 ± 0.2

[a] $n = 3$

Fig. 1. Binding of [^{3}H]DAMGO to brain stem membranes. Panel A: Representative saturation binding isotherms for [^{3}H]DAMGO to membranes from 3 day- (▾) and 7 day- (▽) old guinea pig pups. Experiments were repeated 7 or 8 times at each age. [^{3}H]DAMGO (0.5 to 7 nM) and membranes from the pons and medulla were incubated at 25°C for 45 min, then filtered over glass-fiber filters. Non-specific binding was defined using 1 μM morphine. Binding affinity did not differ between the two ages, however, B_{max} increased 42% from day 3 to day 7. Panel B: Scatchard transformation (Scatchard, 1949) of data illustrated in Panel A (SB=specific bound, F=free drug).

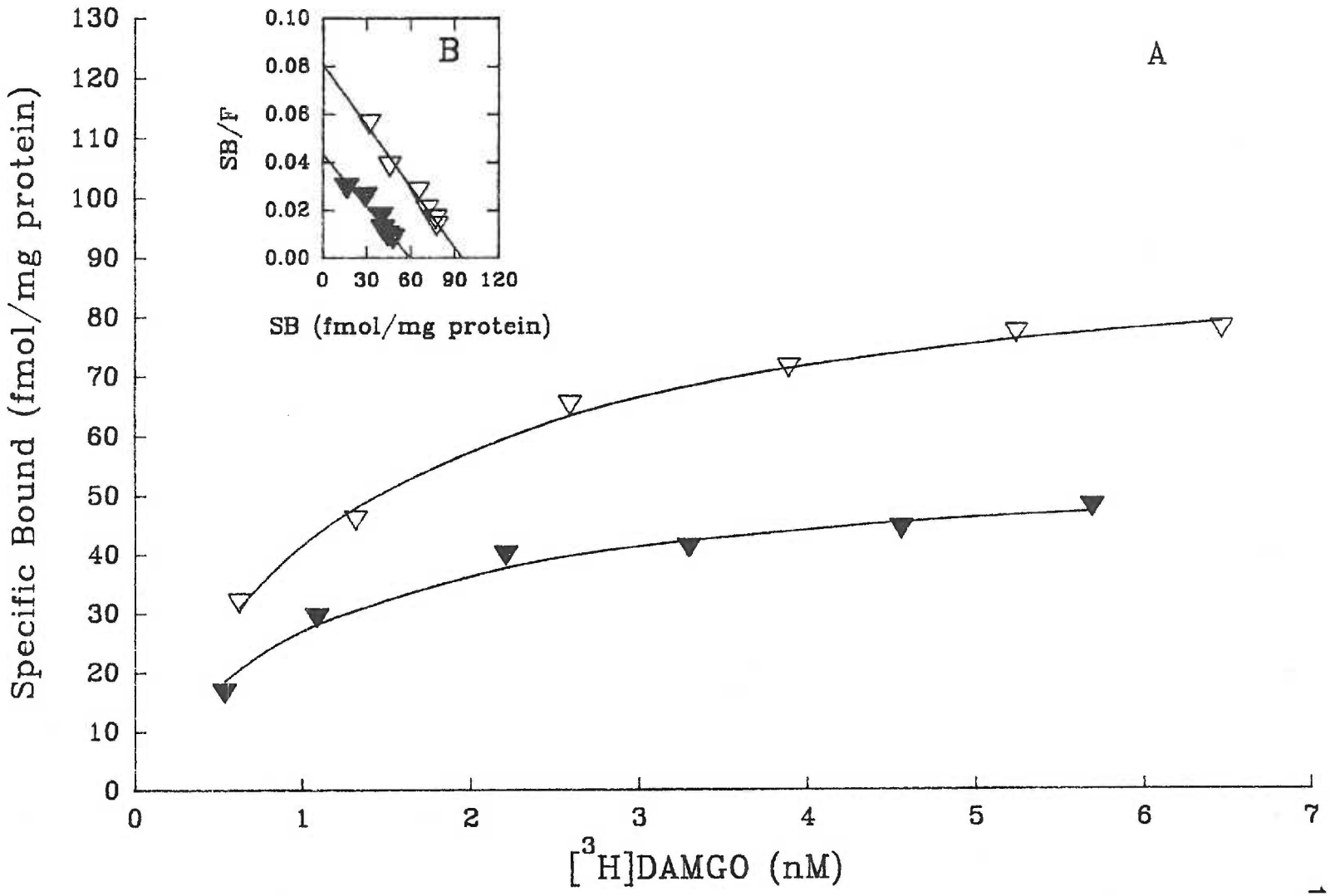
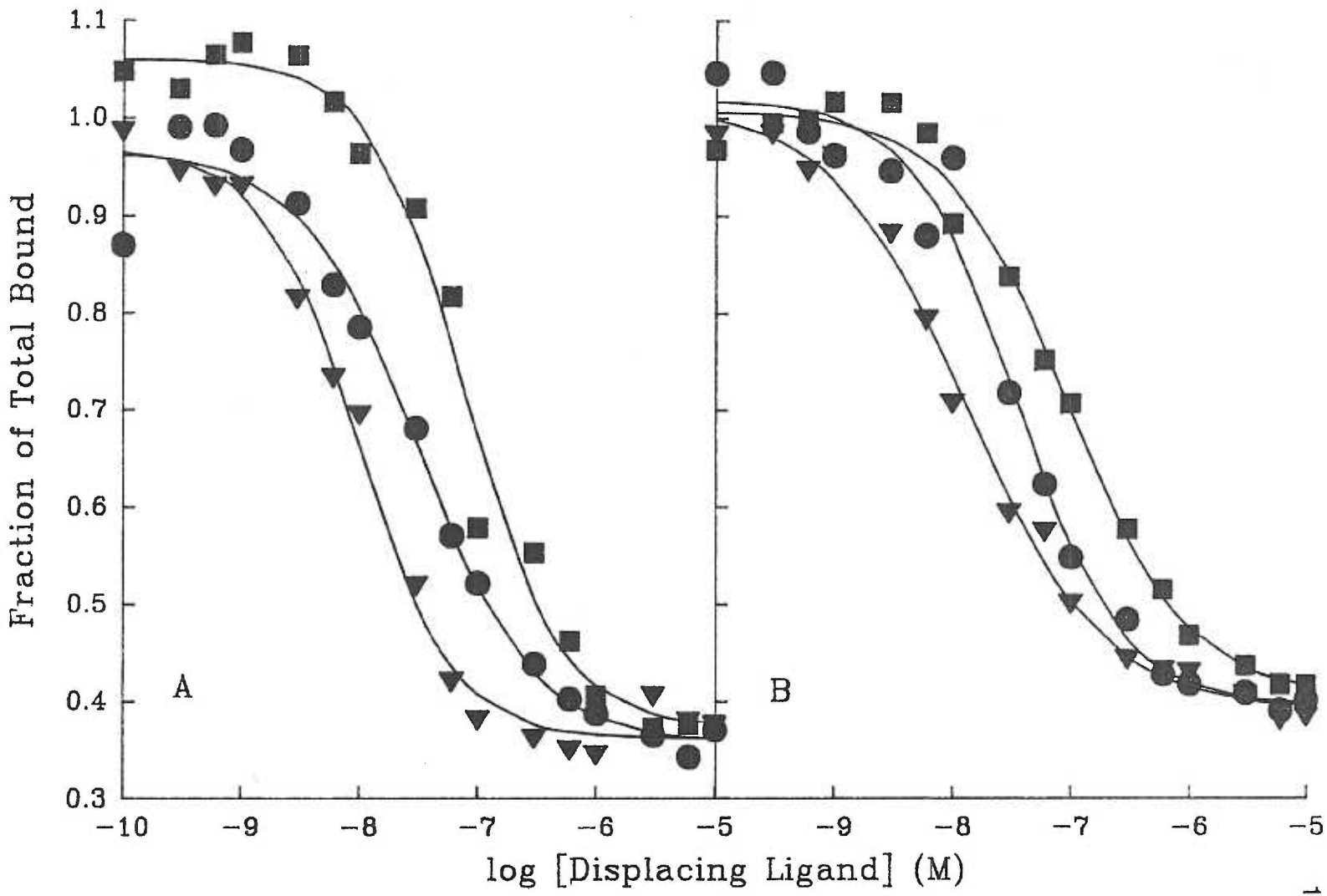

A
B
Specific Bound (fmol/mg protein)
[3H]DAMGO (nM)
SB/F
SB (fmol/mg protein)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
1
2
3
4
5
6
7
0.00
0.02
0.04
0.06
0.08
0.10
30
60
90
120

Fig. 2. Competitive displacement of [^{3}H]DAMGO by morphine (●), M6G (■) and unlabelled DAMGO (▾) in guinea pig pontine and medullary membranes. One experiment for each ligand is shown. At least three observations were made for each competitor. [^{3}H]DAMGO (5 nM) and competitor (100 pM to 10 μM) were incubated for 45 min at 25°C, then filtered over glass-fiber filters. Panel A: 3 day old pups. Panel B: 7 day old pups.


Fraction of Total Bound
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
A
B
−10
−9
−8
−7
−6
−5
log [Displacing Ligand] (M)

Discussion and Conclusions

The pharmacology of M6G has been studied in adult animals of many species by other investigators, including the mouse (Shimomura *et al.*, 1971; Pasternak, *et al.*, 1987; Paul *et al.*, 1989), rat (Yoshimura *et al.*, 1973; Abbott and Palmour, 1988; Abbott and Franklin, 1990; Gong *et al.*, 1991, 1992; Francés *et al.*, 1990, 1992), dog (Pelligrino *et al.*, 1989) and human (Osborne *et al.*, 1988, 1992; Hanna *et al.*, 1990; Peat *et al.*, 1991). The investigations described in this dissertation are the first studies of the pharmacology of M6G in guinea pigs and in neonates of any species.

