NOTE TO USERS

NOP & OFQ: BEHAVIORAL EFFECTS DURING DEVELOPMENT

by

ESTEVAN RUIZ LIMÓN

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

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Abstract

NOP & OFQ: BEHAVIORAL EFFECTS DURING DEVELOPMENT

by

Estevan Ruiz Limón

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The novel opioid peptide receptor (NOP, previously referred to as ORL1) and its endogenous ligand orphanin FQ (OFQ) may provide a non-opioid means of alleviating pain. Experimental evidence using the adult rat suggests that 1) NOP and OFQ are expressed in supraspinal and spinal regions associated with the modulation of nociceptive information and 2) OFQ can induce analgesia when administered spinally and supraspinally. However, due to the lack of data describing the effects of OFQ administration during development and a definitive antagonist for the NOP receptor, the role of NOP and OFQ on nociception during development and the mechanisms involved have yet to be determined. The present studies were intended to evaluate the effects of OFQ administration, both alone and in combination with the purported antagonist, [Nphe1]nociceptin(1-13)-NH_2 (Nphe1), in infants. To study the role of NOP and OFQ on nociception during development, both withdrawal latency to a noxious thermal stimulus and pain intensity scores in the formalin test were examined during development (3-, 10-, and 21-days in the case of thermal testing, 3- and 21-days in the case of the formalin test). Analgesia was produced following spinal administration, but in general did not alter pain sensitivity after supraspinal administration. To study the mechanisms involved in this analgesia, Nphe1 was administered alone and in combination with OFQ and its

effects on pain intensity scores in the formalin test were examined during development (3- and 21-days-old). NOP/OFQ-induced analgesia was unaffected by Nphe1 co-administration, regardless of route of injection (i.e. supraspinal or spinal). Furthermore, when administered alone, Nphe1 appeared to produce an antinociceptive effect. These results suggest that 1) NOP and OFQ play a role in mediating nociception during development that is fundamentally different from that seen in the adult and 2) Nphe1 is not a functional antagonist of the NOP receptor during development, but instead produces antinociceptive effects that are distinct from those of NOP/OFQ.

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Chapter 1: Introduction

The novel opioid peptide (NOP) receptor

The creation of cDNA libraries for the classic opioid receptors (the MOP, or μ-, KOP, or κ-, and DOP, or δ-, receptors), made possible by the discovery of the amino acid sequence for the DOP receptor (Evans, 1992; Kieffer, 1992), provided the bases for low-stringency oligonucleotide probes which, when used in the screening of other cDNA libraries, resulted in the discovery of a previously unknown receptor protein in human, mouse, and rat libraries. This receptor has been given a number of species-specific names [for example, MOR-C (Nishi *et al*, 1994) and KOR-3 (Pan *et al*, 1994) in mice; LC132 (Bunzow *et al*, 1994), Ratxor1 (Chen *et al*, 1994), ROR-C (Fukuda *et al*, 1994), C3 (Lachowicz *et al*, 1995), XOR1 (Wang *et al*, 1994), and Hyp 8-1 (Wick *et al*, 1994) in rats], but is generally referred to by the name of the human receptor, opioid receptor-like 1 (ORL1) (Mollereau *et al*, 1994). The receptor has recently been renamed to NOP at the suggestion of the International Union of Basic and Clinical Pharmacology (IUPHAR); however, use of this moniker has yet to become widespread in the literature. Based on the high degree of homology (roughly 50% identity overall, with transmembrane regions demonstrating up to 80% identity) between the receptor and the classic opioid receptors (Mollereau *et al*, 1994) (Figure 1), it was originally thought to be one of the putative subtypes of the MOP, KOP, or DOP receptors that have been suggested by pharmacological studies (Pasternak, 1993; Fowler & Fraser, 1994; Zaki *et al*, 1998); however, the receptor expressed little or no affinity for several peptide and nonpeptide opioid ligands (Mollereau *et al*, 1994; Lachowicz *et al*, 1995).

The receptor is widely distributed throughout the body (Figure 2A,B): *in situ* hybridization (Bunzow *et al*, 1994; Fukuda *et al*, 1994; Mollereau *et al*, 1994; Wick *et al*, 1994; Lachowicz *et al*, 1995; Neal *et al*, 1999; Houtani *et al*, 2000b; Berthele *et al*, 2003), immunohistochemical (Anton *et al*, 1996; Riedl *et al*, 1996; Houtani *et al*, 2000b; O'Donnell *et al*, 2001), and autoradiographic studies (Letchworth *et al*, 2000) have localized the receptor to the cortical and cortico-limbic areas (amygdala, hippocampus, habenula, septum), hypothalamus (ventromedial and paraventricular nuclei), brain stem (locus ceruleus, parabrachial nucleus, periaqueductal grey, dorsal raphe nucleus) and the spinal cord (dorsal and ventral horns).

Structurally, the NOP receptor is a typical G-protein coupled receptor with seven transmembrane spanning domains (Chen *et al*, 1994; Mollereau *et al*, 1994; Nishi *et al*, 1994), several of which are currently thought to be actively involved in peptide binding (Topham *et al*, 1998; Mouledous *et al*, 2000) (Figure 3). Like those coding for the traditional opioid receptors, the gene coding for the NOP receptor, Oprl1, has three coding exons: the first being responsible for the amino terminus and first transmembrane domain, the second for the next three transmembrane domains, and the third for the last three transmembrane spanning domains and the carboxyl terminus (Pan *et al*, 1996). Original studies into the structure of Oprl1 suggested the gene was composed of five exons (three coding, two non-coding) (Pan *et al*, 1998) (Figure 4), but later studies have discovered the existence of two additional minor coding exons (Xie *et al*, 1999). However, it has yet to be determined if these additional "mini-exons" are omnipresent or situationally and/or anatomically specific.

It has been demonstrated that the NOP receptor, like the traditional opioid receptors, is subject to alternative splicing. Currently, five NOP receptor variants have been identified and, to some degree, characterized. The first of these, named NOP_d, has been seen in mouse (Pan *et al*, 1998), rat (Wick *et al*, 1994; Xie *et al*, 1999), and human (Peluso *et al*, 1998) brains and demonstrates a 15-bp deletion from the 3' end of the first coding exon. The second, NOP_e, is also seen in mouse (Pan *et al*, 1998), rat (Chen *et al*, 1994; Xie *et al*, 1999), and human (Xie *et al*, 1999), and demonstrates the retention of the intron between the second and third coding exons. In mice, NOP_e is non-functional and characterized by an 81-bp insertion and a stop codon that prevents the translation of the last three transmembrane domains (Pan *et al*, 1998). In rats, two versions of NOP_e have been described: the first similar to that seen in the mouse (Xie *et al*, 1999) and the second characterized by an 84-bp insertion that translates into a functional receptor (Chen *et al*, 1994). It has yet to be determined what the relative expression levels of the two rat NOP_e variants are, or what differences, if any, exist between the functional variant and the "normal" NOP receptor. The remaining three variants are the result of translation at the two additional "mini-exons" (Xie *et al*, 1999). NOP_a is the result of a 34-bp insertion between the first and second coding exons that produces a frameshift with a stop codon in the second coding exon, resulting in a shortened receptor that does not demonstrate any transmembrane domains. NOP_c is the result of a different, 139-bp insertion between the first and second exons that, like NOP_a, produces a frameshift and results in a shortened receptor. Finally, NOP_b is the result of a truncated version of the same "mini-exon" responsible for NOP_c. This 98-bp insertion also results in a shortened receptor. The functional significance, if any, of these shortened NOP variants has yet to be determined.

Artificial manipulation of the structure of the NOP receptor has added further support of its close relationship to the traditional opioid receptors. Selective alterations in the last three transmembrane regions of the receptor have no effect on NOP's binding of its own agonists, but do increase its ability to bind the KOP receptor agonist dynorphin A by up to 50-fold (Meng *et al*, 1996). Replacement of the first coding exon for the NOP receptor with that of the KOP receptor produces a hybrid receptor displaying a high affinity for both NOP agonists and dynorphin A (Lapalu *et al*, 1998; Mollereau *et al*, 1999). Similar hybrids using the first coding exons of the MOP and DOP receptors had no effect on NOP agonist binding, nor did they demonstrate any affinity for either MOP or DOP agonists (Pan *et al*, 1996); however, co-immuniprecipitation of NOP and MOP receptors has been reported to create a heterodimer of these receptors that demonstrates high affinity for both NOP and MOP agonists that was unaffected by general opioid receptor antagonists (Pan *et al*, 2002).

The ligand for the orphan opioid receptor, orphanin FQ (OFQ)

The endogenous agonist was discovered independently by two laboratories shortly thereafter, when the fractionation of rat brain (Meunier *et al*, 1995) and porcine hypothalamus (Reinscheid *et al*, 1995) extracts by conventional procedures led to the isolation and identification of a heptadecapeptide whose amino acid sequence supports a close evolutionary relationship to existing endogenous opioid peptides [specifically, dynorphin A (1-17) and γ-dynorphin] (Figure 5). The ligand was named nociceptin by Meunier *et al* (1995) due to a resultant increase in response to painful stimuli when administered supraspinally. It should be noted, however, that this name might prove misleading, as current research casts doubt on the pronociceptive nature of the ligand.

Reinscheid *et al* (1995), on the other hand, named it OFQ, signifying a ligand to an orphan receptor flanked by a phenylalanine (F) residue at the amino terminus and a glutamate (Q) residue at the carboxyl terminus.

OFQ and its precursor transcripts, like the receptor, are also widely distributed throughout the body (Figure 2B). Hybridization (Houtani *et al*, 1996; Kummer & Fischer, 1997; Neal *et al*, 1999; Pettersson, 2002), immunohistochemical (Dickenson, 1996; Riedl *et al*, 1996; Kummmer & Fischer, 1997; Mitsuma *et al*, 1998; Houtani *et al*, 2000a; O'Donnell *et al*, 2001), and autoradiographic (Letchworth *et al*, 2000) studies have localized the peptide and its precursor to cortical layers I-III, claustrum, ventral forebrain, nucleus of the lateral olfactory tract, olfactory bulb, lateral septum, bed nucleus of the stria terminalis, lateral geniculate nucleus, medial habenula, medial preoptic, ventromedial and supramammillary hypothalamic nuclei, amygdala, CA1-3 hippocampal regions, mammillary bodies, inferior colliculus, ventral tegmentum, region dorsal to the central canal, raphe complex, periaqueductal grey, locus ceruleus, substantia nigra, interpeduncular nucleus, nucleus of the lateral lemniscus, pontine nuclei, central tegmental field, superior olive, solitary nucleus, nucleus ambiguous, spinal trigeminal nucleus of the brain stem, reticular formation, and the dorsal and ventral horns of the spinal cord.

As mentioned above, OFQ is composed of 17 amino acids, four of which have been determined to be of critical importance in the binding properties and distinct functionality of the peptide (Dooley & Houghten, 1996). Of these, amino acids 1, 2, and 4 are particularly important. The phenylalanine at position 1 is necessary for OFQ's binding selectivity, as replacing it with a tyrosine results in a peptide demonstrating a

much greater affinity at the traditional opioid receptors (Champion & Kadowitz, 1997a, b; Mathis *et al*, 1998). The glycine and phenylalanine at positions 2 and 4 are part of a complex (the initial FGGF sequence) that has been shown to be critical for activity of the peptide (Guerrini *et al*, 1997). However, this initial sequence is apparently the only portion of the peptide necessary for activity, as modification of its basic structure or shortening of the peptide at the carboxyl terminus has no appreciable effect (Guerrini *et al*, 1997).

The existence of two pairs of basic amino acids (an arginine-lysine pair at positions 8-9 and 12-13) and the role of aminopeptidase and endopeptidase 24.15 in OFQ metabolism suggest the capacity for further processing of OFQ, including the documented fragments OFQ_{1-6}, OFQ_{1-7}, OFQ_{1-9}, OFQ_{1-11}, OFQ_{1-13}, OFQ_{2-17}, OFQ_{3-17}, OFQ_{12-17}, and OFQ_{13-17} (Montiel *et al*, 1997; Suder *et al*, 1999; Terenius *et al*, 2000). Limited data exists characterizing the effects of OFQ_{1-7} and OFQ_{1-11} following supraspinal (Rossi *et al*, 1997) and spinal (King *et al*, 1997) administration, but these studies suggest a solely analgesic nature to these peptides. Similar results are obtained using analogs of OFQ_{1-11} (Mathis *et al*, 1998): placement of a tyrosine at position 1 ($[Tyr^1]OFQ_{1-11}$) results in a peptide with high affinity at the traditional opioid receptors, while placement of a tyrosine at position 10 ($[Tyr^{10}]OFQ_{1-11}$ and $[IodoTyr^{10}]OFQ_{1-11}$) produces peptides with much higher affinity at the NOP receptor than that of OFQ_{1-11} itself. All of these analogs are capable of inducing analgesia of a much longer duration than that of OFQ_{1-11}. OFQ_{1-6} produces an initial analgesia followed by hyperalgesia (Suder *et al*, 1999). The analgesia, but not the hyperalgesia, is reversible by naloxone. Spinal administration of OFQ_{1-9} and OFQ_{1-13} reduces OFQ-induced scratching, licking,

and biting, but these peptides do not exhibit any activity of their own (Sakurada *et al*, 2000) OFQ_{13-17}, on the other hand, apparently acts as a hyperalgesic (Chen *et al*, 2002). Currently, no behavioral data exists regarding the remaining fragments.

Currently, no definitive antagonist for NOP exists to verify the effects of OFQ administration are the result of interaction with NOP and not with co-localized or downstream opioid mechanisms. A number of potential candidates have been proposed over the years, including Ac- RYYRIK-NH_2 (Dooley *et al*, 1997), nocistatin (Okuda-Ashitaka *et al*, 1998), naloxone benzoylhydrazone (NalBzoh) (Dunnill *et al*, 1998), [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]$nociceptin_{1-13}$-$NH_2$ (Guerrini *et al*, 1998), [$Nphe^1$]$nociceptin_{1-13}$-NH_2 (Nphe) (Caló *et al*, 1998), J-113397/CompB (Kawamoto *et al*, 1999), UFP-101 (Caló *et al*, 2002), JTC-801 (Yamada *et al*, 2002), two azacycloalkane amino acid derivatives (Halab *et al*, 2002), SB-612111 (Zeratin *et al*, 2004), and members of a novel series of piperidin-4-yl-1,3-dihydroindol-2-ones (Zaveri *et al*, 2004), but, for the most parts, this antagonism has been limited to *in vitro* studies (azacycloalkane amino acid derivatives, piperidin-4-yl-1,3-dihydroindol-2-ones), inconsistent (nocistatin, NalBzoh, [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]$nociceptin_{1-13}$-$NH_2$), or the compounds themselves have also demonstrated antinociceptive effects of their own ($Nphe^1$, UFP-101, JTC-801).

Behavioral effects of NOP/OFQ

The wide expression of both the NOP receptor and OFQ throughout the nervous system has suggested their involvement in a number of central processes, including learning and memory, attention, emotion, motor function, homeostasis, neuroendocrine function, as well as modulation of all of the sensory modalities. Due to the high homology between NOP/OFQ and the traditional opioid system, as well as their

prominent expression in key neural structures involved in the analysis of and response to noxious stimuli, such as the periaqueductal grey, locus ceruleus, rostroventral medulla, and dorsal horns of the spinal cord, it was originally surmised that both are heavily involved the modulation of supraspinal and spinal mechanisms related to nociception. Initial experiments were supportive of this modulatory role and subsequent studies using antisense oligonucleotide (ASO) probes, changes in patterns of expression, and NOP knockout mice served to strengthened it (Meunier *et al*, 1995; Andoh *et al*, 1997; Nishi *et al*, 1997; Tian *et al*, 1997a,b; Jai *et al*, 1998; Tian *et al*, 1998; Inoue *et al*, 1999; Noda *et al*, 1998; Rosen *et al*, 2000; Clarke *et al*, 2003; Depner *et al*, 2003; Inoue *et al*, 2003; Witta *et al*, 2003). Later experiments demonstrating the ability of NOP/OFQ to regulate transmitter release in the peripheral nervous system expanded this role to the peripheral nervous system as well. The current body of literature, however, fails to come to a final consensus regarding the exact nature of that involvement. Studies have suggested not only an analgesic nature to their actions, but also hyperalgesic and anti-opioid natures as well.

Supraspinal

Hyperalgesia. The initial studies by both Meunier *et al* (1995) and Reinschied *et al* (1995) suggested that supraspinal administration of OFQ in male CD-1 mice results in a heightened nociceptive response. Subsequent studies support this assertation, demonstrating hyperalgesia in response to doses as low as 0.0055 nmol and as high as 30 nmol. This response has been seen in a number of paradigms, including the hot plate (Meunier *et al*, 1995; Lufty & Maidment, 2000), pinch tailflick (Reinschied *et al*, 1995), heat tailflick (Rossi *et al*, 1996; Shimohigashi *et al*, 1996; Rossi *et al*, 1997; Candeletti *et*

al, 2000; Citterio *et al*, 2000; Ozaki *et al*, 2000), immersion tail withdrawal (Caló *et al*, 1998; Wang *et al*, 1999b; Chen *et al*, 2002), and formalin (Zhu *et al*, 1997; Wang *et al*, 1999a) tests, using both single (Meunier *et al*, 1995; Reinschied *et al*, 1995) and multiple observations (Rossi *et al*, 1996; Shimohigashi *et al*, 1996; Rossi *et al*, 1997; Zhu *et al*, 1997; Caló *et al*, 1998; Candeletti *et al*, 2000; Wang *et al*, 1999a,b; Lufty & Maidment, 2000; Chen *et al*, 2002). This response has also been seen in multiple mouse species, including CD-1 (Meunier *et al*, 1995; Rossi *et al*, 1996, 1997; Caló *et al*, 1998; Citterio *et al*, 2000), NMRI (Reinschied *et al*, 1995), ddY (Chen *et al*, 2002), and ICR (Shimohigashi *et al*, 1996; Ozaki *et al*, 2000) strains, and in Sprague-Dawley rats (Zhu *et al*, 1997; Candeletti *et al*, 2000; Wang *et al*, 1999a,b; Lufty & Maidment, 2000). Duration of this hyperalgesia varied, with times reported from 15 to 120 minutes; however, in the majority of cases, this effect was long-lasting (i.e. 30+ minutes; Shimohigashi *et al*, 1996; Zhu *et al*, 1997; Caló *et al*, 1998; Wang *et al*, 1999a,b; Lufty & Maidment, 2000; Ozaki *et al*, 2000; Chen *et al*, 2002). This effect was insensitive to the general opioid antagonists naloxone (Caló *et al*, 1998; Chen *et al*, 2002) and diprenorphine (Rossi *et al*, 1996, 1997), but was reversed by NOP-specific compounds (Meunier *et al*, 1995; Rossi *et al*, 1997).

