POSTNATAL ANALGESIC EFFECTS OF RACEMIC METHADONE AND ITS ISOMERS IN 129/SvEv AND CD-1 MOUSE PUPS

by

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A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requiremnets for the degree of Doctor of Philosophy, The City University of New York

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Abstract

Postnatal Analgesic Effects of Racemic Methadone and Its Isomers in 129/SvEv and CD-1 Mouse Pups

by

Ramkumarie Baliram

Advisor: Professor Gordon A. Barr

Experiment One: The purpose of this experiment was to describe the roles of racemic methadone and its isomers in the 129/SvEv and the CD-1 mouse pups during postnatal ontogeny in the formalin nociceptive test. The CD-1 pups were used as a control comparison for results obtained with the 129/SvEv mouse pups, as the 129/SvEv mouse has been reported to have putative deficits of the μ-opioid and NMDA receptors. Fifteen minutes post drug injection (d-,l-and dl-methadone) pups were injected with 2 % dilute formalin at volumes of 5, 10 and 20 μl at PD 3, 10 and 21 respectively. Behavioral responses were observed for every minute and continued for 45 minutes. The first hypothesis in this thesis, that dl-methadone and its two enantiomers may result in greater analgesic effectiveness in infants than in adults was supported. Greater analgesic effectiveness was observed at PD 3, an age group representative of infants than at PD 21, an age group representative of adults. Further, l- and dl-methadone were effective

analgesics in both phases of the formalin test and d-methadone being only minimally effective in the second phase.

Experiment Two: This study described the analgesic effects of racemic methadone and its isomers in the 129/SvEv and the CD-1 mouse pups during postnatal ontogeny in the thermal test of nociception. In this test, pups were either injected with vehicle or each of four doses of either: d-, l- or dl-methadone in a cumulative dose response paradigm. Thirty minutes post-drug injection, appendage withdrawal latencies were measured and taken as indicators of nociception and antinociception. The findings presented here, in both mouse strains demonstrated that the second hypothesis in this thesis was supported, as all three drugs were more effective at PD 3 than at PD 21. dl-Methadone resulted in potentiated analgesia at the younger ages tested but not at PD 21 and may be explained by an interation of the μ-opioid and the NMDA receptor systems. The analgesic effects exerted by dl-methadone at PD 21, are consistent with those of adults and the three drugs tested were more effective and potent at PD 3 than at PD 21. l-Methadone was more effective than dl-methadone and d-methadone was the least effective of the three drugs tested at PD 21.

Experiment Three: Attempted to provide a mechanism for the potentiated analgesic effects observed with dl-methadone at PD 3 and 10 in the thermal test in both mouse strains on the basis that the μ-opioid and the NMDA receptor systems were interacting in the exertion of the potentaited analgesia observed with this compound. Two compounds; morphine and MK801 were co-administered in this experiment in an attempt to mimic the effects of dl-methadone at PD 3. Like d-methadone, MK801 is an NMDA antagonist and like l-methadone, morphine is a μ-opiate agonist. Results showed that, MK801 did not potentiate the analgesic effects exerted by morphine in both mouse strains suggesting that, the μ-opioid and the NMDA receptor systems were not interacting in the exertion of dl-methadone potentiated analgesic effects. Further, morphine was an effective analgesic by itself but MK801 did not exert any analgesic effects in both mouse strains. The data presented in this experiment, casted a shadow on the role of the NMDA receptor in the potentiated analgesic effect of dl-methadone and in the analgesic effects of d-methadone. d-Methadone was likely exerting minimal analgesia in both phasic and tonic nociception in both mouse strains through other receptors other than the NMDA receptor.

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GLOSSARY OF ABBREVIATED TERMS:

(1) S.C.---------- Subcutaneous.

(2) I.P.---------- Intraperitoneal.

(3) ED_{50} ---------- Median Effective Dose.

(4) %MPE ----------Percent Maximal Effect.

(5) PD 3 ---------- Postnatal Day 3.

(6) PD 10 ----------Postnatal Day 10.

(7) PD 21----------Postnatal Day 21.

(8) NMDA receptor ----------N-methyl-D-aspartate receptor.

(9) MK801 ---------- NMDA receptor antagonist.

Keywords: Racemic methadone, Isomers, D- and L-methadone, Ontogeny, Postnatal development, Opioid receptors, NMDA antagonist, Morphine, MK801, Formalin test, Thermal test, 129/SvEv mouse, CD-1 mouse, Nociception, Antinociception, Strain, Genotype.

CHAPTER ONE:

General Introduction

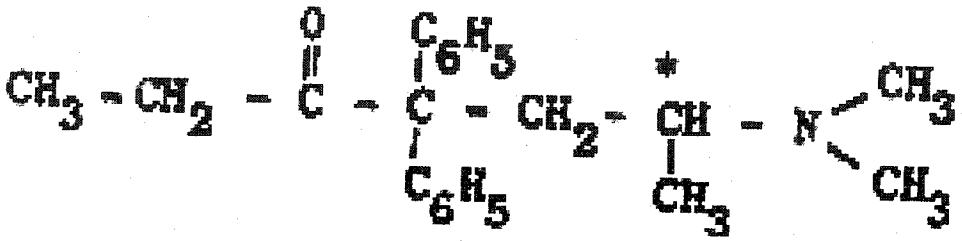
The use of opiates for the treatment of pain in infants was once deemed unnecessary because the presumption was that infants were not able to perceive pain. Today however, there is overwhelming evidence that the neural pain processing systems are present in newborns (Anand, 1993; M Fitzgerald, 1994) and they are capable of perceiving pain (Andrews & Fitzgerald, 1994). This has prompted an increase in the use of opioids in this age group after surgery (de Lima, Lloyd-Thomas, Howard, Sumner, & Quinn, 1996; Purcell-Jones, Dormon, & Sumner, 1987). Effective and efficient pain management is of clinical importance as neonatal and pediatric opioid administration has been shown to decrease stress, decrease recovery times following surgery and decrease morbidity and mortality (Anand & Hickey, 1987; Dilworth & MacKellar, 1987; Olkkola, Hamunen, & Maunuksela, 1995; Orsini, Leef, Costarino, Dettorre, & Stefano, 1996). A detailed understanding of nociception and antinociception of adequately and effectively alleviating pain in infants is necessary in pain management.

Therefore, the overall goal of this thesis is to examine the postnatal ontogeny of nociception and antinociception of dl-methadone and its isomers. Specifically, this thesis examined first, the developmental analgesic effects of d-, l- and dl- methadone in the thermal test, a model of acute pain. Second, this thesis examined the developmental analgesic effects of racemic methadone and its isomers in the formalin test, a model of

both acute and chronic pain. Third, this thesis examined the interaction of the μ-opioid and the NMDA receptor systems as a possible mechanism for dl-methadone's potentiated analgesic effects, in the thermal test during early postnatal ontogeny. The analgesic effects of racemic methadone and its isomers were examined during postnatal development, a developmental period where receptors that are involved in pain modulation, opiate pharmacology and non-opiate pharmacology are dynamically developing.

The following sections review the literature on: the pharmacology of racemic methadone and its isomers, NMDA receptors and opiate receptors interactions in pain/analgesia; the involvement of MK801 in pain and analgesia, infant mouse: a model in pain studies; NMDA or μ-opioid receptor deficits in the 129/SvEv mouse and the effects of dl-methadone and its isomers in different pain states.

Figure 1. Depicted the asymmetric carbon atom of methadone with molecular formula ($C_{21}H_{27}NO$).



Racemic Methadone and its Isomers.

Methadone hydrochloride (6-dimethylamino-4, 4-diphenyl-3-heptanone), a salt form of methadone, is a synthetic opioid that was developed during the second World War in Germany. However, its role as an analgesic was not discovered until a few years later (Troxil, 1948). Methadone is asymmetric meaning that this molecule or compound cannot be divided into symmetrical halves. The asymmetric carbon atom of methadone as depicted in Figure 1. results in two isomeric forms of methadone, levo-methadone (l-methadone) and dextro-methadone (d-methadone). By definition, the l- and d-forms of methadone are often referred to as isomers of methadone because they have the same molecular formula ($C_{21}H_{27}NO$) but different structural formula. These isomers of methadone are also known as enantiomers of methadone due to the fact that equal numbers of l- and d- molecules exist in a 50/50 mixture or an equi-molar mixture, this form of methadone is known as racemic methadone. Racemic methadone is specified by, prefixing the name of the compound with the symbol (+/-), for example +/- methadone. In contemporary literature, the racemate is referred to as *rac*-methadone or RS methadone and the enantiomers as R- and S-methadone. In older literature, they were referred to, respectively, as dl-, l- and d-methadone. It is important to note the nomenclature of the differing forms of methadone to avoid confusion of their stereo-

specific pharmacological effects. The isomers of methadone and the racemate will be referred to as l-,d- and dl-methadone respectively in this entire dissertation. The contemporary naming system has not gained momentum in its use, as such majority if not all of the work cited on methadone have used the older naming system of l-,d- and dl-methadone. Therefore, to be consistent with the literature, I will use the old naming system when referring to racemic methadone and its isomers. It is to be noted that in many instances, the literature does not specify the word dl-methadone when referring to the racemic form of methadone, the word methadone alone stands by itself without the prefix dl- preceeding the word methadone. Early laboratory and clinical studies indicated that dl-methadone was equivalent to morphine and offered no pharmacological advantage as an analgesic (Beaver, Wallenstein, Houde, & Rogers, 1967) and as a result, methadone fell into disuse. However, later studies have shown that, methadone differs from other opioids, for its unique pharmacological characteristics such as its long half-life (Wolff et al., 1997) and its non-opioid, NMDA effects (Ebert, Thorkildsen, Andersen, Christrup, & Hjeds, 1998; Gorman, Elliott, & Inturrisi, 1997). These characteristics may render methadone a superior and a preferred analgesic over commonly used opioids in pain management. Further, as an opioid, methadone is also unique for its pharmacodynamic profile, it is characterized by both opioid and non-opioid properties. For example, like morphine, methadone preferentially binds to the μ-opioid receptors (Neil, 1984b) and produces behavioral effects similar to that of morphine in rodents and in man (Smits &

Myers, 1974). The implications of both the opiate and non-opiate effects in pain management are profound, in that it has been shown that both the μ-opioid receptors and the NMDA receptors are involved in analgesia (Redwine & Trujillo, 2003). Moreover, studies have shown that non-opiate compounds like MK801 (an NMDA antagonist) potentiate low doses of opiates such as morphine (Wong, Liaw, Tung, Su, & Ho, 1996) and enhance the analgesic effects of morphine (Bernardi, Bertolini, Szczawinska, & Genedani, 1996). Both d-methadone and MK801 are NMDA antagonists and may have similar analgesic mechanism of actions. Similarly, both l-methadone and morphine preferentially bind to the μ-opioid receptor and may also have similar analgesic mechanisms of actions.

dl-Methadone is clinically available and is the most commonly used form of this compound in the United States. l-Methadone is also available and its analgesic effects are approximately twice that of dl-methadone (Nauck F, 2001). l-Methadone, is the most potent analgesic of the three forms of methadone and it is twice as potent as dl-methadone. Both isomers have a low affinity for the delta and kappa opioid receptors (Kristensen, Christensen, & Christrup, 1995) and bind with similar affinities to the non-competitive NMDA receptor (Gorman et al., 1997). Moreover, methadone blocks the presynsptic reuptake of serotonin (Codd, Shank, Schupsky, & Raffa, 1995). It binds to

the μ-opioid receptor (Neil, 1984a) with an affinity similar to that of morphine (Kristensen et al., 1995), its behavioral effects are similar to morphine in opioid tests in rodents (Smits & Myers, 1974). dl-Methadone is a 50/50 mixture of the d- and l-isomer with each individual isomer demonstrating stereo-specific pharmacological effects. For example, the l-isomer is responsible for the opioid properties, whereas the d-isomer is weak or inactive as an opioid (Horng, Smits, & Wong, 1976). In comparing the analgesic effects of the individual isomers in adult rodents, the l-isomer possesses analgesic activity where as the d-isomer has little (Ingoglia & Dole, 1970)Both isomers have a low affinity for the μ-opioid receptors. Moreover, dl-methadone and its isomers all have NMDA antagonist effects (Gorman et al., 1997).

NMDA and Opiate Receptor Interaction in Pain and Analgesia

Both μ-opioid and NMDA receptors have been shown to be involved in analgesic mechanisms (Marsh, Hatch, & Fitzgerald, 1997). Further, these receptors are distributed in the same pain processing areas of the central nervous system that includes the dorsal horn of the spinal cord and the mesencephalic periaqueductal grey matter. Moreover, the two receptor populations have been found to anatomically co-localize on the same neurons in the trigeminal dorsal horn (Aicher, Goldberg, & Sharma, 2002), thus setting

the stage for an interaction of the two receptor systems in mechanisms of pain and analgesia.

Peripheral NMDA receptor involvement in pain related responses may lead to reduced opioid sensitivity hence opiate drugs are less effective in neuropatic pain where the NMDA receptors are involved (Dickenson, 1994). Further, a number of pathological and physiological events can result in poor opioid sensitivity and many appear to be operative in neuropatic pain models, thus supporting the clinical view that neuropatic pain can be less sensitive to opioids (Dickenson, 1994). In tonic pain models, such as the formalin test, NMDA receptors are said to play a major role in the second phase, thus opiate drugs alone are not particularly effective (Vaccarino, Clemmons, Mader, & Magnusson, 1997). NMDA receptor activation itself will contribute to poor opioid sensitivity because these receptors will increase excitation in the pain transmitting systems. Therefore, more opioid will be needed to control pain when enhanced by NMDA receptor activation.

Neuronal excitations produced by postsynaptic NMDA receptor activation breaks through the opioid inhibitions so that at moderate doses, opioids delay the onset of wind-up (Dickenson, 1994). By definition, wind up is a phonemenon in pain processing that

involves the abnormal activity of dorsal horn neurons after prolonged stimulation, during this phonemenon the NMDA receptors become abnormally active and opioid sensitivity is reduced. The NMDA receptor antagonist abolishes wind-up thus implicating the NMDA receptor in chronic or tonic pain (Coderre & Melzack, 1992). Powerful synergism arises from a combination of threshold doses of morphine doses with low doses of NMDA antagonist (Dickenson, 1994). In addition, in a model of neuropatic pain, a pain state where morphine is inoperative the coapplication of an NMDA antagonist restores the ability of morphine to inhibit the response (Yamamoto & Yaksh, 1992). Synergism has also been observed with a combination of spinal lignocaine and morphine, partly due to the ability of the former to block NMDA mediated spinal events (Fraser, Chapman, & Dickenson, 1992).

Involvement of MK801 in Pain Analgesia

MK801 is a potent NMDA receptor antagonist (Wong et al., 1986), it has been tested in many animal models, as a prototypic NMDA antagonist with high potency and free CNS penetration. MK801 has a number of unexpected properties, e.g. in delaying or preventing the development of morphine tolerance and dependence (Trujillo & Akil, 1991b), blocking the effect of behavioral sensitization to cocaine and amphetamine (Karler, Calder, Chaudhry, & Turkanis, 1989) and inhibiting alcohol withdrawal signs

(Morrisett et al., 1990). In contrast to the adult effects of the NMDA receptor antagonist in blocking morphine tolerance and dependence, the NMDA antagonist are not effective in blocking tolerance and dependence in infants (Bell & Beglan, 1995; Zhu & Barr, 2000). Moreover, MK801 was not effective in suppressing the expression of opiate withdrawal (Zhu & Barr, 2000, 2001). Like MK801, d-methadone is a compound with NMDA receptor antagonist activities and has been shown to attenuate morphine tolerance in adult animal studies (Davis et al., 1999). However, the pharmacological effectiveness of d-methadone as an analgesic in infants is unknown.

Infant Mouse: A Model in Pain Studies

For more than a decade, there has been a steady increase in the number of studies that utilize the infant rat or mice as animal models to describe the mechanisms in pain and analgesia (Anand, 1993). Much work in animals has described the anatomy, cytochemistry, neuro-physiological and behavioral aspects of pain, while a limited body of data, in man, have shown that even the most preterm infants are capable of complex responses to painful stimuli (Anand & Hickey, 1987).

Animal studies utilizing axonal staining techniques have shown the early development of peripheral sensory nerves into the limbs. In the rat peripheral nerves

grow out to the hind limb buds by the 13 th gestational day (GD 13) and to the toes by GD 19 (Reynolds, Fitzgerald, & Benowitz, 1991). Different fibers develop at different rates: skin innervation occurs before motor nerves and A-fibers develop before C-fibers (Fitzgerald, 1987; Lawson, Caddy, & Biscoe, 1974). Peripheral nerves grow to the surface of the fetal epidermis, forming a dense undifferentiated plexus (Fitzgerald, 1966). Later, at around the time of birth, this plexus is found in the subepidermal layer, and with further maturation, axons penetrate the epidermis to form differentiated sensory endings similar to that in the adult (Gleiss & Stuttgen, 1970) .

Central connections in the spinal cord occur parallel to the development of peripheral afferents. By GD 15, dorsal root afferents started to grow into the dorsal grey (Fitzgerald, 1991). Differentiated cutaneous afferent fibers are seen arriving in the dorsal horn by GD 19 (Beal, Knight, & Nandi, 1988) and have been shown to have complex functional synaptic connections soon after arrival (Fitzgerald, 1991). A-fiber central connections preceded those of c-fibers and may temporarily extend their central connections until the c-fibers mature (M. Fitzgerald, 1994). The ascending spinothalamic connections are anatomically in place by GD 18 (Barr, Miya, & Paredes, 1992). Descending tracts that moderate nociceptive responses develop before birth but their

dorsal horn connections mature later where descending inhibition is not seen in the rat until around postnatal day 14 (Fitzgerald, 1991c).

Human fetal data have shown a remarkable similar developmental timetable in terms of nociceptive processing to the rat. Dorsal horn cells in the spinal cord form synapses with developing sensory neurons by 6 weeks of gestation (Wozniak et al., 1980 and Okado 1981). Peripheral nerves migrate to the skin and limbs by 11 weeks (Humphrey 1964) achieving by birth a density of nociceptive nerve endings similar to that in adults (Gleiss 1970). The first appearance of transmitter vessicles occurs at 13 weeks gestation and development of further synaptic connections and organization of the dorsal horn structure continues up to 30 weeks (Rizvi et al., 1986 and Bijlani et al., 1988). The fetal neocortex has a full complement of cell by 20 weeks and thalamo-cortical tracts can be shown to synapse with dendritic processes of the cells in the neo-cortex by 24 weeks gestation (Rakic and Goldman 1982 and Molliver et al., 1973). In sum, the infant rat and mouse are useful models in the study of mechanisms that are involved in pain and analgesia in humans since the infant animal, like the infant human follow a similar developmental sequence and they are capable at birth to process pain.

Pain can be alleviated with opioid analgesics like morphine and methadone. Much evidence points to the endogenous opiate system as contributing to the analgesic mechanisms during the early postnatal period (Marsh et al., 1998) where l-methadone and dl-methadone may likely exert their analgesic effects at the μ-opiate receptor. As previously mentioned in this dissertation, as an opioid, methadone is unique, in that it is characterized by both opioid and non-opioid NMDA antagonist effects. Therefore, racemic methadone and its isomers may have analgesic actions at both μ-opioid receptors and NMDA receptors. The combination of both μ-opioid, agonistic effects and non-opioid, NMDA antagonists properties of dl-methadone may be particularly effective in pain management, since some NMDA mediated events, as in chronic pain or neuropatic pain states can be difficult to control with opioids alone. However, the postnatal period is marked by substantial μ-opioid receptor and NMDA receptor development, therefore analgesics like dl-methadone and its isomers may likely have differential effects in infants as opposed to adults (Gonzalez, Fuchs, & Droge, 1993; Kar & Quirion, 1995; Rahman, Dashwood, Fitzgerald, Aynsley-Green, & Dickenson, 1998).

Opioid receptors are present from early foetal life in the rat and human, in the brain and spinal cord and displayed pre- and postnatal maturational changes (Attali et al1990). During postnatal ontogeny however, the density of the μ-opioid receptor changes, as the

animal matures to the third postnatal week, the μ-opioid receptor number drops to adult levels (Baume 1980; Pasternack *et al.*, 1980). During postnatal ontogeny, the CNS is both structurally and functionally different from that of the adult and significant changes in opioid actions occur both prenatally and postnatally (Fitzgerald, 1995; Barr 1992, 1993). Furthermore, binding studies during the postnatal period on opioids have shown that this period is characterized by substantial changes of the μ-opioid receptor. For example, autoradiographic studies have shown a higher proportion of the μ-opioid receptor and kappa binding in the newborn than in the adult with peak binding at postnatal day 7. While the delta opioid receptor binding is first seen at around postnatal day 7 (Rahman *et al.*, 1998). The μ-opioid receptor binding peaks at postnatal day (4), then declines gradually to adult levels by the third postnatal week (Kar and Quirion 1995). In sum, the μ-opioid receptor binding peaks during the first postnatal week and then gradually declines.

In addition to the pharmacological involvement of the opiate system in analgesic mechanisms, the glutaminergic system is also implicated in this phonemenon, where the NMDA receptors are said to play a key role in pain transmission by modulating spinal hypersensitivity "wind up" to nociceptive stimuli in adults (Dickenson, 1994). Like the dynamic nature of the opiate system, the glutaminergic system is also developing and

may likely affect the analgesic effects of racemic methadone and its isomers in infants. For example, during postnatal development, the NMDA receptors are in flux, where the spinal cord has a higher concentration of NMDA receptors in the grey matter than those of adults (Gonzlez *et al.*, 1993). Furthermore, the affinity of the glutamate receptors for NMDA decreases with age and the amount of NMDA-evoked calcium influx in the rat substantia gelatinosa neurons are very high in the first postnatal week and declines as the animal matures (Hori and Kanda, 1994). Numerous studies also show that there is significant developmental alterations of the density (Morin, 1989; Represa et al., 1989; Tremblay et al., 1988) and the sensitivity to magnesium (Ben-Ari et al., 1988; Morrisett et al., 1990 Bowe and Nadler, 1990) of NMDA receptors during the course of development. The density of NMDA receptors increases rapidly after birth (P0 - P7) in the cortex, hippocampus and widespread expression of these receptors occurs in other supraspinal areas (Ben-Ari et al.,1997). The composition of NMDA receptors in the infant brain is different from that of the adult, with significantly increased expression of the NR 1, NR 2A and NR 2B subunits in the infant hippocampal and the cerebral cortex (Kim et al., 1998).

The 129/SvEv mouse is hypothesized to have putative deficits of the NMDA or the μ-opioid receptor systems:

Hypothesis 1 and 2:

• It was hypothesized that the NMDA receptors or steps leading up to the NMDA receptors may be defective in the 129/SvEv mouse (Kolesnikov et al., 1997).

• It was also hypothesized that the μ-opiate receptor may be defective in the 129/SvEv (Crain et al., 1999).

Hypothesis 1: The 129/SvEv mice may be defective at the NMDA receptor or steps leading up to the activation of nitric oxide synthase.

Comparative studies in mouse strains have shown significant variations in their sensitivity to morphine. For example, the 129/J and the 129/SvEv mouse appear to be more sensitive to the analgesic effects of morphine (Mogil et al., 1997 & Mogil et al., 1998). In a study, that compared the analgesic effects of morphine in the 129/SvEv mouse and the CD-1 mouse, the 129/SvEv mouse was also more analgesic to morphine than the CD-1 mouse. Additionally, the 129/SvEv mouse did not develop tolerance to morphine but the CD-1 mouse readily develop tolerance (Kolesnikov et al., 1997). It was further showed, that NMDA when given by itself in one paradigm, attenuated

morphine analgesia in the CD-1 mouse and in another paradigm when given daily with morphine enhanced the development of tolerance in CD-1 mouse. In contrast, NMDA administration had no significant effect in the 129/SvEv mouse in either of the paradigms used in the CD-1 mouse.