There are several important considerations in choosing an animal model for research work. This is particularly important for developmental studies in pharmacology, if meaningful extrapolations are to be made to the human condition. The animal model should be similar to the human in regard to CNS development, drug metabolite profile, physiological response for effects which are being studied and placental structure and permeability.

Neonatal guinea pigs were utilized to study the developmental pharmacology of M6G for several reasons. A critical period of CNS development occurs *in utero* for the guinea pig, similar to human CNS development (Dawes, 1968; Olsen and Weil, 1992a). The guinea pig is, therefore, more mature at birth compared to newborn cats, dogs and rats, common animal models for adult respiratory studies. Guinea pigs produce M6G and M3G after morphine administration (Oguri *et al.*, 1970), including neonates of the species (Murphey and

Olsen, 1993, 1994b). This metabolite profile is similar to what is seen in the adult human (Boerner *et al.*, 1975; Svensson *et al.*, 1982; Osborne *et al.*, 1990) and in the neonate (Choonara et al., 1989; Hartley et al., 1993). The control of breathing in the newborn guinea pig is stimulated by inhalation of hypercarbic gas mixtures (Olsen and Weil, 1992a,b; Murphey and Olsen, 1994a,b) as is the breathing in the human neonate (Olsen and Lees, 1980). In addition, opioids depress ventilation in neonatal guinea pigs (Murphey and Olsen, 1994a,b) and humans infants (Way *et al.*, 1965). The guinea pig and human both have hemomonochorial placentae with similar permeability characteristics. While this last point was not directly related to this dissertation, it is important for further studies involving chronic *in utero* morphine and M6G exposure, which follow from the current work.

In order to study drug disposition, metabolism or pharmacokinetics, a reliable, sensitive and specific assay must be available. For most relatively hydrophilic drugs such as morphine and its glucuronide metabolites, reverse phase HPLC technique meets such requirements. A second consideration in the case of developmental pharmacology is the limited sample sizes available from small animals.

The first aim of this dissertation, therefore, was to develop a HPLC microassay for morphine and metabolites. The limitations of many of the currently developed methods, and comparisons to the method resulting from the current work are discussed in Manuscript I. Briefly, most methods utilized prohibitively large sample sizes (500 to 1000 μl) for use with small animals (Svensson et al.,

1982; Svensson, 1986; Joel et al., 1988; Venn et al., 1990; Mason et al., 1991). By comparison, the microassay developed for this work uses an 80 μl sample size. Solid phase extraction also has the advantage of a single clean-up step and multiple sample extraction capability with a vacuum manifold. The assay was used to collect initial data on the *in vivo* and *in vitro* production of morphine metabolites in neonatal and pregnant guinea pigs. These data established that the guinea pig does produce M6G and M3G, and in this respect was a good model for human morphine metabolism. Even though normorphine was not detected *in vivo* in the neonatal guinea pig, hepatic microsomes from pregnant guinea pigs are capable of *in vitro* production of normorphine. This is a only minor (<10%) metabolite in the human, and does not nullify the effectiveness of the guinea pig model.

As dissertation work progressed, an assay for morphine and metabolites in brain became necessary. The original extraction utilizing silica-bonded C-18 solid phase cartridges did not provide sufficient sample clean-up for brain samples. A novel, multi-step extraction of such samples was developed. Initial clean-up of supernatant of brain homogenates used ultrafiltration with a 5000 molecular weight cut-off filter. Further impurities were removed by passing the ultrafiltrate over a silica-bonded cyclohexyl extraction column. This step would serve to selectively retain morphine and metabolites while more hydrophilic impurities pass through and to leave more lipophilic impurities adsorbed to the cyclohexyl packing as the drugs of interest are eluted. The cyclohexyl eluate underwent further clean-up using an extraction column with combination C-18 and strong cation exchange

packing (Clean Screen). During extraction on the Clean Screen column, low pH neutralized the glucuronide carboxyl group, and ionic interaction between drug and the solid phase was maintained through the amine of morphine. Several washes with polar solvents further cleaned the sample and elution was accomplished using a methanol: dichloromethane mixture at high pH to neutralize ionic interactions.

The HPLC assay was developed with UVD. This method of detection accorded adequate sensitivity for the quantitation of plasma, microsomal incubate and injection solution drug levels, reported in Manuscripts I, II and III. For M6G brain distribution studies reported in Manuscript III, UVD lacked adequate sensitivity to quantitate drugs in brain tissue after morphine administration.

A coulometric type detector for ECD was added to the assay to increase sensitivity. The coulometric method of ECD utilizes a porous, flow-through analytical electrode with a large surface area as opposed to amphometric ECD which has a small surface area electrode. The coulometric detector insures that almost all of a sample is oxidized (or reduced), and is more sensitive than amphometric detectors which only oxidize (or reduce) a small fraction of a sample. Because coulometric detectors are very efficient in oxidizing the entire sample, two analytical cells can be used in tandem. The first cell is set at a lower potential, to oxidize extraneous compounds and the second cell, which is connected to the recording device, is set at a higher potential to oxidize the drug of interest. Coulometric detection, therefore, offers advantages of a stronger molar signal, and

of a less noisy base-line, compared to amphometric ECD and UVD, leading to a lower limit of detection, and greater sensitivity.

ECD for morphine utilizes oxidation of the 3 phenolic group. This type of detection, therefore, cannot measure M3G, which has the 3 phenol conjugated to glucuronic acid. UVD, on the other hand, detects morphine, M6G and M3G. In order to quantitate all three, ECD, for increased morphine and M6G sensitivity, in series with UVD, for M3G, was used. The original HPLC assay and its modifications were indispensable for the study of M6G neonatal pharmacology reported in Manuscript III of this dissertation.

There are several compelling reasons to determine M6G neonatal pharmacology. As reviewed in Section II of the Introduction, M6G has a similar pharmacological profile to morphine, but in adults it is many times more potent than the parent compound. M6G is produced by human neonates (Choonara *et al.*, 1989; Hartley *et al.*, 1993). Therefore, as the ability of newborns to perceive and respond to pain has become better appreciated (Bhat *et al.*, 1990), the use of morphine in human neonatal medicine is increased and leads to M6G exposure in this age group. Heroin is rapidly metabolized to morphine in the human (Inturrisi *et al.*, 1984) and ultimately glucuronidated to M6G. Because morphine glucuronide crosses the hemomonochorial placenta (Olsen *et al.*, 1989), and because the human fetus is capable of M6G production from morphine transferred across the placenta (Pacifici *et al.*, 1982; Gawronska-Szklarz *et al.*, 1985), maternal heroin and morphine abuse during late pregnancy could lead to appreciable amounts of M6G

in the fetus, which persists in the newborn for the first few days of life. Thus, knowledge of effects which this active, potent metabolite has in the neonate are vital for the appropriate therapeutic use of morphine, and the treatment of newborn M6G toxicity as a consequence of maternal drug abuse. This is particularly significant because neonates may be inherently more sensitive to the effects of opioids than adults. (Way *et al.*, 1965; Choonara *et al.*, 1989)

The second aim of the dissertation was to determine the neonatal respiratory effects of M6G. Opioid abuse during pregnancy leads to altered breathing movements in the human fetus (Richardson *et al.*, 1984) and altered respiratory control in the human neonate (Olsen and Lees, 1980; Davidson Ward, *et al.*, 1986). Infants who have had *in utero* exposure to opioids have a 5- to 10-fold increased risk of dying from SIDS (Kandall and Gaines, 1991; Kandall *et al.*, 1993). Defining the effects acute exposure to M6G have on breathing control in the previously unexposed neonate adds to the understanding of neonatal respiratory control and may contribute to an explanation of the differences in breathing control of infants who have had *in utero* opioid exposure.

Based upon reports in the literature for adult animals, and limited pilot studies in the laboratory using 2- to 3-week old guinea pigs, it was hypothesized that M6G would be approximately three times more potent than morphine as a respiratory depressant in neonatal guinea pigs. Therefore, doses of morphine which were three-fold greater than those of M6G were used. The acute respiratory effects of M6G and morphine in neonatal guinea pigs were determined using a

non-invasive, placebo-controlled, randomized, cross-sectional study on days 3, 7 and 14 after birth. Plethysmography technique measured breathing in conjunction with computerized data acquisition. Time-action curves for each drug were determined, and the values for peak V_I depression underwent further analysis.

Dose-response curves for V_I at peak depression were remarkable for a change in potency between day 3 and day 7. M6G and morphine were equipotent on day 3, but M6G increased in potency 8-fold by day 7. This increase persisted through day 14. Initial dose-response curves were log-linear, plotting log dose versus V_I. This analysis has been repeated, plotting log dose versus the probit of percent of placebo V_I for the drug treated groups. The probit analysis is presented in Appendix B. The original conclusions of the study are not changed by the reanalysis.

The observed potency change was not due to age-related changes in respiration. In fact, over the two weeks of study, base-line V_I of placebo-treated pups did decrease (Appendix B). Opioid effects on respiration were superimposed upon this normal maturational change. The potency of morphine relative to itself did not change with aging. Only the potency of M6G changed, occurring between day 3 and day 7. The change in potency is, therefore, an age-related phenomenon unique to M6G.

Several different pharmacokinetic or pharmacodynamic components determined potency in this study, and could be involved in the potency change. M6G and morphine were administered *via* s.c. injection into the shaved scruff of

the neck. Age related differences in systemic absorption or elimination could result in lower plasma M6G levels in 3-day olds than in 7-day olds, leading to less drug entering the CNS. Likewise, age related increases in M6G distribution to CNS could explain the increased potency. Once M6G reached its site of action, differences in receptor binding or activation of effectors could also be responsible for the increase in respiratory effects of the drug.