Anti-opioid. Results obtained from other laboratories, however, suggested that these effects were not due to a hyperalgesic response but rather the reversal of opioid-mediated stress-induced analgesia (SIA) (Mogil *et al*, 1996a). Sufficient evidence exists supporting the idea that supraspinal OFQ-administration is capable of antagonizing opioid-induced analgesia: OFQ has been demonstrated to reverse and/or prevent MOP-mediated (Grisel *et al*, 1996; Mogil *et al*, 1996a,b; Tian *et al* 1997a; Zhu *et al*, 1997; Caló

et al, 1998; King *et al*, 1998; Suaudeau *et al*, 1998; Zhu *et al*, 1998; Lutfy *et al*, 1999; Wang *et al*, 1999a,b; Candeletti & Ferri, 2000; Citterio *et al*, 2000; Rady & Fujimoto, 2002), DOP-mediated (Mogil *et al*, 1996b; King *et al*, 1998; Wang *et al*, 1999a), and KOP-mediated (Mogil *et al*, 1996b; King *et al*, 1998; Wang *et al*, 1999a; Citterio *et al*, 2000) analgesia. Furthermore, direct injection into the periaqueductal grey (Morgan *et al*, 1997; Bytner *et al*, 2001) and rostroventral medulla (Heinricher *et al*, 1997; Pan *et al*, 2000; Yang *et al*, 2001) also blocks MOP-mediated analgesia. These reversals occurred at an extremely wide range of dosages (40 fmol-135 nmol). Finally, OFQ administration is also capable of reversing other forms of opioid-mediated analgesia, including acupuncture (Zhu *et al*, 1996; Tian *et al*, 1997b; Du *et al*, 1998) and SIA (Mogil *et al*, 1996a; Suaudeau *et al*, 1998; Rizzi *et al*, 2001). This antagonism can be long lasting, persisting up to six hours (Candeletti & Ferri, 2000), but is subject to tolerance with repeated doses (Lufty *et al*, 1999). It should be noted, however, that a number of these experiments (Zhu *et al*, 1996, 1997; Caló *et al*, 1998; Zhu *et al*, 1998; Wang *et al*, 1999a,b; Citterio *et al*, 2000) demonstrated a hyperalgesic response in addition to the reported anti-opioid activity.

Analgesia. The work of Rossi posits yet another view of the actions of OFQ by presenting data demonstrating a late onset analgesia following supraspinal administration of OFQ (Rossi *et al*, 1996, 1997) and the OFQ_{1-7} and OFQ_{1-11} fragments in CD-1 mice (Rossi *et al*, 1997). Later studies from her lab produced a quicker-onset, longer-acting analgesia in Sprague-Dawley rats (Rossi *et al*, 1998) in response to OFQ administration, as well as a quick-onset, short-acting analgesia following administration of OFQ_{1-7} and OFQ_{1-11}. Additional studies using the OFQ_{1-11} fragment produced similar results (Mathis

et al, 1998). Suder *et al* (1999) also demonstrated analgesia following supraspinal OFQ administration of the OFQ_{1-6} fragment in Wistar rats. Finally, direct injection of OFQ and the OFQ_{1-7} and OFQ_{1-11} fragments into the periaqueductal grey of Sprague-Dawley rats has been shown to result in analgesia (Shane *et al*, 2001, 2002). In studies using OFQ, the analgesia is either of late onset (Rossi *et al*, 1996, 1997) or long lasting (Rossi *et al*, 1998; Shane *et al*, 2001, 2002), while those using OFQ fragments (Rossi *et al*, 1997; Mathis *et al*, 1998; Suder *et al*, 1999; Shane *et al*, 2001) demonstrate a shorter-duration (2-20 m) analgesia. In all of these studies, analgesia was produced by a wide range of dosages (0.015-30 nmol) and was reversible by either NOP receptor specific compounds (Rossi *et al*, 1997) or opioid receptor specific antagonists (Rossi *et al*, 1996, 1997, 1998; Mathis *et al*, 1998; Suder *et al*, 1999; Shane *et al*, 2002).

Spinal

Analgesia. Unlike supraspinal administration, where the data favors either a hyperalgesic or anti-opioid action of NOP/OFQ, the data regarding spinal administration demonstrates a predominantly analgesic response. OFQ administration has been shown to prevent or reverse hyperalgesia or allodynia in variants of the hot plate test (Yamamoto *et al*, 1997b,c,; Yamamoto & Nozaki-Taguchi, 1997; Yamamoto *et al*, 2000a), the heat tailflick test (Xu *et al*, 1996; Hao *et al*, 1997; King *et al*, 1997; Tian *et al*, 1997a,b; Hao *et al*, 1998; Kamei *et al*, 1999a,b; Wang *et al*, 1999b; Ma *et al*, 2003), the immersion tail withdrawal test (Wang *et al*, 1999a); the formalin test (Erb *et al*, 1997; Yamamoto *et al*, 1997a; Hao & Ogawa, 1998; Kamei *et al*, 1999a; Wang *et al*, 1999a; Yamamoto & Sakashita, 1999a; Nakano *et al*, 2000; Yamamoto *et al*, 2000b), the spinal flexor reflex test (Xu *et al*, 1996), the SBL test (Inoue *et al*, 1999; Villaneuva *et al*, 2002; Menéndez *et*

al, 2003), the Von Frey test (Hao *et al*, 1998; Yamamoto & Sakashita, 1999b), and the subcutaneous bee venom test (Sun *et al*, 2004) at doses ranging from 0.055 to 17 nmol. This analgesia was seen in multiple mouse, including CD-1 (King *et al*, 1997), ddY (Inoue *et al*, 1999; Nakano *et al*, 2000), and ICR (Kamei *et al*, 1999a,b), and rat, including Sprague-Dawley (Xu *et al*, 1996; Erb *et al*, 1997; Hao *et al*, 1997; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nazaki-Taguchi, 1997; Hao *et al*, 1998; Hao & Ogawa *et al*, 1998; Wang *et al*, 1999a,b; Yamamoto & Sakashita, 1999a,b; Yamamoto *et al*, 2000a,b; Ma *et al*, 2003; Sun *et al*, 2004) and Wistar (Tian *et al*, 1997a,b; Villaneuva *et al*, 2002; Menéndez *et al*, 2003), species. In the majority of instances, this effect was of long duration, lasting at least 30 minutes (Xu *et al*, 1996; Erb *et al*, 1997; King *et al*, 1997; Tian *et al*, 1997a; Yamamoto *et al*, 1997a,b; Hao & Ogawa, 1998; Kamei *et al*, 1999a,b; Wang *et al*, 1999a,b; Yamamoto & Sakashita, 1999a,b; Yamamoto *et al*, 2000a,b; Ma *et al*, 2003; Sun *et al*, 2004). When co-administered with general opioid antagonists (King *et al*, 1997; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Hao & Ogawa, 1998; Yamamoto & Sakashita, 1999b), these effects were reversed in four of the seven experiments (King *et al*, 1997; Yamamoto *et al*, 1997b; Hao & Ogawa, 1998; Yamamoto & Sakashita, 1999b); however, in two of those instances (Yamamoto *et al*, 1997b; Yamamoto & Sakashita, 1999b) this reversal required extremely high antagonist doses.

Hyperalgesia. While the data regarding spinal administration appears to favor an analgesic response to OFQ administration, a body of data also exists suggesting a hyperalgesic response as well. OFQ-induced hyperalgesia has been demonstrated in the hot plate (Hara *et al*, 1997; Minami *et al*, 1997; Sakurada *et al*, 1999b), heat tailflick

(Zhang *et al*, 1997; Zhu *et al*, 1997; Sakurada *et al*, 1999a,b), spinal reflex (Xu *et al*, 1996, 1999), and SBL (Inoue *et al*, 1999) tests at a dosage range of 2.75×10^{-9} to 0.055 nmol. This hyperalgesic response does not appear to be affected by opioid antagonists, but can be reversed by the administration of NK1 receptor antagonists (Inoue *et al*, 1999; Sakurada *et al*, 1999a) and the OFQ fragment OFQ_{13-17} (Sakurada *et al*, 1999b). The majority of these studies involved limited observation periods (i.e. $\leq$ 30 minutes), but the one study using an extended observation period demonstrated hyperalgesia up to 60 minutes post-administration (Hara *et al*, 1997).

Anti-opioid. A body of data, though not as large as that for supraspinal administration, also exists demonstrating an anti-opioid nature to NOP/OFQ in the spinal cord. Spinal OFQ administration at doses from 0.0055-20 nmol has been shown to antagonize MOP- (Zhang *et al*, 1997; Jhamandas *et al*, 1997; Zhu *et al*, 1998; Rady *et al*, 2001), DOP- (Dawson-Basoa & Gintzler, 1997), and KOP- (Dawson-Basoa & Gintzler, 1997; Zhu *et al*, 1998) mediated analgesia. These anti-opioid effects can be long-lasting, with antagonism being maintained up to 140 minutes post-injection (Dawson-Basoa & Gintzler, 1997).

Peripheral

Analgesia. Administration of a wide range of OFQ doses (1 nmol-100μmol) in the periphery has also been shown to induce a quick and short-acting analgesia in the heat tailflick (Kolesnikov & Pasternak, 1999), immersion tail withdrawal (Ko *et al*, 2002), spinal reflex (Inoue *et al*, 1998), and SBL tests (Inoue *et al*, 1998), as well as during hyper-rotation of in acutely inflamed knees (McDougall *et al*, 2000). This response was seen not only in mice (Inoue *et al*, 1999; Kolesnikov & Paternak, 1999) and rats, but also

in monkeys (Ko *et al*, 2002), and was most prominent in cases involving local inflammation (Inoue *et al*, 1999; McDougall *et al*, 2000; Ko *et al*, 2002).

Hyperalgesia. There are a limited number of studies, however, that demonstrate a hyperalgesic response to local OFQ administration. This was generally in response to a much lower dose range of OFQ (0.01-100 fmol; Inoue *et al*, 1998, 1999); however, in the case of McDougall *et al* (2000), administration of 100 μmol resulted in a sensitization during hyper-rotation of normal rat knee joints. In all of these cases, however, the hyperalgesia was short acting.

Significance

While subject to some argument, the results above seem to demonstrate, for the most part, that both the NOP receptor and OFQ are capable of producing analgesia when administered supraspinally and spinally. There is also the suggestion that, at least in the case of spinal OFQ administration this mediation may not involve the opioid system. Taken together, these two items suggest that this receptor-ligand system might show promise as a clinical analgesic capable of bypassing the addictive properties inherent in opioid analgesics. These facts could prove useful since the use of traditional opioids for the management of pain, while still extremely effective, can be problematic for both adults and infants. Thus, non-opioid treatment alternatives that are capable of avoiding the problems associated with traditional opioid treatment, such as the development of tolerance and the potential for addiction, could be of great benefit. If this is the case, it may be important that OFQ be investigated for use in cases involving the clinical treatment of neonates, since perinatal development of the endogenous opioid systems makes treatment of pain during infancy with opiate drugs problematic. Unfortunately,

there is limited data the role of NOP/OFQ action on nociception during development. Currently, this data is limited to anatomical expression of the receptor and its ligand (Wu *et al*, 1997; Ikeda *et al*, 1998; Neal *et al*, 2001) and their effects on cellular mechanisms (Faber *et al*, 1996; Liebel *et al*, 1997; Laudenbach *et al*, 2001; Kim *et al*, 2002). This anatomical data does suggest that NOP/OFQ could also be involved in pain mediation during neonatal development. As a result of the similarities between expression of NOP and OFQ throughout the developing and mature nervous systems, we hypothesized that the behavioral effects of OFQ administration during development would be very similar to those of adults. However, current knowledge regarding the anatomical and behavioral development associated with nociception, such as differences in activity and expression of the traditional opioid receptor/ligand systems, as well as the immaturity of aspects of both the ascending sensory and descending inhibitory neural pathways, led us to consider the potential for some variation in those effects. This variation, should it exist, could help to shed light on the seemingly "dichotomous" nature of the adult data, as changes in physiological and behavioral processes during development can provide clues about the neural organization of these processes, and be helpful in the development of new methods to treat premature and seriously ill infants. To begin such a task, a basic foundation of work must be laid down to establish that the results obtained in the adults are, in fact, applicable to the developing organism. A current lack of these types of experiments has provided us with a key opportunity to establish the behavioral effects of OFQ administration during development.

Chapter 2: Methodology and Design

Rationale

The discovery of NOP and OFQ may prove to be of help in treating neonatal pain, since recent research seems to demonstrate their involvement in the modulation and/or transmission of nociceptive sensory information. As stated in the previous chapter, there is experimental evidence suggesting that, in spite of the similarity of both NOP and OFQ to their opioid cousins, this modulation may be accomplished without the involvement of opioid systems (Rossi *et al*, 1996; Erb *et al*, 1997; Rossi *et al*, 1997; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Caló *et al*, 1998; Yamamoto & Sakashita, 1999b; Chen *et al*, 2002). Anatomical studies in adult rats have demonstrated the expression of both the NOP receptor and the OFQ ligand in a number of supraspinal and spinal regions associated with the modulation and transmission of nociceptive sensory information, such as the postcentral gyrus, ventral posterolateral nucleus of thalamus, pontine and medullary reticular formations, periaqueductal grey, nucleus raphé magnus, and the dorsal horns of the spinal cord (Bunzow *et al*, 1994; Fukuda *et al*, 1994; Mollereau *et al*, 1994; Wick *et al*, 1994; Lachowicz *et al*, 1995; Anton *et al*, 1996; Dickenson, 1996; Houtani *et al*, 1996; Riedl *et al*, 1996; Kummer & Fischer, 1997; Mitsuma *et al*, 1998; Neal *et al*, 1999; Houtani *et al*, 2000a,b; Letchworth *et al*, 2000; O'Donnell *et al*, 2001; Pettersson, 2002; Berthele *et al*, 2003). Also, behavioral studies have demonstrated that the ligand itself is capable of inducing both analgesic (i.e. a decreased response to painful stimuli) and hyperalgesic (i.e. an enhanced response to painful stimuli) responses in adult rats depending on the site of administration, with the general consensus being that spinal administration results in analgesia (Xu *et al*, 1996;

Erb *et al*, 1997; Hao *et al*, 1997; King *et al*, 1997; Tian *et al*, 1997a,b; Yamamoto *et al*, 1997a,b,c) and supraspinal administration results in hyperalgesia (Meunier *et al*, 1995; Reinschied *et al*, 1995; Rossi *et al*, 1996; Rossi *et al*, 1997; Tian *et al*, 1997b; Zhu *et al*, 1997). In an attempt to verify that these effects are, in fact, the result of NOP/OFQ activity, a number of potential NOP receptor antagonist candidates have been investigated. Of the more recent of these, Nphe1 appears to show great promise, demonstrating an ability to reverse the effects of OFQ administration in multiple in vitro (Rizzi *et al*, 1999; Berger *et al*, 2000; Caló *et al*, 2000; Hashimoto *et al*, 2000; Pheng *et al*, 2000; Albrecht *et al*, 2001; Guerrini *et al*, 2001; Chiou *et al*, 2002) and in vivo (Caló *et al*, 2000; Guerrini *et al*, 2000; Rizzi *et al*, 2000; Bytner *et al*, 2001; Corradini *et al*, 2001; Di Giannuario *et al*, 2001; Lu *et al*, 2001; Rizzi *et al*, 2001; Xu *et al*, 2002) assays. However, similar experiments have yet to be conducted on the infant. Such experiments could be of great importance, since the fact that the adult data demonstrates 1) that the NOP receptor and OFQ are in some way capable of mediating the transmission of nociceptive input and 2) that this mediation may not involve the opioid system suggests that this receptor-ligand system shows promise as a potential clinical analgesic capable of bypassing the addictive properties inherent in opioid analgesics.

Design

Effects of OFQ administration on behavioral response to noxious stimuli

To determine if and to what extent the behavioral effects observed in the mature nervous system are indeed applicable to the developing system, OFQ was administered both supraspinally and spinally to the immature rat and resultant behaviors were measured in two paradigms of nociception. Rats were tested at 3-, 10-, and 21-days of age, ages that act as

models for the neural developmental state of the late third trimester human fetus (and thus also the premature human infant), the newborn human infant, and the human toddler, respectively (Anand, 1999). The specific paradigms, a variant of the hot plate test and the formalin test, were chosen due to both their ability to simulate the types of noxious stimuli that newborns medical intervention could encounter and the fact that, in these paradigms, infant rats demonstrate the same stereotypical responses seen in the adult (Fitzgerald & Gibson, 1984; Hughes & Barr, 1988; McLaughlin *et al*, 1990; Fitzgerald, 1991; Guy & Abbott, 1992; Barr, 1998). Furthermore, the biphasic nature of the formalin response allows for the differentiation between acute and chronic mechanisms of nociception. Due to the lack of reference experimental work using OFQ during development, it should be noted that the dosages used were estimates extrapolated from the adult data. Eight litters per age and ten pups per litter were run for all ages, according to the general design detailed in Table 1. Two controls, untreated and vehicle-treated, were included in this design to account for the possibility of SIA resulting from either the injection or surgical procedures (Mogil *et al*, 1996a) and the effects of the drugs themselves. Litters were used for one age only and are run in random order.

Experiment 1: Effects of OFQ administration on behavioral response to thermal noxious stimuli. To determine the nature and extent of OFQ administration on behavioral response to thermal noxious stimuli, OFQ was administered to the immature rat according to the general design for behavioral studies mentioned above. Behavioral response was tested using a variant of the hot plate paradigm, in which the right hindpaw is placed in contact with exposed resistors used to generate an age-appropriate range of temperatures. Pups were exposed to the different stimulus intensities in a random order of presentation

every 15 minutes for a period of 1 hour, allowing for the observation of both short- and long-term effects of OFQ administration.

Experiment 2: Effects of OFQ administration on behavioral response to inflammatory noxious stimuli. To determine the nature and extent of OFQ administration on behavioral response to inflammatory noxious stimuli, OFQ was administered to the immature rat according to the general design for behavioral studies mentioned above. Behavioral response was tested using a formalin paradigm, in which the right hindpaw was injected with formalin (2%, 10 μl) and the resultant behavior scored on a scale of 0-4 at one-minute intervals for the period of one hour. This extended period of observation allowed for the observation of both short- and long-term effects of OFQ administration, as well as its effect on both acute and chronic mechanisms of nociception.