Furthermore, at the second messenger level, activation of NMDA receptors result in the production of nitric oxide (NO), which is involved in morphine tolerance. It has been established that sodium nitroprusside (Nitric oxide donor) and l-arginine (an excitatory amino acid) have both increased nitric oxide levels and hence decreased morphine's analgesia in both CD-1 mouse and 129/SvEv mouse (Kolesnikov et al., 1997). These results suggested that the defects in the 129/SvEv mouse is at the NMDA receptor itself (Babey et al., 1994; Kolesnikov et al., 1992, 1993b, 1994). Therefore, evidence suggested that the difference in tolerance and analgesia seen in the 129Sv/Ev mouse and the CD-1 mouse is specific to the NMDA receptor pathway itself or steps leading up to the activation of nitric oxide synthase (Kolesnikov et al.,1997). It was suggested that kappa tolerance may not be mediated through the NMDA/NO pathway like the μ- and delta receptors (Babey et al., 1994; Elliott, Minami, Kolesnikov, Pasternak, & Inturrisi, 1994; Kolesnikov, Maccechini, & Pasternak, 1994; Kolesnikov, Pick, Ciszewska, & Pasternak, 1993; Kolesnikov, Pick, & Pasternak, 1992) and that the deficits in the

129/SvEv mouse may not be at the kappa receptor. Tolerance to both $kappa_1$ and $Kappa_3$ drugs in the 129Sv/Ev mouse developed at a similar rate to previously reported for the CD-1 mouse (Babey et al., 1994; Kolesnikov et al., 1994; Kolesnikov et al., 1993; Kolesnikov et al., 1992). Therefore, evidence suggested that the difference in tolerance and analgesia seen in the 129/SvEv and CD-1 mouse is specific to the NMDA receptor pathway.

Hypothesis #2: Deficiency in GM1 ganglioside-regulated excitatory opioid receptor functions in 129/SvEv mouse.

Under normal μ-opiate receptor functioning, a low dose of an opioid antagonist and an opioid agonist results in enhanced analgesic potency and reduced tolerance and dependence (Crain & Shen, 1995, 1998; Shen & Crain, 1997). For example, competitively antagonizing the G_s -coupled excitatory opioid receptor functions by co-treatment with extremely low doses of opioid antagonists, e.g. naloxone or naltrexone, enhances morphine's analgesic potency and simultaneously attenuated opioid tolerance and dependence (Crain & Shen, 1995, 1998; Shen & Crain, 1997). Further, clinical studies of post-surgical pain patients co-treated with morphine plus ultra-low doses of naloxone or malmefene, a 6-methylene analog of naltrexone have demonstrated

significant enhancement of morphine's analgesic potency (Gan et al., 1997; Joshi et al., 1999).

However evidence suggested, that the μ- opiate receptor system in the 129/SvEv mouse may be defective. When morphine was co-administered with naltrexone, it enhanced and also prolonged morphine's antinociceptive effects in the Swiss Webster mouse (SW) but attenuated antinociception in the 129/SvEv mouse suggesting that the μ-opiate receptor is defective. Further, when morphine, a μ- opiate receptor was given alone, it yielded greater antinociceptive potency in the 129/SvEv mouse than in the SW mouse. Also, chronic treatment of the129/SvEv mouse with morphine results in much less tolerance than occurs in the SW mouse or the CD-1 mouse. Acute administration of the GM1 ganglioside in the 129/SvEv mouse resulted in marked attenuation of morphine antinociception. These findings (Crain & Shen, 2000) suggested a deficiency of the excitatory opioid receptor function in the 129/SvEv mouse. The involvement of GM1 ganglioside in morphine's analgesic mechanisms is to attenuate morphine's analgesic effect and rapidly increase the efficacy of excitatory G_s-coupled opioid receptor functions.

It is to be noted that that the 129/SvEv mouse was not always consistently more analgesic to morphine than the SW mouse at all ages (Crain & Shen, 2000). The antinociceptive potency of morphine was considerably weaker in the younger 129/SvEv mouse (1 month old; 10-15 grams) compared to the older mouse, 2 months old 20-25 grams (Crain & Shen, 2000). Further, it was reported that the young 129/SvEv mouse did not develop tolerance during chronic morphine treatment, resembling that observed in the SW mouse (Crain & Shen, 2000). Thus, suggesting that the effects seen in the 129/SvEv mouse across age may be a developmental anomaly.

Effects of dl-methadone and its Isomers may differ in different Pain States (Formalin Test versus Thermal Tests).

The thermal and formalin tests are two animal models of nociception that are mediated by differing mechanisms. These two models have been extensively used in the study of different kinds of pain. The thermal test is a model of acute pain and the formalin teast is a model of both acute and inflammatory pain. Opioid analgesics may exert differential antinociceptive effects in different pain states (Dickenson, 1994). To examine this, the thermal and the formalin tests will be investigated in this dissertation in infants. Subcutaneous injection of dilute formalin produces a biphasic nociceptive response, an early phase and a late phase in behavioral studies in adult rats (Dubuisson &

Dennis, 1977; Wheeler-Aceto, Porreca, & Cowan, 1990) and in adult mice (Shibata, Ohkubo, Takahashi, & Inoki, 1989). Each phase represents different pathological processes (Dickenson & Sullivan, 1987b; Woolf & Thompson, 1991). Moreover, each phase is differentially sensitive to centrally acting and peripherally acting analgesic agents in adult mice (Hunskaar & Hole, 1987; Murray & Cowan, 1991).

Overwhelming evidence implicated the excitatory amino acids in injury induced sensitization in the dorsal horn. Intrathecal administration of the EAA, l-glutamate and l-aspartate produced an increase in the excitability of flexor afferents (Woolf & Wiesenfeld-Hallin, 1986). On the other hand, both competitive and non-competitive NMDA antagonists reduce facilitation of flexion reflexes induced by either a brief electrical C-fiber stimulation or cutaneous application of the chemical irritant mustard oil (Woolf & Thompson, 1991). Repetitive C-fiber stimulation produces a "wind-up" of dorsal horn neuron activity that is mimicked by the application of l-glutamate (Zieglgansberger & Herz, 1971) and NMDA (King, Thompson, Urban, & Woolf, 1988), and is blocked by application of either competitive (Dickenson & Sullivan, 1987a, 1990; Woolf & Thompson, 1991) or non-competitive (Davies & Lodge, 1987; Woolf & Thompson, 1991) NMDA antagonists. Subcutaneous injection of formalin produces an

increased release of glutamate and aspartate in the spinal cord dorsal horn (Haley, Sullivan, & Dickenson, 1990).

The thermal test is an animal model of acute or phasic pain. Phasic pain is defined as short lasting pain that is felt immediately when triggered by an injury. On the other hand, tonic pain is defined as long lasting, often ill defined that develops subsequent to an injury. An example of acute or phasic pain in man, is a burn from a hot object. Acute activation of small afferents result in clearly defined pain behavior in humans and in animals. This type of pain behavior may be mediated by the release of excitatory afferent transmitters and by the subsequent depolarization of projection neurons. The magnitude of the response to acute pain is typically proportional to the intensity of the stimulus (or to the magnitude of the injury). The organization of this acutely driven system is typically modeled in terms of a linear relationship between activity in the peripheral afferent and the activity of neurons that project out of the spinal cord to the brain.

A-delta afferent fibers are small-diameter rapidly conducting myelinated nociceptive fibers that conduct thermal or mechanical information at the rate of 5 to 30 m/sec. The pain ascribed to these fibers has been described as being of a sharp pricking nature (C-fiber afferent nociceptive neurons are small diameter slowly conducting unmyelinated

polymodal receptors that are activated by a wide variety of high-intensity stimuli, such as mechanical, chemical and thermal, at a rate of 0.5 to 2 m/s.

Opiates are differently effective in the thermal versus the formalin models of pain. On the one hand, morphine is quite potent in surppressing phasic pain, where 0.5 mg/kg to 4.0 mg/kg produces a dose-related increase in hind limb withdrawal latency to a hotplate from postnatal day 2 with maximum sensitivity at postnatal day 6 (Fitzgerald, 1995). On the other hand, morphine is less effective in tonic pain models than in phasic pain models, where 1-2 mg/kg of morphine is needed to suppress formalin induced pain in 3 day old rat pups (McLaughlin, Lichtman, Fanselow, & Cramer, 1990). Also, the effects of opiates such as morphine and ketocyclazocine are dependent on the type of stimulus thermal versus mechanical, the age of the animal tested, the appendage tested and the route of administration. Opiates such as morphine exert its analgesic effects not only by central actions but also by peripheral actions in the immature rat in the formalin test (Barr, 1999).

Goals of this thesis

Experiments in this thesis were designed to:

- Describe the nociceptive and the anti-nociceptive effects of dl-methadone and its isomers during postnatal ontogeny in the thermal and formalin noceceptive tests in the 129/SvEv and CD-1 mouse pups.

- Provide a possible mechanism for the potentiated analgesic effects exerted by dl-methadone during early postnatal development, by the co-administration of an NMDA antagonist (MK801) and and a μ-opiate agonist (morphine) to mimic the effects of dl-methadone.

The experiments in this thesis are the first to describe the analgesic effects of dl-methadone and its isomers during the postnatal period. The postnatal period is a period, where both the μ-opioid and the NMDA receptors continue to develop. Also, they are the first to describe a possible mechanism for the potentiated analgesic effects exerted by dl-methadone during early postnatal development in the thermal test, by the co-administration of MK801 and morphine. These two compounds mimic the pharmacological effects of dl-methadone. Like l-methadone, morphine is a μ-opiate agonist and like d-methadone, MK801 is an NMDA antagonist.

The following hypotheses were tested in this thesis:

(1) That, dl-methadone and its isomers may result in greater analgesic effectiveness in infants than in adults in both 129/SvEv and CD-1 mouse pups in the formalin nociceptive test.

(2) That, dl-methadone and its isomers may result in increased analgesic effectiveness in infants than in adults in both 129/SvEv and CD-1 mouse pups in the thermal nociceptive test.

(3) That, an NMDA antagonist such as MK801 may potentiate the analgesic effects of a μ-opiate agonists such as morphine in both the 129/SvEv and the CD-1 mouse pups in the thermal nociceptive test at PD 3. A potentiated analgesic effect is defined as a leftward shift in a dose response curve that results from the interactive effects of two drugs co-administered.

The first hypothesis was tested in both the 129/SvEv and CD-1 mouse pups by examining each appendage (forepaw, hindpaw and tail) in water baths that served as a thermal stimulus. By examining the behavioral responses of each appendage, the analgesic effectiveness of dl-methadone and its isomers were studied in an acute pain model. The second hypothesis was tested by injecting formalin into the plantar surface of a paw of the animal (for this dissertation, the left hind paw was used in all studies) in the

129/SvEv and CD-1 mouse pups. By examining the behavioral responses to formalin, the analgesic effectiveness of dl-methadone and its isomers were examined in an inflammatory pain model. The third hypothesis was tested by a co-administration procedure, where an NMDA antagonist was co-administered with a μ-opiate agonist. The NMDA antagonist, MK801 was administered firstly and subsequently, the μ-opiate agonist was administered and the combined effects of the two compounds were studied in the 129/SvEv and the CD-1 mouse pups in the thermal test of nociception. By examining the behavioral responses of the forepaw, hindpaw and tail in water baths that served as thermal noxious stimuli, the potentiated analgesic effects of co-administered morphine and MK801 were studied.

General Methods

Experimental Animals: Subjects were mouse pups at postnatal day (PD) 3,10 and 21 from 129/SvEv, also referred to as 129/S6 (Taconic Farms, German Town NY) and from CD-1 dams (Charles River; Wilmington MA) in our animal facilities. All dams were checked twice daily for new births, at approximately 0900 and 1800 hours. Pups found at either time were designated 0 days of age. Parental animals and pups were housed in standard laboratory cages in a colony room maintained between 22 - 24 ^{0}C with a 12 hour light-dark reverse photocycle with light onset at 1030 hours. Food and water were

available ad libitum. For all experiments, it was required that no more than one pup per litter was in any given treatment condition, therefore the litter was the unit of analysis. Whenever, more than one pup within a single litter were given the same treatment condition, as was sometimes done in the thermal test, the data was averaged and the single mean was used for further data analysis. A treatment condition consists of a pup receiving all of the doses of a single drug.

All experiments were approved by Hunter College IACUC and followed Ethical Guidelines of the Society for Neuroscience, National Institute of health and the International Society for Developmental Psychobiology.

Doses of Racemic Methadone and Its Isomers

The subcutaneous doses of racemic methadone and its isomers were chosen for studies in this dissertation by extrapolating from the literature on adult rodents (Chizh, Schlutz, Scheede, & Englberger, 2000; Ingoglia & Dole, 1970; Jage, 1989; Olsen et al., 1977; Scott, 1948; Scott & Orr, 1969; Thorp, 1949). This is the first developmental research that addresses the pharmacological analgesic effects of racemic methadone and its isomers on the developing μ-opioid and on the developing NMDA receptors in infants. There is no knowledge of the analgesic effects of these compounds in the

developing animal and this was the first study that addressed the analgesic effects of these compounds in infants.

Doses of MK801 and Morphine

Morphine doses were selected based on both the adult literature and by extrapolating from studies done on infant animals (Auguy-Valette, Cros, Gouarderes, Gout, & Pontonnier, 1978; Blass, Cramer, & Fanselow, 1993; Fanselow & Cramer, 1988; Giordano & Barr, 1987; Johannesson & Becker, 1973; Kehoe & Blass, 1986; Marsh, Dickenson, Hatch, & Fitzgerald, 1999; McLaughlin & Dewey, 1994; Spear, Enters, Aswad, & Louzan, 1985; Thornton, Compton, & Smith, 1998). The MK801 doses were selected based on the adult literature (Allen & Dykstra, 2001; Bhargava & Matwyshyn, 1993; Carlezon, Kosten, & Nestler, 2000) and in infant animals (Bell & Beglan,1995; Trujillo & Akil, 1991a; Zhu & Barr, 2000).

Thermal Nociceptive Test (Acute Model)

In this acute nociceptive paradigm, the test compounds studied (that is dl-methadone) and its isomers were subcutaneously injected thirty minutes before behavioral testing. Behavioral testing was accomplished by random immersion of each appendage in thermal

water baths at temperatures of 48^{0}C, 50^{0}C, and 52^{0}C. Appendage withdrawal latencies were taken as indicators of pain and analgesia. A cutoff latency of 20 seconds was used to avoid any tissue damage. All drugs were studied in a cumulative dose response paradigm. In this paradigm, each pup was treated with incremental doses of a single drug and was also tested at fixed time intervals. All data was collapsed over temperature.

Formalin Nociceptive Test (Tonic Model)

Each of the different forms of methadone (d-, l- and dl-methadone) was subcutaneously administered 15 minutes before dilute formalin was administered into the plantar surface of a paw (all experiments utilized the left hind paw). The volumes of formalin used were 5, 10 and 20 μl at PD 3, 10 and 21 respectively. These volumes were derived from comparable studies in adult animals (Abbott, Melzack, & Samuel, 1982; Coderre, Fundytus, McKenna, Dalal, & Melzack, 1993; Dickenson & Sullivan, 1987b; Morita et al., 1990), prior development work (Abbott, Melzack, & Leber, 1982; Guy & Abbott, 1992; Williams, Evan, & Hunt, 1990; Yi & Barr, 1995) and from pilot studies. The volumes used here were decreased of necessity because of the smaller size of the paw during the postnatal period. Pain rating behavior began immediately after fromalin injection and was scored on a scale of 0 – 4 for every minute for 45 minutes. The

formalin test has been previously described (Barr,1998) and the behavioral scale is also described below.

Behavioral Scale: Assessment of formalin Nociception:

0 = Injected paw rests flat on surface and animal applied pressure.

1 = Injected paw favor.

2 = Injected paw lift.

3 = Injected paw shake.

4 = Injected paw lick.

Co-administration of Morphine and MK801

This paradigm was used to test whether an NMDA receptor antagonist, can potentiate the analgesic effects of a μ-opiate agonist in early postnatal development. In this paradigm, one of four doses of MK801 an NMDA antagonist was subcutaneously administered 15 minutes before the first of five doses of morphine was administered to each pup and behavioral testing occurred 15 minutes post morphine drug injection. All t doses of morphine and MK801 were studied in a single litter. That is each pup with in a single litter was treated with one dose of MK801 and all of the four doses of morphine at 15 minutes time intervals in a cumulative dose response procedure.

Drug Preparation and Administration

Methadone

The isomers, d-methadone [(S)-(+)-methadone], l-methadone [(R)-(-)-methadone] and dl-methadone [(+/-)-methadone HCl] were generously donated by the National Institute on Drug Abuse (NIDA). dl-Methadone was completely dissolved in 0.9% saline solution. However, the free base of the individual isomer was completely dissolved with the aid of 1 N HCl in 0.9% saline solution and adjusted to a final pH of 6.0. All drugs were administered subcutaneously (s. c.) in the nape of the neck.

Morphine and MK801

Morphine sulfate was obtained from (H.Schein) and was diluted with 0.9% saline. The NMDA antagonist, MK801 was obtained from Tocris and was also dissolved in 0.9% saline solution.

The 129/SvEv and CD-1 Mouse

The 129/SvEv mouse has been hypothesized to have NMDA receptor deficits (Kolesnikov, Jain, Wilson, & Pasternak, 1998) or μ-opioid receptor deficits (Crain & Shen, 2000) because they did not develop tolerance to morphine. The 129/SvEv mouse

strain was used in this thesis because of the putative μ-opioid and NMDA receptor deficits and thus, will serve as a useful model in the study of the μ-opioid and the NMDA receptor involvement in the analgesic mechanisms of racemic methadone and its isomers. The CD-1 mouse was used as a control comparison for results obtained with the 129/SvEv mouse. This CD-1 mouse strain was chosen as a model for comparison in this entire dissertation for results obtained in the 129/SvEv mouse because they readily develop tolerance to morphine (Kolesnikov et al., 1997) and are widely used in behavioral studies on nociception and antinociception.

Data Analysis

Thermal data: All data was collapsed over temperature, as there was no drug effect on temperature. Drug, age and strain were compared by means of a factorial analysis of variance (ANOVA), followed by the contrast for post hoc analyses. Repeated measures were done for drug, as all five doses were administered to the same animal. The effects of strain and age was analyzed separately using a between subjects analysis. The data were converted to the percentage of maximum possible effect (%MPE) according to the method of (Harris & Pierson, 1964). The % MPE is represented as follows; % MPE = Test Latency – Baseline Latency / Cutoff Time – Baseline Latency X 100. The test latency represents the time it takes the animal to withdraw its appendage from the

stimulus. The baseline latency was derived from the saline treated animals. The cutoff latency represents the time after which the animal was not tested any further. The transformed %MPE data was then used to construct dose-response curves for d-, l- and dl-methadone's analgesic effect. The ED_{50} values and 95% Confidence limits (95% CL) of d-, l- and dl-methadone's analgesic effect were determined using a nonlinear analysis (Prism 3, Graph Pad Software Inc., San Diego, CA).

Formalin data. The data was averaged into 3-minute bins to minimize minute by minute variability and a factorial analysis of variance (ANOVA) was performed on the behavioral scores to decrease variability. The 15 3-min bins was a within subject variable and age was a between subject variable. The variables age and strain was analyzed individually. The two main effects dose and bins and the interaction of these two main effects were also analyzed. The doses were all tested within a single litter at any given age, and therefore, drug dose, bins and the interaction were analyzed by a within subject design. The post hoc analysis was performed for drug dose effects using the contrasts as post hoc analyses. Statistical tests were considered significant if $p < 0.05$. Data were expressed as means +/-standard error (S.E.M.).

CHAPTER 2

Postnatal Analgesic Effects of Racemic Methadone and Its Isomers in 129/SvEv and CD-1 Mouse Pups in the Formalin Nociceptive Test

The formalin test was first introduced by ((Dubuisson & Dennis, 1977) and later modified by (Tjolsen, Berge, Hunskaar, Rosland, & Hole, 1992). It is a useful tool for obtaining neurogenic inflammation and continuous pain (Brown, Kissel, & Lish, 1968). The nociceptive behavioral biphasic response to formalin has been widely accepted as a model of prolonged noxious stimulation and is characterized by both behavioral (Wheeler-Aceto & Cowan, 1991) and electrophysiological measures (Haley et al., 1990). Both in adult rats (Dubuisson & Dennis, 1977) and mice (Shibata et al., 1989), the formalin nociceptive assay consists of an "early" first phase (acute phase) and a "late" second phase (tonic phase). The acute "early" phase (lasts about 0 -5 min), involves direct activation of monosynaptic primary afferent input into the superficial laminae of the spinal cord, that is then followed by a quiescent period of 5-10 min. The activity in the first phase appears to trigger a subsequent polysynaptic response involving sensitization of deeper laminae dorsal horn neurons and manifested in behavioral models as a more prolonged, tonic phase of activity, lasting up to approximately 60 min. The tonic phase is also accompanied by the onset of a persistent inflammatory response that lasts beyond the cessation of the nociceptive response (Tjolsen et al., 1992).

Both the μ-opioid (Bennett, 2000; Murray & Cowan, 1991; Ravert, Bencherif, Madar, & Frost, 2004; Seltzer, Cohn, Ginzburg, & Beilin, 1991) and NMDA receptors (Bennett,

2000; Seltzer et al., 1991; Woolf, 1994; Woolf & Thompson, 1991) have been implicated in tonic or formalin nociception. However, since these receptors undergo dramatic maturational changes (Bardoni, Magherini, & MacDermott, 2000; Ben-Ari, Khazipov, Leinekugel, Caillard, & Gaiarsa, 1997; Kar & Quirion, 1995; Rahman et al., 1998) they may impart differential analgesia in tonic pain in infants.

Despite the functionality of the NMDA receptors in postnatal ontogeny (Bardoni et al., 2000), the behavioral correlates of this functionality in relation to pain and analgesia in a model of both acute and inflammatory pain are unknown. Further, there is no knowledge on the functional interactions of racemic methadone and its isomers, on the developing μ-opioid and NMDA receptors in immature pain pathways, in this pain model.

The 129/SvEv mouse has been hypothesized to have putative deficits of the μ-opioid or NMDA receptors, therefore the CD-1 mouse pups was used as control comparisons in describing the antinociceptive effects of d, l- and dl-methadone, during postnatal ontogeny in the fomalin test.

METHODS

Behavioral Formalin Stimulation

Formalin. Both 129/SvEv and CD-1 pups were used in this study (8 litters per age at 5 pups per litter were used for each drug and each age). Each of the three drugs (d-, l- and dl-methadone) was tested at each age group in a single litter in a dose response protocol. A small volume [5,10 and 20 μl at PD 3,10 and 21 respectively] of 2 % formalin was subcutaneously injected into the plantar surface of the left hind paw. The volumes and concentrations of formalin used in this study, were derived from comparable studies in adult animals, prior developmental work and from pilot studies. Pain rating behavior began immediately after formalin injection for every 60-s for 45 minutes and was rated on a 5-point scale previously described (Barr, 1998).

Statistics

The design of this experiment was a mixed design with age being a between subject variable, while dose and bin being within subject variables. The minute by minute data were averaged into 3-minute bins to decrease variability. A three way analysis of variance (ANOVA) were performed on dose, age, and bin and a Four way ANOVA were performed on dose, age, strain and bin. The 15 3-minute bins and drug dose were repeated measure variables and age and strain were between measure variables. The

contrast was used as a post hoc analysis for between subject variables and for factors in the repeated measures ANOVA, the analysis made the following comparisons for the repeated measure variables, all doses versus saline doses, bin1 (Phase 1) versus each bin of 7-10 (phase two).

RESULTS

The effects of d-, l- and dl-methadone on formalin-induced nociceptive responding in the 129/SvEv and CD-1 mouse pups at PD 3,10 and 21 are depicted in Figures: 2 - 4 and Figures 5 - 7 respectively. The effects of dl-methadone and its isomers on formalin-induced nociceptive responding in the first and second phase in the 129/SvEv and CD-1 mouse pups at PD 3,10 and 21 are depicted in Figures: 8 - 9 and Figures: 10 -11 respectively.