The pharmacokinetic hypotheses were tested first. Neonates on day 3 and 7 of age were injected with M6G. Blood and brain tissue were collected at the average time of maximal M6G depression and analyzed by HPLC. Contrary to the observed potency change, 3-day old pups had significantly higher levels of M6G in plasma than did 7-day old pups. In brain tissue, M6G concentrations were also higher in 3-day olds than in 7-day olds, but the difference did not reach significance. From this study, pharmacokinetic differences were excluded as a cause of M6G respiratory depression potency changes.

The pharmacodynamic hypothesis was partially explored by examining the affinity of M6G and morphine for the *mu* opioid receptor. Competitive displacement binding of the *mu* specific radioligand, [^{3}H]DAMGO, by M6G and morphine was done on days 3 and 7. In addition, the affinity and number of [^{3}H]DAMGO binding sites was determined for the two ages. There was no difference in the IC_{50} of either M6G or morphine between day 3 and 7. However, the B_{max} for [^{3}H]DAMGO binding increased by 42% between the two days, while the K_D remained constant. It is possible that this increase in receptor number plays a role in the M6G

respiratory depression potency change.

A perplexing finding of this study was that the affinity of M6G for the *mu* receptor, as reflected by the IC_{50}, was 3- to 4-times lower than the *mu* receptor affinity of morphine. However, the two drugs are equipotent in mediating respiratory depression on day 3, and M6G is 8-fold more potent on day 7. Many investigators have shown that greater M6G potency than morphine for analgesia is not reflected by a greater receptor affinity for M6G (Pasternak *et al.*, 1987; Abbott and Palmour, 1988; Francés *et al.*, 1990, 1992). This is true also for respiratory depression in the neonatal guinea pig (Murphey and Olsen, 1994a,c). Perhaps further investigation into the basis of the affinity versus potency discrepancy may shed light upon the cause of the observed potency change for respiratory depression in the neonatal guinea pig.

Data collected for the pharmacokinetic study suggested that M6G crosses the BBB by diffusion. Several lines of evidence led to this conclusion. Brain concentrations of M6G were a linear function of the plasma M6G levels. The brain to plasma concentration ratio for M6G did not change with dose, indicating M6G entry into brain was not a saturable process over the dose range studied (which caused a physiologically significant range of effects). The brain to plasma ratios also did not change with age, showing there was no indication of induction of maturation of M6G permeability into the CNS during the period of study, consistent with diffusion.

The most intriguing evidence for diffusion involves comparisons of *in vivo*

lipophilicity of M6G and M3G, as reflected by crossing tight membranes, and *in vitro* lipophilicity of M6G and M3G, as reelected by HPLC isocratic capacity factors (k_i) (Carrupt *et al.*, 1991). A new index was proposed, calculated, and presented in Manuscript III, which normalized the permeability of M6G or M3G across tight membranes to the permeability of morphine. The index was named the Relative Hydrophilicity Index and it is the inverse of lipophilicity. Hydrophilicity calculations resulted in the comparison of integers, rather than the comparison of fractions, which resulted from lipophilicity calculations. Therefore, hydrophilicity was used for this index. Data for M6G and M3G crossing several tight membranes from the literature, and from the current study were compared to the k_i for M6G or M3G, normalized to the k_i of morphine. The *in vivo* and *in vitro* data were all in the same range and rank order (table 4, Manuscript III). M6G and M3G cross tight membranes in agreement with their lipophilicity. M6G is about 5- to 10-times more hydrophilic than morphine while M3G about 10- to 15-times more hydrophilic than morphine.

The conclusion that M6G and M3G are only 5- to 15-times more hydrophilic than morphine is surprising. Glucuronic acid is a very polar molecule and in theory morphine glucuronide should be approximately 1700 times more hydrophilic than morphine (Carrupt *et al.*, 1991). However, molecular modeling indicates that M6G and M3G can exist in two conformations (Carrupt *et al.*, 1991). In aqueous environments, they exist as extended molecules. In lipid environments, the molecule folds, and intramolecular interactions between either the free 3- or 6-

hydroxyl of morphine and the carboxyl of glucuronic acid mask the polarity of the later group.

Traditional octanol: water partition coefficient (log P) determination of lipophilicity does not adequately reflect the properties of molecules that can undergo conformational changes such as peptides, M6G and M3G (Bernard Testa, University of Lausanne, Switzerland, Personal Communication). In fact, measurement of the log P for M6G and M3G was attempted, but could not be determined precisely due to the very small amount of drug that entered the octanol phase. A rough estimate of -2.4 was made for the log P of M6G and M3G. In contrast to the octanol: water partition measurements, the k_i method utilizes a stationary lipid phase and a moving aqueous phase. This better models biologic systems than does the liquid to liquid interface of the octanol: water system (Braumann, 1986; Carrupt *et al.*, 1991). In addition, relationships between lipophilicity and pharmacologic activity break-down at the extremes of the log P scale (Corwin Hansch, Pomona College, Claremont, CA, Personal Communication). This may be due in part because log P does not reflect the *in vivo* lipophilicity of compounds similar to M6G and M3G.

Respiratory effects of M3G were also examined. M3G had been reported to cause respiratory stimulation in adult dogs (Pelligrino *et al.*, 1989) and rats (Gong *et al.*, 1992). Doses less than 75 mg/kg given to 7-day old guinea pigs by s.c. injection had no effect on breathing. Pups given 75 mg/kg M3G experienced mild respiratory depression, rather than stimulation. Analysis of the injection

solution revealed a very small (0.6%) morphine impurity that is present in the drug stock. However, at the high concentration of M3G used, the impurity amounted to 0.5 mg/kg morphine, and this impurity was probably responsible for the observed respiratory depression observed in the M3G-treated animals.

In humans, M6G contributes to the analgesia produced after administration of morphine (Portenoy *et al.*, 1992). M6G is probably also responsible for some of the respiratory depression seen after morphine administration to neonatal guinea pigs. Because of the potency change with aging, M6G would have an even larger contribution to morphine respiratory effects on days 7 and 14 than on day 3, when the two drugs were equipotent. M3G apparently plays no role in functional antagonism of morphine respiratory effects. The following calculations of M6G contributions to morphine effect are done on the basis of brain drug concentrations, and the known dose-response relationships of each drug. Concentrations of M6G in brain tissue of day 3 or 7 neonates treated with 15 mg/kg M6G, s.c., were normalized to amount of respiratory depression per 1 ng/g of drug. Using this relationship the levels of M6G in the brain of pups given 15 mg/kg, s.c., morphine were used to calculate the theoretic respiratory depression attributable to the glucuronide. For day 7 pups, M6G accounts for approximately 20% of the respiratory effect seen 45 min after a dose of morphine is given. For day 3 pups, this contribution is 14%.

This dissertation has led to the following conclusions: 1) M6G and M3G are produced by the neonatal and pregnant guinea pig and can be measured in

plasma and brain tissue by HPLC; **2)** M6G and morphine cause decreases in f, V_T and V_I in neonatal guinea pigs after s.c. administration; **3)** the peak respiratory effect of M6G occurs at a later time than does the peak respiratory effect of morphine after s.c. drug administration; **4)** the potency of M6G in depressing respiration, relative to morphine and to itself, increases 8-fold between day 3 and 7 after birth and this change persists through at least day 14; **5)** after s.c. injection, more M6G is in the plasma and brains of 3 day pups than 7 day pups at the time of average peak respiratory depression, and, therefore, distribution cannot explain the observed potency change; **6)** the entry of M6G into the brain of neonatal guinea pigs is a gradient-driven, linear function of plasma M6G concentrations; **7)** brain to plasma ratios of M6G are independent of dose and of age; **8)** the ability of M6G to cross membranes is in agreement with *in vitro* lipophilicity determinations; **9)** M3G does not stimulate respiration in the neonatal guinea pig; **10)** the maximal number of radiolabelled DAMGO binding sites in the neonatal guinea pig brain stem increased 42% between day 3 and day 7.

The observed M6G potency change for respiratory depression that occurs between days 3 and 7 after birth remains unexplained. However, the increase in number of *mu* opioid receptors with aging that was reported in Manuscript IV is intriguing. It is possible that the receptor for M6G is a different entity than the receptor which mediates morphine-induced respiratory depression in the older neonate, and that the M6G receptor develops after birth while the morphine receptor is fully developed at birth. M6G does cause some respiratory depression

on day 3 because of interaction with the morphine receptor, but its effect is enhanced by day 7 due to the appearance of a M6G specific receptor.

Further studies of opioid receptors is the logical progression of the work started in this dissertation. Four paths of investigation need pursuit. First, the interaction of M6G with opioid receptors requires more complete investigation using radiolabelled M6G. Competitive displacement studies of *mu* specific radioligands reflect only the equilibrium affinity of M6G for the radioligand binding site. Characterization of the kinetics of M6G association and dissociation or of the number of binding sites may reveal an age related change that could explain the potency change noted for respiratory depression.

Second, in conjunction with direct binding studies of M6G, characterization of the binding site is needed. The increase in receptor number between day 3 and 7 after birth may represent more of the same receptor, or the expression of a separate gene product that is recognized differently by M6G than by morphine. Western blot analysis, using a specific antibody for the *mu* receptor, of day 3 and day 7 tissue would be the first step to characterize the receptor by size and see if any age-related changes exist.

Third, the interaction of M6G with *delta* opioid receptors may also be important in its respiratory effects. Characterization of this M6G affinity by competitive displacement of *delta* specific radioligands, such as cyclic [D-Pen^2,D-Pen^5]enkephalin, in conjunction with *mu* binding data should be undertaken.