Effects of OFQ administration on behavioral response to noxious stimuli in the presence of NOP antagonists

To determine the extent to which the observed behavioral changes are the result of activity at either the NOP or opioid receptors, as has been suggested due to the similarity between the NOP receptor and the opioid family of receptors, OFQ was administered in combination with the purported full antagonist for the NOP receptor [Nphe1]Nociceptin(1-13)-NH_2 both supraspinally and spinally to the immature rat. Due to the inconsistent nature of the 10-day-old data in Experiments 1 and 2, rats were tested at 3- and 21-days of age only. Also, as a result of the pattern of behavioral response in both paradigms seen in Experiments 1 and 2, behavior was tested using the previously mentioned formalin paradigm only. Due to the lack of reference experimental work using OFQ and Nphe1 during development, it should be noted that the dosages used were estimates extrapolated

from the adult data. Eight litters per age and ten pups per litter were run for all ages, according to the general design detailed in Table 2. Two controls, untreated and vehicle-treated, were included in this design to account for the possibility of SIA resulting from either the injection or surgical procedures (Mogil *et al*, 1996a) and the effects of the drugs themselves. Litters were used for one age only and are run in random order.

Experiment 3: Effects of OFQ administration on behavioral response to noxious stimuli in the presence of [Nphe1]Nociceptin(1-13)-NH_2. To determine the nature and extent of NOP influences, OFQ was administered to the immature rat in combination with the purported full antagonist for the NOP receptor [Nphe1]Nociceptin(1-13)-NH_2 according to the general design for behavioral studies mentioned above.

Subjects

Subjects were awake Long-Evans hooded rat pups, 3- to 21-days of age, bred and born in our facilities. Dams were checked for new births twice daily at 9 AM and 5 PM. The day of birth was designated as age 0 (P0). All pups were housed with the dam and their siblings in plastic cages measuring 40 × 20 × 24 cm with bedding. Food and water were available at all times. The animals were maintained under a 12-hour light:dark cycle (lights on at 7:30 AM) and the colony room temperature at 24±1°C. Pups were removed from the cage and kept warm (30-32°C) in an incubator until testing. After testing, animals were returned to the incubator until they were sacrificed.

Behavioral Testing

Intracerebroventricular (i.c.v.) injection

The procedure for injection, adapted from Ellis *et al* (1983), is minimally stressful and has been used extensively in our lab (Barr *et al*, 1992; McPhie & Barr, 2000; Zhu &

Barr, 2000). Drug doses were loaded into a 10 μl syringe with a 30-gauge needle fitted with a guard. The length that the needle extends beyond the guard corresponds to the depth of the third ventricle at each age. The pup was hand-held and gently restrained with its head held perpendicular to the axis of its spinal cord, exposing the cleft between the base of the skull and the spinal column. The injection was made into the third ventricle via this cleft. Injections were verified for each age during the development of the procedure by injecting India ink into eight pups of each age and removing the brains to take note of the location of the deposited ink. With practice, the "hit rate" is virtually 100%. Drug doses were administered over a 1-minute period.

Intrathecal (i.t.) surgery

Using methods developed in our lab that allow introduction of small quantities of drug into the spinal cerebrospinal fluid of infant rats with minimal trauma, we implanted intrathecal catheters (Paredes *et al*, 1990; Barr *et al*, 1992; Hughes & Barr, 1988). This surgery is performed on fully anesthetized animals (methoxyflurane) and typically requires less than 30 minutes to complete. Briefly, a small incision was made above the spinal column and a laminectomy performed on one or two vertebrae. The dura was lifted and a small cut made. The catheter was threaded caudally on the side contralateral to the tested paw, thus avoiding the decreased sensitivity to touch that may occur on the side of implant. The catheter was cemented to an intact spinal vertebra and the skin was closed around it. Following surgery, pups were kept in a warm, moist (30-32°C) incubator and observed until ambulatory. The time for recovery from surgery, 4-6 hours, is standard in our lab (Giordano & Barr, 1987; Hughes & Barr, 1988; Barr *et al*, 1992; King *et al*, 2000a,b) and, where expected, results were equivalent to the adult model (as an added

precaution, untreated pups were used to control for any effects of the surgical and/or injection procedures). Longer rest periods are problematic since pups can neither be returned to their dams (for fear of having the dams remove the catheter) nor kept from their dams for extended periods of time (for fear of starvation and/or dehydration). There is little, if any, damage to the spinal cord with this procedure.

Intrathecal injection

After surgery, pups, with the exception of the untreated controls, were injected with drugs dissolved in saline. To inject, a 10 μl microsyringe was filled with 10 μl of drug solution and inserted 0.25 cm into the exposed end of the catheter. To fill the dead space in the catheter, 4 μl of solution were injected, followed by a steady injection of 4-6 μl, depending on the age, at the rate of 1 μl per 15 seconds. A speck of cyanoacetate cement ("Crazy Glue®") was used to seal the exposed end of the silastic to prevent the remaining solution from seeping into the cord.

Thermal stimuli

Five minutes following initial injection, pups were tested with four intensities of a thermal stimulus (36, 38, 41, or 43°C for 3- and 10-day-old pups and 41, 43, 45, or 48°C for 21-day-old pups) using a variation of the hot plate test that has been used with success in our lab (King *et al*, 2000b; King & Barr, 2003). The pup was gently held while the right hindpaw was placed in contact with exposed resistors used to generate an age-appropriate range of temperatures (generated by the adjustment of the electrical current passed through the resistors and based on reaction times obtained using untreated pups in each age group). After 20 seconds, the paw was removed to prevent tissue damage.

Animals were exposed to all stimulus intensities in a random order every 15 minutes for a period of 1 hour.

Inflammatory stimuli

Five minutes after initial injection, pups were injected with the biphasic inflammatory agent formalin (2%, 10 μl) into the plantar pad of the left hindpaw. Immediately following formalin injection, behavioral scoring was performed at one-minute intervals for a period of one hour. The behavioral rating scale was as follows: 0 = injected paw was not favored at all; 1 = injected paw was slightly off, but still in contact with, the floor ("favored"); 2 = injected was elevated off of the floor ("paw lift"); 3 = injected paw was elevated and vigorously shaken ("paw shake"); 4= animals lick the injected paw ("paw lick").

Statistical Analysis

For studies using thermal noxious stimuli, reaction time data was analyzed using a repeated measures one-way analysis of variance (ANOVA). All doses were administered within a single litter, and the drug dose effect was thus treated as a within-subjects variable.

For studies using inflammatory noxious stimulus, a repeated measures one-way analysis of variance (ANOVA) was conducted for each dose schedule. The 60-minute observation period was divided into 20 different time periods (bins), each consisting of 3 minutes. Behavioral responses were summed in each time period, which was treated as a within-subjects variable. All doses were administered within a single litter, and the drug dose effect was thus treated as a within-subjects variable.

Chapter 3: Behavioral Studies – Thermal and Inflammatory Paradigms

Introduction

Although once a controversial topic subject to heated debate, it is now accepted that infants feel pain and alleviating that pain is important to reduce morbidity and mortality associated with serious medical problems (Anand *et al*, 1987; Yaster, 1987). Opiate drugs are the analgesics of choice but there is concern about using opiates because of the potential for dependency and/or tolerance to develop. Furthermore, perinatal development of the endogenous opioid systems makes treatment of pain during infancy with opiate drugs problematic since the relationship of unwanted side effects to therapeutic effects changes with age. Using non-opiate drugs to treat pain may alleviate these concerns. Therefore, a detailed understanding of the non-opioid aspects of nociception (i.e. the perception of pain) is necessary. There is some, albeit incomplete, information regarding the role of the NOP and OFQ on the mediation of nociception in adults that suggests that they are capable of inducing analgesia (Rossi *et al* 1996; Xu *et al*, 1996; Erb *et al*, 1997; Hao *et al*, 1997; King *et al*, 1997; Rossi *et al*, 1997; Tian *et al*, 1997a,b; Hao *et al*, 1998; Hao & Ogawa, 1998; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Mathis *et al*, 1998; Rossi *et al*, 1998; Inoue *et al*, 1999; Kamei *et al*, 1999a,b; Suder *et al*, 1999; Wang *et al*, 1999a,b; Yamamoto & Sakashita, 1999a,b; Nakano *et al*, 2000; Yamamoto *et al*, 2000a,b; Shane *et al*, 2001, 2002; Villaneuva *et al*, 2002; Ma *et al*, 2003; Menéndez *et al*, 2003; Sun *et al*, 2004) and that they may be doing so via non-opioid mechanisms (Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Yamamoto & Sakashita, 1999b); however, no corresponding data exists for early ages. This information could prove important because of the growing

recognition that adequate and continued alleviation of pain is a critical factor in the survival of human infants after invasive medical procedures (Anand *et al*, 1987; Yaster, 1987).

Thus, the present experiments attempt to lay down a basic foundation of work determining the developmental time course of the behaviors associated with OFQ administration. Experiments I & II examined the effects of supraspinal and spinal OFQ administration on the behavioral response of 3-, 10-, and 21-day-old rat pups in the thermal model of nociception, while Experiments III & IV noted the effects of supraspinal and spinal administration of OFQ on the behavioral response of 3- and 21-day-old rat pups in the inflammatory model of nociception.

Methods

Subjects

Subjects were awake Long-Evans hooded rat pups, age 3-, 10-, and 21-days-old, bred and born in our facilities. Dams were checked for new births twice daily at 9 AM and 5 PM. The day of birth was designated as age 0 (P0). All pups were housed with the dam and siblings in plastic cages. Food and water were available at all times. The animals were maintained under a 12-hour light:dark cycle (lights on at 7:30 AM) and the colony room temperature at 24±1°C. The litter was removed from the cage and pups were randomly assigned to either of two administration methods: supraspinal (i.c.v.) or spinal (i.t.). Pups assigned to the supraspinal administration condition were kept warm (30-32°C) in an incubator until injection. Pups assigned to the spinal administration condition were implanted with spinal catheters prior to being placed in the incubator and were allowed a minimum of four hours to recover before injection. Animals were returned to

the incubator until sacrificed by an intraperitoneal (i.p.) injection of an overdose of sodium pentobarbital (150 mg/kg).

Injection procedures

Intracerebroventricular injection. The procedure for injection was adapted from Ellis *et al* (1983) and has been used extensively in our lab (Barr *et al*, 1992; McPhie & Barr, 2000; Zhu & Barr, 2000). Drug doses were loaded into a 10 μl syringe with a 30-gauge needle fitted with a guard. The length that the needle extends beyond the guard corresponds to the depth of the third ventricle at each age. The pup is hand-held and gently restrained with its head held perpendicular to the axis of its spinal cord, exposing the cleft between the base of the skull and the spinal column. The injection is made into the third ventricle via this cleft. Injections were verified for each age during the development of the procedure by injecting India ink into eight pups of each age and removing the brains to take note of the location of the deposited ink. With practice, the "hit rate" was or neared 100%. Drug doses are administered over a 1-minute period.

Intrathecal surgery. Using methods developed in our lab that allow introduction of small quantities of drug into the spinal cerebrospinal fluid of infant rats with minimal trauma, we implanted intrathecal catheters (Paredes *et al*, 1990; Barr *et al*, 1992; Hughes & Barr, 1988). This surgery is performed on fully anesthetized animals (methoxyflurane) and typically requires less than 30 minutes to complete. Briefly, a small incision is made above the spinal column and a laminectomy performed on one or two vertebrae. The dura is lifted and a small cut made. The catheter is threaded down the cord towards the tail on the side contralateral to the tested paw, thus avoiding the decreased sensitivity to touch that tends to occur on the side of implant. It is cemented to an intact spinal vertebra and

the skin is closed around the catheter. Following surgery, pups are kept in a warm, moist (30-32°C) incubator and observed until ambulatory. The time for recovery from surgery, 4-6 hours, is standard in our lab (Giordano & Barr, 1987; Hughes & Barr, 1988; Barr *et al*, 1992; King *et al*, 2000a,b) and, where expected, results are equivalent to the adult model. Longer rest periods are problematic since pups can neither be returned to their dams (for fear of having the dams remove the catheter) nor kept from their dams for extended periods of time (for fear of starvation and/or dehydration). There is little, if any, damage to the spinal cord with this procedure.

Intrathecal injection. After surgery, pups were injected with drugs dissolved in saline. To inject, a 10 μl microsyringe was filled with 10 μl of drug solution and inserted 0.25 cm into the exposed end of the catheter. To fill the dead space in the catheter, 4 μl of solution were injected, followed by a steady injection of 4-6 μl, depending on the age, at the rate of 1 μl per 15 seconds. A speck of cyanoacetate cement (e.g. "Crazy Glue®") was used to seal the exposed end of the silastic to prevent the remaining solution from seeping into the cord.

Behavioral testing

Thermal. Five minutes following initial injection, pups were tested with four intensities of a thermal stimulus (36, 38, 41, or 43°C for 3- and 10-day-old pups and 41, 43, 45, or 48°C for 21-day-old pups) using a variation of the hot plate test that has been used with success in our lab (King *et al*, 2000b; King & Barr, 2003). Eight litters per age and ten pups per litter were tested for all ages. Subjects were used for one age only and tested in random order, with a period of roughly 15 seconds between intensities. One pup was used for each dose (vehicle, 0.1, 1, 10 nmol) in each injection route (i.c.v. or i.t.).

Also, to control for any effects of surgical and/or injection procedures, an untreated (i.e., no surgery, no injection) pup was included in each administration group (i.c.v. or i.t.). The pups were gently held while the right hindpaw was placed in contact with exposed resistors used to generate an age-appropriate range of temperatures (generated by the adjustment of the electrical current passed through the resistors and based on reaction times obtained using untreated pups in each age group). After 20 seconds, the paw was removed to prevent tissue damage. Animals were exposed to all stimulus intensities in a random order every 15 minutes for a period of 1 hour.

Inflammatory. Five minutes after initial injection, pups were injected with the biphasic inflammatory agent formalin (2%, 10 μl) into the plantar pad of the left hindpaw. Eight litters per age and ten pups per litter were tested for all ages. Subjects were used for one age only and tested in random order, with a period of roughly 15 seconds between intensities. One pup was used for each dose (vehicle, 0.1, 1, 10 nmol) in each injection route (i.c.v. or i.t.). Also, to control for any effects of surgical and/or injection procedures, an untreated (i.e., no surgery, no injection) pup was included in each administration group (i.c.v. or i.t.). Immediately following formalin injection, behavioral scoring was performed at one-minute intervals for a period of one hour. The behavioral rating scale was as follows: 0 = injected paw was not favored at all; 1 = injected paw was slightly off, but still in contact with, the floor ("favored"); 2 = injected was elevated off of the floor ("paw lift"); 3 = injected paw was elevated and vigorously shaken ("paw shake"); 4= animals lick the injected paw ("paw lick").

Statistical analyses

Reaction time (RT) data and pain intensity score (PIS) data (collapsed into 3-min bins for ease of analysis) were analyzed using a repeated measures multifactorial analysis of variance (ANOVA). All doses were administered within a single litter, and the drug dose effect was thus treated as a within-subjects variable. Effects of stimulus temperature and testing time/bins were also within-subjects variables. The Newman-Keuls test was used for *post hoc* comparisons. All significance was set at $p < 0.05$.

Results

Control Effects

In the formalin test, differences between controls were seen in the 21-day-old pups via both supraspinal ($F_{4, 28} = 7.98$, $p < 0.001$) and spinal ($F_{4, 28} = 8.50$, $p < 0.001$) routes of administration only (Table 3). Saline-treated controls demonstrated lower pain intensity scores (PIS), suggesting the presence of a stress-related analgesia resulting from the injection itself. No differences were seen between routes of injection in either uninjected or saline-treated controls at 3- ($F_{1,7} = 2.46$, $p > 0.05$; $F_{1,7} = 1.43$, $p > 0.05$) (Figure 6, Figure 7) or in uninjected controls at 21-days of age ($F_{1,7} = 1.50$, $p > 0.05$) (Figure 8); however, differences were seen between routes of injection in saline-treated controls at 21-days of age ($F_{19,133} = 2.23$, $p < 0.005$) (Figure 9), with greater PISs following spinal administration. Furthermore, differences were also seen between ages in both uninjected ($F_{1,7} = 12.61$, $p < 0.01$) (Figure 10) and saline-treated ($F_{1,7} = 8.01$, $p < 0.05$) (Figure 11) controls following spinal injection and in uninjected controls following supraspinal injection ($F_{19,133} = 7.43$, $p < 0.005$) (Figure 12), with 21-day-olds

demonstrating greater PISs in all three cases. In the thermal paradigm, differences were seen in the 3- ($F_{4, 28} = 3.59$, $p < 0.05$) and 10-day-old ($F_{4, 28} = 5.42$, $p < 0.005$) ages for the spinal injection route only (Table 3). Saline-treated controls demonstrated higher reaction times (RT), again suggesting the presence of a stress-related analgesia. Differences between routes of injection were seen in 21-day-old uninjected controls only ($F_{1,7} = 10.58$, $p < 0.05$) (Figure 13), with lower RTs following spinal injection. In uninjected controls, differences were seen between ages following supraspinal ($F_{2,14} = 11.71$, $p < 0.005$) (Figure 14) and spinal ($F_{2,14} = 12.63$, $p < 0.001$) (Figure 15) administration. In both cases, 10-day-olds demonstrated much higher RTs than both 3- and 21-day-olds; however, differences between 3- and 21-day-olds were only seen following spinal administration, with 21-day-olds demonstrating lower RTs. In saline-treated controls, differences were seen between ages following supraspinal ($F_{2,14} = 6.41$, $p < 0.05$) (Figure 16) and spinal ($F_{2,14} = 13.87$, $p < 0.001$) (Figure 17) administration. In both cases, both 3- and 10-day-olds demonstrated higher RTs than 21-day-olds; however, differences between 3 and 10-day-olds were only seen following spinal administration.

Dose Effects

Intrathecal – Thermal. Differences were seen between drug-treated pups and uninjected controls in 3-day-olds only ($F_{4, 28} = 3.59$, $p < 0.05$) (Figure 18). All treated conditions demonstrated higher RTs, implying a potential analgesia. However, no differences were seen between drug-treated pups and injected controls. Differences were seen between the middle and high doses and uninjected controls, but not between the low dose and uninjected controls, in 10-day-olds ($F_{4, 28} = 5.42$, $p < 0.005$) (Figure 19). Both the middle and high doses produced higher RTs, suggesting a possible analgesia

following these higher doses. Again, no differences were seen between drug-treated pups and injected controls. No significant dose-related effects were seen in 21-day-olds ($F_{4, 28} = 2.44$, $p > 0.05$) (Figure 20).

Intrathecal – Inflammatory. Although there were differences between drug-treated 21-day-old pups and uninjected controls ($F_{4, 28} = 8.50$, $p < 0.001$) (Figure 21), there were no differences between these OFQ-treated pups and the saline-treated controls: all injected pups demonstrated lower PIS values. However, there was a strong trend suggesting a difference between the high dose and the injected control, which would imply that high doses are necessary to produce analgesia in this paradigm. No significant dose effects were seen in the 3-day-olds ($F_{4, 28} = 1.50$, $p > 0.05$) (Figure 22), suggesting that OFQ has little effect on inflammatory pain early in development when administered spinally.