The three way ANOVA revealed a significant main effect of age in the 129/SvEv pups $\underline{F}$ (2,19)=32.284, $p < 0.05$ and in the CD-1 pups $\underline{F}$ (2,19)=32.284, $p < 0.05$. A significant main effect of dose was also revealed in the 129/SvEv mouse pups $\underline{F}$ (4,76)=92.867, $p< 0.05$ and in the CD-1 pups $\underline{F}$ (4,88)=104.772 $p< 0.05$, as l-methadone dose dependently decreased nociceptive responding at all ages tested. To determine whether analgesia observed at PD 3,10 and 21 were more pronounced in the first phase or

the second phase, a separate ANOVA was performed that compared bin 1 with bins 7 -10 across drug doses. The three way ANOVA revealed a significant phase effect in the 129/SvEv pups F (14,266)=13.629, $p< 0.05$ and in the CD-1 pups (14,308)=11.551, $p< 0.05$. Also, a significant dose by phase by age interaction was revealed in the 129/SvEv pups F (112,1064)=1.632, $p< 0.05$ and for the CD-1 mouse pups F (112,1232)=1.595, $p< 0.05$. The contrast for post hoc analyses revealed that, l-methadone had a greater effect of decreasing nociceptive responding in the first phase, than in the second phase, at all ages ages tested in both mouse strains. Also, that l-methadone exerted significant analgesic effects at 1.0 to10.0 mg/kg doses, $p < 0.05$ at PD 3 and 10 in both mouse strains. At PD 21, only the highest dose of l-methadone resulted in significant analgesic effect in both mouse strains, $p < 0.05$.

Three way ANOVA revealed that dl-Methadone dose dependently decreased nociceptive responding at all ages tested, in the 129/SvEv F (4,60) 42.693, $p< 0.05$ and in the CD-1 mouse pups F (4,84)= 40.965, $p< 0.05$. To determine whether antinociception observed at PD 3,10 and 21, were more pronounced in the first phase or the second phase, a separate ANOVA was performed that compared bin 1 with bins 7 -10 across drug doses. dl-Methadone had a greater effect of decreasing nociceptive responding in the first phase than in the second phase, at each age tested in the 129/SvEv mouse pups

(dose by age by phase interaction) F (112,840)=1.545, $p < 0.05$. The contrast for post hoc analyses revealed that at PD 3 and 10 in both mouse strains, dl-methadone resulted in significant analgesia compared to saline treated animals at the two highest doses tested, $p < 0.05$. This compound rsulted in significant analgesic effect only at the highest dose tested at PD 21, $p < 0.05$.

d-Methadone was totally ineffective in both 129/SvEv and CD-1 mouse pups at PD 21, ($p > 0.05$) and was only minimally effective at PD 3 and 10, (dose by age interaction, F (8,144)=80.806, $p < 0.05$, Figures 2 and 6. A significant main effect of age was revealed in the 129/SvEv pups F (2,15)=10.608, $p < 0.05$ but not for the CD-1 pups, $p > 0.05$. ANOVA also revealed, that phase two responses was significantly different from phase one responses in the 129/SvEv pups, F (14,210)=28.372, $p > 0.05$; and in the CD-1 pups F (14,294)=7.677, $p > 0.05$. A significant interaction of age by phase by dose was revealed for the CD-1 pups F (14,112)=1.249, $p > 0.05$, indicating that the dose effects were significantly different at each age in phase one versus phase two of the formalin test. The contrast for post hoc analyses revealed that, at PD 3 and 10 in both mouse strains, d-methadone resulted in significant analgesia compared to saline treated animals only at the highest dose tested at 10.0 mg/kg, $p < 0.05$.

Figure 2. Data presented are means (+/- SEM) and showed the time course of l-methadone's effect at each age tested in the 129/SvEv mouse pups on formalin induced nociceptive responses. This compound was more effective at PD 3 and 10 than at PD 21 in reducing formalin evoked responses and was the most efficacious form of methadone tested at all age.

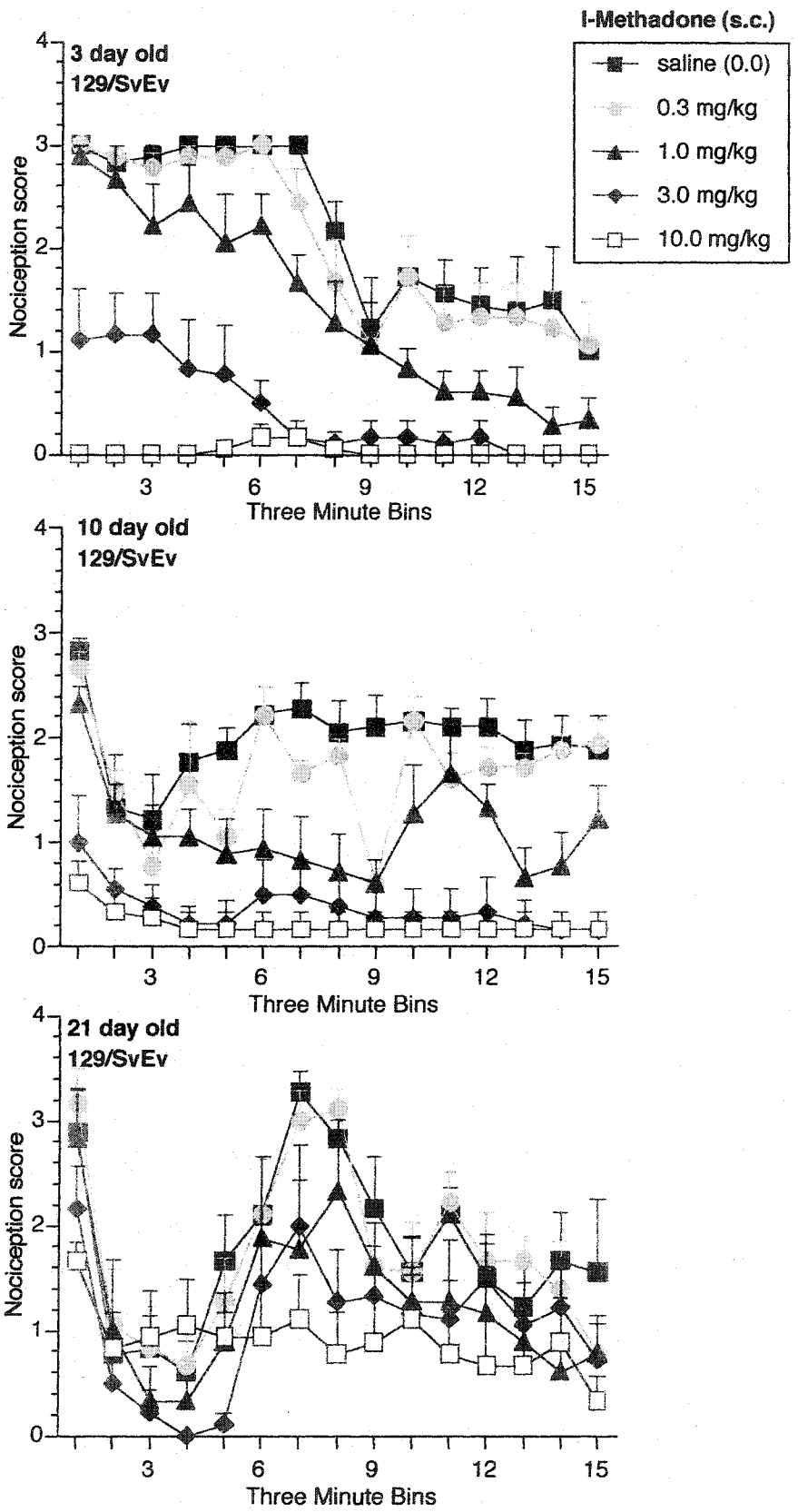


Figure 3. Data presented are means (+/- SEM) and showed the time course of d-methadone's effect at each age tested in the 129/SvEv mouse pups on formalin induced nociceptive responses. This compound was minimally effective at PD 3 and 10 and totally ineffective at PD 21 in reducing formalin evoked responses.

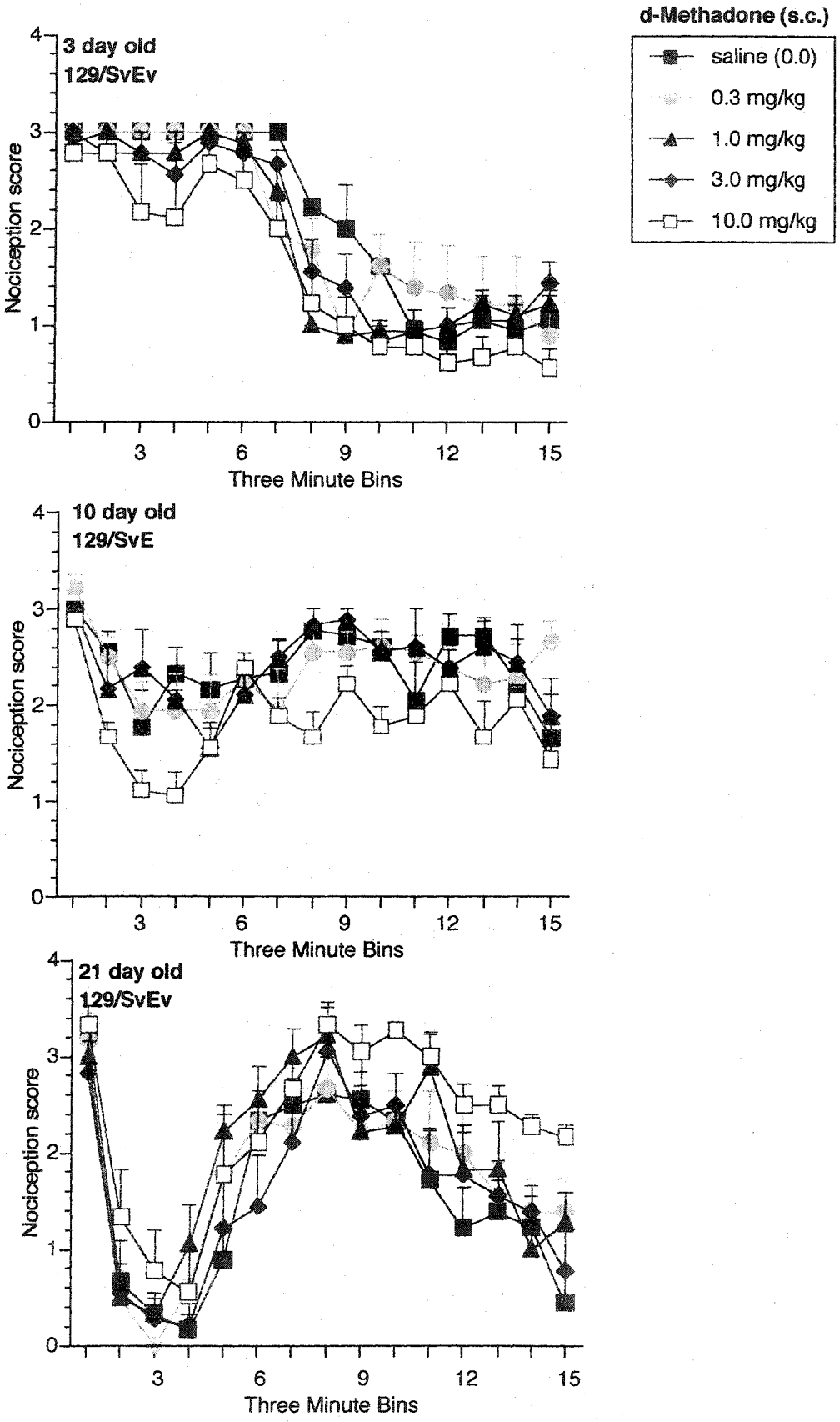


Figure 4. Data presented are means (+/- SEM) and showed the time course of dl-methadone's effect at each age tested in the 129/SvEv mouse pups on formalin induced nociceptive responses. This compound was more effective at PD 3 and 10 than at PD 21 in reducing formalin evoked responses and was less effective than l-methadone at each age studied.

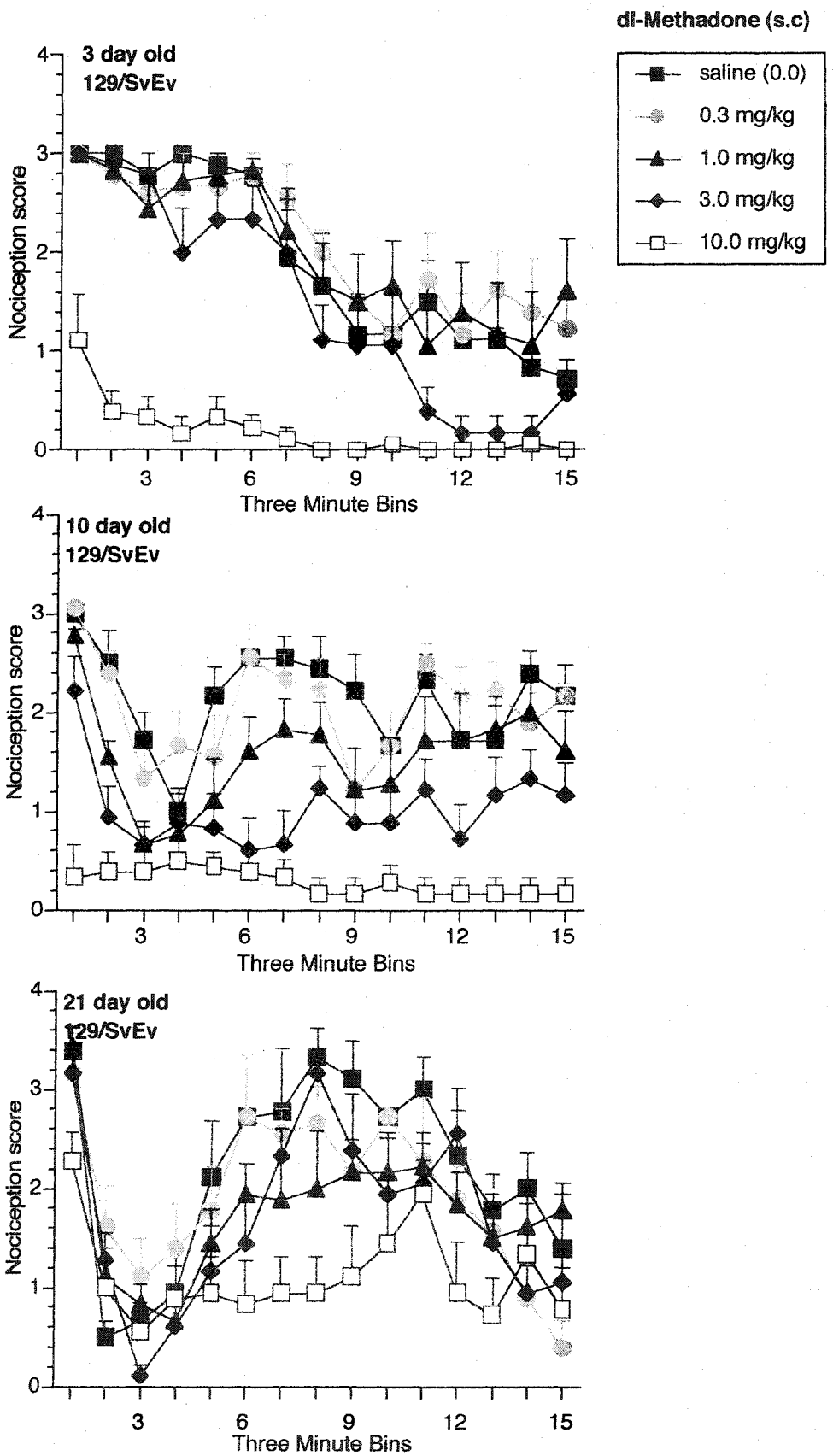


Figure 5. Data presented are means (+/- SEM) and showed the time course of l-methadone's effect at each age tested in the CD-1 mouse pups on formalin induced nociceptive responses. This compound was more effective at PD 3 and 10 than at PD 21 in reducing formalin evoked responses and was the most efficacious form of methadone tested at each age.

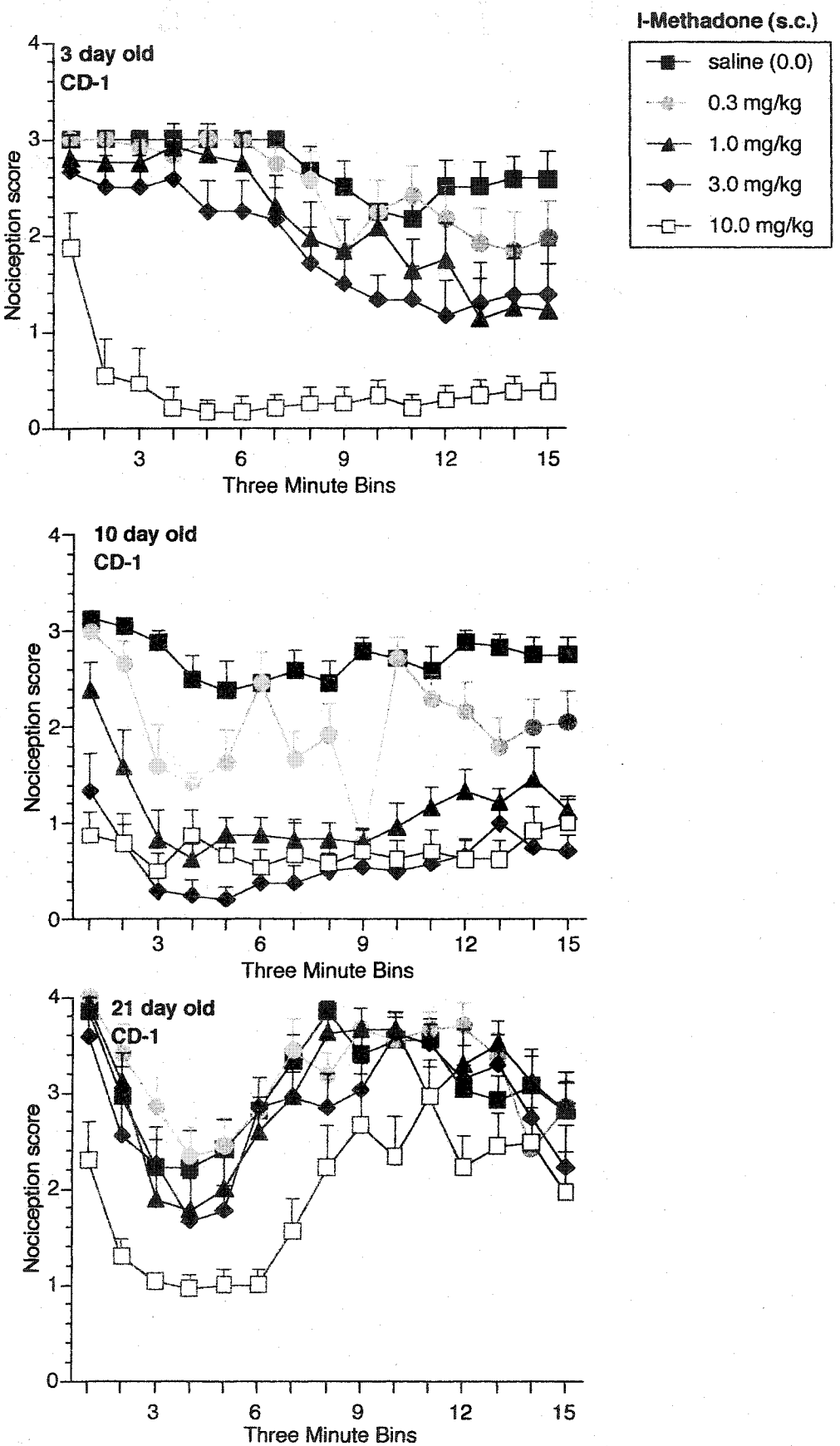


Figure 6. Data presented are means (+/- SEM) and showed the time course of d-methadone's effect at each age tested in the CD-1 mouse pups on formalin induced nociceptive responses. This compound was minimally effective at PD 3 and 10 and totally ineffective at PD 21 in reducing formalin evoked responses.

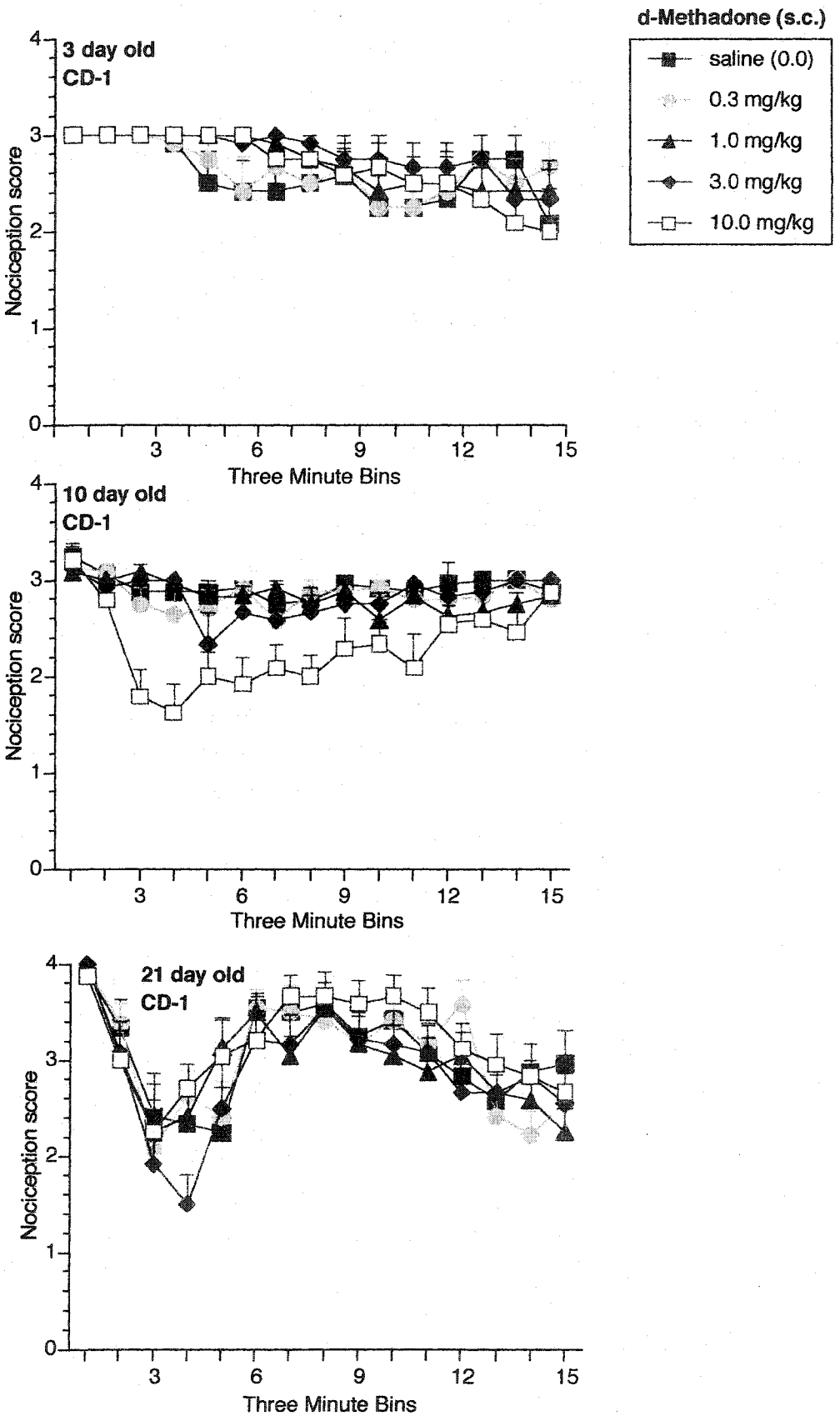


Figure 7. Data presented are means (+/- SEM) and showed the time course of dl-methadone's effect at each age tested in the CD-1 mouse pups on formalin induced nociceptive responses. This compound was more effective at PD 3 and 10 than at PD 21 in reducing formalin evoked responses and was less effective than l-methadone at each age studied.