A final path of investigation should be the examination of effectors. The

ability of M6G to inhibit adenylyl cyclase in brain stem tissue should be determined. M6G may be a better effector once bound to opioid receptors on day 7 than on day 3, which could explain the potency change.

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Appendix A

Additional studies related to Manuscript I.

TABLE 1. **Concentration of morphine and glucuronide metabolites in maternal and fetal plasma after multiple s.c. morphine administration to late gestation guinea pigs to test whether M3G and M6G were present in the fetus after maternal exposure.** A total of 61.5 mg/kg of morphine in 9 injections three times a day over a 48 hr period from day 65 to 67 of gestation (term = 69 days), including a final dose of 7.5 mg/kg given immediately prior to sampling[a]. Following ether anesthesia, maternal and fetal blood were collected. Mean ± S.E. for fetal concentrations (n=4).

	Morphine ng/ml	M6G ng/ml	M3G ng/ml
Maternal Plasma	3,740	1,450	6,430
Fetal Plasma	1,080 ± 169	264 ± 104	2,310 ± 429

[a]Doses of 7.5, 7.5 and 0.75 mg/kg were given on day 65. Doses of 0.75, 7.5, 7.5 and 7.5 were given on day 66, after discovery of an error in calculations which resulted in giving 0.75 mg/kg for two treatments. On day 67, 15 mg/kg was given 1 hr prior to and 7.5 mg/kg immediately before sampling.

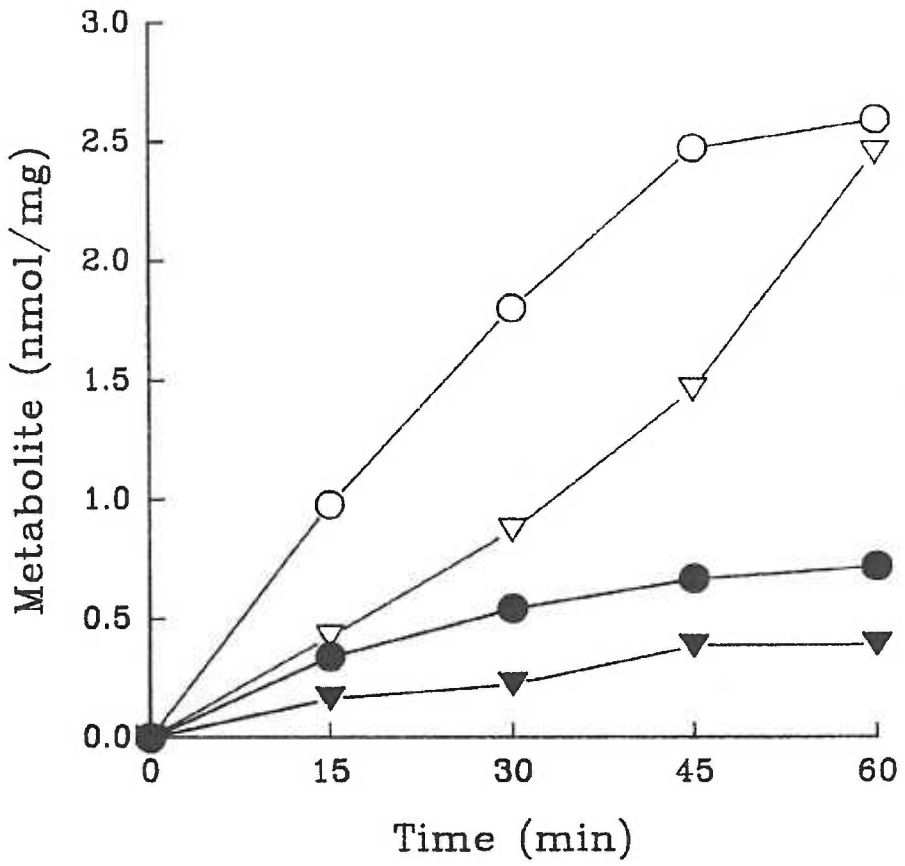

Fig. 1. *In vitro* formation of M6G and M3G from morphine by hepatic microsomes from two day 60 gestation (term = 69 days) pregnant guinea pigs. Animals were pre-treated with saline (○,●) or 6 mg/kg cocaine (▽,▼) before microsome preparation, as described in Sandberg *et al.* (1993). Morphine (0.1 mM), UDP glucuronic acid (UDPGA; 1.0 mM) and protein (2 mg/ml) were incubated at 39°C (guinea pig body temperature) according to the method of Pacifici *et al.* (1982). Open symbols are M3G, closed symbols are M6G.

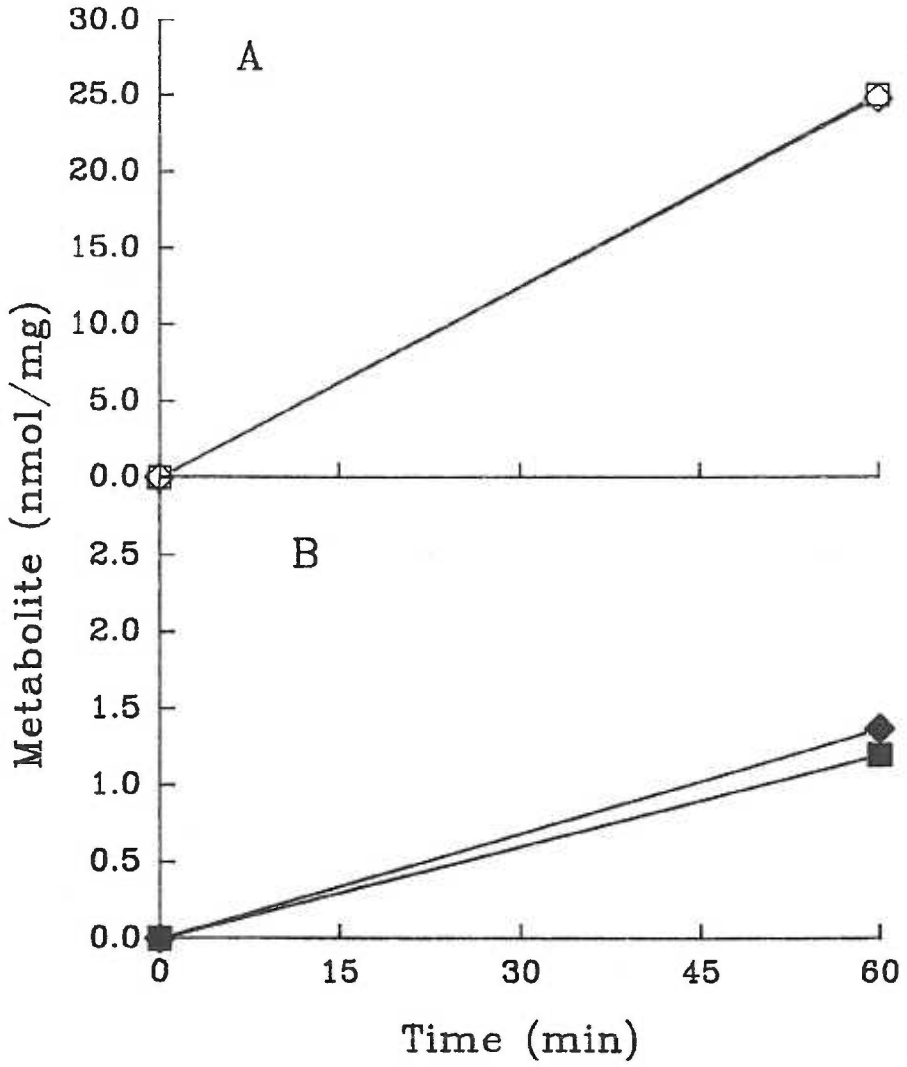

Fig. 2. Morphine glucuronidation by hepatic microsomes from day 60 gestation (term = 69 days) guinea pig fetuses. Animals were treated with saline before microsome preparation (Sandberg *et al.*, 1993). Fetal tissue from the same litter was pooled. Incubations with 6 mM morphine, 1.5 mM UDPGA and 3 (♦,◊) or 4 (■,□) mg/ml protein were done as described in figure 1. Panel A: M3G formation. Panel B: M6G formation. Note differences in ordinate scale. M3G formation exceeded M6G formation by about 20 fold.