Intracerebroventricular – Thermal. Differences were seen between the high dose and uninjected, but not injected, controls only in the 3-day-olds ($F_{4, 28} = 4.92$, $p < 0.005$) (Figure 23), with the high dose demonstrating higher RTs, suggesting that a high OFQ dose may produce analgesia early development in this paradigm. This idea could be supported by strong trends suggesting differences between the high dose and the injected control and low dose, which would imply that the analgesia seen is distinct from that produced by saline injection. No significant dose related effects were seen in 10- ($F_{4, 28} = 0.80$, $p > 0.05$) (Figure 24) and 21-day-olds ($F_{4, 28} = 1.19$, $p > 0.05$) (Figure 25).

Intracerebroventricular – Inflammatory. Differences were seen between the high dose and both controls, as well as between the high dose and the low and middle doses, in both the 3- ($F_{4, 28} = 5.42$, $p < 0.005$) (Figure 26) and 21-day-old ($F_{4, 28} = 7.98$, $p < 0.001$)

(Figure 27) pups, with the high dose demonstrating lower PISs. No differences were seen between the low and middle doses, or between the low and middle doses and the injected control, at either age, implying that a high dose of OFQ is capable of producing a distinct analgesia during development.

Interaction Effects

Intrathecal – Thermal. Significant dose × time interactions were demonstrated in 21-day-olds ($F_{16, 112} = 2.84$, $p < 0.001$) (Figure 28). The 21-day-olds showed an increase in RTs in a dose-dependent manner during the first 30 minutes of testing, suggesting a quick onset and relatively long duration of effect. No significant dose × bin interactions were seen in 3- ($F_{16, 112} = 0.60$, $p > 0.05$) (Figure 29) and 10-day-olds ($F_{16, 112} = 1.23$, $p > 0.05$) (Figure 30).

Intrathecal – Inflammatory. Significant dose × bin interactions were demonstrated in 21-day-olds ($F_{76, 532} = 2.11$, $p < 0.001$) (Figure 31). The 21-day-olds showed a dose-dependent decrease in PIS values that demonstrated later onsets and shorter durations (with a maximum period of 24 minutes for the high dose, beginning at bin 2), implying that OFQ administration is capable of producing analgesia in both phases of the formalin response. No significant dose × bin interactions were seen following spinal administration in 3-day-olds, again suggesting that OFQ has no effect on inflammatory pain early in development when administered spinally ($F_{76, 532} = 1.14$, $p > 0.05$) (Figure 32).

Intracerebroventricular – Thermal. Significant dose × time interactions were demonstrated in 3- ($F_{16, 112} = 1.86$, $p < 0.05$) (Figure 33) and 10-day-olds ($F_{16, 112} = 2.65$, $p < 0.005$) (Figure 34). The 3-day-olds showed an increase in RTs between the high dose

and controls at the 35-minute test time, implying a quick-acting and long duration analgesic effect of OFQ. On the other hand, the 10-day-olds showed a decrease in RTs during the first 15 minutes of testing between the two high doses and controls, implying a quick-acting and short duration hyperalgesic effect of OFQ. No significant dose × bin interactions were seen in 21-day-olds ($F_{16, 112} = 1.37$, $p > 0.05$) (Figure 35).

Intracerebroventricular – Inflammatory. Significant dose × bin interactions were demonstrated in 3- ($F_{76, 532} = 1.47$, $p < 0.01$) (Figure 36) and 21-day-olds ($F_{76, 532} = 3.31$, $p < 0.001$) (Figure 37). The 3-day-olds showed a decrease in PIS values between the high dose and controls during the first 12 minutes of testing, suggesting that OFQ is only capable of influencing the first phase of the formalin response at this age. The 21-day-olds showed a dose-dependent decrease in PIS values that demonstrated an earlier onset and longer duration as doses increased (reaching a maximum period of the first 33 minutes of testing with the high dose), implying a greater influence of OFQ that affects both phases of the formalin response.

Discussion

The data from the inflammatory paradigm are fairly straightforward: both routes of administration result in analgesia. In the case of supraspinal administration, analgesia appeared at both 3- and 21-days of age and, in spite of differences between the controls in the 21-day-olds, seemed to demonstrate a marked influence of NOP/OFQ: OFQ was significantly different from both controls at both ages. This analgesia was quick acting, but was limited to the equivalent of the first phase of formalin behavior in the 3-day-olds while affecting both phases in the 21-day-olds. The analgesia seen following spinal administration, however, was limited to older pups and seemed to be mediated more by a

stress-related response than the action of NOP/OFQ, as no differences were demonstrated between any of the treatment doses and the saline-injected control. The time-course of this stress-induced analgesia differed slightly from that seen following supraspinal injection: although still quick acting, the time of onset was slightly later and the duration was shorter. The shift in onset of analgesic response, both for age and time, between routes of administration may reflect a change in NOP receptor density/maturation of the NOP system and its associated neural connections between supraspinal and spinal populations, with spinal populations being less dense and/or immature relative to the supraspinal ones. This point could be clarified by the use of anatomical techniques, such as *in situ* hybridization and immunohistochemistry, to verify receptor population density during development. On the other hand, the differences seen in timing and duration of analgesic response in both routes of administration are to be expected in light of the response to formalin during development and could indicate that the mechanisms underlying NOP/OFQ-induced analgesia do not involve NMDA activation (Barr, 1998). Finally, the fact that a stress-mediated response occurred in 21-day-olds as a result of both routes of administration suggests an age-related mechanism rather than one related to the route of administration.

The thermal paradigm data are more difficult to interpret. The data from spinal OFQ administration show an analgesic response in 3-, 10-, and 21-day-olds; however, in the two younger ages, differences were seen between controls, but not between the saline-treated control and the treatment doses, suggesting that this analgesia was mediated more by SIA than NOP activity. These younger ages also showed no significant dose × time interaction. The 21-day-olds, on the other hand, demonstrated a time-course

of action that was quick and fairly long lasting. These differences may be the result of low NOP receptor population density at these ages, something that would correspond with the above-mentioned spinal administration behavioral data from the inflammatory paradigm. The change in response as the rat matures may indicate a marked increase in that density between the ages of 10- and 21-days. The data from supraspinal administration are more confusing. The 3-day-old pups demonstrated a quick and short-acting analgesia in response to the high dose, but the 10-day-olds showed an unexpected hyperalgesic response in response to the two high doses while the 21-day-olds showed no significant response at all. The analgesia seen in the 3-days-olds once again suggests low NOP receptor population density early in development, but the radical shift in response seen in 10- and 21-day-olds also suggests that either supraspinal and spinal NOP/OFQ populations interact with different mechanisms during development or, perhaps more likely, the data represent a statistical anomaly.

In the case of both routes of administration, the general lack of effectiveness of anything but the high dose stands in marked contrast to the adult data, where doses as low as the fmol range have been shown to elicit responses. This once again suggests that differences exist in NOP receptor populations across ages, with population densities increasing with age. Furthermore, the difference in age-onset of response between routes of administration (i.e. analgesia demonstrated at later ages following spinal administration relative to supraspinal administration) suggests different development time-courses for each population of receptors, with supraspinal populations being expressed earlier than spinal ones. Finally, differences between paradigms in the ages at which stress-related responses are seen suggest that each paradigm may be acting on

different neural pathways and/or systems, or that different interactions may exist between the nociceptive pathways being activated by the paradigm and those mediating the stress-related response.

During development, there is, for the most part, no dichotomy between behavior following supraspinal and spinal injection: both routes of administration are capable of producing analgesia. Based on the adult data, analgesia following spinal administration of OFQ is expected, but analgesia following supraspinal injection comes as a bit of a surprise. These result are not overwhelmingly conclusive, however, as this analgesia appears to be paradigm-dependent at certain ages and one example exists in which hyperalgesia, rather than analgesia, occurs. In spite of this, the data as a whole remains fairly consistent and suggest that the NOP receptor and its endogenous ligand OFQ can have a sizable influence on the mediation of nociception during development.

Conclusion

Combined with the body of adult data suggesting that the actions of NOP and OFQ may not involve the activation of the opioid system (Rossi *et al*, 1996; Erb *et al*, 1997; Rossi *et al*, 1997; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Caló *et al*, 1998; Yamamoto & Sakashita, 1999b; Chen *et al*, 2002), our data may be relevant as it applies to the treatment of neonates. There is a growing recognition that the alleviation of pain is critical to the survival of human infants after invasive medical procedures (Anand *et al*, 1987; Yaster, 1987). Unfortunately, perinatal development of the endogenous opioid system makes treatment of pain during infancy with the current popular opiate-based analgesics problematic. This is further complicated by the fact that the relationship of unwanted side effects to therapeutic effects of opioids changes with

age. NOP/OFQ-based clinical analgesics might be useful in bypassing these problems since the OFQ system is capable of suppressing nociceptive input at early ages and has yet to demonstrate any of the addictive properties inherent in opioid analgesics. However, little is known currently about the mechanisms by which nociception is mediated in the developing organism, and almost nothing about the roles of NOP/OFQ in that mediation. While our results are a first step in that direction, further studies are required to establish the developmental time course of the behaviors associated with OFQ administration, as well as the anatomical distribution of NOP/OFQ and the existence of a definitive NOP antagonist. Combined with current knowledge regarding the anatomy and behavior associated with nociception during development, this could prove invaluable in the discovery of the mechanisms governing nociception and its mediation during development and the development of new methods to treat premature and seriously ill infants.

Chapter 4: Behavioral Studies – Antagonist

Introduction

Data regarding the behavioral effects of NOP/OFQ are complicated. First, in the adult effects of OFQ seem to be dependent on the site of injection, with spinal injection generally resulting in analgesia and supraspinal in hyperalgesia. The data demonstrating a spinal analgesia are consistent (Xu *et al*, 1996; Erb *et al*, 1997; Hao *et al*, 1997; King *et al*, 1997; Tian *et al*, 1997a,b; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Hao *et al*, 1998; Hao & Ogawa, 1998; Inoue *et al*, 1999; Kamei *et al*, 1999a,b; Wang *et al*, 1999a,b; Yamamoto & Sakashita, 1999a,b; Nakano *et al*, 2000; Yamamoto *et al*, 2000a,b; Villaneuva *et al*, 2002; Ma *et al*, 2003; Sun *et al*, 2004); however, there is substantial variability in the supraspinal data (Rossi *et al*, 1996; Zhu *et al*, 1996; Rossi *et al*, 1997; Zhu *et al*, 1997; Caló *et al*, 1998; Zhu *et al*, 1998; Wang *et al*, 1999a,b; Citterio *et al*, 2000). Based on the data presented in the previous chapter, this dichotomy of action may not exist during development, as an analgesic response is seen regardless of injection paradigm. Second, there is uncertainty about whether these effects result from NOP/OFQ activity alone or involve opioid mechanism co-activation. Data exist in which the effects of OFQ are reversed by non-specific opioid antagonists administration (Rossi *et al*, 1996; King *et al*, 1997; Rossi *et al*, 1997; Yamamoto *et al*, 1997b; Hao & Ogawa, 1998; Mathis *et al*, 1998; Rossi *et al*, 1998; Suder *et al*, 1999; Yamamoto & Sakashita, 1999b; Shane *et al*, 2001, 2002), but, again, these results are inconsistent.

The behavioral effects of NOP/OFQ are also difficult to interpret because no definitive antagonist for NOP exists to verify that the effects of OFQ administration result from an interaction with NOP and not from co-localized or downstream opioid

mechanisms. A number of potential candidates have been proposed over the years, including Ac- RYYRIK-NH_2 (Dooley *et al*, 1997), nocistatin (Okuda-Ashitaka *et al*, 1998), NalBzoh (Dunnill *et al*, 1998), [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]$nociceptin_{1\text{-}13}$-$NH_2$ (Guerrini *et al*, 1998), [$Nphe^1$]$nociceptin_{1\text{-}13}$-NH_2 (Caló *et al*, 1998), J-113397/CompB (Kawamoto *et al*, 1999), UFP-101 (Caló *et al*, 2002), JTC-801 (Yamada *et al*, 2002), two azacycloalkane amino acid derivatives (Halab *et al*, 2002), SB-612111 (Zeratin *et al*, 2004), and members of a novel series of piperidin-4-yl-1,3-dihydroindol-2-ones (Zaveri *et al*, 2004), but, for the most parts, this antagonism has been limited to in vitro studies (azacycloalkane amino acid derivatives, piperidin-4-yl-1,3-dihydroindol-2-ones), inconsistent (nocistatin, NalBzoh, [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]$nociceptin_{1\text{-}13}$-$NH_2$), or the compounds themselves have also demonstrated antinociceptive effects of their own ([$Nphe^1$]$nociceptin_{1\text{-}13}$-NH_2, JTC-801, UFP-101). Of these, the two most promising seem to be J-113397/CompB (CompB) and [Nphe1]nociceptin(1-13)-NH_2 (Nphe1). CompB demonstrates antagonistic effects both in vitro (Kawamoto *et al*, 1999, Ozaki *et al*, 2000a; Ichikawa *et al*, 2001; Chiou & Fan, 2002) and in vivo (Ozaki *et al*, 2000b; Yamamoto *et al*, 2001), but is a proprietary compound and access to it is limited. Nphe1, on the other hand, has been suggested to have antinociceptive properties of its own (Caló *et al*, 2000; Di Giannuario *et al*, 2001), but is readily available commercially and thus presents a more practical option for study.

Keeping these facts in mind, the present experiments attempt to investigate the effectiveness of Nphe1 as a NOP antagonist during development by noting the effects of OFQ when co-administered with Nphe1 via either spinal or supraspinal routes on the

behavioral response of 3- and 21-day-old rat pups in the inflammatory paradigm of nociception.

Methods

Subjects

Subjects were awake Long-Evans hooded rat pups, age 3-, 10-, and 21-days-old, bred and born in our facilities. Dams were checked for new births twice daily at 9 AM and 5 PM. The day of birth was designated as age 0 (P0). All pups were housed with the dam and siblings in plastic cages. Food and water were available at all times. Animals were maintained under a 12-hour light:dark cycle (lights on at 7:30 AM) and the colony room temperature at 24±1°C. The litter was removed from the cage and pups were randomly assigned to either of two administration methods: supraspinal (i.c.v.) or spinal (i.t.). Pups assigned to the supraspinal administration condition were kept warm (30-32°C) in an incubator until injection. Pups assigned to the spinal administration condition were implanted with spinal catheters prior to being placed in the incubator and were allowed a minimum of four hours to recover before injection. Animals were returned to the incubator until sacrificed by an intraperitoneal (i.p.) injection of an overdose of sodium pentobarbital (150 mg/kg).

Injection procedures

Intracerebroventricular injection. The procedure for injection, adapted from Ellis *et al* (1983), is minimally stressful and has been used extensively in our lab (Barr *et al*, 1992; McPhie & Barr, 2000; Zhu & Barr, 2000). Drug doses were loaded into a 10 μl syringe with a 30-gauge needle fitted with a guard. The length that the needle extends beyond the guard corresponds to the depth of the third ventricle at each age. The pup was

hand-held and gently restrained with its head held perpendicular to the axis of its spinal cord, exposing the cleft between the base of the skull and the spinal column. The injection was made into the third ventricle via this cleft. Injections were verified for each age during the development of the procedure by injecting India ink into eight pups of each age and removing the brains to take note of the location of the deposited ink. With practice, the "hit rate" is virtually 100%. Drug doses were administered over a 1-minute period.

Intrathecal surgery. Using methods developed in our lab that allow introduction of small quantities of drug into the spinal cerebrospinal fluid of infant rats with minimal trauma, we implanted intrathecal catheters (Paredes *et al*, 1990; Barr *et al*, 1992; Hughes & Barr, 1988). This surgery is performed on fully anesthetized animals (methoxyflurane) and typically requires less than 30 minutes to complete. Briefly, a small incision was made above the spinal column and a laminectomy performed on one or two vertebrae. The dura was lifted and a small cut made. The catheter was threaded caudally on the side contralateral to the tested paw, thus avoiding decreased sensitivity to touch that may occur on the side of implant. The catheter was cemented to an intact spinal vertebra and the skin was closed around it. Following surgery, pups were kept in a warm, moist (30-32°C) incubator and observed until ambulatory. The time for recovery from surgery, 4-6 hours, is standard in our lab (Giordano & Barr, 1987; Hughes & Barr, 1988; Barr *et al*, 1992; King *et al*, 2000a,b) and, where expected, results were equivalent to the adult model. Longer rest periods are problematic since pups can neither be returned to their dams (for fear of having the dams remove the catheter) nor kept from their dams for

extended periods of time (for fear of starvation and/or dehydration). There is little, if any, damage to the spinal cord with this procedure.

Intrathecal injection. Four to six hours after surgery, pups were injected with drugs dissolved in saline. To inject, a 10 μl microsyringe was filled with 5-7 μl of drug solution and inserted 0.25 cm into the exposed end of the catheter. Three μl of solution were injected into the catheter to fill the dead space followed by a steady injection of 2-4 μl, depending on age, at the rate of 1 μl per 15 seconds. A speck of cyanoacetate cement ("Crazy Glue®") was used to seal the exposed end of the silastic to prevent the remaining solution from seeping into the cord.

Behavioral testing

Five minutes after the central injection, pups were injected with the biphasic inflammatory agent formalin (2%, 10 μl) into the plantar pad of the left hindpaw. Eight litters per age and ten pups per litter were tested for all ages. Subjects were used for one age only and tested in random order, with a period of roughly 15 seconds between intensities. One pup was used for each dose (vehicle, 0.1, 1, 10 nmol) in each injection route (i.c.v. or i.t.). Also, to control for any effects of surgical and/or injection procedures, an untreated (i.e., no surgery, no injection) pup was included in each administration group (i.c.v. or i.t.). Immediately following formalin injection, behavioral scoring was performed at one-minute intervals for a period of one hour. The behavioral rating scale was as follows: 0 = injected paw was not favored at all; 1 = injected paw was slightly off, but still in contact with, the floor ("favored"); 2 = injected was elevated off of the floor ("paw lift"); 3 = injected paw was elevated and vigorously shaken ("paw shake"); 4= animals lick the injected paw ("paw lick").

Statistical analyses

Pain intensity score (PIS) data (collapsed into 3-minute bins for ease of analysis) was analyzed using a repeated measures multifactorial analysis of variance (ANOVA). All doses were administered within a single litter, and the drug dose effect was thus treated as a within-subjects variable. Effect of bins was also treated as a within-subjects variable. In all analyses, *post hoc* tests of significant interactions were Newman-Keuls tests using the appropriate within-subjects error term. All significance was set at $p < 0.05$.