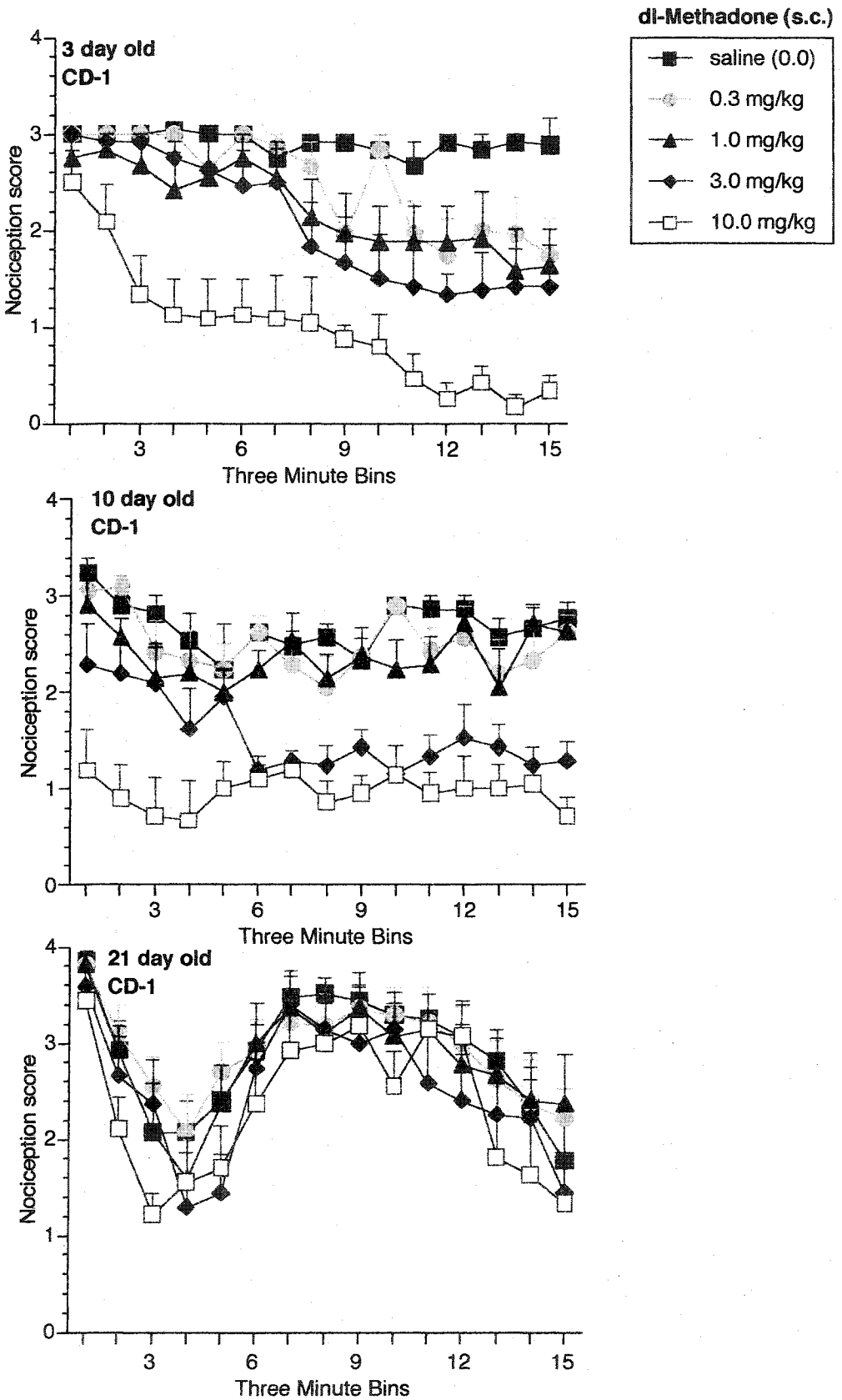


Figure 8. Data are means (+/- SEM). Graphs represent dose response curves for phase one of the formalin test in the 129/SvEv mouse pups at PD 3, 10 and 21 for the three forms of methadone studied. l and dl-Methadone were effective analgesics in the first phase but were less effective at PD 21 than at the younger ages tested. l-Methadone was the most effective analgesic and d-methadone was totally ineffective in reducing formalin induced nociceptive responses at all ages in phase one responses.

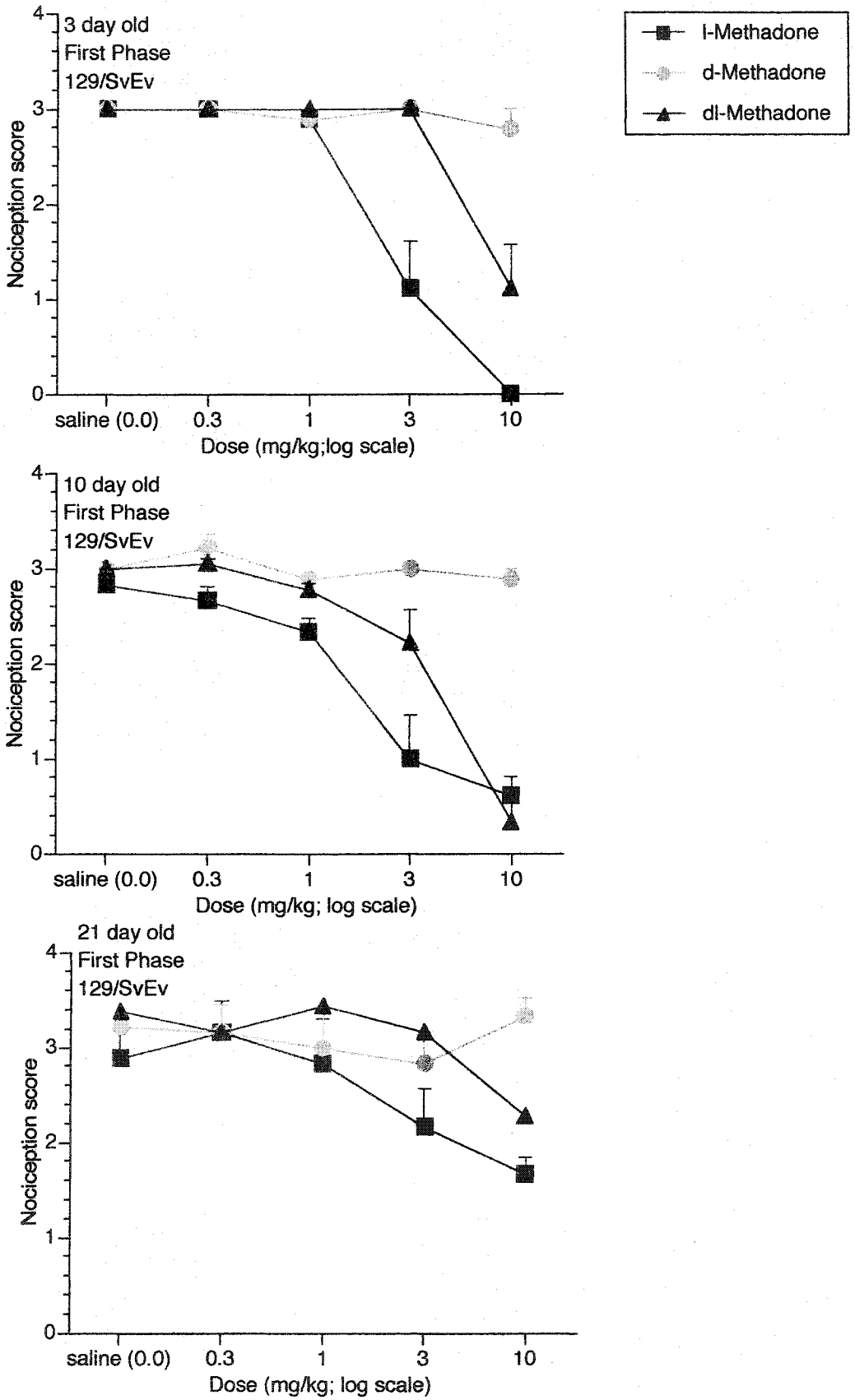


Figure 9. Data are means (+/- SEM). Graphs represent dose response curves for phase two of the formalin test in the 129/SvEv mouse pups at PD 3, 10 and 21 for the three forms of methadone studied. d-Methadone was totally ineffective at PD 3 and 10. l- and dl-Methadone were both effective in reducing formalin induced nociception across all ages studied with dl-methadone being less effective than l-methadone. Further, l- and dl-methadone were less effective at PD 21 than at PD 3 and 10.

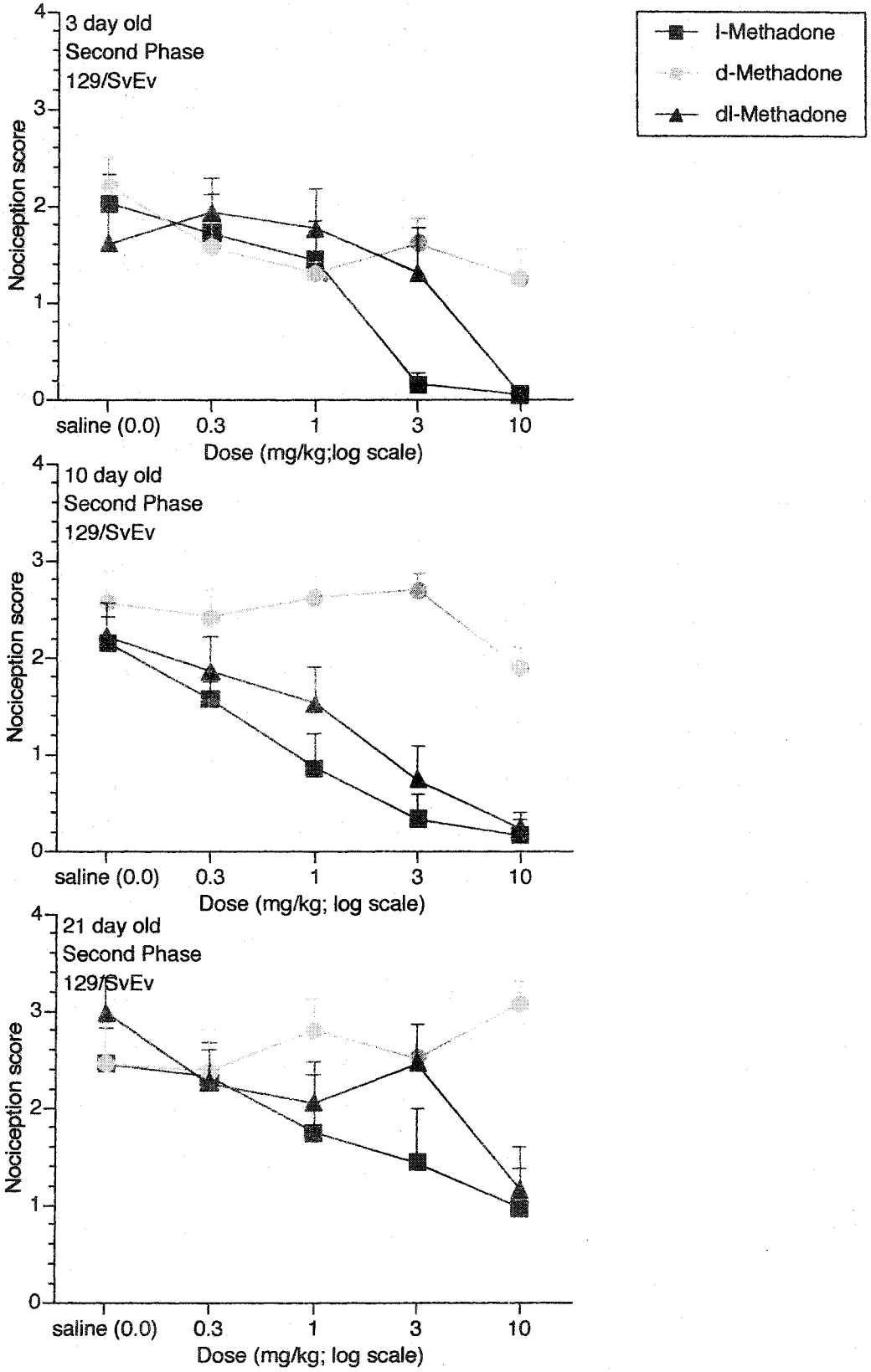


Figure 10. Data are means (+/- SEM). Graphs represent dose response curves for phase one of the formalin test in the CD-1 mouse pups at PD 3, 10 and 21 for the three forms of methadone studied. d-Methadone was totally ineffective in reducing formalin induced nociception at all ages tested in the first phase. l- and dl-Methadone were both effective in reducing formalin induced nociception across all ages studied with dl-methadone being less effective than l-methadone. Further, l- and dl-methadone were less effective at PD 21 than at PD 3 and 10.

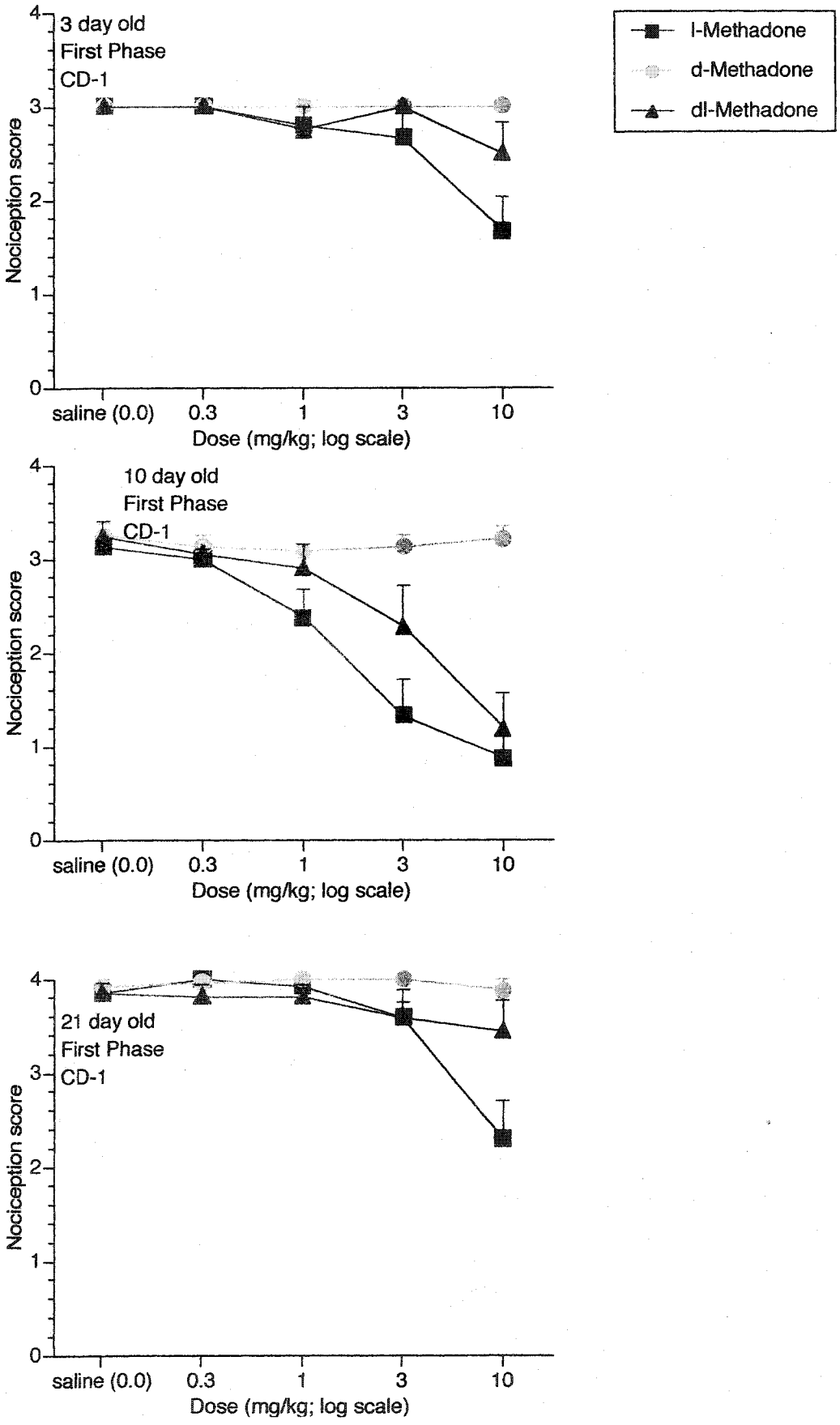
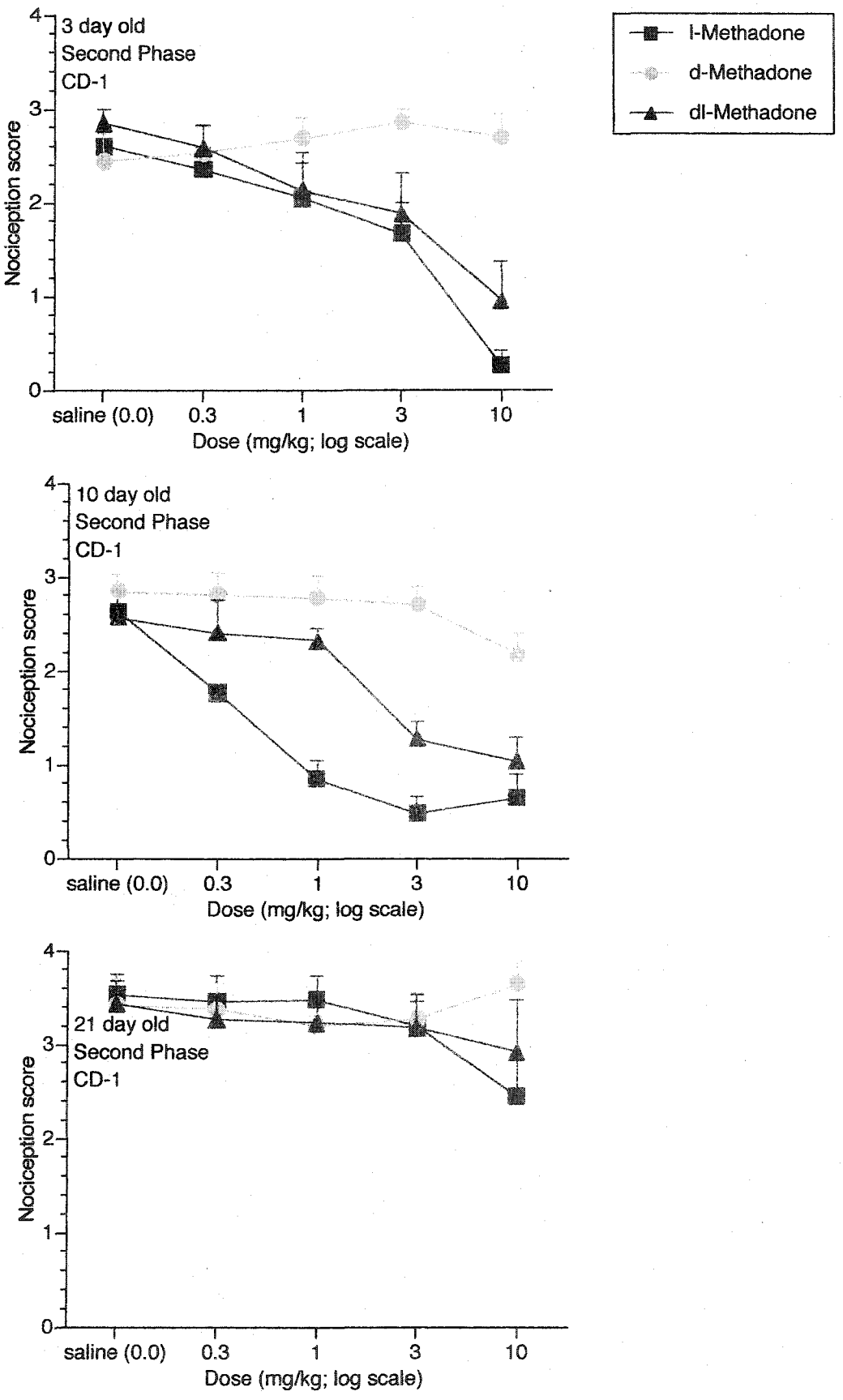


Figure 11. Data are means (+/- SEM). Graphs represent dose response curves for phase two of the formalin test in the CD-1 mouse pups at PD 3, 10 and 21 for the three forms of methadone studied. d-Methadone was totally ineffective in reducing formalin induced nociception at PD 21 and was only minimally effective at PD 3 and 10. l- and dl-Methadone were both effective in reducing formalin induced nociception across all ages studied with dl-methadone being less effective than l-methadone. Further, l- and dl-methadone were effective at PD 21 than at PD 3 and 10.



DISCUSSION

The study in this chapter, investigated the analgesic effects of d-, l- and dl-methadone during postnatal ontogeny at PD 3,10 and 21 in the 129/SvEv and CD-1 mouse pups in the formalin nociceptive test. The findings presented here, revealed that subcutaneous formalin significantly induced differential nociceptive responding between the 129/SvEv and CD-1 mouse pups. The CD-1 pups at each age tested, displayed greater nociceptive responding in the formalin test, than the 129/SvEv pups. However, no strain differences were observed between the 129/SvEv and CD-1 pups on the analgesic effects of racemic methadone and its isomers. The observed strain differences in nociceptive responding between the 129/SvEv and CD-1 pups, may be explained by a genetic difference between the two mouse strains or may be an effect only seen during postnatal ontogeny. Results also showed that, the rank order of analgesic effectiveness of dl-methadone and its isomers in both mouse strains was similar to those of adults and was consistent with substantial prior findings (Scott, 1948; Smits & Myers, 1974; Thorp, 1949). The findings of this study are in good agreement with theoretical predictions and previous findings, that l-methadone is the most effective analgesic of the three forms of methadone and is almost twice as effective as dl-methadone, with d-methadone being the least effective (Scott, 1948; Smits & Myers, 1974; Thorp, 1949). Therefore, the putative μ-opioid or

NMDA receptor deficits in the 129/SvEv mouse may not be influencing dl-methadone and its isomers analgesic effectiveness in infants.