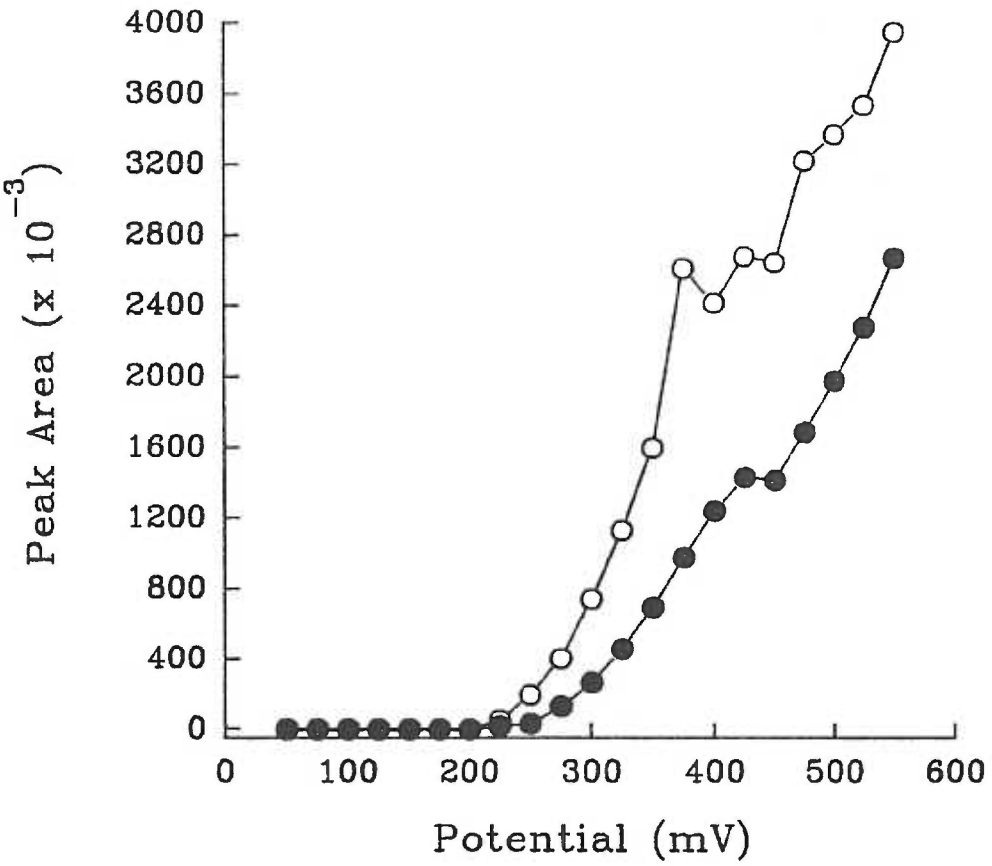

Fig. 3. Voltammogram for morphine and M6G using coulometric ECD with HPLC assay from Manuscript I. Morphine (○) and M6G (●), 20 ng each were injected onto column and peak areas recorded. Potential for the analytical cell was increased between each injection. Voltage for monitoring eluant was ultimately set at +450 mV, to provide a strong signal with minimal background noise.

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Appendix B

Additional data related to Manuscript II.

Calculation of resistance of screens in rear (body) chamber of plethysmograph.

1. Volume of rear chamber: 10 x 10 x 18 cm = 1800 cm^3 = 1800 ml
2. Calibration of transducer by injection of 5 ml of air within a 2 sec period: 7.846 ml/sec = 1 V
3. Block screens with cork and inject 0.45 ml of air into rear of chamber with syringe. This will increase the volume in the rear chamber by 0.025%. (0.45 ml ÷ 1800 ml box volume = 0.00025).
4. Recorded output for 0.45 ml injection of air = 3.76 V
5. Calculate flow: 3.76 V x 7.846 ml/sec/V = 29.5 ml/sec. Therefore, injection of 0.45 ml yields a pressure change corresponding to a flow of 29.5 ml/sec.
6. Injection of 0.025% of chamber volume should increase pressure by 0.26 cm H_2O[a].
7. Resistance = Pressure/Flow

   Resistance = 0.26 cm H_2O ÷ 29.5 ml/sec = 0.0088 cm H_2O/ml/sec

[a]See calculation on pages 3 and 4 of Appendix B.

Calculation of pressure change in body chamber of plethysmograph when calculating resistance of screens.

1. Ideal gas law: $PV = nRT$

   P = pressure, V = volume, n = moles of gas, R = universal gas constant, T = absolute temperature

2. In determining resistance of screens, assume T is constant, R and V are also constants, therefore, the relationship reduces to:

$$P \propto n \text{ or } \frac{P_1}{n_1} = \frac{P_2}{n_2}$$

3. After injection of 0.025% of plethysmograph volume, the amount of gas in chamber increases. $n_2 = n_1 + 0.00025 \bullet n_1$

4. Substituting for equation 2.

$$\frac{P_1}{n_1} = \frac{P_2}{n_1 + 0.00025 \bullet n_1}$$

5. Rearrange to solve for P_2.

$$\frac{P_1 (n_1 + 0.00025 \bullet n_1)}{n_1} = P_2$$

$$\frac{P_1 \bullet n_1 + P_1 \bullet 0.00025 \bullet n_1}{n_1} = P_2$$

6. Divide through by n_1. $P_1 + 0.00025\ P_1 = P_2$; $P_2 = 1.00025\ P_1$.

7. P_1 is 1 atm = 1030 cm H_2O

$P_2 = 1.00025 \bullet P_1 = 1.00025 \times 1030 = 1030.26$ cm H_2O

$P_2 - P_1 = 1030.26 - 1030 = 0.26$ cm H_2O

Calculation of time constant[a] for rear chamber of plethysmograph.

1. Volume of rear chamber: 10 x 10 x 18 cm = 1800 cm^3 = 1800 ml

2. Estimated volume of smallest animal: 45 g = 45 ml

   Estimated volume of largest animal: 280 g = 280 ml

3. Free volume in rear chamber

   Small animal: 1800 ml - 45 ml = 1755 ml

   Large animal: 1800 ml - 280 ml = 1520 ml

4. Atmospheric pressure = 1 atm = 1030 cm H_2O

5. Resistance of screens: 0.0088 cm H_2O/ml/sec

6.

$$\text{Time constant} = \text{free volume} \times \frac{\text{Resistance}}{\text{Pressure}} = \text{free volume} \times \frac{1}{\text{flow}}$$

$$\text{Small animal: } 1755 \text{ ml} \times \frac{0.0088 \text{ cm } H_2O/\text{ml/sec}}{1030 \text{ cm } H_2O} = 0.0150 \text{ sec}$$

$$\text{Large animal: } 1520 \text{ ml} \times \frac{0.0088 \text{ cm } H_2O/\text{ml/sec}}{1030 \text{ cm } H_2O} = 0.0130 \text{ sec}$$

7. Average time constant = 0.0140 sec = 14.0 msec

[a]The time constant is the time required for 63% of pressure change to return to baseline.

TABLE 1. **Time of peak depression.** Overall time to peak effect on room air and 5% CO_2 (mean ± S.E., n=6) was 55 ± 3 and 67 ± 3 min respectively for M6G, and 40 ± 4 and 46 ± 3, respectively for morphine. There was a significant difference between the time to peak action on either CO_2 or room air between morphine and M6G ($P<.01$). There was no difference with aging by 3-way ANOVA.

Day		0.5 mg/kg M6G	1.5 mg/kg M6G	5.0 mg/kg M6G	1.5 mg/kg Morphine	4.5 mg/kg Morphine	15 mg/kg Morphine
3	Room air[a]	-	-	-	-	-	-
	CO_2	63±9	58±5	73±10	38±5	28±3	40±6
7	Room air[b]	58±7	50±12	53±9	43±11	28±10	38±7
	CO_2	65±5	70±10	78±10	53±12	48±10	40±8
14	Room air[b]	70±5	53±6	45±7	58±7	35±5	38±8
	CO_2	75±9	63±11	63±7	78±7	45±4	43±8

[a] There was no statistically significant respiratory depression on room air for either drug on day 3.

[b] There was a significant effect of dose for room air peak depression by 3-way ANOVA ($P<.05$).

TABLE 2. **Minute ventilation of morphine and M6G treated pups at the time of peak effect.** Overall, morphine and M6G treatment decreased V_I compared to placebo ($P<.0001$). There was no difference between the effects of morphine and M6G breathing room air or 5% CO_2 by 3-way ANOVA of z-scored data. There were no 2-way or 3-way interactions for either analysis. Placebo animal's data were averaged over the 90 min time-action study. Data are given as mean ± S.E. (n=6 per treatment).

	V_I ($ml \cdot min^{-1} \cdot 100g^{-1}$)						
Day	Placebo	0.5 mg/kg M6G	1.5 mg/kg M6G	5.0 mg/kg M6G	1.5 mg/kg Morphine	4.5 mg/kg Morphine	15.0 mg/kg Morphine
Room air[a]							
3[b]	87±5.6	75±5.7	79±12.7	87±5.3	82±2.0	86±4.2	85±10.1
7	84±3.6	64±2.6	59±5.3	51±6.5	68±2.5	73±3.0	53±1.3
14	69±3.8	43±2.3	46±2.1	50±2.8	51±2.2	48±2.5	40±3.0
5% CO_2[a,c]							
3	348±26.7	250±21.2	250±26.6	188±29.9	245±19.1	206±23.5	146±18.9
7	289±20.1	161±23.1	167±20.4	96±19.3	188±17.3	166±13.0	100±19.6
14	207±18.4	111±8.1	94±12.7	75±14.7	125±14.3	111±8.6	79±10.0

[a] Significant effect of aging by 3-way ANOVA of Z-scored data.
[b] Day 3 animals breathing room air were not significantly different from placebo.
[c] Significant effect of dose by 3-way ANOVA of Z-scored data.

TABLE 3. **Mean inspiratory flow peak data.** There was not a difference between the drugs. Placebo values are averaged over the 90 min time-action study. Data represent mean ± S.E. (n=6 per treatment).

	V_T/T_I (ml•sec^{-1})						
Day	Placebo	0.5 mg/kg M6G	1.5 mg/kg M6G	5.0 mg/kg M6G	1.5 mg/kg Morphine	4.5 mg/kg Morphine	15.0 mg/kg Morphine
Room Air[a,b]							
3	4.01±0.23	2.66±0.42	2.26±0.30	2.74±0.28	2.88±0.11	2.74±0.19	2.72±0.36
7	5.66±0.57	3.03±0.25	2.61±0.19	2.20±0.26	3.37±0.15	3.12±0.30	2.02±0.22
14	5.86±0.82	2.95±0.17	3.01±0.31	3.23±0.37	4.28±0.70	2.69±0.21	2.38±0.22
5% CO_2[a,b]							
3	13.07±0.71	8.44±1.08	7.12±0.81	5.85±0.90	8.17±0.52	6.88±0.77	5.14±0.76
7	14.99±0.75	7.69±0.94	7.96±0.68	4.26±0.80	9.43±0.72	7.96±0.99	4.04±0.79
14	14.59±0.94	8.24±0.67	8.07±0.92	5.86±0.73	10.45±0.87	7.31±0.42	5.04±0.45

[a] Significant effect of dose by 3-way ANOVA of Z-scored data.
[b] Significant effect of day by 3-way ANOVA of Z-scored data.