Results

Control Effects

Differences between controls were seen in the 21-day-old pups following spinal routes of administration ($F_{3,21} = 7.86$, $p < 0.001$) only (Table 4). Saline-treated controls demonstrated lower PISs. No differences were seen between routes of injection in either uninjected or saline-treated controls at this age ($F_{1,7} = 0.28$, $p > 0.05$; $F_{1,7} = 4.4$, $p > 0.05$) (Figure 38, Figure 39) or in uninjected controls at 3-days of age ($F_{1,7} = 3.77$, $p > 0.05$) (Figure 40). However, differences were seen between injection routes in saline-treated controls at 3-days of age ($F_{19,133} = 2.47$, $p < 0.005$) (Figure 41), with spinal administration resulting in greater PISs. In uninjected controls, differences were seen between ages following both supraspinal ($F_{1,7} = 51.01$, $p < 0.0005$) (Figure 42) and spinal ($F_{1,7} = 79.71$, $p < 0.001$) (Figure 43) administration. In both cases, 21-day-olds demonstrated much higher PISs than 3-day-olds. In saline-treated controls, differences between ages were also seen following both supraspinal ($F_{1,7} = 34.51$, $p < 0.001$) (Figure 44) and spinal ($F_{19,133} = 5.03$, $p < 0.001$) (Figure 45) administration. The results were opposite, however,

with supraspinal injection resulting in greater PISs in 21-day-olds and spinal injection resulting in greater PISs in 3-day-olds.

Dose Effects

Intrathecal. Differences were seen between Nphe1 co-administration (saline/Nphe1, and OFQ/Nphe1) groups and uninjected controls in 21-day-old pups ($F_{3,21} = 7.86$, $p < 0.001$) (Figure 46), implying a possible analgesic effect. However, there were no differences between Nphe1 co-administration groups and the saline-treated controls: all injected pups demonstrated lower PIS values, suggesting that the observed analgesia is stress-mediated. No significant dose effects were seen in 3-day-olds ($F_{3,21} = 1.43$, $p > 0.05$) (Figure 47).

Intracerebroventricular. In 3-day-olds, significant differences were seen between the injected control and the saline/Nphe1 co-administration group only ($F_{3,21} = 3.94$, $p < 0.05$) (Figure 48). The saline/Nphe1 co-administration group demonstrated lower PISs, suggesting an analgesic nature of Nphe1 and hinting at its possible antagonism of OFQ. However, there were strong trends suggesting additional differences between the uninjected controls and Nphe1 co-administration groups, as well as between the injected control and the OFQ/Nphe1 co-administration group. These trends add to the idea of an analgesic effect of Nphe1, but may argue against its possible antagonism of OFQ. No differences were seen between controls, arguing against the presence of a SIA, or the two Nphe1 co-administration groups. The lack of differences in the co-administration groups could indicate that 1) the analgesia produced by Nphe1 is equal to that produced by OFQ or 2) the analgesia seen in both cases is mediated by Nphe1. In 21-day-olds, differences were seen between the uninjected controls and Nphe1 co-administration groups ($F_{3,21} =$

11.35, $p < 0.001$) (Figure 49), with the co-administration groups demonstrating lower PISs. These results again suggest a possible analgesic response to the co-administration groups. However, differences were seen between the injected control and the OFQ/Nphe1 co-administration group only, with the OFQ/Nphe1 co-administration group also demonstrating lower PISs. Taken together, these two sets of results could imply that differences in the nature of the analgesia produced by each of the co-administration groups, with the saline/Nphe1-produced analgesia being the result of a SIA and the OFQ/Nphe1-produced analgesia the result of OFQ activity. Once again, there were strong trends suggesting additional differences between the injected control and the saline/Nphe1 co-administration group, and between the Nphe co-administration groups only. If these two points prove to be true, they would suggest that the Nphe1-induced analgesia and OFQ-induced analgesia are distinct.

Interaction Effects

Intrathecal. Significant dose × bin interactions were demonstrated in 21-day-olds ($F_{57,399} = 1.748$, $p < 0.005$) (Figure 50). The 21-day-olds showed a decrease in PIS values that demonstrated early onsets and long durations (with a maximum period of 39 minutes), implying that spinal OFQ administration is capable of producing analgesia in both phases of the formalin response. No significant dose × bin interactions were seen in 3-day-olds, suggesting that OFQ has no effect on inflammatory pain early in development when administered spinally ($F_{57,399} = 1.297$, $p > 0.05$) (Figure 51).

Intracerebroventricular. Significant dose × bin interactions were demonstrated in 21-day-olds ($F_{57,399} = 1.525$, $p < 0.05$) (Figure 52). The 21-day-olds showed a decrease in PIS values that demonstrated a slightly later onset (except in the case of OFQ/Nphe1 co-

administration) and longer duration (in the case of OFQ/Nphe1 co-administration, as long as 57 minutes), suggesting that supraspinal OFQ administration is also capable producing analgesia in both formalin phases. No significant dose × bin interactions were seen in 3-day-olds ($F_{57,399} = 1.086$, $p > 0.05$) (Figure 53), again implying a lack of effect by OFQ on inflammatory pain early in development.

Discussion

Following supraspinal administration, Nphe1 demonstrates mixed effects that appear to be age-dependent. In 3-day-olds, the lack of differences in PISs between untreated controls and saline-injected controls indicates a lack of any stress-related effects. This fact, combined with the differences between the saline-injected controls and saline/Nphe1 co-administration groups, suggests that Nphe1 may be capable of analgesic effects at this age. Those effects may not work synergistically with the OFQ-induced analgesia described in the previous chapter. Keeping in mind the lack of difference between the Nphe1 co-administration groups, it could be argued that the analgesia seen following OFQ/Nphe1 co-administration is, in fact, mediated by Nphe1, which, as a purported antagonist, should also be blocking OFQ activity. However, the data from the 21-day-olds, in which an analgesic effect above and beyond that demonstrated by Nphe1 alone may be seen under the same circumstances, argues against any antagonistic effect of Nphe1 on OFQ, thereby suggesting that the analgesic response seen may be OFQ-mediated (a fact which would be more in line with the data presented in the previous chapter). If the trends seen in this data are taken into consideration, a stronger argument is made for an analgesic effect of Nphe1, but no additional clarification regarding the mechanisms underlying the analgesia following OFQ/Nphe1 administration is made. In

the case of 21-day-olds, no differences are demonstrated between controls, once again arguing against any SIA. Furthermore, the lack of differences between injected controls, as well as between Nphe1 co-administration groups, again argues that there is an analgesic effect following Nphe1 administration and once again makes it difficult to determine if the observed analgesia is due to the actions of either Nphe1, OFQ, or a combination of both. However, the trends seen in this data, specifically the suggestion of a difference between the Nphe1 co-administration groups, can be used to argue that there is, in fact, an analgesia resulting from OFQ administration and that this analgesia is above and beyond that seen with Nphe1 alone. As stated above, this would also provide further argument that Nphe1 is incapable of reversing the effects of OFQ.

In the case of spinal administration, on the other hand, no effects are seen in any groups at 3-days of age, suggesting that the mechanisms by which Nphe1 elicits effects here are not yet mature. At 21-days of age, the equivalent analgesia levels seen between injected controls, saline/Nphe1 co-administration, and OFQ/Nphe1 administration imply that this analgesia is not mediated by either OFQ or Nphe1 administration. Combined with the differences between controls, as well as the lack of differences between Nphe1-treated groups and the saline-treated control, it can be argued that the observed analgesia is instead the result of a stress-related mechanism and that Nphe1 is, once again, not eliciting any effects, antagonistic or analgesic.

Conclusion

Behavioral responses in this study seem to be in line with those presented in the last chapter: spinal OFQ administration at 3- and 21-days does not appear to have any effect, while supraspinal administration at those ages produces analgesia. The fact that

OFQ is still apparently capable of producing analgesia regardless of Nphe1 co-administration suggests that Nphe1 is not demonstrating any antagonistic effects at the tested ages. This is a marked departure from the behavioral studies done in the adult (Caló *et al*, 2000; Guerrini *et al*, 2000; Rizzi *et al*, 2000; Bytner *et al*, 2001; Corradini *et al*, 2001; Di Giannuario *et al*, 2001; Lu *et al*, 2001; Rizzi *et al*, 2001; Xu *et al*, 2002). Furthermore, the differences in PISs between the 3- and 21-day-old saline controls and saline/Nphe1 co-administration groups suggest that Nphe1 is capable of producing an analgesic effect of its own at early ages, a fact that has been suggested in the adult data (Caló *et al*, 2000; Di Giannuario *et al*, 2001).

The observed lack of antagonistic effect at these ages, combined with the data demonstrating an analgesic effect of Nphe1 administration, argue that Nphe1 is, in fact, a partial agonist, rather than an antagonist, of the NOP receptor. Unlike full agonists, which are capable of full receptor activation and thus maximal system response, partial antagonists result in partial receptor activation, submaximal system response, and the potential blockade of full agonist activation of the receptor (Kenakin *et al*, 2001). This would go a long way in explaining both the analgesia and purported antagonism seen in adults following Nphe1 administration. If differences exist in NOP receptor expression between 3- and 21-day-olds, and assuming differential binding at the NOP receptor for OFQ and Nphe1 in favor of Nphe1, it could also go a long way in explaining the differences seen during development. Lower receptor populations in 3-day-olds would increase the likelihood of OFQ blockage by Nphe1 and thus any resultant analgesia would mainly be due to Nphe1 activity. In 21-day-olds, increased receptor expression would allow for increased competition for binding sites between Nphe1 and OFQ and the

potential of an analgesia mediated more by OFQ. On the other hand, the lack of significant differences in analgesia demonstrated by the Nphe1 co-administration groups at early ages could imply that the activity of Nphe1 and OFQ are mediated by different and distinct pathways. If that is the case, the mechanisms associated with each pathway do not appear to work in either an additive or synergistic manner, but act instead in an antagonistic manner. This type of interaction between analgesic mechanisms has been suggested by the collateral inhibition model (Kirshgessner *et al*, 1982), which argues that relative levels of activation of multiple pain inhibitory systems will result in one system inhibiting the other(s).

As a result, it seems that Nphe1 is a poor choice of antagonist for use in the study of developmental effects of NOP/OFQ and that other potential antagonist candidates, such as CompB or SB-612111, should be utilized to further investigate the mechanisms underlying NOP/OFQ-mediated analgesia in both adults and infants.

Chapter 5: Discussion

A large body of evidence exists demonstrating that pain is a serious clinical problem for the human neonate (Anand *et al*, 1987). Premature and seriously ill full-term infants are subjected to a number of physical insults as a routine part of their treatment, including incisions, needle pricks, and burns. These procedures can and do cause tissue damage and localized inflammation. Furthermore, the effects of these procedures are not limited to the short-term, but can also result in permanent alterations in the structure and function of the pain system that extend from development into adulthood. These alterations include increased sensitivity to noxious stimuli (Anand *et al*, 1999), hyperinnervation in areas of tissue damage (Reynolds & Fitzgerald, 1995), increased sprouting of primary afferent fibers (Ruda *et al*, 2000), hyperexcitability of dorsal horn neurons (Rahman *et al*, 1998), and a decrease in receptive field size of dorsal horn afferents (Rahman *et al*, 1998). As a result, greater import has been given to the treatment of pain during development, but safe and effective treatments have proven difficult since the most effective analgesics, traditional opioid drugs, do not produce the same effects in the infant as in the adult (Fitzgerald, 1993; Barr, 1995). The differences in effect are most easily characterized by a change in the ratio of therapeutic effects to unwanted side effects, with benefits of treatment decreasing relative to costs at younger ages. Consequently, traditional opioid analgesics are not regularly used for infants in intensive care (Anand & Clancy, 1994).

Though a number of alternative anesthetics exist to treat neonatal pain, none are as effective as the opioid analgesic for the management of severe or chronic pain. As a result, an equivalent non-opioid mechanism of analgesia needs to be discovered to

provide a viable alternative to the use of opioids. The NOP/OFQ receptor/ligand system may provide the basis for such an analgesic. In the adult, both anatomical (Bunzow *et al*, 1994; Fukuda *et al*, 1994; Mollereau *et al*, 1994; Wick *et al*, 1994; Lachowicz *et al*, 1995; Anton *et al*, 1996; Dickenson, 1996; Houtani *et al*, 1996; Riedl *et al*, 1996; Kummmer & Fischer, 1997; Mitsuma *et al*, 1998; Neal *et al*, 1999; Houtani *et al*, 2000a,b; Letchworth *et al*, 2000; O'Donnell *et al*, 2001; Pettersson, 2002; Berthele *et al*, 2003) and behavioral (Rossi *et al* 1996; Tian *et al*, 1996; Xu *et al*, 1996; Erb *et al*, 1997; Hao *et al*, 1997; King *et al*, 1997; Rossi *et al*, 1997; Tian *et al*, 1997a,b; Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Hao *et al*, 1998; Hao & Ogawa, 1998; Mathis *et al*, 1998; Rossi *et al*, 1998; Inoue *et al*, 1999; Kamei *et al*, 1999a,b; Suder *et al*, 1999; Wang *et al*, 1999a,b; Yamamoto & Sakashita, 1999a,b; Nakano *et al*, 2000; Yamamoto *et al*, 2000a,b; Shane *et al*, 2001, 2002; Villaneuva *et al*, 2002; Ma *et al*, 2003; Menéndez *et al*, 2003; Sun *et al*, 2004) data have suggested their involvement in the modulation of nociceptive sensory input. Furthermore, additional studies have suggested that this modulation may not involve the opioid systems (Yamamoto *et al*, 1997a,b,c; Yamamoto & Nozaki-Taguchi, 1997; Yamamoto & Sakashita, 1999b).

The experiments described here have provided behavioral evidence – although not definitive, certainly suggestive – that NOP/OFQ are also involved in the mediation of nociception during development. In Experiment 1, testing using the thermal paradigm demonstrated a NOP/OFQ-mediated analgesia following supraspinal OFQ administration in 3-day-olds. However, this was followed by a varied pattern of response in older pups that shifted from hyperalgesia at 10-days-of-age to no response at 21-days. Spinal administration, on the other hand, did not produce a definitive NOP/OFQ-mediated

response until 21-days-of-age (though analgesia was seen in both 3- and 10-day-olds, it was more likely the result of a stress-related mechanism rather than the action of NOP/OFQ). Experiment 2, using an inflammatory paradigm of nociception, demonstrated more straightforward results: supraspinal administration resulted in analgesia in 3- and 21-day-olds, but spinal administration did not appear to have any effect, regardless of age (a stress-mediated analgesia, however, was seen at 21-days-of-age). Experiment 3 produced results remarkably similar to those of Experiment 2. Once again, supraspinal OFQ administration in the inflammatory paradigm resulted in analgesia at both 3- and 21-days-of-age, while spinal administration produced no response beyond a stress-mediated analgesia in 21-day-olds. Neither the co-administration of the putative antagonist Nphe1 with OFQ nor Nphe1's administration alone resulted in any antagonism of analgesia. In fact, Nphe1 administration resulted in an analgesic response in both circumstances. This analgesia did not, however, appear to work in either an additive or synergistic nature with OFQ-mediated analgesia.

As a whole, these results differ qualitatively from those of adults in two significant ways. First, NOP/OFQ activity produces analgesia in the developing rat regardless of the site of injection. Despite apparent differences at particular data points between the thermal and inflammatory experiments, these results remain fairly consistent (in fact, if one were to further examine these data points and factor in the abnormal nature of the 10- and 21-day-old thermal supraspinal data, as well as the confounding nature of the SIA seen in the spinal administration conditions, then the differences between the paradigms shrinks considerably and suggests a more consistent effect of NOP/OFQ during development). In all three experiments, the observed OFQ-mediated analgesia was

demonstrated at an earlier age following supraspinal injection than spinal injection. Furthermore, the duration of the analgesia changed during development, in that it increased with age. These results suggest that differences exist in the maturation of NOP receptor populations during development, with a general trend of supraspinal populations developing earlier than spinal ones. Developmental variation is not without precedent in the opioid family of receptors, since each of the classical receptors demonstrates a unique course of development. For example, DOP receptors do not reach maturity until well after birth.

Furthermore, the co-occurrence of a SIA seems to also follow a consistent pattern. In both paradigms, SIA only occurred following spinal injection. This difference between routes of administration may be explained by differences in receptor population densities in supraspinal and spinal sites of action suggested earlier. Lower population densities in the spine could allow for a stress-mediated analgesia to be expressed instead of a NOP/OFQ-mediated one. This is certainly plausible in the case of an opioid-mediated SIA, as spinal MOP and KOP receptors and their peptides are expressed and functional at these ages (Leslie & Loughlin, 1992; Barr, 1995). This would be consistent with both the literature regarding SIA during development (Wiedenmayer & Barr, 1998) and the behavioral data presented here; however, it would run contrary to the published data existing regarding NOP/OFQ expression during development (Wu *et al*, 1997; Ikeda *et al*, 1998; Neal *et al*, 2001). It should be noted, however, that this data is limited and may not be definitive. Another potential explanation could be related to the nature of the spinal administration method. While catheter implantation has been used with great success in both adults and infants in our lab and others, it is not difficult to imagine that

the surgery itself and, more importantly, the relatively short recovery times made necessary by the age of the test subjects could contribute to the appearance of a SIA: the 4-6 hour recovery time simply may not be sufficient for complete removal of influences of the surgical procedure.