The CD-1 pups at PD 3, 10 and 21 displayed greater formalin induced nociceptive responding than the 129/SvEv pups at these ages tested. A possible explanation for the differences in nociceptive responding may lie in the explanation, that the genotype of each individual mouse strain may have dictated the behavioral phenotypic nociceptive expression evoked by formalin. The findings presented here, is not the first to demonstrate genetic differences in terms of nociceptive responding in the formalin test. A previous study, (Mogil, Lichtensteiger, & Wilson, 1998) have shown strain differences in nociceptive responding in the formalin test, between the moderately resistant 129/J, a substrain of the 129 mouse and the sensitive (C57BL/6J) inbred mouse strains. Converging, lines of evidence are accumulating that the phenotypic behavioral expression of pain sensitivity and pain inhibition may be governed in part by genetic influence.

l-Methadone was an effective analgesic at PD 3, 10 and 21 and dose dependently reduced formalin induced nociceptive responding, in both mouse strains in this study. This drug was also effective, in both phase of the formalin test, suggesting a possible role

of the μ-opioid receptor in both phase in this test. Previous reports have implicated, the μ-opioid receptor in the inhibition of dorsal horn activity induced by subcutaneous formalin injection (Dickenson & Sullivan, 1987b). In particular, the μ-opioid receptor agonist, morphine has been shown to reduce pain scores in both phase of the formalin test in infants (Abbott & Guy, 1995; Gupta, Cheng, Wang, & Barr, 2001; McLaughlin & Dewey, 1994). The data in the present study are consistent with previous reports, that revealed robust antinociceptive effects of opiate drugs in neonates (Enters, Guo, Pandey, Ko, & Robinson, 1991; McLaughlin & Dewey, 1994). These findings suggest, that the high density of μ-opioid receptor seen early in postnatal life (Rahman et al., 1998) may have influenced the analgesic effects exerted by l-methadone at PD 3 and 10 in this study.

d-Methadone was found to be minimally effective at PD 3 and 10 and totally ineffective at PD 21 in both mouse strains in the formalin test. Further, this compound was minimally effective only in the second phase of the formalin test, at PD 3 and 10. These results are suggestive of a possible role of the NMDA receptor in the formalin test, at the younger ages tested. The NMDA receptors have been shown to play a role in the second phase of the formalin test (Medvedev et al., 2004). d-methadone has been known to have functional in vivo NMDA receptor antagonistic activity (Ebert et al., 1998; Gorman et al., 1997). Additionally, this compound has been shown to be antinociceptive,

in the second phase of the formalin test in adult rodents (Shimoyama, Shimoyama, Elliott, & Inturrisi, 1997). The spinal cord has a greater number of NMDA receptors, during postnatal ontogeny (Gonzalez et al., 1993), in addition, electrophysiological studies revealed that during all ages, the NMDA receptors are functionally effective (Bardoni et al., 2000). The findings at the ages tested are consistent with the expression of the NMDA receptors during postnatal ontogeny. It is likely that the high density of NMDA receptors during early postnatal ontogeny correlates with there being more functional NMDA receptors, hence there are more d-methadone and NMDA receptor complexes, subsequently more analgesia.

dl-Methadone was less effective than l-methadone in the formalin test at all ages tested in this study, suggests that the effects seen with dl-methadone may be primarily due to the pharmacological activity of the l-enantiomer as the d-enantiomer has been shown to be weakly analgesic. As previously shown in adult rodents, the l-enantiomer possesses analgesic activity where as the d-enantiomer is weak or inactive as an analgesic (Ingoglia & Dole, 1970; Scott, 1948). Therefore, most of the analgesic effects seen with dl-methadone in the formalin test may be due to the effects of the l-enantiomer. The fact that dl-methadone chemically consists of 50% of the pharmacologically inactive

enantiomer, suggests that the inactive d-component of dl-methadone may be a contributing factor in dl-methadone being less effective as an analgesic than l-methadone.

dl-Methadone was effective in both phase of the formalin test, suggests at a first glance, that both μ-opioid and NMDA receptors may be playing a role in both phase of this test, as this compound has been shown to have pharmacological actions, at both μ-opioid and NMDA receptors (Ebert, Andersen, & Krogsgaard-Larsen, 1995). The results of this thesis showed that, the d-isomer was only minimally effective in the second phase of the formalin test, at the youngest ages tested, suggests that d-methadone may not be playing much of a role in dl-methadone's analgesic effectiveness in both phase of the formalin test. Therefore, the analgesic effects seen with dl-methadone may be attributed to the effects of the pharmacologically active l-enantiomer. Previous evidence have shown, that the analgesic effects obtained with dl-methadone may be due to the pharmacologically active l-enantiomer (Scott, 1948; Smits & Myers, 1974).

d-, l- and dl-Methadone were all found to be more efficacious at the two youngest age group tested than at the older age tested, at PD 21, this finding is consistent with previous findings that opiate drugs are generally more efficacious in infants (Abbott, Melzack, & Leber, 1982; Blass et al., 1993; Enters et al., 1991; Gupta et al., 2001; Johannesson &

Becker, 1973; McLaughlin & Dewey, 1994; McLaughlin et al., 1990). This finding suggests that the efficacy of d-, l- and dl-methadone during postnatal ontogeny may depend on the pharmacodynamics of the μ-opioid and the NMDA receptor densities. As the animal matures the densities of μ-opioid and the NMDA levels drop to adult levels, hence the resultant analgesic effectiveness is less. These finding are in direct support of the first hypothesis in this thesis that d-, l- and dl- methadone may result in greater analgesic effectiveness in infants than in adults. Other alternative explanations that may account for the greater analgesic efficacy of d-, l- and dl-methadone at PD 3 and 10 than at PD 21 may be explained that the analgesic effectiveness at these ages may be influenced by pharmacokinetic factors or an interaction of pharmacokinetic and pharmacodynamic factors.

In summary, the findings in this chapter supported the hypothesis, that dl-methadone and its isomers may result in greater analgesic effectiveness in infants than in adults. Also, the findings presented here have demonstrated, that l- and dl-methadone are effective analgesics in the formalin test, a rodent model of inflammatory pain. That these two compounds, are effective analgesics in both phase of the formalin test, are arguing for possible roles of the μ-opioid and the NMDA receptors in the formalin test. That d-methadone is not a particularly effective analgesic in infants in tonic nociception, as this

compound resulted in minimal analgesic effectiveness in the second phase of the formalin test. That d-, l- and dl-methadone resulted in comparable analgesia in both mouse strains suggested that the putative receptor deficits were not influencing dl-methadone and its isomers analgesic effectiveness in infants.

CHAPTER 3

Postnatal Analgesic Effects of Racemic Methadone and its Isomers in the Thermal Test in the 129/SvEv and the CD-1 Mouse Pups

The aim of this chapter was to describe the analgesic abilities of racemic methadone and its isomers in an acute or phasic model of thermal nociception. This model differs from the tonic model of formalin nociception, as anatomical data suggested that phasic and tonic pain may be differentially mediated. Thermal nociception was first suggested to be mediated, along specific anatomical sites in the central nervous system (Ryan, Watkins, Mayer, & Maier, 1985). Lesions of the caudal periaqueductal gray and the nucleus raphae magnus (NRM) attenuated morphine-induced antinociception in phasic nociception but not in tonic nociception (Abbott, Melzack, & Leber, 1982). Further, pharmacological data suggested that phasic and tonic pain may also be differentially mediated. Opiate compounds may exert differential analgesic effects in different pain states in infants than in adults (McLaughlin et al., 1990).

Opiate drugs have been shown to be effective analgesics in infant animal models of pain (Abbott, Melzack, & Samuel, 1982; Auguy-Valette et al., 1978; Dubuisson & Dennis, 1977; M Fitzgerald, 1994; Giordano & Barr, 1987; McLaughlin et al., 1990; Thornton et al., 1998) and for the critically ill neonates (Anand & Craig, 1996; Johnston, Collinge, Henderson, & Anand, 1997). In particular, the μ-opioid receptor has been shown to be involved in thermal nociception, an acute nociceptive test (Auguy-Valette et al., 1978; Thornton et al., 1998). Morphine, a μ-opioid receptor agonist, is the prototypic

opiate, with powerful analgesic effects at the μ-opioid receptor, however, simultaneously exerting unwanted side effects such as; withdrawal, dependence and tolerance (Suresh & Anand, 1998; Zahorodny et al., 1998) which render its use as an analgesic very limited. The NMDA receptor has also been implicated in phasic nociception and may potentiate the effects of μ-opioid agonists (Redwine & Trujillo, 2003), thus reducing the addictive side effects of μ-opiate agonists, such as morphine. Therefore, it is necessary to investigate compounds with both μ-opioid agonistic and NMDA antagonistic effects in maximizing analgesia, while simultaneously reducing unwanted side effects. One such compound with pharmacological effects at both μ-opioid and NMDA receptors that may maximize analgesia, while simultaneously reducing unwanted side effects is dl-methadone. Further it is also necessary to elucidate the analgesic effects of each enantiomers of dl-methadone in phasic pain, so as to determine the individual contribution of each enantiomer of the dl-mixture in analgesia. For example, dl-methadone and its two enantiomers are unique opiate compounds with pharmacological actions at both the μ-opioid and NMDA receptors (Ebert et al., 1995; Gorman et al., 1997). Additionally, each enantiomer of methadone may exert stereospecific analgesic effects at either the μ-opioid and NMDA receptors and may be adequately effective in alleviating pain of differing origins while minimizing potential side effects (Smits & Myers, 1974).

The 129/Sv/Ev mouse pups will be used in conjunction to the CD-1 pups to describe the roles of dl-methadone and its isomers during postnatal ontogeny, as the 129/SvEv mouse has been hypothesized to have putative deficits of the μ-opioid or the NMDA receptors.

METHODS

Acute Thermal Test

Thermal tests. The 129/SvEv and the CD-1mouse Pups (n= 8 litters per age and per drug) were administered saline (0.0), or one of the four doses 0.3, 1.0, 3.0 and 10.0 mg/kg of each drug in a cumulative dose response paradigm. The cumulative dose response paradigm, entailed each animal being subcutaneously injected with the same drug at incremental doses at half-hour intervals, until all five doses were administered to the same animal.

RESULTS

Four way analysis of variance (ANOVA) revealed no strain differences between the 129/SvEv and the CD-1 mouse pups, on nociceptive and antinociceptive responding in the thermal test for l-methadone $\underline{F}$ (1,30)=1.493, $p > 0.05$; for dl-methadone $\underline{F}$ (1,38)=1.117, $p > 0.05$; and d-methadone. Figures: 12 -14 and 15 -16. depicted the dose

response relationships of the effects of dl-methadone and its isomers in the three appendages tested in the 129/SvEv and CD-1 mouse pups respectively, at PD 3,10 and 21 in the thermal test of nociception. ANOVA also revealed no significant interaction of dose, age, strain and limb for l-methadone, for d-methadone and for dl-methadone $p<$ 0.05. Figures 17 and 18 depicted comparable analgesic effects of dl-methadone and its isomers at all ages tested in both mouse strains in 3 and 21 day old respectively. Figures 19 and 20; demonstrated the % MPE and is a linear display of the data. The % MPE values were used in the constuction of the ED_{50} values. Table 1 displayed the ED_{50} values, lower ED_{50} values were obtained at the younger ages tested in both mouse strains for all drugs tested indicating that all drug was more effective at the younger ages. dl-Methadone resulted in potentiated analgesia at PD 3 and 10 but not at PD 21 is extrapolated from similar ED_{50} value of l-metahdone and dl-methadone at PD 3 and 10 but not at PD 21.

Four way ANOVA revealed that l-methadone administered subcutaneously in both mouse strains, significantly increased nociceptive latencies relative to vehicle treatment across doses $\underline{F}$ (4,120)=233.19, $p<$ 0.005, across age $\underline{F}$ (1,30)=66.823, $p<$ 0.005, across limb $\underline{F}$ (2,60)=19.887, $p<$ 0.005. The three way ANOVA revealed a significant main effect of age in the 129/SvEv pups $\underline{F}$ (1,16)=15.327, $p<$ 0.005 and in the CD-1 pups $\underline{F}$

(1,14)=50.967, $p< 0.005$. Also, a significant main effect of dose was also revealed in the 129/SvEv pups F (4,64)=160.899, $p< 0.005$ and for the CD-1 pups F (4,56)=85.376, $p< 0.005$. The contrast for post hoc analyses revealed that at PD 3, in both mouse strains, l-methadone exerted significant analgesic effects at 1.0 to 10.0 mg/kg doses, as the analgesic effects at these doses were statistically different from saline control, $p < 0.05$. At PD 21 only the highest dose of l-methadone exerted significant analgesic effect in both mouse strain, $p < 0.05$.

dl-methadone administered subcutaneously, significantly increased nociceptive behavioral latencies relative to vehicle treatment for both mouse strains across doses F (4, 152)=130.269, $p< 0.005$; across age F (2,30)=40.393, $p< 0.005$ and across limb F (2,760=32.239, $p< 0.005$. Subcutaneous administration of dl-methadone at PD 3 and 10 significantly shifted the dose response curve to the left (Figure 12 and 13). The three way ANOVA revealed a significant main effect of age in the 129/SvEv pups F (2,24)=15.396, $p< 0.005$ and in the CD-1 pups F (1,14)=68.028 $p< 0.005$. Also, a significant main effect of dose was also revealed in the 129/SvEv pups F (4,96)=139.692, $p< 0.005$ and for the CD-1 pups F (4,56)=39.963, $p< 0.005$. The contrast for post hoc analyses revealed that dl-methadone exerted significant analgesia at all ages tested, $p< 0.005$ and at lower doses in the younger animals than at PD21.

d-Methadone was totally ineffective at PD 21 in both mouse strains when administered subcutaneously. Subcutaneous administration of this compound significantly increased behavioral nociceptive latencies relative to vehicle treatment across doses in the CD-1 pups $\underline{F}$ (4,56)=7.575; $p< 0.005$, across age in the 129/SvEv pups $\underline{F}$ (2,15)=9.208 < 0.005 and in the CD-1 pups $\underline{F}$ (1,14)=11.173; $p < 0.005$. The contrast for post hoc analyses revealed that this compound exerted significant analgesia only at the highest dose tested at PD 3 and 10 in the 129/SvEv pups and at PD 3 in the CD-1 pups as CD-1 pups were not tested at PD 10 in this mouse strain. A significant interaction of dose by age was observed in the 129/SvEv pups $\underline{F}$ (8,84)=7.386; $p< 0.005$ and in the CD-1 pups $\underline{F}$ (4,56)=10.253; $p< 0.005$.

Figure 12. Showed the effects of subcutaneous administration of d-, l- and dl-methadone on forepaw, hindpaw and tail withdrawal latencies from thermal water baths at temperatures of 48^0 C, 50^0 C, and 52^0 C in 129/SvEv mouse pups at PD 3. Data are collapsed over temperature and are means (+/- one SEM). l-Methadone was the most effective of the forms of this compound tested in all appendages and d-methadone was totally ineffective.

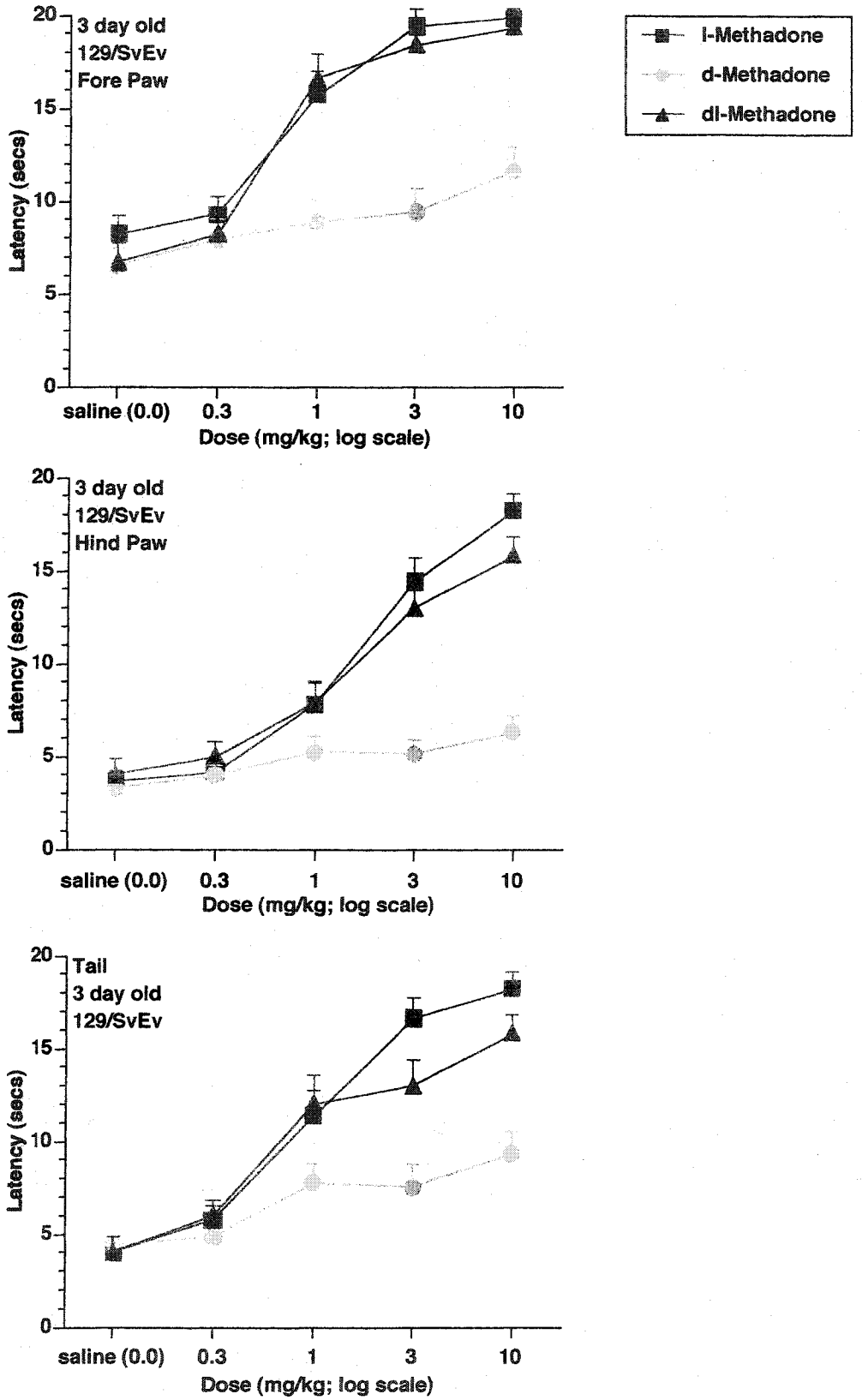


Figure 13. Showed the effects of subcutaneous administration of d-, l- and dl-methadone on forepaw, hindpaw and tail withdrawal latencies from thermal water baths at temperatures of 48^0C, 50^0C, and 52^0C in CD-1 mouse pups at PD 10. Data are collapsed over temperature and are means (+/- one SEM). dl-Methadone was as effective as l-methadone and d-methadone was the least effective drug tested at this age in all appendages.

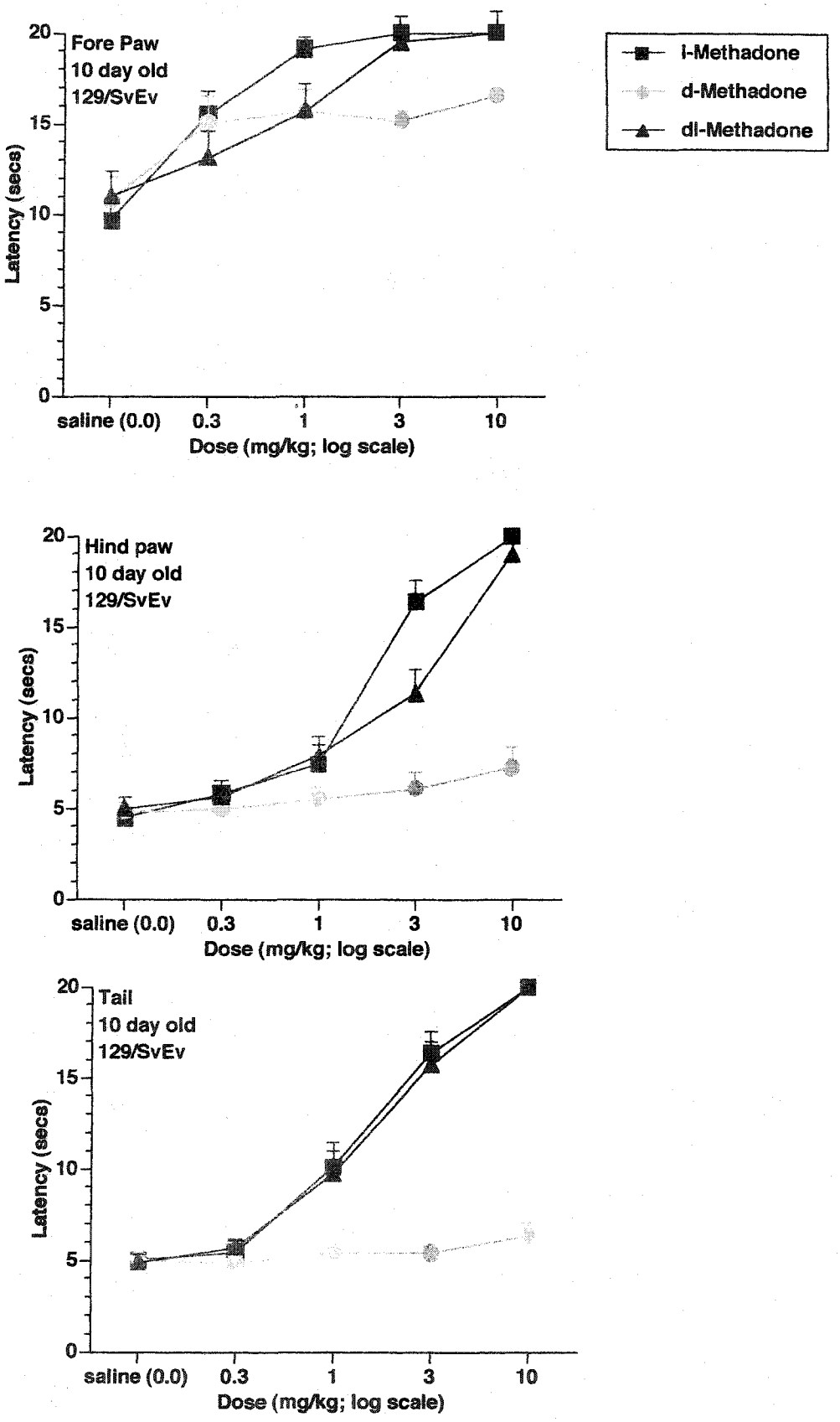


Figure 14. Showed the effects of subcutaneous administration of d-, l- and dl-methadone on forepaw, hindpaw and tail withdrawal latencies from thermal water baths at temperatures of 48^0 C, 50^0 C, and 52^0 C in CD-1 mouse pups at PD 21. Data are collapsed over temperature and are means (+/- one SEM). l-Methadone was the most effective of the forms of this compound tested in all appendages and d-methadone was totally ineffective.

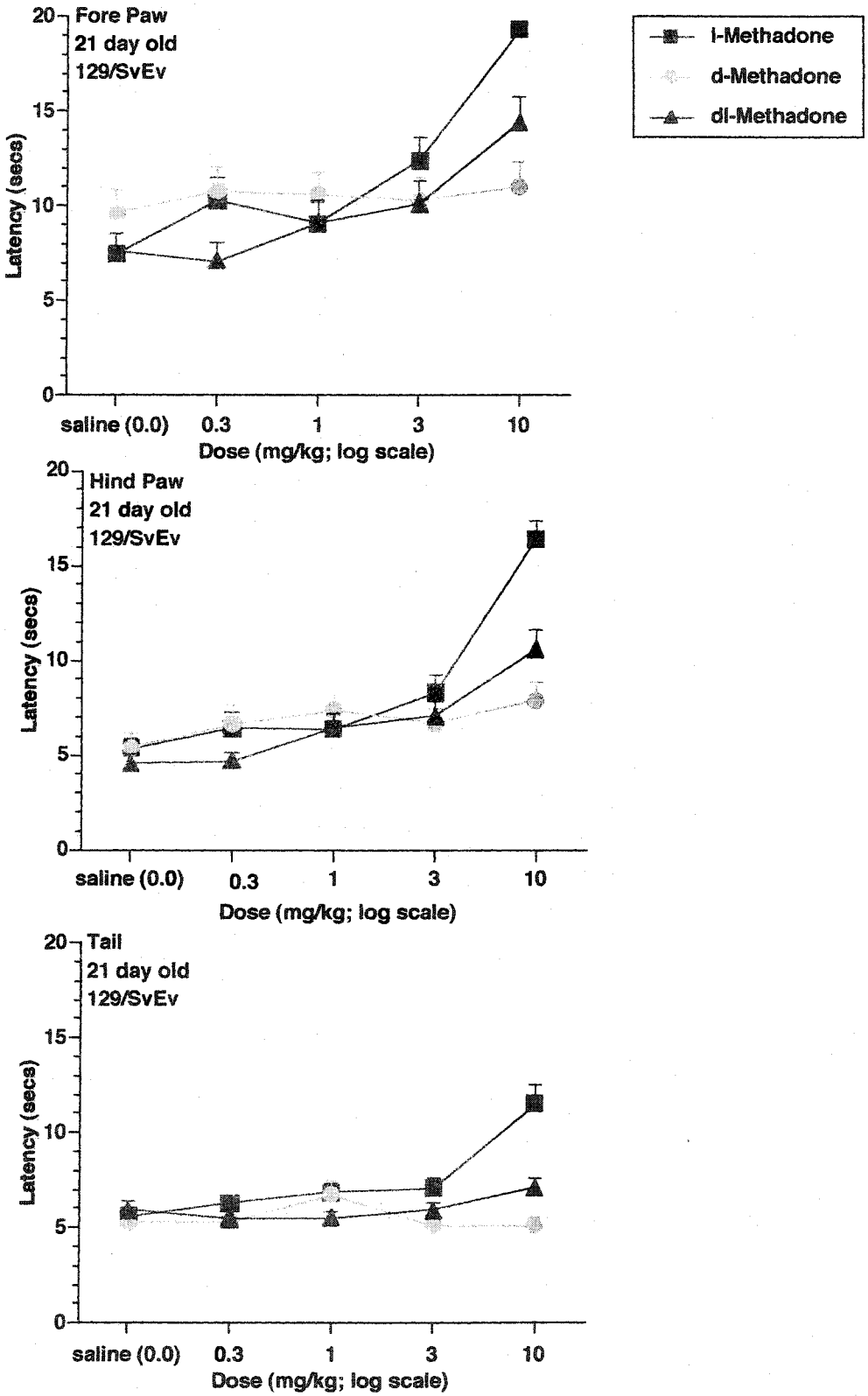


Figure 15. Showed the effects of subcutaneous administration of d-, l- and dl-methadone on forpaw, hindpaw and tail withdrawal latencies from thermal water baths at temperatures of 48^0 C, 50^0 C, and 52^0 C in CD-1mouse pups at PD 3. Data are collapsed over temperature and are means (+/- one SEM). l-Methadone was the most effective of the forms of this compound tested in all appendages and d-methadone was totally ineffective.