TABLE 4. **Frequency data at time of peak effect while breathing room air or 5% CO_2.** Overall, morphine and M6G decreased f compared to placebo ($P<.001$), with no difference between the two drugs by 3-way ANOVA of the z-scored data for both breathing room air and 5% CO_2. Placebo data are average values over the 90 min time-action study. Data are expressed as mean ± S.E. (n=6 per treatment).

	f (breaths•min^{-1})						
Day	Placebo	0.5 mg/kg M6G	1.5 mg/kg M6G	5.0 mg/kg M6G	1.5 mg/kg Morphine	4.5 mg/kg Morphine	15.0 mg/kg Morphine
Room air[a,b]							
3	122±9	116±10 [c]	103±6	107±7 [c]	119±6 [c]	104±7	96±12
7	143±10	103±5	93±17	70±9	115±4	95±10	75±5
14	106±8	47±7	57±13	62±14	80±13 [c]	66±7	53±6
5% CO_2[a,b]							
3	178±8	146±8	138±6	123±9	151±5	124±12	103±13
7	171±6	114±8	115±17	84±11	123±8	105±12	83±11
14	126±13	72±6	73±11	84±15	104±16 [c]	89±8	65±5

[a] Significant effect of dose by 3-way ANOVA of Z-scored data.
[b] Significant effect of day by 3-way ANOVA of Z-scored data.
[c] Not significantly different from placebo.

TABLE 5. **Tidal volume data at time of peak effect.** V_T was increased in drug-treated animals breathing room air on all days ($P<.001$). No effects of dose, day and no difference between drugs were found by 3-way ANOVA of z-scored room air data. Overall, while breathing 5% CO_2, V_T was depressed by morphine and M6G ($P<.001$) with exceptions noted below and no difference was found between the drugs. Placebo data are averages for the 90 min time-action study. Data are expressed or mean ± S.E. (n=6 per treatment).

	V_T ($ml \cdot 100g^{-1}$)						
Day	Placebo	0.5 mg/kg M6G	1.5 mg/kg M6G	5.0 mg/kg M6G	1.5 mg/kg Morphine	4.5 mg/kg Morphine	15.0 mg/kg Morphine
Room air							
3	0.74 ±0.08	0.66 ±0.05	0.76 ±0.09	0.83 ±0.04	0.70 ±0.04	0.84 ±0.06	0.90 ±0.06
7	0.61 ±0.03	0.64 ±0.05	0.80 ±0.17	0.76 ±0.08	0.61 ±0.03	0.83 ±0.12	0.73 ±0.07
14	0.69 ±0.06	1.04 ±0.16	1.07 ±0.26	0.93 ±0.12	0.81 ±0.21	0.81 ±0.14	0.81 ±0.08
5% CO_2 [a,b]							
3	1.99 ±0.17	1.73 [c] ±0.13	1.85 [c] ±0.24	1.52 ±0.18	1.64 ±0.16	1.66 ±0.11	1.46 ±0.15
7	1.70 ±0.09	1.39 ±0.15	1.52 [c] ±0.14	1.11 ±0.22	1.52 ±0.09	1.63 [c] ±0.15	1.15 ±0.17
14	1.66 ±0.07	1.60 [c] ±0.18	1.44 [c] ±0.16	0.96 ±0.12	1.33 ±0.15	1.28 ±0.09	1.21 ±0.13

[a] Significant effect of dose by 3-way ANOVA of Z-scored data.
[b] Significant effect of aging by 3-way ANOVA of Z-scored data.
[c] Not significantly decreased compared to placebo.

TABLE 6. **Minute ventilation data from diurnal variation study.** Neonates were studied at 9:00 a.m., noon and 3:00 p.m. at age 3, 5, 7 and 14 days. After 10 min acclimatization to the plethysmograph, data while breathing room air were recorded, followed by data while breathing a 5% CO_2, 30% O_2, balance N_2 gas mixture. There was no time of day effect, but there were significant age and CO_2 effects by 3-way ANOVA. Mean ± S.E., n=6.

	V_I ($ml \cdot min^{-1} \cdot 100\ g^{-1}$)			
	day 3	day 5	day 7	day 14
Room Air				
9:00 a.m.	84 ± 5	85 ± 8	88 ± 4	84 ± 6
Noon	86 ± 11	90 ± 16	95 ± 6	78 ± 6
3:00 p.m.	77 ± 9	79 ± 7	86 ± 4	83 ± 8
5% CO_2				
9:00 a.m.	266 ± 9	246 ± 13	231 ± 10	217 ± 15
Noon	264 ± 17	262 ± 29	251 ± 17	207 ± 13
3:00 p.m.	256 ± 25	268 ± 33	261 ± 28	229 ± 25

[a]Significant age effect by ANOVA ($F_{3,45}$=3.82, P < .05).
[b]Significant CO_2 effect by ANOVA ($F_{1,15}$=554, P < .0001).

Fig. 1. Log dose-response with linear regression for peak depression of V_I while breathing a 5% CO_2 gas mixture. Extended study data for day 3 (from Panel A, figure 5, Manuscript II) are plotted with the three-dose study data for days 7 and 14 (from Panels B and C, figure 3, Manuscript II). Further study details are described in Manuscript II. ○ = M6G, ● = morphine.

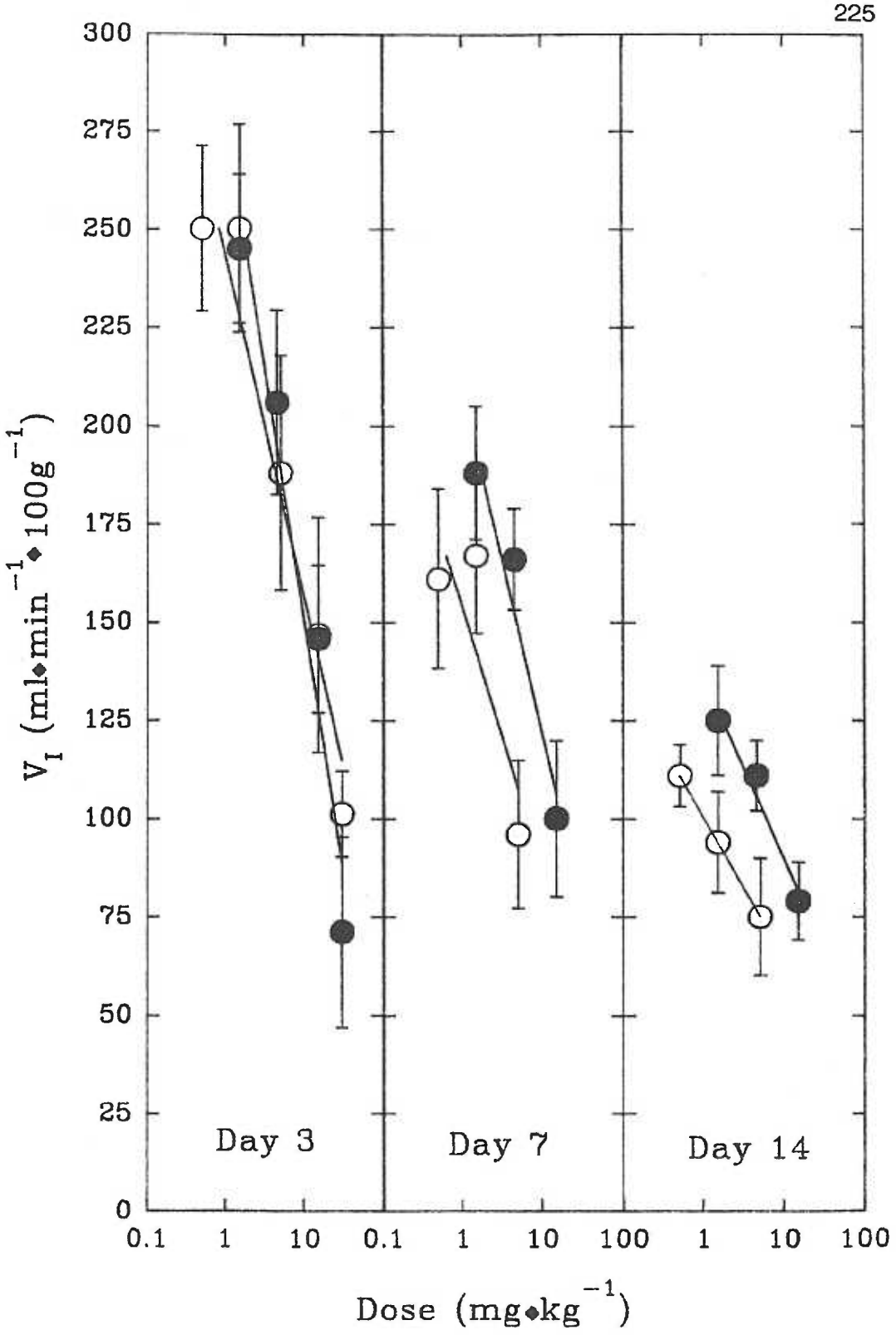
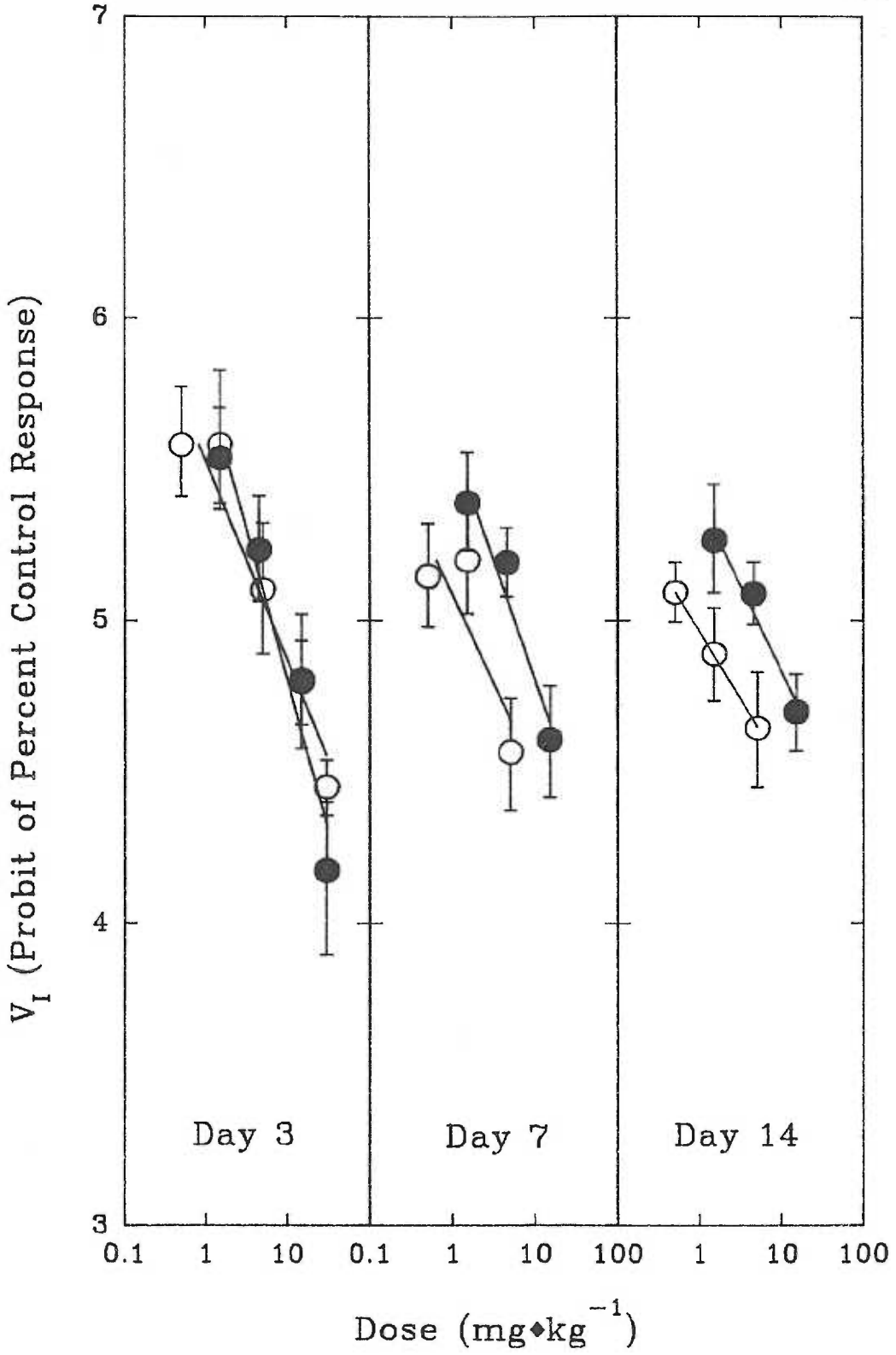