In the thermal paradigm, SIA was limited to the younger animals, while in the inflammatory paradigm it was limited to the oldest pups. This particular pattern of appearance makes sense in the case of the inflammatory paradigm, but becomes more difficult to explain with the thermal paradigm results. The response elicited in the formalin test is composed of a range of motor behaviors, the complexity of which increases with increased nociception. As the result of differences in motor abilities between 3- and 21-day-olds, where a lack of motor development makes the execution of some of these behaviors by 3-day-olds difficult, an analgesic effect that is easily seen at 21-days may be difficult to see at 3-days. One could argue that this is made more difficult by the existence of data demonstrating that OFQ/NOP also affects motor activity in adult rats (Reinschied *et al*, 1995; Devine *et al*, 1996; Murphy *et al*, 1996; Noble & Roques, 1997; Sandin *et al*, 1997; Walker *et al*, 1998; Berger *et al*, 2000; Lutfy *et al*, 2001; Rizzi *et al*, 2001; Kuzmin *et al*, 2004; Narayanan *et al*, 2004). It should be noted, however, that this data can be contradictory depending on dosage used, with hypolocomotor effects being seen at doses 2- to 20-times higher than those used in nociception and analgesia studies (Florin *et al*, 1996; Kuzmin *et al*, 2004). Furthermore, additional evidence suggests this suppression of motor activity is readily habituated to (Devine *et al*, 1996; Walker *et al*, 1998; Lutfy *et al*, 2001). This sort of explanation, however, does not work in the case of the thermal paradigm, where the pattern of SIA expression is reversed. This

may instead be related to a combination of the nature of the noxious stimulus and the physical development of the 3- and 10-day-olds. Considerable differences can be seen in the thickness of the hindpaw skin of 3-, 10-, and 21-day-olds, which may make 3- and 10-day-olds more sensitive to tissue damage and neural activation, and thus response, as a result of the thermal stimulus. If this proved to be the case, it may be easier to see the effects of SIA at these ages due to that sensitivity, as a mild analgesic effect would then be likely to produce a greater change in response than in 21-day-olds. Another explanation may involve differences in the nature of the stress involved or in the mechanisms mediating it. For example, a number of factors have been shown to contribute to the expression of SIA and that this analgesia can be mediated by both opioid and non-opioid mechanisms (Lewis *et al*, 1980; Watkins & Mayer, 1982). In the case of these particular experiments, such factors could include the trauma of the supraspinal injection and the introduction of the injection volume itself into the cerebroventricular system (Grisel *et al*, 1996; Mogil *et al*, 1996a,b). Similar circumstances could be argued for the spinal catheter and injection procedures as well (Grisel *et al*, 1996; Mogil *et al*, 2000b). Additionally, there is the suggestion of an anxiolytic nature to OFQ (Jenck *et al*, 1997; Mamiya *et al*, 1998; Kaster *et al*, 1999; Reinschied *et al*, 2002), which could imply the importance of the "psychological" state of the test subject. This state could be influenced by the subject's environment immediately prior to the test, the test itself, or even genetic factors (Mogil & Pasternak, 2001). Finally, there is also the possibility that each paradigm is resulting in a distinct and different stress response. Recent data has demonstrated that specific stimuli can activate noradrenergic pathways involved in stress differentially (Myers & Rinaman, 2003).

Regardless of the actual mechanisms of this stress effect, the existence of stress during development has important implications. Prenatal stress exposure results in alterations of a number of hormonal and neurochemical mechanisms, including testosterone release (Ward & Weisz, 1980), endogenous opioids (Ward *et al*, 1986), catecholamines (Rohde *et al*, 1989), hypothalamic and pituitary gonadotrophins (Rohde *et al*, 1989), and hypothalamo-pituitary-adrenocortical (HPA) axis hormones (Takahashi *et al*, 1998). These changes result in the demasculinization of sexually dimorphic behaviors during adulthood, such as the demonstration of lordosis (Dahlof *et al*, 1977; Ward *et al*, 1994) and the reduction of aggressive responses (Kinsley & Svare, 1986, 1988). The expression of stress-related behaviors is also altered in adulthood. Traditional markers of heightened stress response, such as HPA hyperreactivity, increased freezing, and decreased exploratory behavior, are seen (Rosencrans *et al*, 1984; Takahashi *et al*, 1990; Szuran *et al*, 1991). Furthermore, these adults demonstrate alterations in the expression of SIA (Kinsley *et al*, 1988; Szuran *et al*, 1991; Sternberg, 1999). Similar effects are seen following neonatal stress exposure. Stress exposure in the preweaning period decreases stress response, measured by low corticosteroid levels (Vallee *et al*, 1997), decreased glucocorticoid release, and altered opioid activity (Larson, 1982; Bernardi *et al*, 1986; Kehoe & Blass, 1986; Kalinichev *et al*, 2001). As a result, alterations in nociceptive latencies (including changes in SIA expression) and morphine analgesia are demonstrated in adulthood (Pieretti *et al*, 1991; Sternberg & Ridgway, 2003). These alterations are of particular importance, since SIA magnitude can reflect the strength of synaptic connections and the density of receptors in the modulatory pathways

involved in pain inhibition, thus providing evidence for the neural organizing effects of neonatal experience (Sternberg & Ridgway, 2003).

Second, co-administration of Nphe1 did not antagonize NOP/OFQ activity but, rather, appeared to result in an analgesia of its own. This analgesia only occurred following supraspinal administration and may involve different mechanisms than those mediating NOP/OFQ action. If the earlier proposition that receptor/peptide densities increase with age is correct, then we might speculate that the differences between age groups seen following supraspinal administration suggest the mechanisms mediating Nphe1 analgesia mature earlier than those mediating NOP/OFQ analgesia, but produce a weaker overall effect that is either overshadowed or replaced by NOP/OFQ. This would be consistent with the model of collateral inhibition (Kirshgessner *et al*, 1982). Since the data presented here suggest an analgesic nature to Nphe1, it could be argued that the antagonism seen in the adult is the result of the competition of two systems mediating analgesia (i.e. NOP/OFQ and Nphe1), rather than of an inhibitory system counteracting an excitatory one. This conclusion could be complicated by the differences seen between the analgesia following Nphe1 administration alone and that following Nphe1/OFQ co-administration. The fact that OFQ was able to elicit an analgesic response greater than that of Nphe1 alone, in spite of co-administration with Nphe1, would suggest that Nphe1 was, in fact, not antagonizing NOP/OFQ action. In light of this, a better explanation might be the idea that Nphe1 is a partial agonist at the NOP receptor, rather than an antagonist. The analgesia seen in both infants and adults, as well as the antagonism seen in adults, could then be explained as the result of submaximal activation of the NOP receptor by Nphe1 and a reduction of OFQ effect due to competition with Nphe1 for

NOP receptor binding sites. This explanation, however, is still not without its own problems. While working very well for explaining the effects of Nphe1 in adults, and, to some degree, the effects seen during development, it does not explain the differences seen between 3- and 21-day-old co-administration groups, which suggest that the analgesia Nphe1 produces is mediated by mechanisms distinct from those of NOP/OFQ.

The unique pattern of action demonstrated by NOP/OFQ during development shows promise for its consideration as the basis for a future clinical pediatric analgesic. However, the present data is yet incomplete and the exact mechanism(s) of the produced analgesia remain unclear. Additional anatomical work needs to be done to address the disparity between the current anatomical data and the behavioral data presented here. Anatomical data demonstrating co-localization of NOP/OFQ with neural regions involved in the mediation of nociception during development exist, but are limited. Also, the lack of a definitive antagonist for the NOP receptor makes it hard to determine if the results observed are due to activity at the NOP receptor, as opposed to other sites or mechanisms. In the case of these studies, this fact is further complicated by the analgesic nature demonstrated by the tested antagonist candidate. Further investigation into the nature of this analgesia, as well as a direct comparison of the analgesia produced by OFQ alone with that produced by Nphe1/OFQ co-administration, could help clarify the differences between the infant and adult antagonist results. Third, no data exists observing the effects of the co-administration of general opioid antagonists with OFQ during development. These studies could help determine if the actions of NOP/OFQ during development are, as suggested by the adult data, independent of the opioid system. Fourth, in the attempt to apply these results to clinical pediatrics, additional paradigms

modeling other insults suffered by infants, such as mechanical stimuli (as well as a more consistent thermal paradigm), should be investigated. Finally, studies investigating the effects of NOP/OFQ at additional ages, particularly those between adulthood and the ages tested here, should be undertaken to pinpoint the exact age at which these differences in infant and adult NOP/OFQ activity occur and provide a more detailed view of the developmental time-course of their effects.

So while the initial enthusiasm shown for the analgesic potential of NOP/OFQ has proven valid, this enthusiasm should remain tempered until further research is done. The research presented here, as well as the work that preceded it, seem to argue that further research is certainly warranted.

Table 1: General design for thermal (Experiment 1) and inflammatory (Experiment 2) paradigms.

Pup	Drug	Dose	Admin
1	saline	n/a	i.c.v.
2	OFQ	0.1 nmol	i.c.v.
3	OFQ	1 nmol	i.c.v.
4	OFQ	10 nmol	i.c.v.
5	control	n/a	n/a
6	saline	n/a	i.t.
7	OFQ	0.1 nmol	i.t.
8	OFQ	1 nmol	i.t.
9	OFQ	10 nmol	i.t.
10	control	n/a	n/a

Table 2: General design for the antagonist studies (Experiment 3).

Litter	Drug	Dose	Admin
1	saline + antag	n/a + 10 nmol	i.c.v.
2	OFQ + antag	10 nmol + 10 nmol	i.c.v.
3	saline	n/a	i.c.v.
4	control	n/a	n/a
5	saline + antag	n/a + 10 nmol	n/a
6	OFQ + antag	10 nmol + 10 nmol	i.t.
7	saline	n/a	i.t.
8	control	n/a	i.t.

Table 3: OFQ administration appears to be capable of producing analgesia regardless of site of injection; however, this analgesia appears to be paradigm specific at particular ages.

Route	Paradigm	Age	Significant Effects	Controls*	Doses**	Behavior	Notes
i.t.	thermal	3 days	d, s	yes	no	analgesia	SIA
i.t.	thermal	10 days	d, s	yes	no	analgesia	SIA
i.t.	thermal	21 days	s, d x t, d x s, d x t x s	n/a	yes (high - 1,2)†	analgesia	
i.t.	inflammatory	3 days	b	n/a	n/a	n/a	
i.t.	inflammatory	21 days	d, b, d x b	yes	no	analgesia	SIA
i.c.v.	thermal	3 days	d, s, d x t	no	yes (high - 1,4)	analgesia	
i.c.v.	thermal	10 days	t, s, d x t, d x s, t x s	n/a	yes (high - 1,2,3)†	hyperalgesia	
i.c.v.	thermal	21 days	t, s, t x s	n/a	n/a	n/a	
i.c.v.	inflammatory	3 days	d, b, d x b	no	yes (high)	analgesia	
i.c.v.	inflammatory	21 days	d, b, d x b	yes	yes (high)	analgesia	SIA (analgesia not affected)

Legend: d = dose, b = bin, t= time, s = stim, SIA = stress-induced analgesia

*** differences between controls, ** differences between doses**

† differences based on interactions

Table 4: Supraspinal administration of Nphe1 does not affect NOP/OFQ-induced analgesia at 3- or 21- days; however, it does appear to produce an analgesic effect of its own that is independent of that of OFQ.

Route	Age	Significant Effects	Controls*	Doses**	Behavior	Notes
i.t.	3 days	b	n/a	n/a	n/a	
i.t.	21 days	d, b, d x b	yes	no	analgesia	SIA
i.c.v.	3 days	d, b	no	no	analgesia	Nphe1-mediated analgesia
i.c.v.	21 days	d, b, d x b	no	yes	analgesia	Nphe1- & OFQ-mediated analgesias?

Legend: d = dose, b = bin, t= time, s = stim, SIA = stress-induced analgesia

*** differences between controls, ** differences between doses**

† differences based on interactions

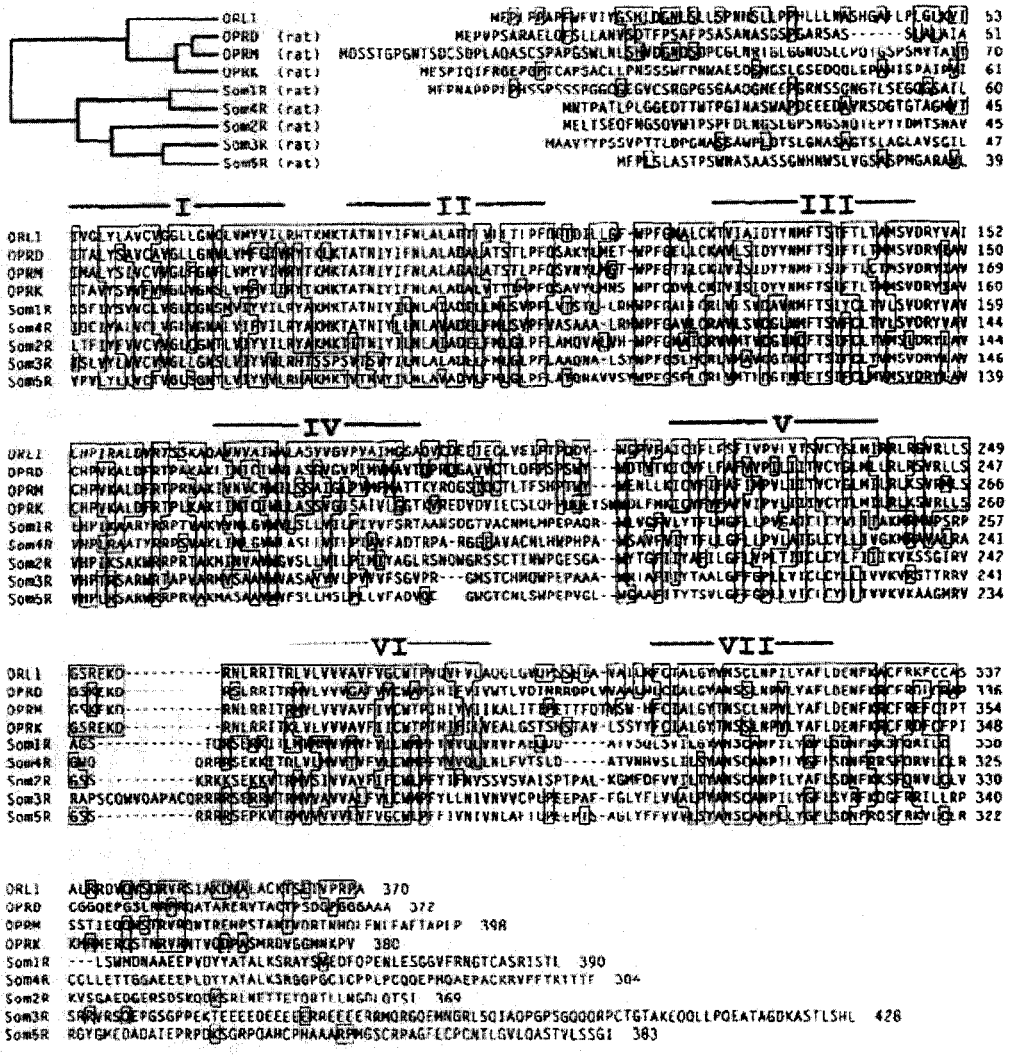

Figure 1: Comparison of rat ORL1, opioid (OPRD, OPRM, and OPRK corresponding to the δ-, μ-, and κ-opioid receptors, respectively), and somatostatin receptor amino acid sequences. Membrane-spanning domains are numbered I-VII and similarities between receptor sequences are boxed (reproduced from Mollereau *et al*, 1994).

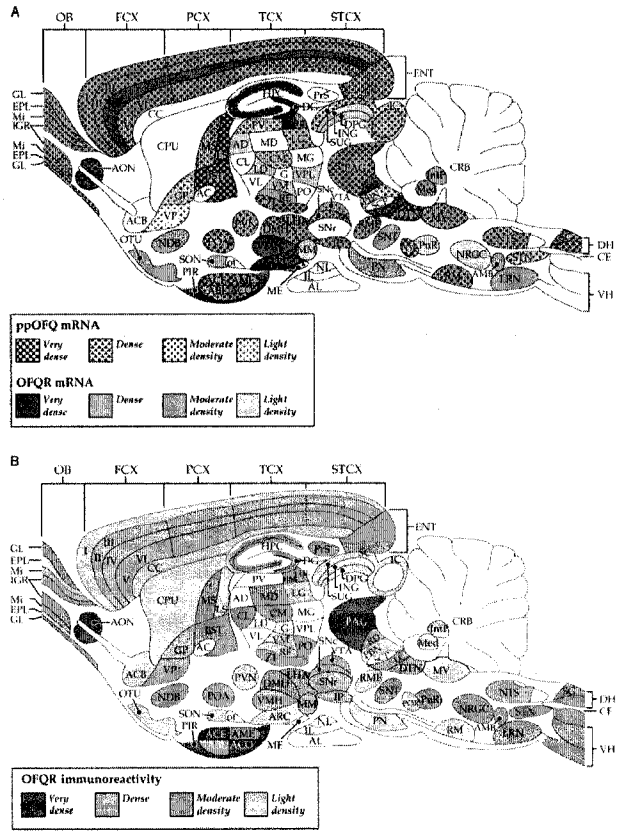

Figure 2: Anatomical distribution of NOP (A & B) and OFQ (A) in the adult rat CNS as determined by both *in situ* (A) and histochemical (B) analyses (reproduced from Darland *et al*, 1998).

Abbreviations: I–VI, cortical layers I–VI; ABL, basolateral amygdaloid nucleus; AC, anterior commissure; ACB, nucleus accumbens; ACE, central amygdaloid nucleus; ACO, cortical amygdaloid nucleus; AD, anterodorsal thalamus; AL, anterior lobe, pituitary; AMB, nucleus ambiguus; AME, medial amygdaloid nucleus; AON, anterior olfactory nucleus; ARC, arcuate nucleus, hypothalamus; BST, bed nucleus, stria terminalis; CC, corpus callosum; CE, central canal; CL, centrolateral thalamus; CM, centromedial thalamus; CPU, caudate putamen; CRB, cerebellum; DG, dentate gyrus; DH, dorsal horn, spinal cord; DMH, dorsomedial hypothalamus; DPG, deep gray layer, superior colliculus; DTN, dorsal tegmental area; ENT, entorhinal cortex; EPL, external plexiform layer, olfactory bulb; FCX, frontal cortex; G, nucleus gelatinosus, thalamus; GL, glomerular layer, olfactory bulb; GP, globus pallidus; HL, lateral habenula; HM, medial habenula; HPC, hippocampus; IC, inferior colliculus; IGR, intermediate granular layer, olfactory bulb; IL, intermediate lobe, pituitary; ING, intermediate gray layer, superior colliculus; IntP, interposed cerebellar nucleus; IP, interpeduncular nucleus; LC, locus coeruleus; LD, laterodorsal thalamus; LG, lateral geniculate thalamus; LHA, lateral hypothalamic area; LRN, lateral reticular nucleus; LS, lateral septum; MD, mediodorsal thalamus; ME, median eminence; Med, medial cerebellar nucleus; MG, medial geniculate thalamus; Mi, mitral cell layer, olfactory bulb; MM, medial mammillary nucleus; MS, medial septum; MV, medial vestibular nucleus; NDB, nucleus diagonal band; NL, neural lobe, pituitary; NRGC, nucleus reticularis gigantocellularis; NTS, nucleus tractus solitarii; OB, olfactory bulb; ot, optic tract; OTU, olfactory tubercle; PAG, periaqueductal gray; PBN, parabrachial nucleus; PCX, parietal cortex; PIR, piriform cortex; PN, pons; PnR, pontine reticular; PO, posterior nucleus thalami; POA, preoptic area; POR, periolivary region; PrS, presubiculum; PV, paraventricular thalamus; PVN, paraventricular hypothalamus; RD, dorsal raphé; RE, reuniens thalami; RM, raphé magnus; RME, median raphé; SC, superior colliculus; SCP, superior cerebellar peduncle; SG, substantia gelatinosa; SNc, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulata; SNT, sensory trigeminal nucleus; SON, supraoptic nucleus; STCX, striate cortex; STN, spinal trigeminal nucleus; SUG, superficial gray layer, superior colliculus; TCX, temporal cortex; VH, ventral horn, spinal cord; VL, ventrolateral thalamus; VM, ventromedial thalamus; VMH, ventromedial hypothalamus; VP, ventral pallidus; VPL, ventroposterolateral thalamus; VTA, ventral tegmental area; and ZI, zona incerta.