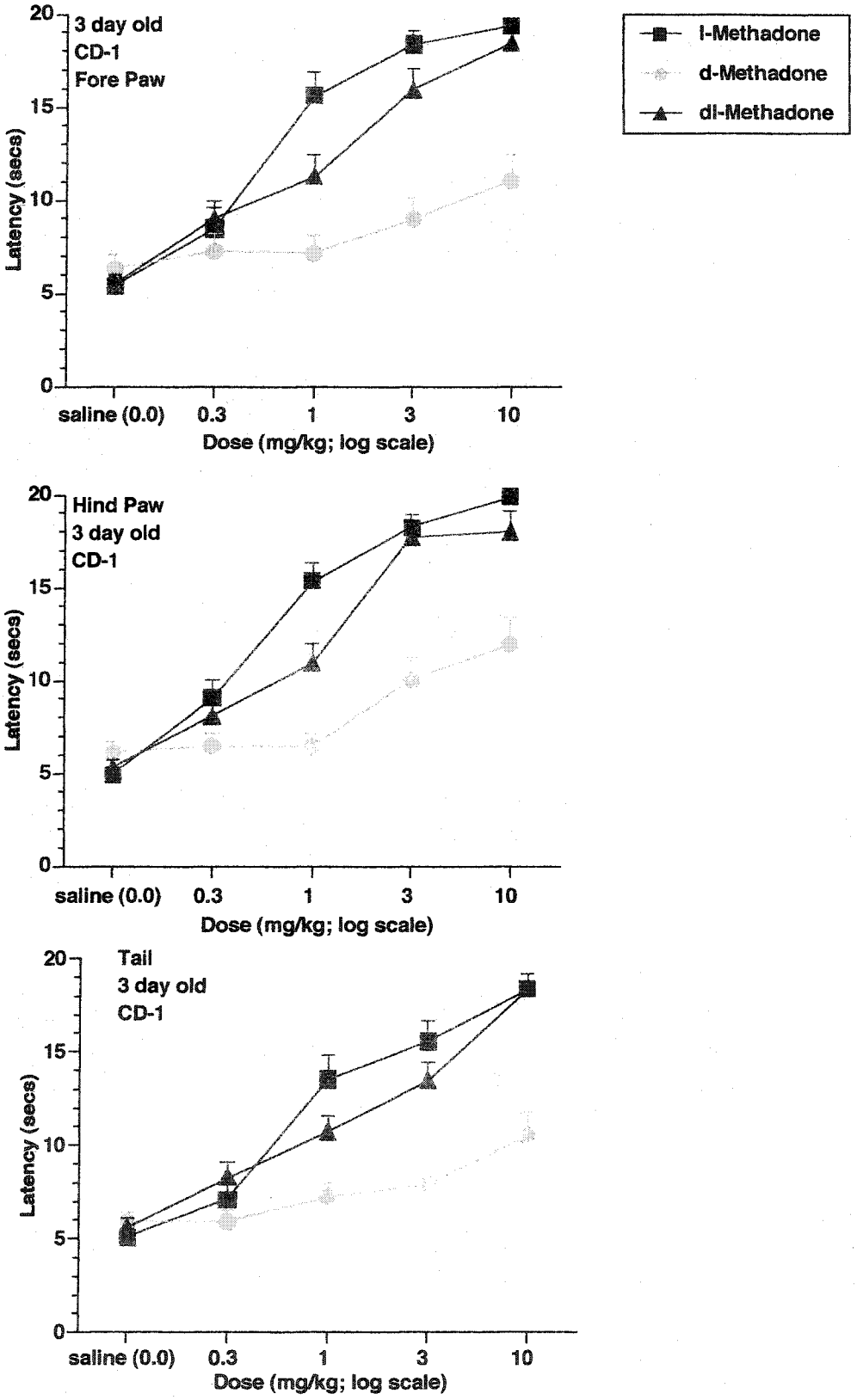


Figure 16. Showed the effects of subcutaneous administration of d-, l- and dl-methadone on forepaw, hindpaw and tail withdrawal latencies from thermal water baths at temperatures of 48^0C, 50^0C, and 52^0C in CD-1 mouse pups at PD 21. Data are collapsed over temperature and are means (+/- one SEM). l-Methadone was the most effective of the three forms of this compound in all appendages tested and d-methadone was totally ineffective.

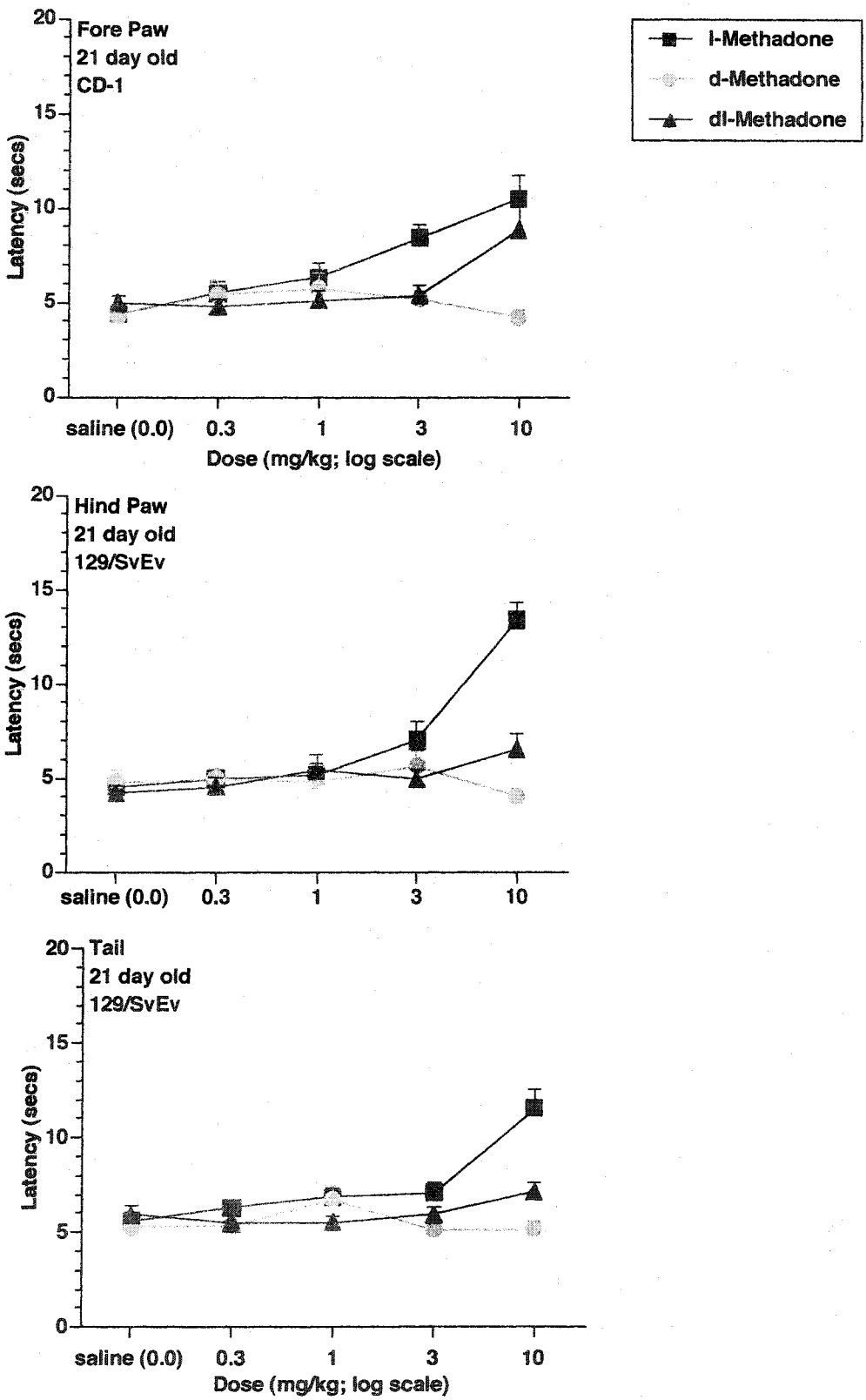


Figure 17. A comparison of the effects of subcutaneous administration of l-,d- and dl-methadone in both 129/SvEv and CD-1 mouse pups at PD 3. Data are collapsed over temperature and are means (+/- one SEM). dl-methadone was as effective as l-methadone and d-methadone was the least effective drug at PD3 in both mouse strains. l-Methadone was the most effective drug and d-methadone was ineffective at PD 21 in both mouse strains.

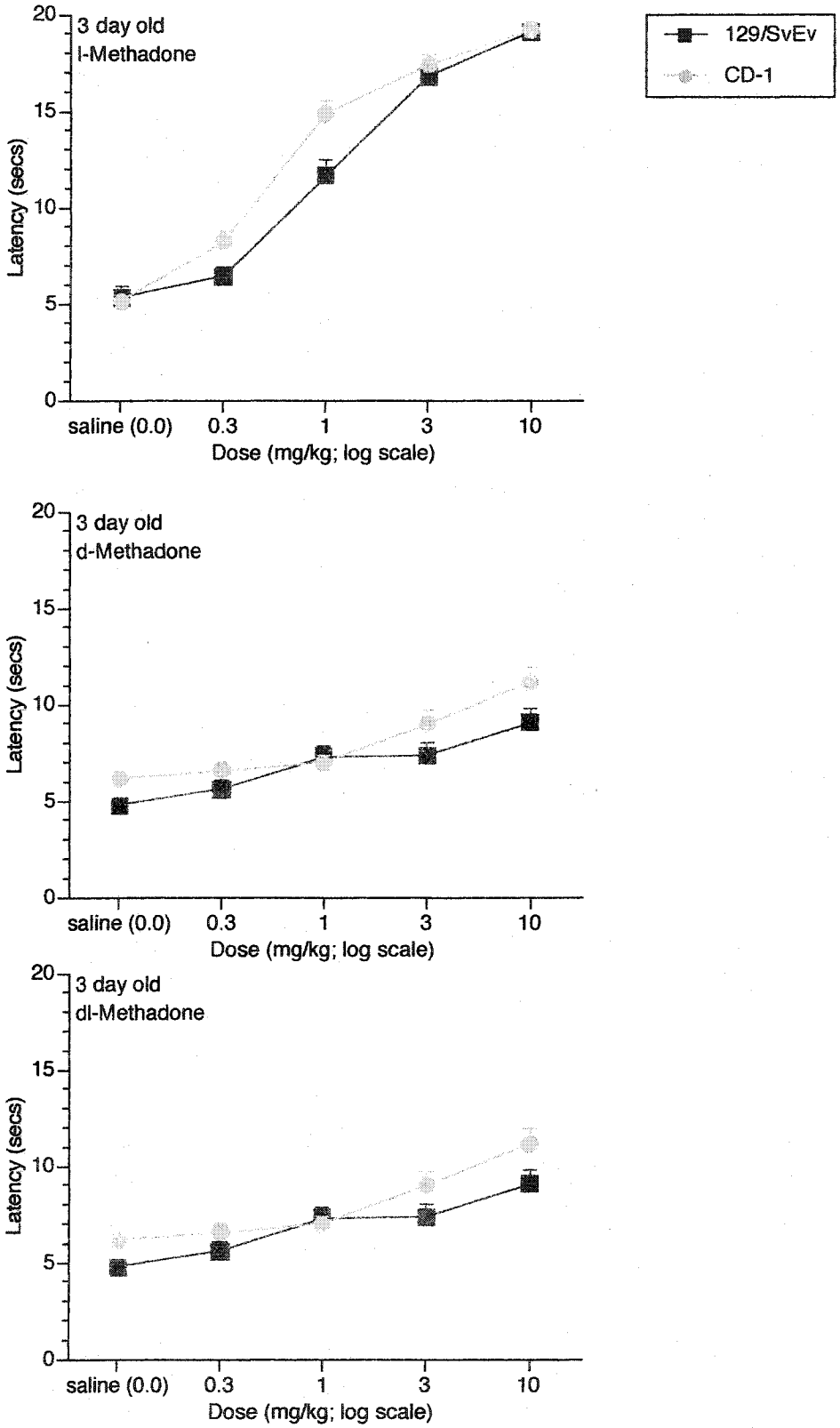


Figure 18. A comparison of the effects of subcutaneous administration of l-,d- and dl-methadone in both 129/SvEv and CD-1 mouse pups at PD 21. Data are collapsed over temperature and are means (+/- one SEM). dl-Methadone was as effective as l-methadone and d-methadone was the least effective drug at PD 21 in both mouse strains. l-Methadone was the most effective drug and d-methadone was ineffective at PD 21 in both mouse strains.

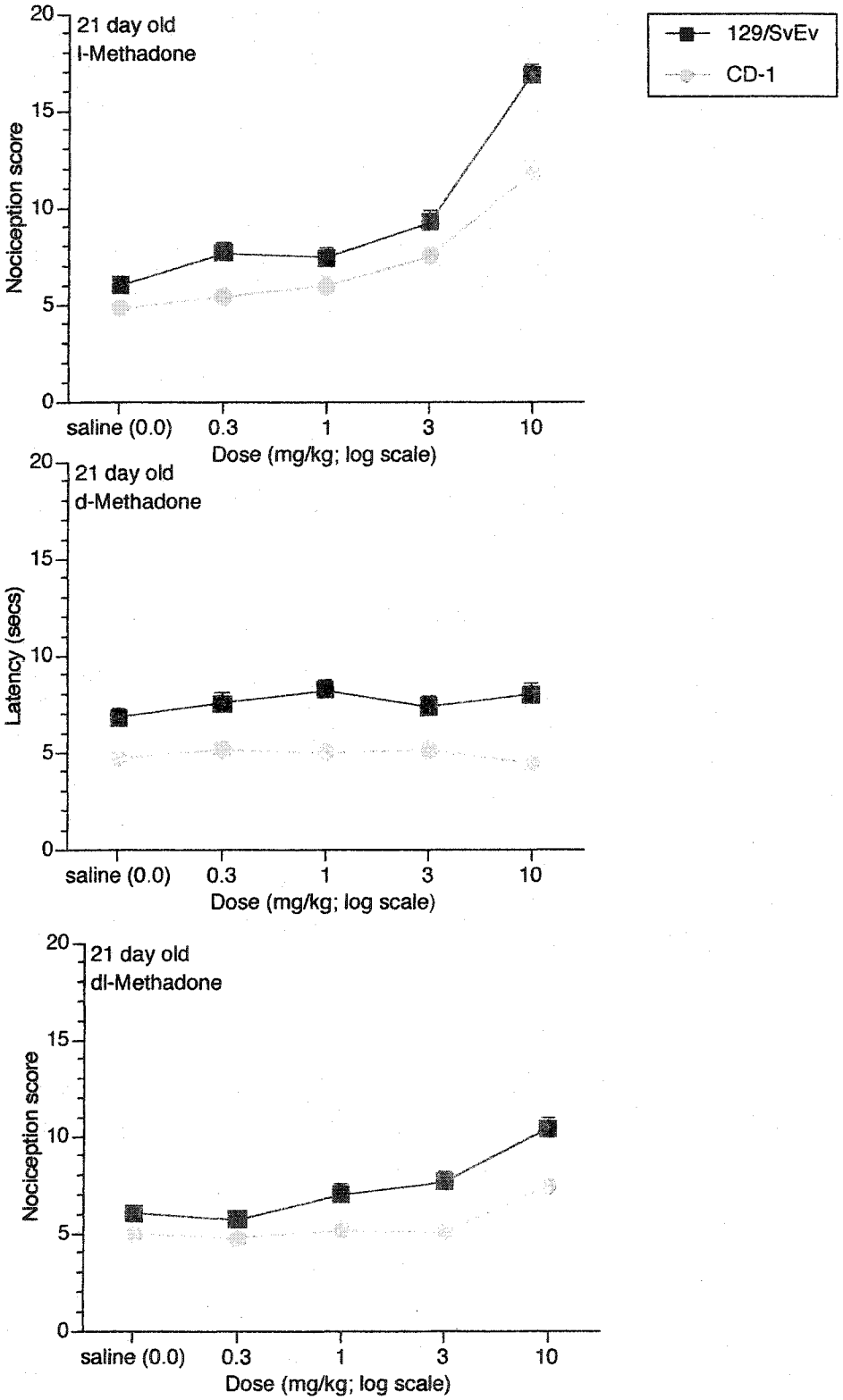


Figure 19. A comparison of the % MPE of l-, d- and dl-methadone in 129/SvEv and CD-1 mouse pups at PD 3. Data are collapsed over temperature and are means (+/- one SEM). dl-Methadone was as effective as l-methadone and d-methadone was the least effective drug at PD 3 in both mouse strains.

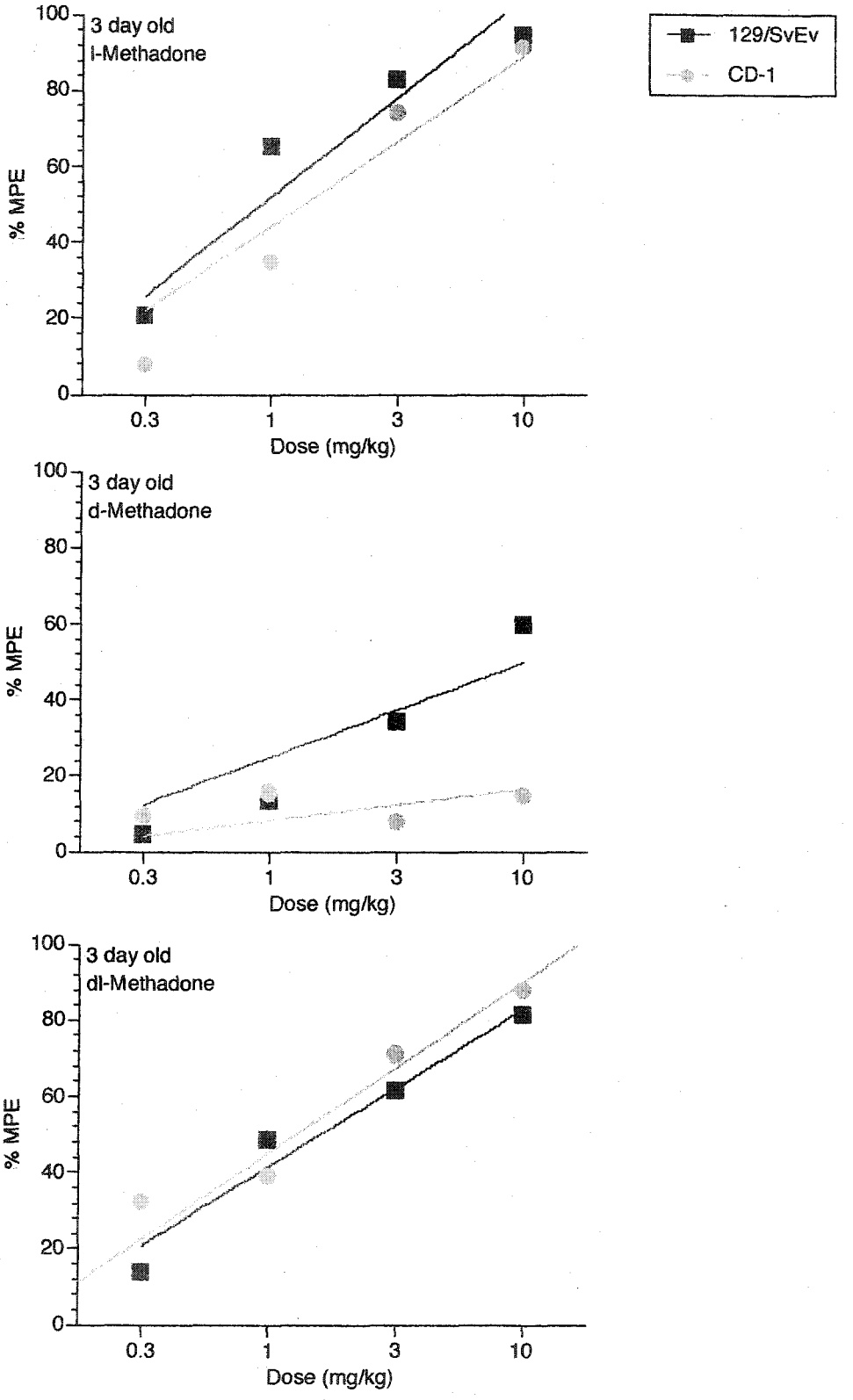


Figure 20. A comparison of the % MPE of l-, d- and dl-methadone in 129/SvEv and CD-1 mouse pups at PD 21. Data are collapsed over temperature and are means (+/- one SEM). l-Methadone was the most effective drug and d-methadone was ineffective at PD 21 in both mouse strains.

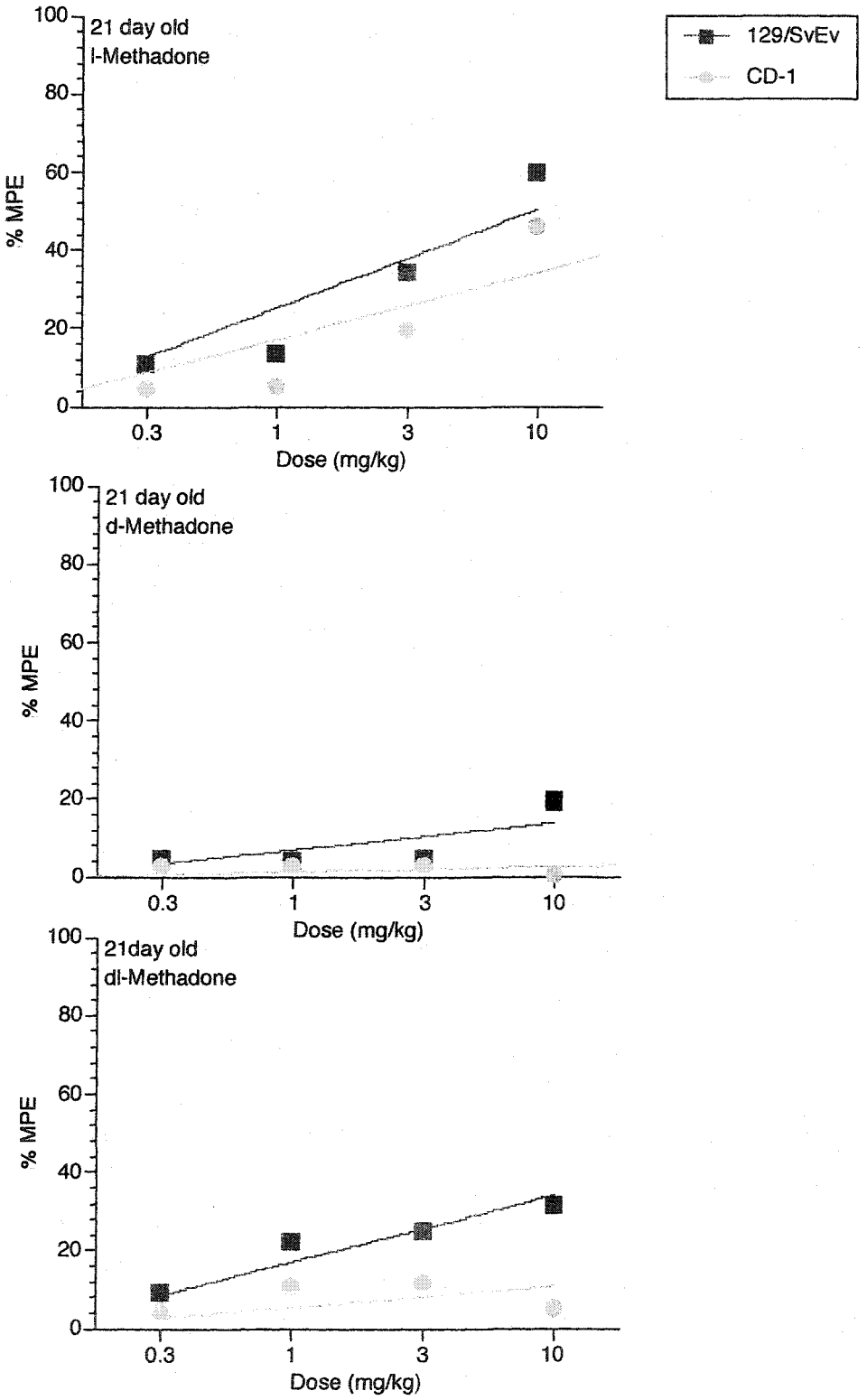


Table 1. A Comparison of the Antinociceptive Potencies of d-, l- and dl-methadone in the thermal test in 129/SvEv and CD-1 mouse pups at 3 and 21 days old.