300
275
250
225
200
175
150
125
100
75
50
25
0
V_I (ml•min^{-1}•100g^{-1})
Day 3
Day 7
Day 14
0.1
1
10
0.1
1
10
100
1
10
100
Dose (mg•kg^{-1})

Fig 2. Log dose-response with probit transformation of V_I at peak depression compared to placebo response, while breathing a 5% CO_2 gas mixture. Data from figure 1 of this appendix are plotted as the probit of percent depression of V_I. Percent depression was calculated by dividing the V_I of each drug treated pup by the average V_I of placebo-treated pups, and multiplying by 100. Probit is a linear transformation of sigmoid dose-response curves. Probits were calculated and plotted using the SigmaPlot program (Jandel Scientific, Corte Madera, CA). ○ = M6G, ● = morphine. (Day 3) V_I (○) = 5.53 - 0.66 • log (M6G); V_I (●) = 5.80 - 0.99 • log (morphine); (Day 7) V_I (○) = 5.08 - 0.59 • log (M6G); V_I (●) = 5.59 - 0.79 • log (morphine); (Day 14) V_I (○) = 4.96 - 0.50 • log (M6G); V_I (●) = 5.40 - 0.57 • log (morphine).


7
6
5
4
3
V_I (Probit of Percent Control Response)
Day 3
Day 7
Day 14
0.1
1
10
0.1
1
10
100
1
10
100
Dose (mg•kg^{-1})

Appendix C

Additional data related to Manuscript III.

Predicted V_I response for 0.45 mg/kg morphine using Probit dose-response relationship for day 7 (Appendix B).

Probit V_I = 5.59 - 0.79 • log (morphine)

Probit V_I = 5.59 - 0.79 • log (0.45)

Probit V_I = 5.86

Change Probit to percent control (Fisher, 1963)

Probit 5.86 = 80.5% of placebo response

Placebo V_I = 289 $ml \cdot min^{-1} \cdot 100\ g^{-1}$

V_I for 0.45 mg/kg morphine = 289 • 0.805

= 233 $ml \cdot min^{-1} \cdot 100\ g^{-1}$

Observed V_I in treated animals = 231 $ml \cdot min^{-1} \cdot 100\ g^{-1}$

TABLE 1. **Brain weights of neonates administered morphine, M6G or M3G.** Drug concentration data are reported in Manuscript III. For the M6G study, pups were injected s.c. with 5 or 15 mg/kg M6G and brains collected at average time of peak respiratory depression (70 min). Morphine animals received 15 mg/kg of drug s.c., and brains collected at average time of peak depression (45 min). The M3G pups were given 75 mg/kg s.c. of metabolite, and brains collected at the end of a 90 min time-action study. Weight did not differ significantly between male and female pups or among treatments for a given day. Average day 7 brain weight was significantly greater than day 3 (2.85 ± 0.14 and 2.61 ± 0.13 g, respectively, $P<.0001$). N.D., Not Done. Mean ± S.E.

Day	5 mg/kg M6G (n=8)	15 mg/kg M6G (n=8)	15 mg/kg Morphine (n=6)	75 mg/kg M3G (n=8)
3	2.62 ± 0.04	2.62 ± 0.04	2.61 ± 0.06	N.D.
7	2.90 ± 0.05	2.90 ± 0.03	2.79 ± 0.04	2.81 ± 0.06

References

Fisher, R.A.: Statistical Tables for Biological, Agricultural and Medical Research, 6th ed., pp. 68-70, Hafner Publishing, New York, 1963.

Appendix D

Additional data related to Manuscript IV.

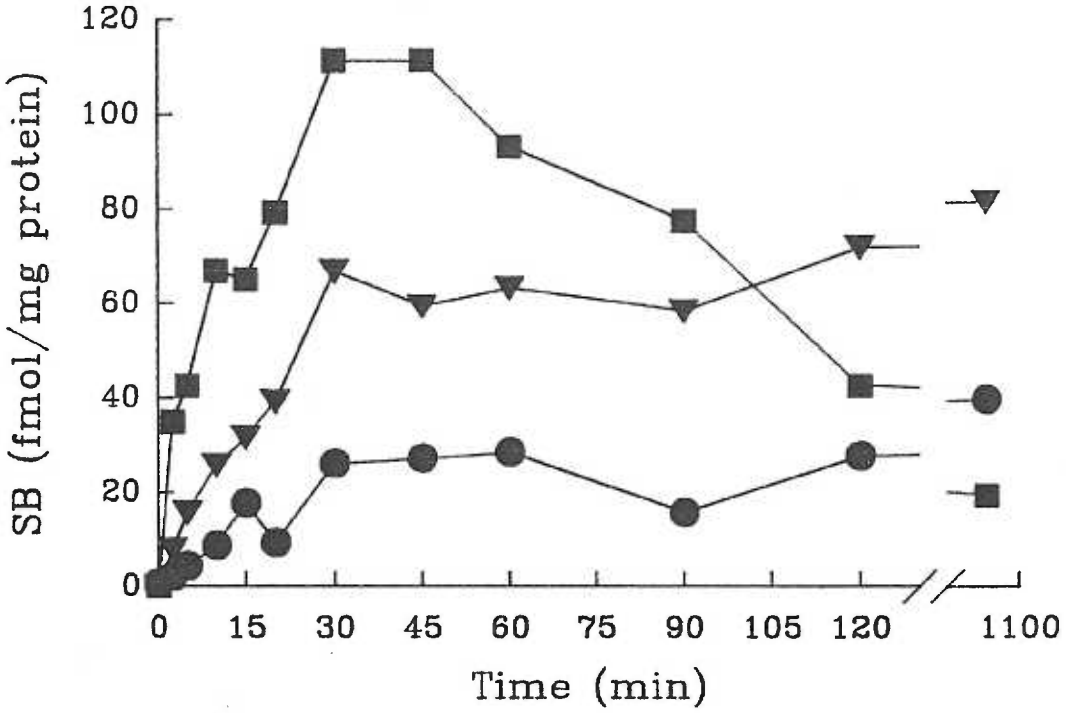


Fig. 1. Time course for binding of 5 nM [^{3}H]DAMGO to 7 day old neonatal guinea pig brain stem membranes at various temperatures. Membranes and ligand were incubated as 4° (●), 25° (▾) and 37° (■). Experiments were stopped at various times over an 18 hr period by rapid filtration over glass fiber filters as described in METHODS of Manuscript IV. Each point was done in duplicate and non-specific binding was determined using 1 μM morphine. Equilibrium at 25°C was reached by 45 min, and this time point was used in further experiments. Exploration of apparent differences in maximal binding are described in figure 2 of this appendix. SB: Specific binding.