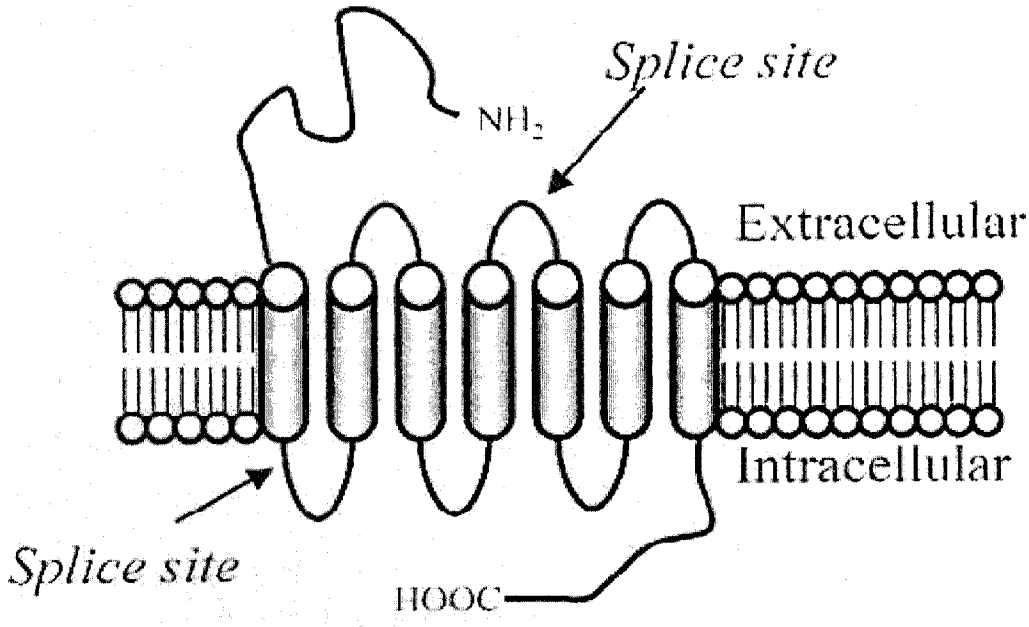

Figure 3: Schematic of NOP receptor (reproduced from Mogil & Pasternak, 2001).

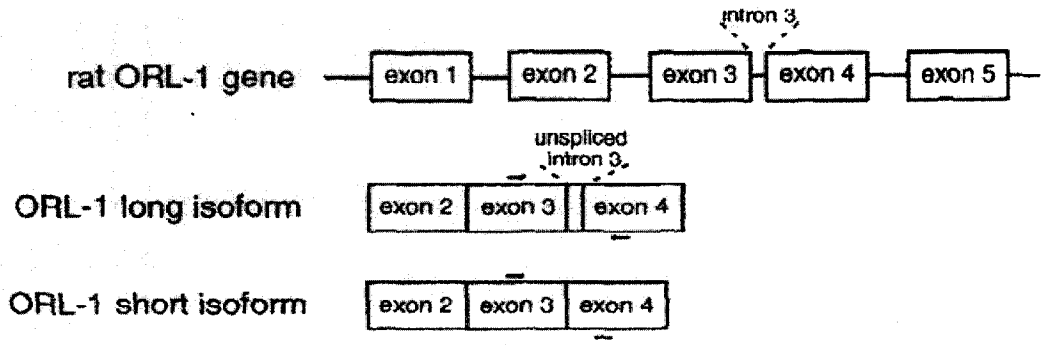

Figure 4: Schematic of ORL1 gene structure and alternate splicing. Arrows represent the primers used in RT-PCR reaction to clone the splicing variants (reproduced from Xie *et al*, 1999).

Receptor:	Ligand:	Precursor Peptide:	Ligand Sequence:
MOP (μ)	β-endorphin	proopiomelanocortin	Tyr-**Gly**-**Gly**-**Phe**-Met-Thr-Ser-Glu-**Lys**-**Ser**-Gln-Thr-Pro-**Leu**-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala(Val)-Tyr(His)-Lys-Lys-Gly-Glu
DOP (δ)	met-enkephalin	proenkephalin	Tyr-**Gly**-**Gly**-**Phe**-Met
	leu-enkephalin		Tyr-**Gly**-**Gly**-**Phe**-Leu
KOP (κ)	dynoprhin A	prodynorphin	Tyr-**Gly**-**Gly**-**Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Lue-Lys-Trp-Asp-**Asn**-**Gln**
	dynorphin B		Tyr-**Gly**-**Gly**-**Phe**-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	α-neodynorphin		Tyr-**Gly**-**Gly**-**Phe**-Leu-Arg-Lys-Arg
NOP (ORL1)	orphanin FQ	pronociceptin	Phe-**Gly**-**Gly**-**Phe**-Thr-Gly-Ala-Arg-**Lys**-**Ser**-Ala-Arg-Lys-**Leu**-Ala-**Asn**-**Gln**

Figure 5: General overview of opioid receptors and their endogenous ligands.

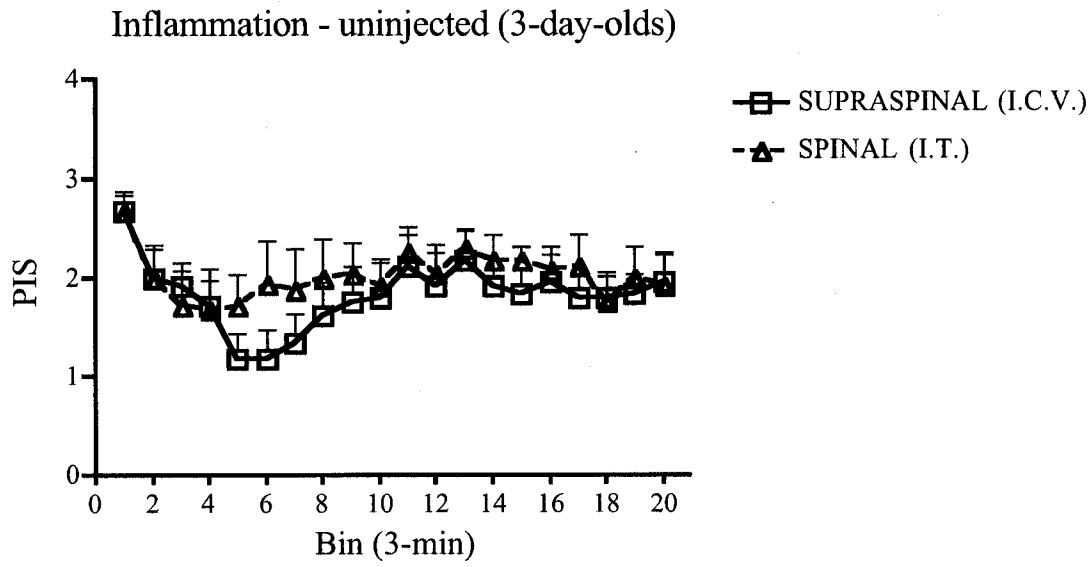

Figure 6: In the agonist experiments, as expected, no differences were seen between 3-day-old uninjected controls assigned to the supraspinal and spinal administration groups in the formalin test.

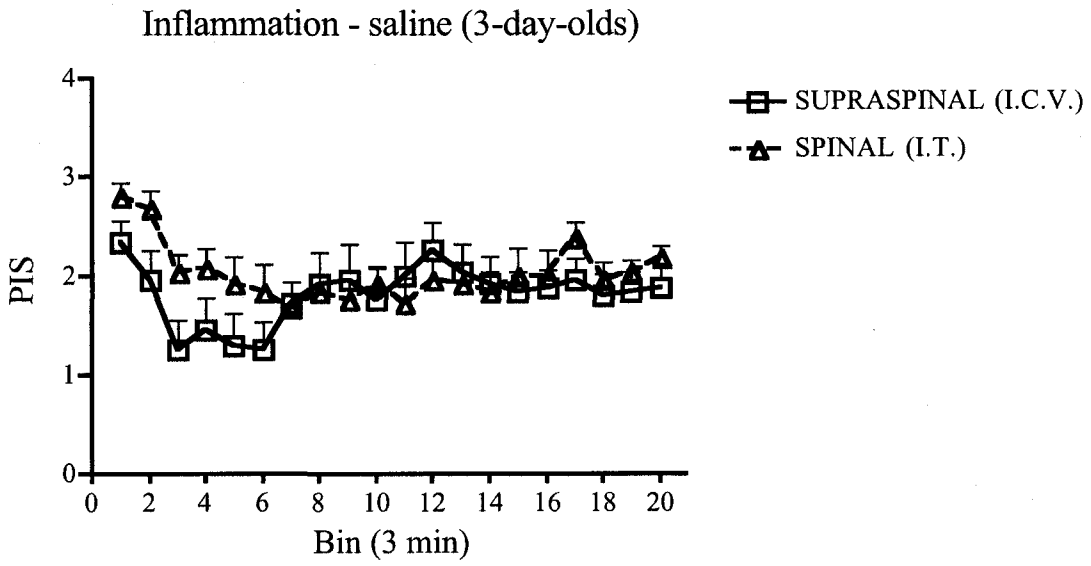

Figure 7: Like the uninjected controls, no differences were seen between routes of injection in 3-day-old saline-treated controls in the formalin test.

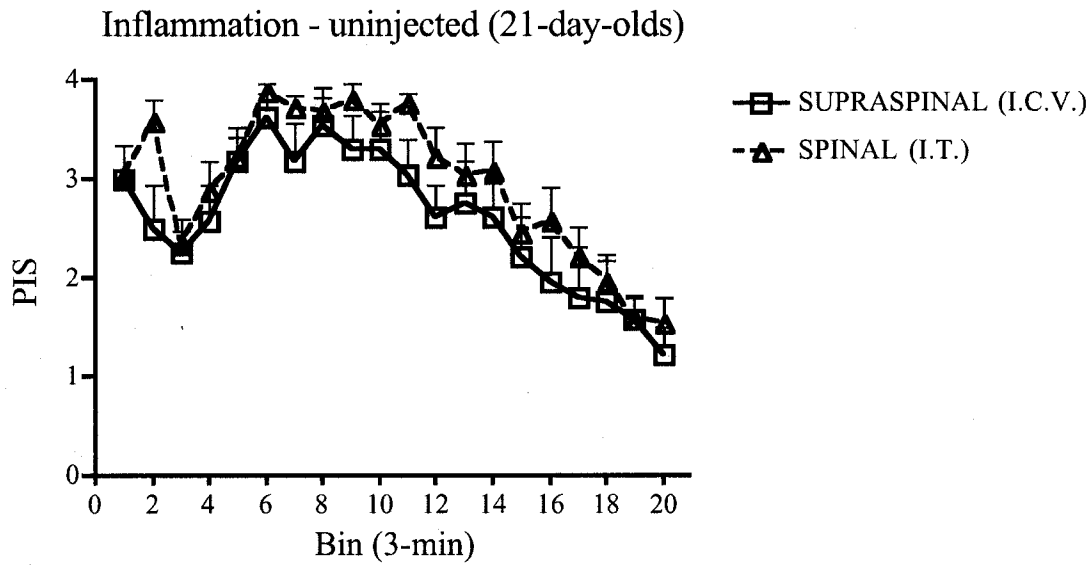

Figure 8: In the formalin test, no differences were seen between 21-day-old uninjected controls assigned to the supraspinal and spinal administration groups in the agonist experiments.

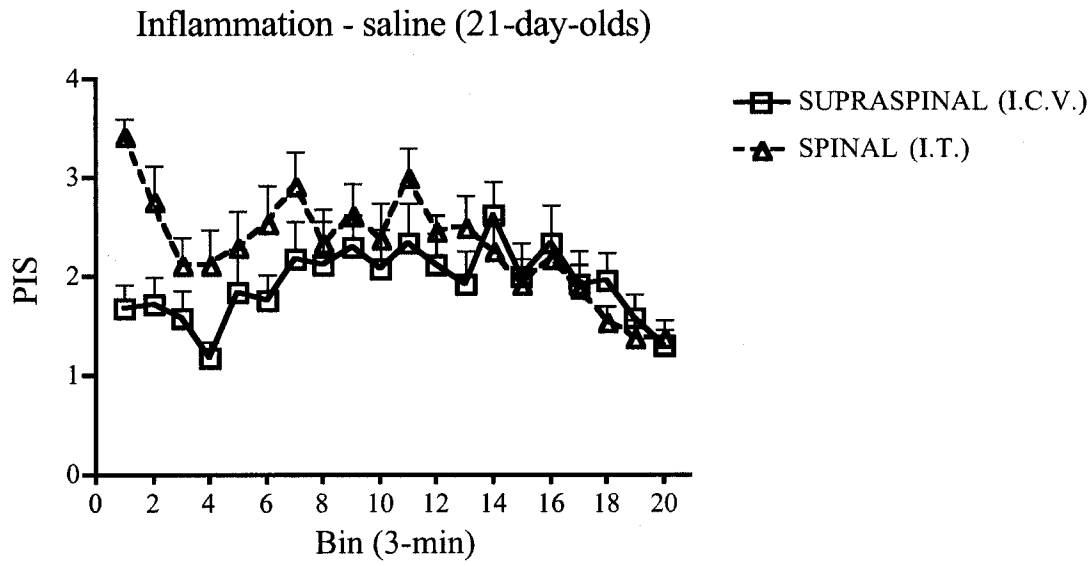

Figure 9: 21-day-old saline-treated controls demonstrated differences between injection routes, with supraspinal administration resulting in lower PISs during the first 6 minutes of the formalin test (suggesting that these differences are limited to the first phase of the formalin response).

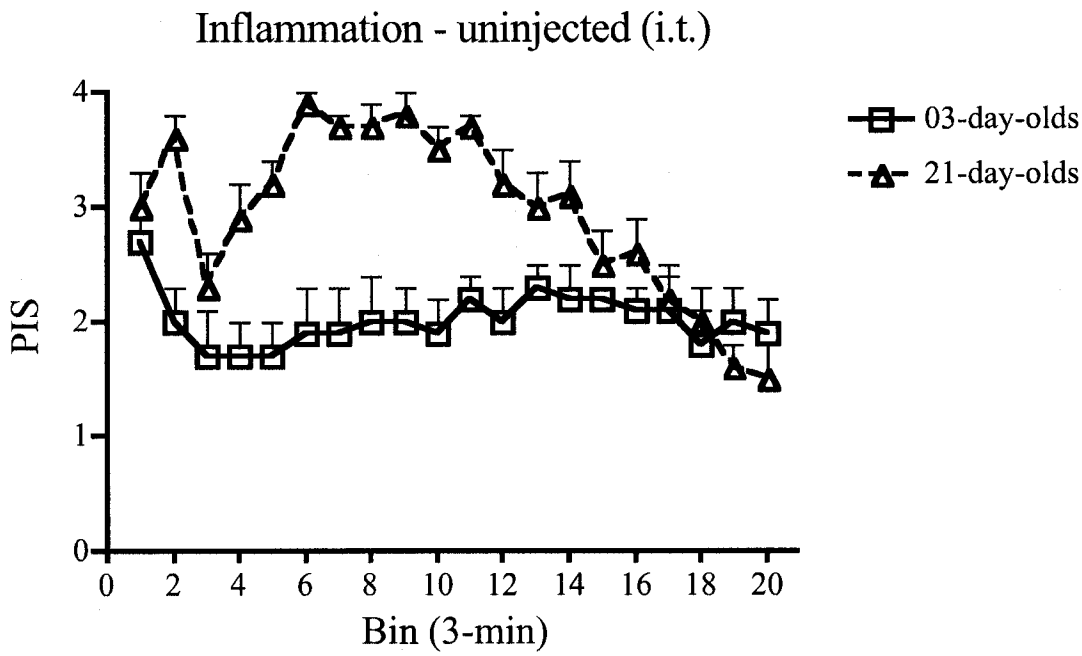

Figure 10: Differences were seen between 3- and 21-day-old uninjected controls assigned to the spinal administration group in the formalin test, suggesting differences in the mechanisms governing inflammatory response at these ages.

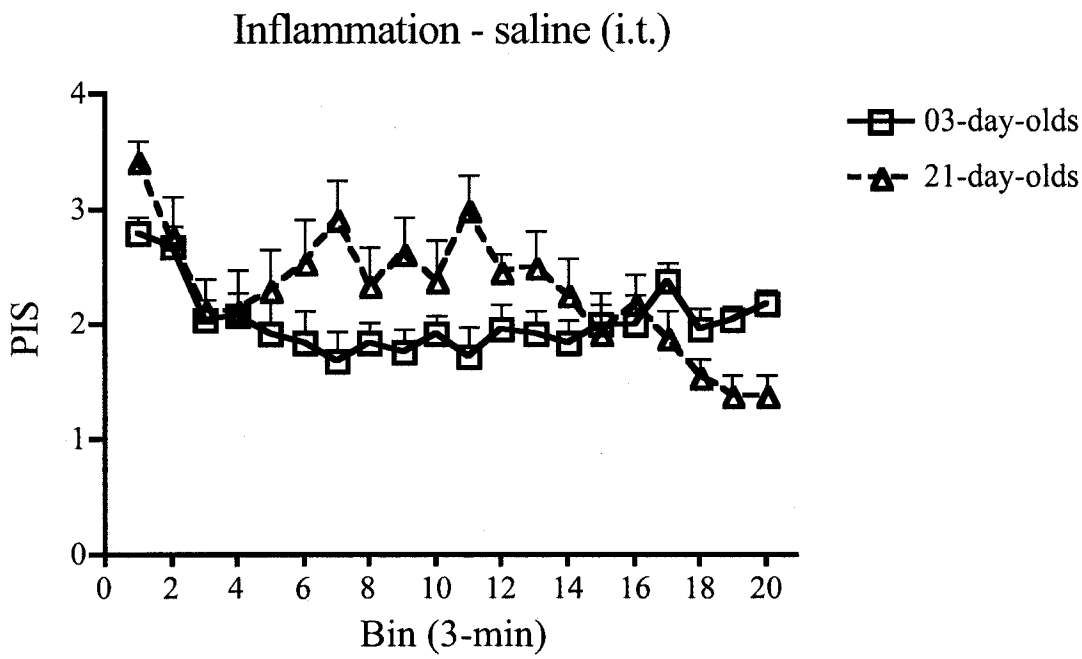

Figure 11: Differences were also seen between 3- and 21-day-old saline-treated controls following spinal administration in the formalin test. Combined with the information from Table 11 and Figures 7 and 9, these results suggest the occurrence of a stress-mediated analgesia following spinal administration.