Table 1

Strain	Drug	3 day old	21 day old
129/SvEv	**l-Methadone**	0.065 (-0.052-0.182)	0.772 (0.584-0.857)
129/SvEv	**dl-Methadone**	0.145 (-0.024-0.313)	1.325 (1.185-1.465)
129/SvEv	**d-Methadone**	1.274 (1.080-1.468)	1.782 (1.552-2.012)
CD-1	**l-Methadone**	0.156 (-0.315-0.002)	1.070 (0.937-1.204)
CD-1	**dl-Methadone**	0.135 (-0.029-0.299)	1.650 (1.441-1.859)
CD-1	**d-Methadone**	1.154 (0.930-1.378)	2.778 (2.130-3.426)

DISCUSSION

The study in this chapter, investigated the analgesic abilities of l-,d- and dl-methadone, during postnatal ontogeny, at PD 3,10 and 21 in the 129/SvEv and CD-1 mouse pups, in the thermal or phasic nociceptive test. dl-Methadone and its isomers exerted comparable analgesia in both mouse strains at all ages tested, therefore the analgesic effects seen with dl-methadone and its isomers may be due to the developmental effects of the μ-opioid and NMDA receptors rather than the deficits of these receptors. In general, the present results showed that, d-, l- and dl-methadone were more effective at PD 3 and10 than at PD 21 in both mouse strains (PD 3 and PD 10 age groups are representative of infants and the PD 21 age group is representative of adults). These findings are in support of the second hypothesis, that dl-methadone and its isomers may exert greater analgesic effectiveness in infants than in adults. Further, these results are consistent with prior findings, that showed that opiate drugs are generally more efficacious in infants, than in adults (M Fitzgerald, 1994; Fitzgerald, 1995). Additionally, the results of this study showed, that the relative effectiveness of the three compounds tested at PD 21, is consistent with substantial prior data (Scott, 1948; Smits & Myers, 1974; Thorp, 1949), where l-methadone was more effective than dl-methadone and d-methadone was the least effective. An interesting new finding of this study was that dl-methadone resulted in potentiated analgesia at PD 3 and 10, its analgesic effects

were similar to those of l-methadone at these two age groups. This was the first finding that demonstrated, that dl-methadone resulted in potentiated analgesia, previous studies in adults have shown that, l-methadone was twice as effective as dl-methadone (Ingoglia & Dole, 1970; Scott, 1948; Thorp, 1949). Apart from this unique finding at PD 3 and 10, that dl-methadone resulted in potentiated analgesia in both mouse strains, the remaining findings in this chapter are consistent with those of adults or are consistent with those of infants.

dl-Methadone resulted in potentiated analgesia and was almost as effective as l-methadone at PD 3 and PD 10 but not at PD 21 in the three appendages tested (fore paw, hind paw and tail) in both mouse strains. At a first glance, this finding seemed to suggest that the effects at PD 3 and 10 may be as a result of the interaction of the μ-opioid and NMDA receptor systems. The idea that the μ-opioid receptors are functionally interacting with the NMDA receptors in analgesia is not new. Previous studies have shown that the NMDA receptor antagonists have potentiated the analgesic effects of opiate agonists (Chapman & Dickenson, 1992; Mao, Price, Caruso, & Mayer, 1996). This dissertation attempted to provide a possible mechanism for dl-methadone's potentiated analgesic effect in the study in the following chapter, based on the idea, that the μ-opioid and the NMDA receptors were functionally interacting at PD 3 in the exertion of the potentiated analgesia seen with dl-methadone. In this study morphine, a μ-opiate agonist and

MK801, an NMDA antagonist were co-administered in an effort to mimic the effects of dl-methadone. Like morphine, l-methadone is an opiate agonist with analgesic actions at the μ-opioid receptor (Neil, 1984b). Like d-methadone, MK801 is an NMDA antagonist (Ebert et al., 1995; Gorman et al., 1997) and the effects of these two compounds, when co-administered together, may result in potentiated analgesia, as was seen with dl-methadone at PD 3.

l-Methadone, dose dependently reduced thermal nociception at all ages tested (PD 3,10 and 21) in all three appendages (fore paw, hind paw and tail). Like morphine, l-methadone, an opiate drug with analgesic actions at the μ-opioid receptor (Neil, 1984a). The fact that l-methadone was shown to be analgesic in this dissertation, in the thermal test, at PD 3,10 and 21 are suggestive of a possible role of the μ-opioid receptor in the analgesic mechanism in infants, in the thermal test. This drug was more efficacious at PD 3 and 10, suggesting that opiate drugs are more efficacious in infants and is consistent with prior data (Blass et al., 1993).

d-Methadone was only minimally effective as an analgesic at PD 3 and PD 10 and totally ineffective at PD 21 in the thermal test of nociception, in all three appendages tested (fore paw, hind paw and tail). These findings may be explained, by a possible mechanism of action of d-methadone at the NMDA receptor, during postnatal ontogeny.

d-Methadone has been shown to have NMDA receptor antagonist activity (Ebert et al., 1995; Gorman et al., 1997) and during postnatal ontogeny, the spinal cord has a higher concentration of NMDA receptors in the grey matter than those of adults where the density of NMDA receptors decline as the animal matures. (Gonzalez et al., 1993). The data presented here suggests a relationship between the density of NMDA receptors expressed in the spinal cord and the antinociceptive potency of d-methadone. As the animal matures, the density of NMDA receptor level drops and antinociceptive potency of d-methadone is subsequently reduced.

In sum, this chapter has shed some light on the following; it has casted some doubt that the μ-opioid and NMDA receptor deficits were influencing l-, d- and dl-methadone's analgesic effectiveness during postnatal ontogeny, as there were no strain differences in the analgesic effectiveness of l-, d-and dl-methadone. The unique and interesting finding, that dl-methadone resulted in potentiated analgesia at PD 3 but not at PD 21 in the two mouse strains, provides strong support for the potentiated effect seen with dl-methadone. Both the analgesic effectiveness of l-, d- and dl-methadone and the rank order of analgesic effectiveness of l-methadone, being the most effective and d-methadone being least effective drug is convincing as these effects has been shown in both mouse strains. This study provides strong support, that μ-opiate drugs are involved in analgesic

mechanisms of l-and dl-methadone as these compounds exerted analgesic effects in both mouse strains. This study provides strong support, that d-methadone is not a particularly effective analgesic in infants.

CHAPTER 4

Co-administration of Morphine and MK801 at Postnatal Day 3 in 129/SvEv and CD-1 Mouse Pups

This chapter will be an attempt to provide a possible mechanism for dl-methadone's potentiated analgesic effect during early postnatal ontogeny in the thermal test of nociception. A likely mechanism for this potentiated analgesic effect may be explained by a possible interaction of the μ-opioid and the NMDA receptor systems during early postnatal development. Both the μ-opioid and the NMDA receptors have been shown to be involved in pain and analgesic mechanisms (Marsh et al., 1997). Further these receptors have been shown to co-localize on the same neuron in the dorsal horn, an anatomical structure that is involved in pain processing (Aicher et al., 2002). dl-Methadone's potentiated analgesic effect may be explained by an interaction of these two receptor systems. Moreover, the involvement of the interaction of the μ-opioid and the NMDA receptor systems in analgesic mechanisms had been previously demonstrated in adult rodents. For example, previous studies have shown that NMDA receptor antagonists have potentiated the analgesic effects of opiate agonists (Chapman & Dickenson, 1992; Mao et al., 1996), although not in infants. Spinally administered opioids are thought to diminish the release of neurotransmitters such as glutamate or peptides from small primary afferent fibers (Dickenson & Sullivan, 1986; Kangrga & Randic, 1991; Malmberg & Yaksh, 1992). The ability of opioids to act presynaptically on C-fiber terminals to reduce transmitter release produces synergistic inhibitions with postsynaptically acting NMDA receptor antagonists (Dickenson, 1997). NMDA antagonists significantly increase morphine's antinociceptive effect on thermal stimuli

(Plesan, Hedman, Xu, & Wiesenfeld-Hallin, 1998) in adults.

Racemic methadone is a 50/50 mixture of the pharmacologically inactive d- and the pharmacologically active l-enantiomers of methadone. This compound may exert pharmacological actions at both the μ-opioid and the NMDA receptors due its characteristic chemical composition. For example, the l-enantiomer is primarily a μ-opioid receptor agonist (Ingoglia & Dole, 1970; Kristensen et al., 1995; Scott & Orr, 1969) and the d-enantiomer is an NMDA receptor antagonist (Chizh et al., 2000; Ebert et al., 1998; Gorman et al., 1997).

Morphine, a μ-opioid receptor agonist with both pre- and post synaptic action to the primary afferent is well known to have antinociceptive effects on acute pain (Yaksh & Rudy, 1976). MK801, a potent NMDA receptor antagonist has been tested in many animal models, as a prototypic NMDA antagonist with high potency and free CNS penetration (Wong et al., 1986). The co-administration of the μ-opiate agonist, morphine and the NMDA antagonist, MK801 will be studied here, as the co-administration of these compounds may mimic the effects of dl-methadone. Like l-methadone, morphine is a μ-opiate agonist (Yaksh & Rudy, 1976) and like d-methadone, MK801 is an NMDA antagonist (Wong et al., 1986). It was hypothesized that by combining these two agents,

morphine and MK801, they may exert potentaited analgesia similar to that of dl-methadone during early postnatal development.

The 129/SvEv mouse has been reported to have putative deficits of either the μ-opioid receptor or the NMDA receptor, therefore the CD-1 mouse was used in conjunction to the 129/SvEv mouse, to describe the potentaited analgesic effects of co-administered morphine and MK801 in the thermal test of nociception.

Method

Morphine and MK801 Co-administration

Co-administration: Each of four 129/SvEv or CD-1 mouse pups in a single litter (n= 8 129/SvEv and n= 6 CD-1 litters) at PD 3 were subcutaneously injected with either saline (0.0), or one of three doses (0.001, 0.003 and 0.03 mg/kg) of MK801. Fifteen minutes post MK801 injection, each animal was intraperitoneally injected with saline and one of four doses of morphine (0.003, 0.01, 0.03 and 0.3 mg/kg). Behavioral testing occurred 15 minutes post morphine injection, in water baths at temperatures of 48°, 50°, and 52°C. A cut off latency of 20 seconds was used to avoid tissue damage.

Experimental Design

The experiment in this chapter, was designed to determine the combined dose-dependence analgesic actions of morphine and MK801 in the thermal test of nociception in the 129/SvEv mouse and CD-1 mouse pups at PD 3. All doses of morphine and MK801 were studied in a single litter.

Statistics

The variable, mouse strain was treated as a between group variable and the combined effects of MK801 and saline together with all of the morphine doses were treated as a repeated measures variable. A four way ANOVA was performed on morphine doses, MK801 doses, limb and strain and a three way ANOVA was performed on morphine dose, MK801 dose, limb followed by the contrast for post hoc analyses. All data was collapsed over temperature, as there was no drug effect on temperature.

RESULTS

The co-administered effects of MK801 on morphine's analgesia at PD 3 in the 129/SvEv and the CD-1 mouse pups are depicted in Figures 21 and 22 respectively. The four way ANOVA revealed a significant main effect of morphine in both mouse strains F

(4,48)=25.376, $p < 0.05$ and also a significant main effect of MK801 in both mouse strains F (3,36)=5.312, $p < 0.05$. MK801 significantly antagonized morphine's analgesic effects in both mouse strains (morphine by MK801 by strain) F (4,48)=12.485, $p < 0.05$, (Figure 21 and 22). The contrast for post hoc analyses revealed that morphine induced significant analgesia in pups of both mouse strains. Morphine's effect was significantly greater in the 129/SvEv mouse strain than in the CD-1 mouse strain (morphine by strain interaction) F (4,48)=12.485, $p< 0.05$, followed by contrast for post hoc analyses $p< 0.05$. MK801 antagonistically reduced morphine's analgesic effects Figures 21 and 22. Morphine and MK801 did not result in potentiated analgesia in either mouse strains Figures 21 and 22.

Figure 21. Depicting data on the co-administered effects of morphine and MK801 in 129/SvEv mouse pups at PD 3. Data presented are means (+/- SEM). Morphine was an effective analgesic but MK801 was not. MK801 antagonized the antinociceptive effects of morphine.

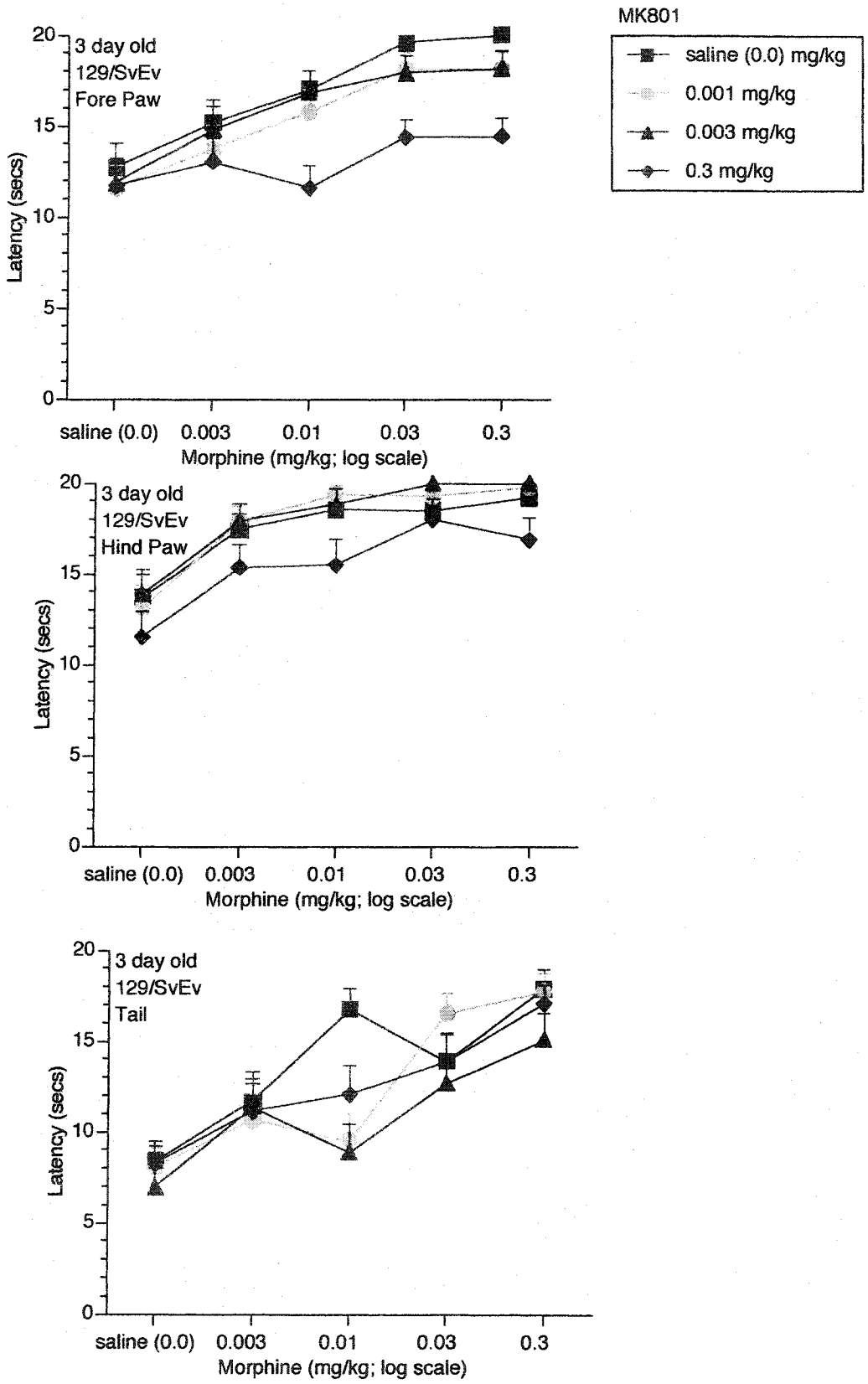
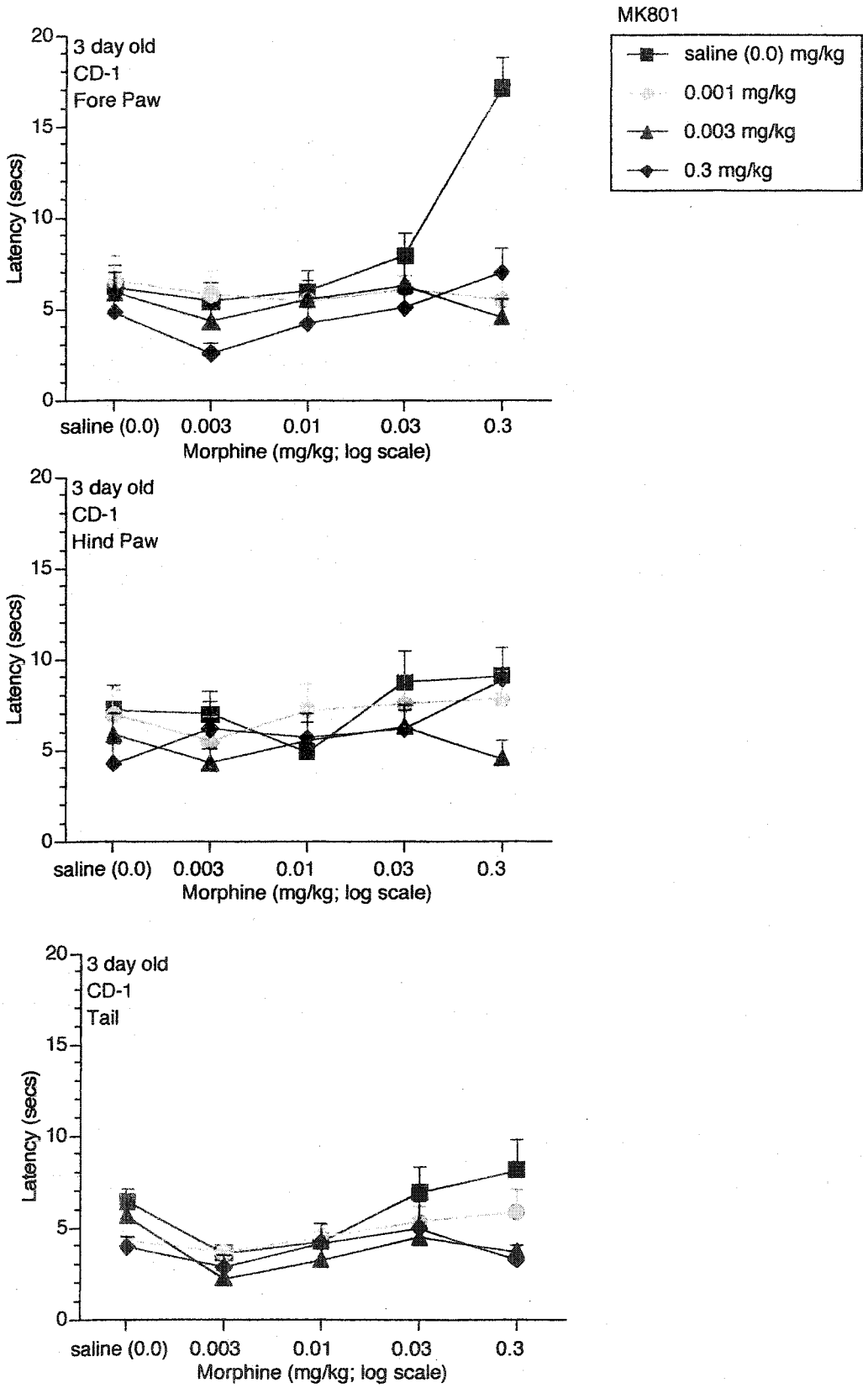


Figure 22. Depicting data on the co-administered effects of morphine and MK801 in CD-1 mouse pups at PD 3. Data presented are means (+/- SEM). Morphine was an effective analgesic but MK801 was not. MK801 antagonized the antinociceptive effects of morphine.



DISCUSSION

The co-administration of morphine and MK801 were studied at PD 3 in the 129/SvEv and CD-1 mouse pups in the thermal nociceptive test. This experiment was pursed in an attempt to replicate the findings in the 129/SvEv and CD-1 mouse pups at PD 3 that dl-methadone resulted in potentiated analgesia. It was hypothesized that MK801 and morphine may result in potentiated analgesia at PD 3, however, the results of this study did not support this hypothesis. The following paragraphs will discuss the findings of the study in this chapter.

Effects of Morphine

Results of this study revealed that morphine was an effective and potent analgesic at PD 3 in both the 129/SvEv and CD-1 mouse pups in the thermal test and was more effective in the 129/SvEv pups than in the CD-1 mouse pups. These results are consistent with those of previous studies in adults, that the opiate, morphine was a more effective analgesic in the 129/SvEv mouse than in the CD-1 mouse or than in the Swiss Webster mouse (Crain & Shen, 2000; Kolesnikov et al., 1998). However, comparable analgesia was obtained with the opiates, dl-methadone and its isomers at PD 3. These findings suggest that, different opiates may exert differential effects in mouse strains of varied

genetic background. A previous study have reported that the opiate fentanyl, was effective in producing opiate tolerance in the 129/J substrain (Varnado-Rhodes, Gunther, Terman, & Chavkin, 2000) while morphine was reported not to produce tolerance in this mouse strain (Crain & Shen, 2000; Kolesnikov et al., 1998). These differences in effects of the differing opiates in different mouse strains may be explained by unique pharmacokinetic factors associated with each opiate rather than pharmacodynamics.

The present findings demonstrated that morphine was an effective analgesic in pups of both mouse strains are consistent with previous findings that morphine is a potent and effective analgesic in neonates (Blass et al., 1993; Fanselow & Cramer, 1988). These results implies that the endogenous opiate system is functional, at least in terms of the availability of receptors and their potential for binding an exogenous opiate and transducing the signal arising from that change into behavioral modulation (i.e. increased paw lift behavior). The effectiveness of the opiate, morphine in suppressing nociceptive responses is not limited to this compound in neonates (Thornton et al., 1998). Further this dissertation has shed some light on racemic methadone and its isomers being effective opiates in analgesia in neonates.

Effects of MK801

This was the first study that investigated the analgesic effects of MK801 in neonatal mice and implicated the NMDA receptor in analgesia in infants. MK801 is a potent NMDA receptor antagonist (Wong et al., 1986) and the NMDA receptors are functional during postnatal development (Bardoni et al., 2000). However, the functional correlates of the NMDA receptors in terms of the behavioral expression of analgesia are not known. The present finding was the first to demonstrate that subcutaneous administration of MK801 was not an effective analgesic in infants in the two mouse strains; and that the NMDA receptor may not be involved in the analgesic mechanism in infants. The fact that this study demonstrated that the NMDA receptors were not effective in infants suggest, that the analgesic effects seen with d-methadone in the thermal test at PD 3 and 10 may not be as a result of the effects of the NMDA receptor. That d-methadone may likely exerting its effects at other receptors other than the NMDA receptor in infants.