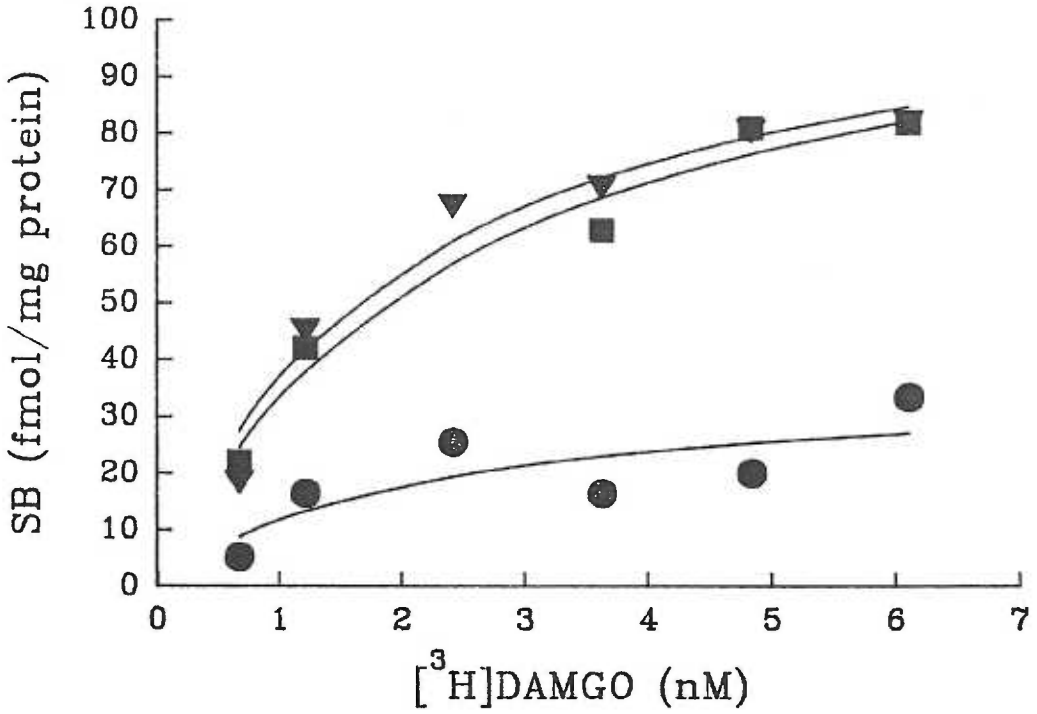


Fig. 2. Temperature influences on saturation binding of [^{3}H]DAMGO to pontine and medullary membranes of 7 day old neonatal guinea pigs. Radioligand was incubated in duplicate with membranes at 4°C (●, 60 min), 25°C (▾, 45 min) or 37°C (■, 30 min) and rapidly filtered over glass fiber filters, as described in METHODS in Manuscript IV. B_{max} increased between 4° and 25°C and remained constant at 37°C. However, K_D was not affected by temperature.

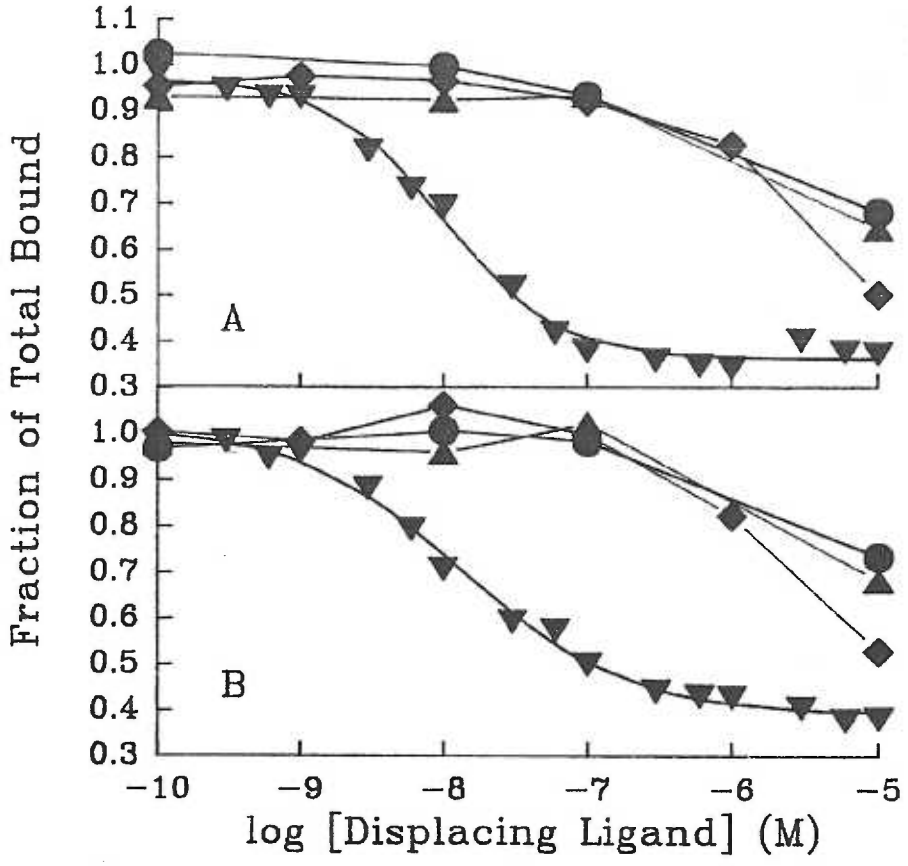


Fig 3. Competitive displacement of [^{3}H]DAMGO from neonatal guinea pig brain stem membranes by M3G, dextrorphan, and cyclic [D-Pen^2, D-Pen^5]enkephalin (DPDPE). A saturating concentration of the *mu* opioid receptor specific agonist, [^{H}]DAMGO, (5 nM) was incubated with membranes at 25°C for 45 min in the presence of M3G (◆), which has very low affinity for the *mu* receptor, dextrorphan (▲), an inactive opioid isomer and DPDPE (●), a *delta* opioid receptor agonist. Experiments were done in duplicate and terminated by rapid filtration over glass fiber filters, as described in METHODS section of Manuscript IV. For comparison, data from figure 2 of Manuscript IV for DAMGO (▼) competitive displacement of [^{H}]DAMGO is included. Panel A: day 3. Panel B: day 7.

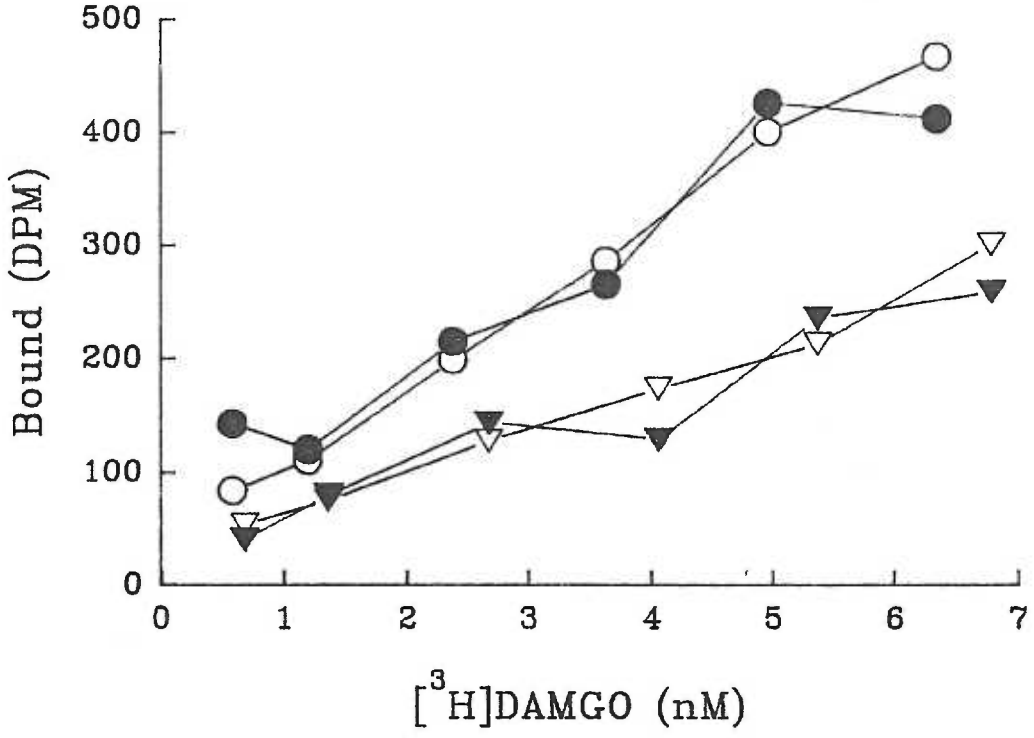


Fig. 4. Saturation binding isotherms for [^{3}H]DAMGO to liver tissue or in the absence of tissue. Various concentrations of radioligand were incubated in duplicate for 45 min followed by rapid filtration over glass fiber filters as described in the METHODS section of Manuscript IV. Non-specific binding was determined using 1 μM morphine. No specific binding was observed with liver tissue (○,●) or when tissue was omitted (△,▲). Total binding are open symbols, non-specific binding are closed symbols.