MK801 did not Potentiate Morphine's Analgesic Effects

The findings of this study did not replicate the findings that dl-methadone resulted in potentiated analgesia at PD3 in the 129/SvEv and CD-1 mouse pups at this age. The effects of MK801 on morphine's analgesic effects in adult animals have been controversial. Some have found that MK801 potentiate the analgesic effects of morphine

in mice (Bernardi et al., 1996) and in squirrel monkeys (Allen & Dykstra, 2001). Some others have found that MK801 blunt the analgesic effects of morphine (Celerier, Laulin, Larcher, Le Moal, & Simonnet, 1999). Yet others have found that MK801 attenuated the effects of morphine (Maeda et al., 2002). Also, the findings of this study showed that MK801 an NMDA antagonist did not potentaite the analgesic effects at PD 3 in both the 129/SvEv and CD-1 mouse strains, suggesting that the hypothesized receptor deficits are not influencing morphine 's analgesic effects.

In conclusion, the hypothesis that dl-methadone resulted in potentiated analgesia at PD 3 due to the interaction of the μ-opioid and NMDA receptors was not replicated in both the 129/SvEv and CD-1 mouse pups by employing the compounds morphine and MK801 with pharmacological properties that mimic those of dl-methdone.

CHAPTER 5

GENERAL DISCUSSION

One goal of this dissertation was to determine nociception and antinociception of the effects of dl-methadone and its isomers, during postnatal ontogeny, in the 129/SvEv and the CD-1 mouse pups, in two different nociceptive tests, the thermal and the formalin tests. Another goal of this dissertation was to determine, if the μ-opioid and the NMDA receptor systems were interacting, in the exertion of dl-methadone's potentiated analgesic effect. This compound resulted in potentiated analgesia during the early postnatal period, at PD 3 in both mouse strains in the thermal test of nociception.

The results of the experiments in this thesis are the first to elucidate the antinociceptive effects of racemic methadone and its isomers during the postnatal developmental period, a developmental period where both μ-opioid and NMDA receptors continue to develop. The findings presented here, are also the first insights on nociception in models of inflammatory and acute pain during postnatal ontogeny in the 129/SvEv and CD-1 mouse pups. Further, the results of this dissertation are the first attempt to provide an insight, on the interaction of the μ-opioid and the NMDA receptor systems in dl-methadone analgesic mechanisms during postnatal development.

A comparison of Nociception and Antinociception on the effects of d-, l- and dl-methadone in the 129/SvEv and the CD-1 Mouse Pups in an Acute Model of Nociception

In general, no strain differences were found between the 129/SvEv and the CD-1 mouse pups on nociceptive responding and on the analgesic effects of d-, l- and dl-methadone during postnatal ontogeny in the thermal test, an acute model of nociception. It has been established that, the pharmacologically active enantiomer, l-methadone and the racemic mixture are characterized by both μ-opioid and NMDA antagonist effects (Ebert et al., 1995; Gorman et al., 1997). The d-enantiomer is inactive at the μ-opioid receptor and is characterized by NMDA antagonistic effects (Ebert et al., 1995; Gorman et al., 1997). Therefore, the analgesic effects observed with the compounds studied in this dissertation, may be attributed to the ligands (d-, l- and dl-methadone) interacting with the μ-opioid and the NMDA receptors during the postnatal period. A developmental period, where both μ-opioid (Bardoni et al., 2000; Ben-Ari et al., 1997; Kar & Quirion, 1995; Rahman et al., 1998) and NMDA receptors are dynamically developing (Gonzalez et al., 1993; Hori & Kanda, 1994; Hrabetova et al., 2000). The fact that d-, l- and dl-methadone resulted in comparable analgesia in both 129/SvEv and CD-1 mouse pups casted a doubt, that the putative μ-opioid or NMDA receptor deficits were influencing the analgesic effects exerted by these compounds during postnatal ontogeny.

The analgesic effects exerted by d-, l- and dl-methadone in the acute model of nociception are destined to have differential analgesic effects in infants due to the dynamically developing μ-opioid and NMDA receptors on which these compounds interact. The first hypothesis in this thesis was supported, as d-, l- and dl-methadone resulted in greater analgesic effects at PD 3, and PD 10 (Ages that are representative of infants) than at PD 21 (An age group representative of adults). At PD 21, the μ-opioid and the NMDA receptor densities are similar to those of adults (Rahman et al., 1998; Sircar, 2000, hence analgesia observed in this age group is consistent with adults.

A comparison of Nociception and Antinociception on the effects of dl- methadone in the 129/SvEv and the CD-1 Mouse Pups in the Acute versus the Inflammatory Models of Nociception

dl-Methadone resulted in comparable analgesic efficiency and potency to l-methadone at PD 3 but not at PD 21 in both mouse strains, in the thermal test, even though, dl-methadone chemically consists of 50% of the pharmacologically active l-isomer. The exact mechanism by which dl-methadone exerted greater analgesic effects at PD 3 than would be predicted by the separate effects of the two isomers remains enigmatic. However, a possible explanation for such a finding, may rest on an interaction of the μ-opioid and the NMDA receptor systems in the analgesic mechanisms of dl-

methadone during early postnatal ontogeny. The idea that the μ-opioid receptors functionally interact with the NMDA receptors in analgesia is not new. Previous studies have shown that NMDA receptor antagonists have potentiated the analgesic effects of opiate agonists in thermal nociception (Chapman & Dickenson, 1992; Mao et al., 1996), although never tested in infants. An attempt was made in this dissertation to provide a possible mechanism that may account for the potentiated analgesic effect obtained with dl-methadone, during early postnatal ontogeny, on the basis that the μ-opioid and the NMDA receptor systems were interacting in the analgesic mechanisms of dl-methadone. This experiment was investigated, in a co-administration procedure by employing a μ-opiate receptor agonist and an NMDA receptor antagonist, in the thermal test of nocicepton. Morphine and MK801 were employed for the purpose of mimicking the pharmacological effects of dl-methadone as morphine is a μ-opiate agonist like l-methadone and MK801 is an NMDA antagonist, like d-methadone. However, the results of this experiment did not support the hypothesis, that the μ-opioid and the NMDA receptor systems were interacting in the potentiated analgesic effect of dl-methadone, as the co-administration of MK801 and morphine did not result in potentiated analgesia.

The data in this thesis revealed differential nociceptive and antinociceptive effects in the thermal versus the formalin nociceptive assays in both mouse strains at both ages. No

strain differences were seen between the 129/SvEv and CD-1 mouse pups in terms of nociception and antinociception in the thermal test at PD 3 and 21. However, strain differences were seen in the formalin test, on the nociceptive responding induced by subcutaneous formalin at PD 3, 10 and 21 but not on the antinociceptive effects induced by d-, l- and dl-methadone. Additionally, dl-methadone resulted in potentiated analgesia at PD 3 in the thermal test but not in the formalin test, suggesting a different mechanism of action of nociception and antinociception in the thermal versus the formalin nociceptive tests. The first phase in the formalin test, represents an acute model of nociception, however the data presented here suggests, that this acute first phase differs from the acute thermal test, as dl-methadone resulted in potentiated analgesia in the thermal test but not in the formalin test. Further, the first line of evidence in support of a differential mechanism as the basis for nociception was presented, when it was first suggested, that anatomically distinct pain modulatory systems might exist for thermal versus formalin nociception (Vaccarino & Melzack, 1989). The data presented here supported previous evidence that the thermal and formalin tests are differentially mediated (Vaccarino & Melzack, 1989).

A comparison of Nociception and Antinociception on the effects of l- methadone in the 129/SvEv and the CD-1 Mouse Pups in the Acute versus the Inflammatory Models of Nociception

The findings of this dissertation demonstrated, that l-methadone was an effective analgesic in both the thermal and the formalin tests, in both mouse strains at all ages tested and is consistent with prior findings in adults (Scott & Orr, 1969; Shimoyama et al., 1997; Smits & Myers, 1974). These findings suggest a possible role of the μ-opioid receptor in both the thermal and formalin tests of nociception. Previous studies in adult rodents, have shown that l-methadone exerted its analgesic action through the μ-opioid receptor (Shimoyama et al., 1997; Smits & Myers, 1974). Further it was speculated in this entire dissertation that l-methadone was exerting its effects through the μ-opioid receptor in infants, a receptor population that continue to develop during postnatal ontogeny. Therefore, to ascertain the roles of the μ-opioid receptor in the analgesic mechanisms of l-methadone, further studies needed to be done. Future studies should investigate the role of the μ-opioid receptor in l-methadone's analgesic effects by either pharmacologically blocking the μ-opioid receptor or at the molecular level by manipulating μ-opioid receptor knock mouse models. Once the receptor involvement is investigated, then it can be said with certainty, whether l-methadone was having its effects through the μ-opioid receptor.

As hypothesized in the first and second hypotheses in this thesis, l- methadone exerted greater analgesic effects at PD 3 (as representative of infants) than at PD 21. In particular, this compound was more potent and effective as an analgesic at PD 3 than at PD 21, this finding is a constant with the sensitivity of other opiate drugs such as morphine in infants (Marsh et al., 1997). The fact that l-methadone was more potent and effective at PD 3 may be attributed to the higher μ-opioid receptor density at PD 3 than at PD21 (Rahman et al., 1998). At PD 3, there may be more receptors available for l-methadone binding, hence more ligand receptor complexes, subsequently greater analgesic effectiveness. The finding that, l-methadone was more effective and potent as an analgesic at PD 3 can be as a result of pharmacodynamic factors, or pharmacokinetic factors or an interaction of pharmacodynamic and pharmcokinetic factors. Further experiments are needed to tease apart these factors, so as to ascertain the contributing factor in l-methadone being more effective and potent as an analgesic at PD 3 than at PD 21.

A comparison of Nociception and Antinociception on the effects of d-methadone in the 129/SvEv and CD-1 Mouse Pups in the Acute Versus the Inflammatory Models of Nociception

The results of this thesis revealed, that d-methadone exerted minimal analgesic effects at PD 3 and 10 in both mouse strains and was totally ineffective at PD 21 in both the

thermal and formalin tests of nociception. Additionally, in the thermal test, d-methadone was minimally effective at PD 3 in the CD-1 mouse strain and totally ineffective at PD 21, this contrasts with the findings in the formalin test. The fact that d-methadone exerted some effects in both the thermal and formalin tests at PD 3 and 10 may argue for a possible role of the NMDA receptor in the thermal and formalin test, during early postnatal ontogeny when the NMDA receptor density is high (Rahman et al., 1998). However, the findings presented here have demonstrated that MK801 was not an effective analgesic at PD 3 suggests, that d-methadone may likely exerting its effects through other receptors other than the NMDA receptor at PD 3 and may not likely exerting its effects at the NMDA receptor.

The fact that d-methadone was a totally ineffective analgesic at PD 21, suggests, that the analgesic effects seen with dl-methadone at this age group in both mouse strains may be as a result of the l-component in dl-methadone. Further experiments will be needed to ascertain the roles of each enantiomer in dl-methadone's analgesic effectiveness. The results of this dissertation demonstrated that d-methadone is not a promising analgesic in infants in either phasic or tonic nociception.

A comparison of Nociception and Antinociception on the effects of l-, d- and dl-Methadone in the 129/SvEv and CD-1 Mouse Strains in an Inflammatory Model of Nociception

Strain differences were observed between the 129/SvEv and the CD-1 pups at all three ages tested in the formalin test but no strain differences were observed on the antinociceptive effects of dl-methadone and its isomers between these two mouse strains in this test. The CD-1 pups showed a higher rate of nociceptive responding than the 129/SvEv pups at all ages tested. The fact that the findings on the analgesic effectiveness of dl-methadone and its isomers were consistent between the two mouse strains strengthens the evidence on the analgesic effectiveness of these compounds observed in this dissertation. The findings in this dissertation, also lend support that the putative receptor deficits in the 129/SvEv mouse pups were not influencing methadone's analgesia in the thermal test, as the results obtained in this mouse strain were also seen in the CD-1 mouse strain. As such, strain differences observed in the formalin test on nociceptive responding to formalin, will be attributed to genetic influence rather than the receptor deficits in the 129/SvEv pups at all ages tested. In animal studies, using mouse strains of varied genetic background have shown that differences in nociception may be due to genotype (Mogil et al., 1999).

Absence of the Quiescent Period in the Formalin test during Early Postnatal Ontogeny

Results of this dissertation revealed an absence of the characteristic quiescent period at PD 3 and 10 but not at PD 21 in both the 129/SvEv and CD-1 mouse pups in the behavioral nociceptive response evoked by formalin. This finding is consistent with substantial prior studies in rats and mice, that the behavioral expression of the quiescent is not expressed before the second postnatal week (Barr, 1998; Gupta et al., 2001; Guy & Abbott, 1992; King, Cheng, Wang, & Barr, 2000; Teng & Abbott, 1998; Yi & Barr, 1995). The absence of the quiescent period in infants may be explained by the late maturing nociceptive dampening mechanisms that are absent at birth but emerge postnatally, during the first three weeks of life (Fitzgerald & Koltzenburg, 1986; Gilbert & Stelzner, 1979) and might promote the quiescent period seen in 21 day olds. Further axons descending from the brain stem are present on the day of birth (Leong, Shieh, & Wong, 1984). The delayed functional maturity of the descending inhibitory pathway has been attributed in part to the late development of serotonin and noradrenergic transmitter terminals in these pathways (Fitzgerald & Koltzenburg, 1986; Tive & Barr, 1992) and insufficient levels of serotonin for effective function of the descending inhibitory pathway (Schul & Frenk, 1991).

Effects of l- and d- Methadone, Morphine and MK801

The fact that both l-methadone and morphine resulted in analgesia in both mouse strains, suggest that the μ-opioid receptors are involved in analgesic mechanisms in neonates. The fact that d-methadone was only minimally effective at PD 3 in this dissertation and that MK801 was not an effective analgesic at this age suggests, that d-methadone is likely having its effects through other receptors other than the NMDA receptors in infants. Therefore this dissertation suggests, that the μ-opioid receptors but not the NMDA receptors were likely playing a role in the analgesic mechanism in infants.

Overall Perspective and Limitations of this Dissertation

This dissertation has taken the behavioral approach in testing the compounds, dl-methadone and its two enantiomers, MK801 and morphine as analgesics during the postnatal developmental period. During this developmental period, both the μ-opioid and NMDA receptors are dramatically developing, as such analgesia may be defined by the dynamic nature of the developing receptors. Firstly, this section will briefly describe some advantages in taking the behavioral approach in the study of drug receptor interactions during a dynamic period of receptor development. Secondly, this section will discuss some limitations of this dissertation.

Firstly, the behavioral approach is an excellent one in the study of drug/ receptor interactions during critical developmental periods when receptor populations are dynamically developing (Gonzalez et al., 1993; Kar & Quirion, 1995; Rahman et al., 1998). The postnatal period is a developmental period that is marked by receptor development in terms of: receptor density, maturity of specific receptor types and subtypes, functional involvement of receptors, neurotransmitter concentration in relation to receptor density, receptor specificity and affinity (Anand, 1993; Fitzgerald, 1991, 1995; Tive & Barr, 1992). During this unique time during development, the only one ideal approach in the study of the effects of ligands at receptors is the behavioral approach, as there are no other approach that can effectively mimic the resultant ligand/ receptor effects in an environment that are dynamically changing.

Also, during postnatal ontogeny, pharmacokinetic factors are influx and may include; drug distribution, drug elimination (Robillard, Segar, Smith, & Jose, 1992; Stonestreet, Bell, Warburton, & Oh, 1983), dug metabolism and enzyme kinetics (Krauer & Dayer, 1991). These conditions cannot be mimicked in an in vitro environment, therefore during postnatal development; the drug effect at a receptor must be studied in in-vivo. In addition, both pharmacodynamic and pharmacokinetic factors may interact to affect the drug/ receptor interaction and hence the effect of the drug. It may be impossible to

mimic the effect of drug receptor interactions in vitro when a vast amount of factors are dynamically changing.

This dissertation has placed a lot of emphasis on the pharmacodynamic factors that may interact in the exertion of analgesia. Each hypothesis tested in this dissertation was built solely on a pharmacodynamic platform. However, it is not only pharmacodynamic factors that may affect the effect of a drug (Fitzgerald, 1991) but also pharmacokinetic factors may also affect the effect of a drug (Larsson et al., 1996). This dissertation has focused only on pharmacodynamic factors and as such it was a limitation of this dissertation. Therefore, the pharmacokinetics of drug receptor interactions should be taken into account in the interpretation of any data presented here. Another limitation of this dissertation is that the involvement of the μ-opioid and NMDA receptors in the analgesic actions of the compounds studied here were not directly investigated, as such this was a limitation of this dissertation. However, future studies should investigate the involvement of the μ -opioid and NMDA receptor involvement in the compounds studied in this thesis. In addition, future studies should also investigate the analgesic effects of racemic methadone and its isomers beyond the behavioral level.

The knowledge gained in this dissertation is by no means comprehensive on the topic of the analgesic effects of racemic methadone and its isomers during postnatal ontogeny and on the involvement of the μ-opioid and NMDA receptors during postnatal ontogeny. However, the knowledge gained here can be used as a foundation for future investigations and to gain insights in elucidating the opiate system in vivo.

GENERAL CONCLUSIONS

The present dissertation examined the antinociceptive effects of dl-methadone and its isomers during postnatal ontogeny, in the thermal and formalin nociceptive tests, in the 129/SvEv and the CD-1mouse pups and the following conclusions were made:

(1) That no strain differences were observed between the 129/SvEv and the CD-1 mouse in the thermal test suggests, that analgesia observed with dl-methadone and its isomers may be due to the developmental effects of the receptors rather than their deficits.

(5) That l-, d- and dl-methadone were effective analgesics at the younger ages tested in both 129/SvEv and CD-1 mouse pups suggests, that the μ-opiate receptor are functional early in postnatal ontogeny.

(6) That dl-methadone resulted in potentiated analgesia at PD 3 in both mouse strains in the thermal test but not in the formalin test suggests, a different mechanism of action as the basis for the thermal versus the formalin tests.

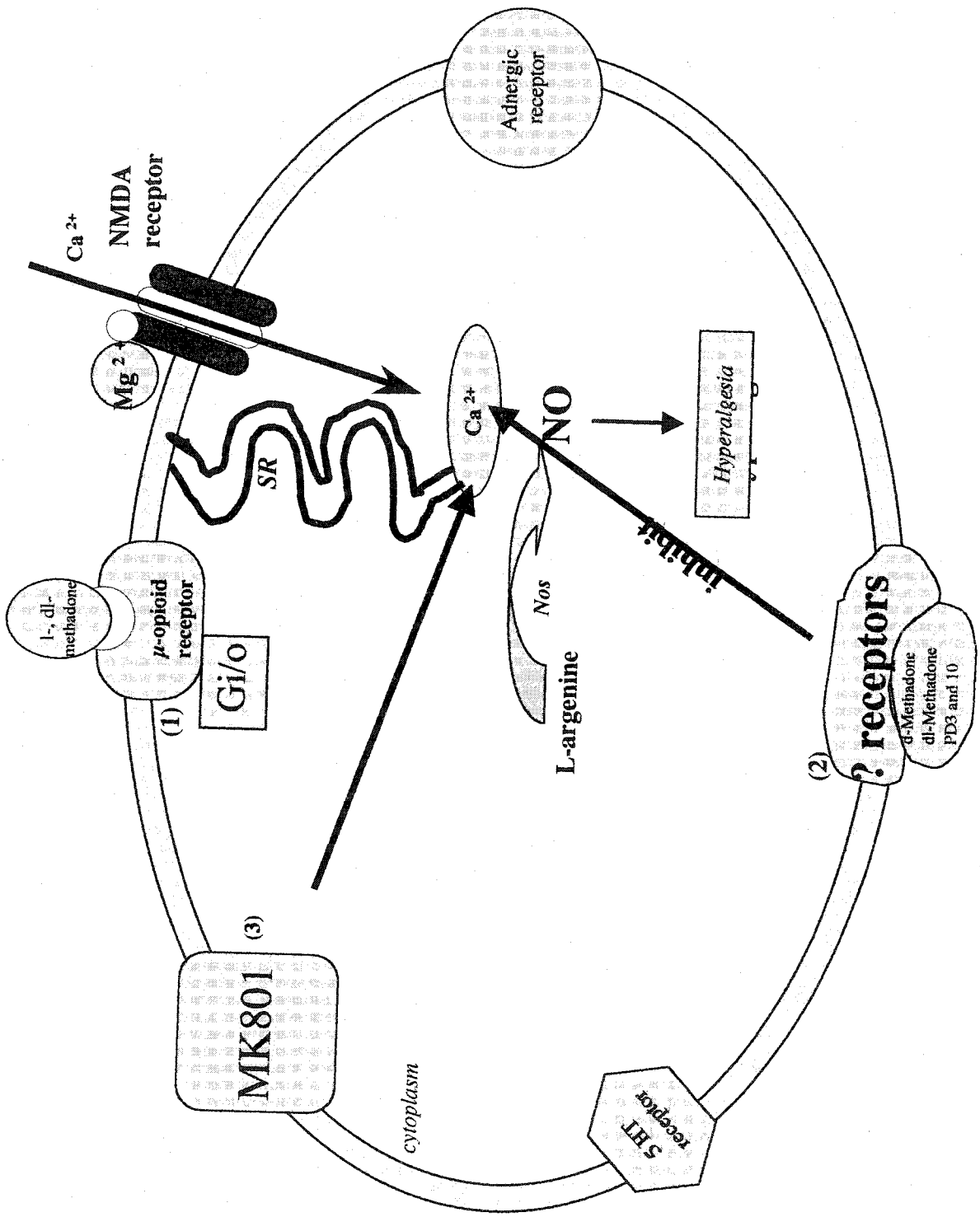
(7) That strain difference was observed on nociceptive responding in the formalin test but not in the thermal test, also suggests that the thermal and formalin tests are mediated by different mechanisms of action.

(8) That MK801 did not result in analgesia at PD 3 and it did not potentiate the analgesic effects of morphine suggests, that the NMDA receptors were not contributing to the analgesic mechanisms of dl-methadone and that d-methadone was likely acting, through other receptors other than the NMDA receptors in infants.

(9) That the opiate, morphine was more effective in the 129/SvEv pups than in the CD-1 pups at PD 3 and the opiates; l-, d- and dl- methadone resulted in comparable analgesia in both mouse strains suggests, that different opiates exert differential effects probably due to pharmacokinetics.

Figure 23. Scheme illustrating possible mechanisms of actions of l-, dl-,d-methadone, morphine and MK801 in analgesia in infant 129/SvEv and CD-1 mouse pups. dl-Methadone resulted in potentiated analgesia possibly by non-specific interactions of the μ-opioid receptor together with receptors other than the NMDA receptors in early postnatal development in the thermal test (1 and 2). l- and dl-Methadone probably induced analgesia by uncoupling of the μ-opioid receptor from its underlying G proteins which appears to be mediated by protein kinase C-dependent phosphorylation in both thermal and formalin tests in both mouse strains. A possibility that d-methadone probably exerted minimal analgesia at other receptors than NMDA receptors by inhibiting ca $^{2+}$ influx from SR thereby, suppressing NOS activity and subsequently reduction of NO production as activation of NOS activity is not specific to NMDA receptor activation in both nociceptive tests in both mouse strains (2). Another possibility, may be that d-methadone may have exerted non-specific actions at non-glutamate receptors, hence uncoupling G-protein mediated receptors and subsequent receptor desensitization in both nociceptive tests and in both mouse strains. MK801 was not an effective analgesic in the thermal test, probably due to the non-involvement of the NMDA receptor in analgesia in infants, this compound may have antagonized morphine's analgesia through non-specific interactions with other receptors or this compound may have caused an increase in ca $^{2+}$ influx from SR, further increasing NOS activity and subsequent NO production hence its antagonizing effects (3). l-methadone, dl-methadone and morphine were more effective as analgesics at PD 3 and 10 in both mouse strains in both nociceptive tests probably due to an interaction of both pharmacodynamic and pharmacokinetic factors.

Abbreviations: NMDA; N-methyl-D-aspartate, ca $^{2+}$; calcium, PD; postnatal day, NO; nitric oxide, NOS; nitric oxide synthase, SR; sarcoplasmic reticulum, G_{i}; G-protien inhibitory subunit, 5HT; serotonin receptor, Mg^{2+}; magnesium.



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