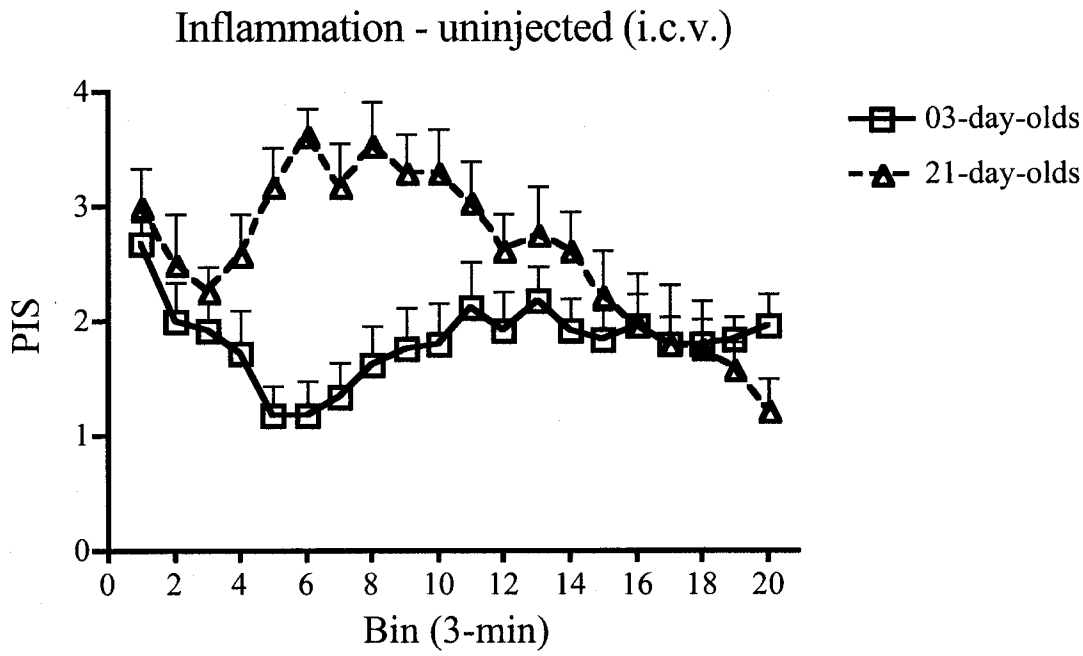

Figure 12: Differences were seen between 3- and 21-day-old uninjected controls assigned to the supraspinal administration group in the formalin test, again suggesting differences in the mechanisms governing inflammatory response at these ages.

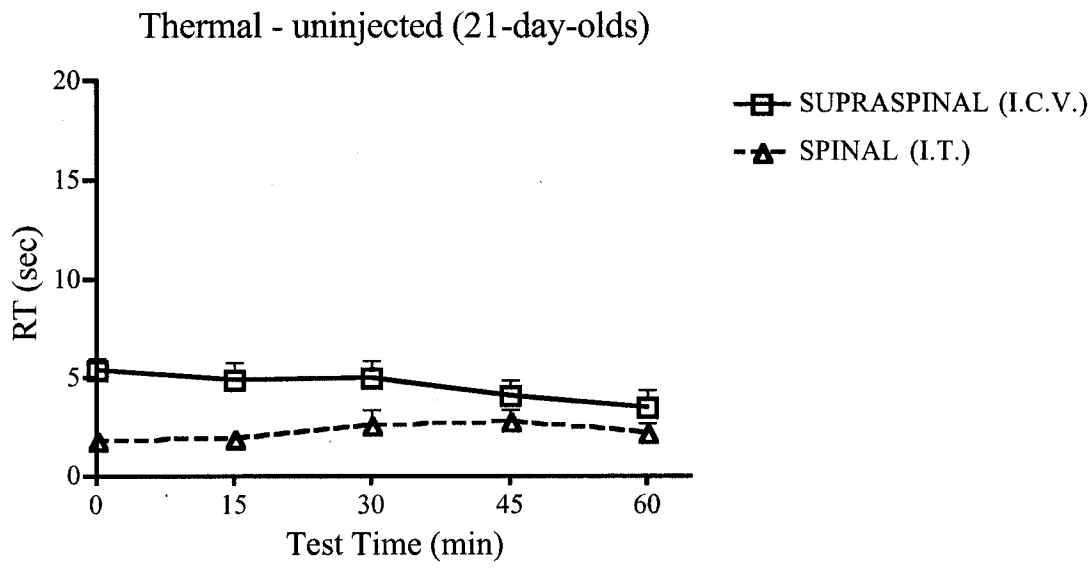

Figure 13: In the thermal paradigm, differences were seen between routes of administration in 21-day-old uninjected controls, with pups assigned to the supraspinal administration group demonstrating greater RTs. These results seem odd, however, in light of the lack of any actual administration procedure being used and may be indicative of inconsistencies in the thermal testing paradigm.

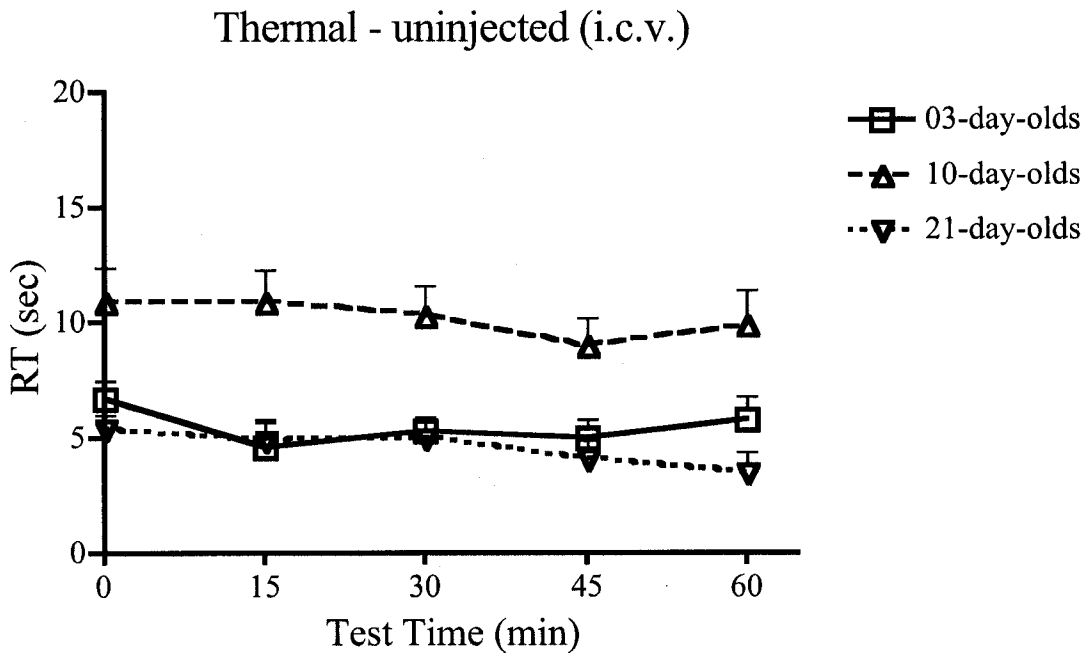

Figure 14: Differences were seen between 3-, 10-, and 21-day-old uninjected controls assigned to the supraspinal injection group in the thermal paradigm, with 10-day-olds demonstrating much greater RTs than both 3- and 21-day-olds (no differences were seen between 3- and 21-day-olds). These results may suggest either the additional presence of a stress-mediated analgesia or, like the 21-day-old uninjected controls, reflect inconsistencies in the thermal testing paradigm.

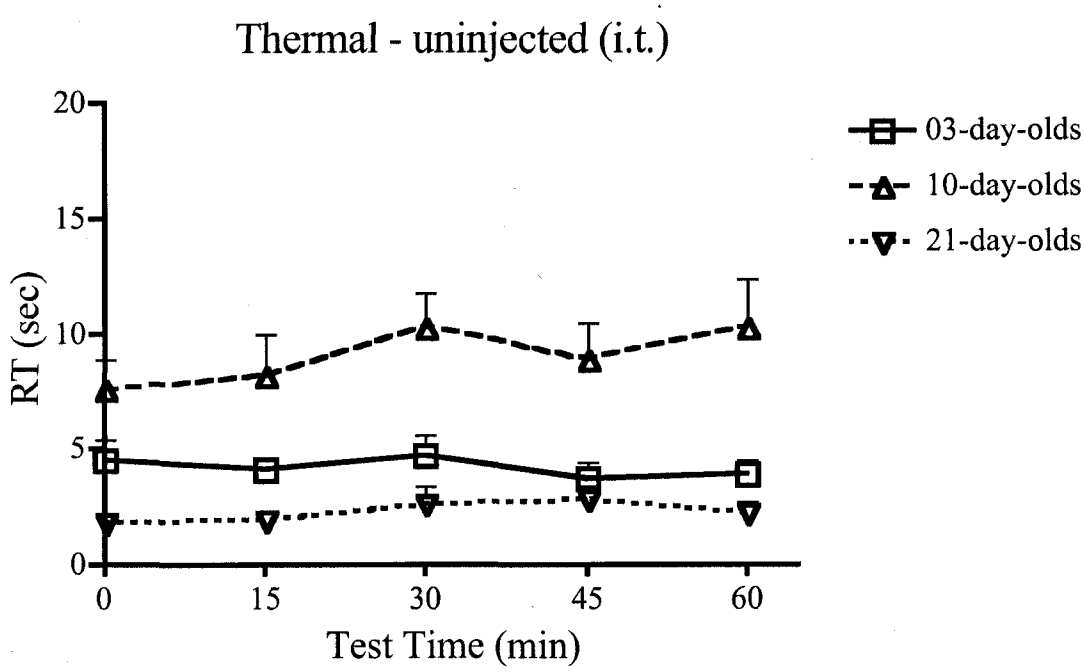

Figure 15: Differences were seen between 3-, 10-, and 21-day-old uninjected controls assigned to the spinal injection group in the thermal paradigm, with 10-day-olds again demonstrating much greater RTs than both 3- and 21-day-olds. Unlike the uninjected controls assigned to the supraspinal injection group, differences were also seen between the 3- and 21-day-olds, with 3-day-olds demonstrating greater RTs. Again, these results may suggest either the additional presence of a stress-mediated analgesia in 10-day-olds or reflect inconsistencies in the thermal testing data.

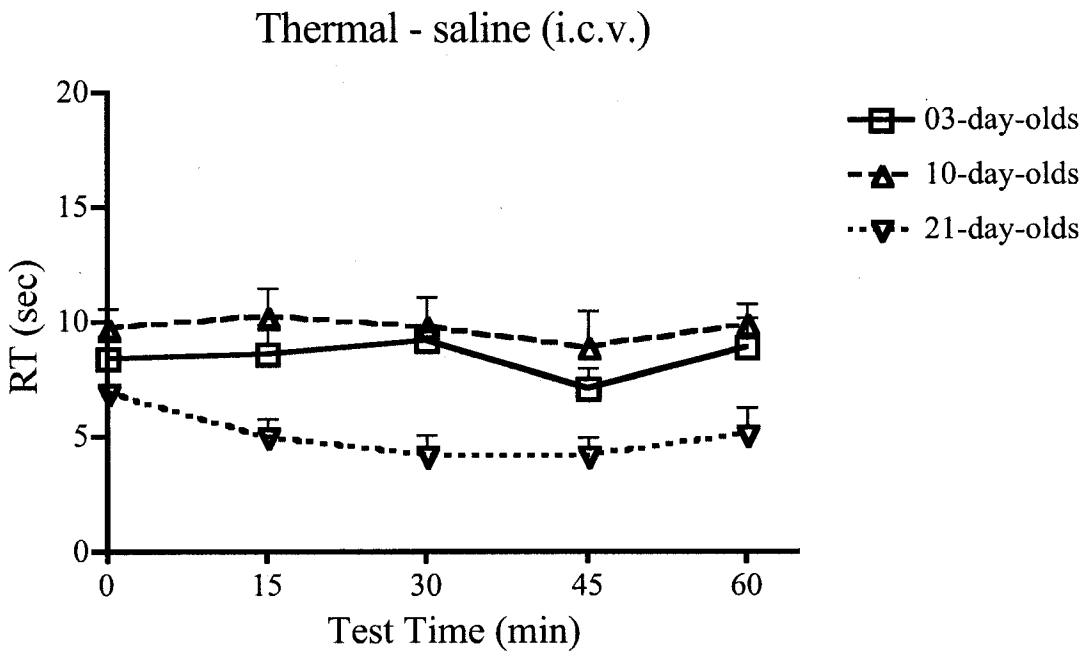

Figure 16: Differences were seen between 3-, 10-, and 21-day-old saline-treated controls following supraspinal administration in the thermal paradigm, with 3- and 10-day-olds demonstrating much greater RTs than 21-day-olds (no differences were seen between 3- and 10-day-olds). Like the results of the uninjected controls, these results could indicate either the additional presence of a stress-mediated analgesia at the two younger ages or irregularities the thermal testing data.

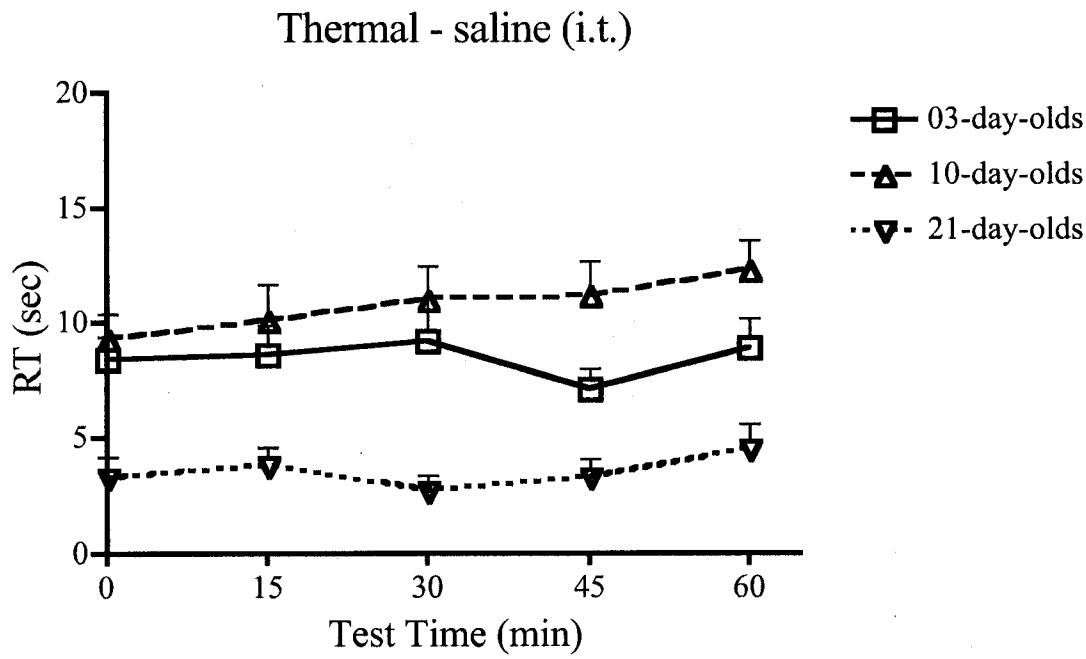

Figure 17: Differences were seen between 3-, 10-, and 21-day-old saline-treated controls following spinal administration in the thermal paradigm, with 3- and 10-day-olds demonstrating much greater RTs than 21-day-olds. Unlike the uninjected controls and supraspinal saline-treated controls, differences were also seen between the 3- and 10-day-olds, with 10-day-olds demonstrating greater RTs. Like the results of the other thermal paradigm controls, these results could indicate either the additional presence of a stress-mediated analgesia at the two younger ages or irregularities the thermal testing data.

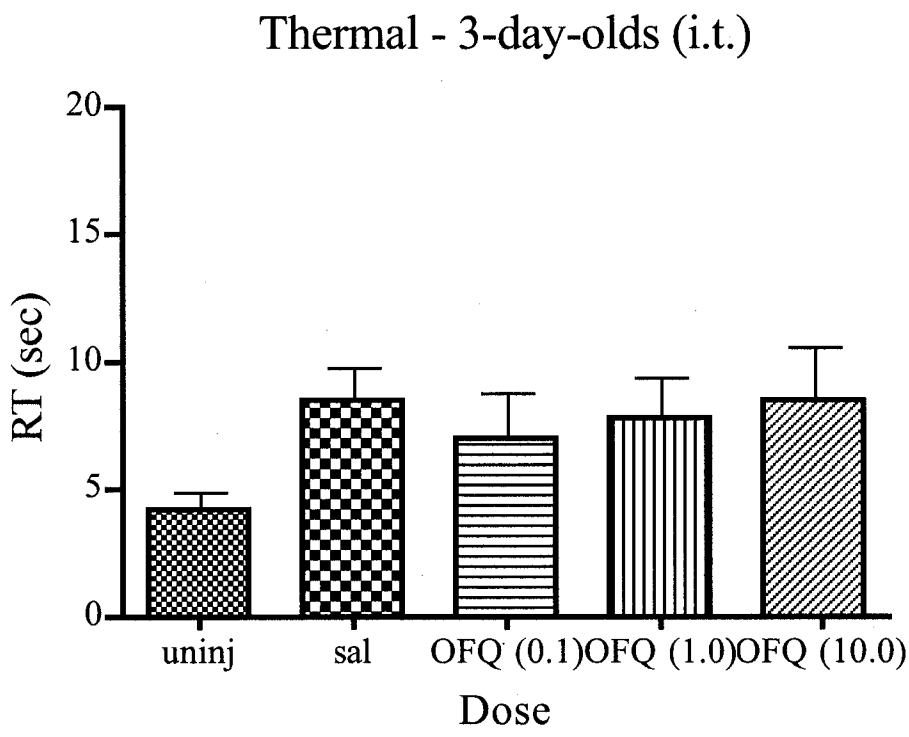

Figure 18: In the thermal paradigm, differences were seen between the injection groups and uninjected controls at 3-day of age following spinal administration, with all injection groups demonstrating greater RTs. No differences were seen between injection groups, suggesting that the analgesia seen was mediated more by stress-related mechanisms than NOP/OFQ activity.

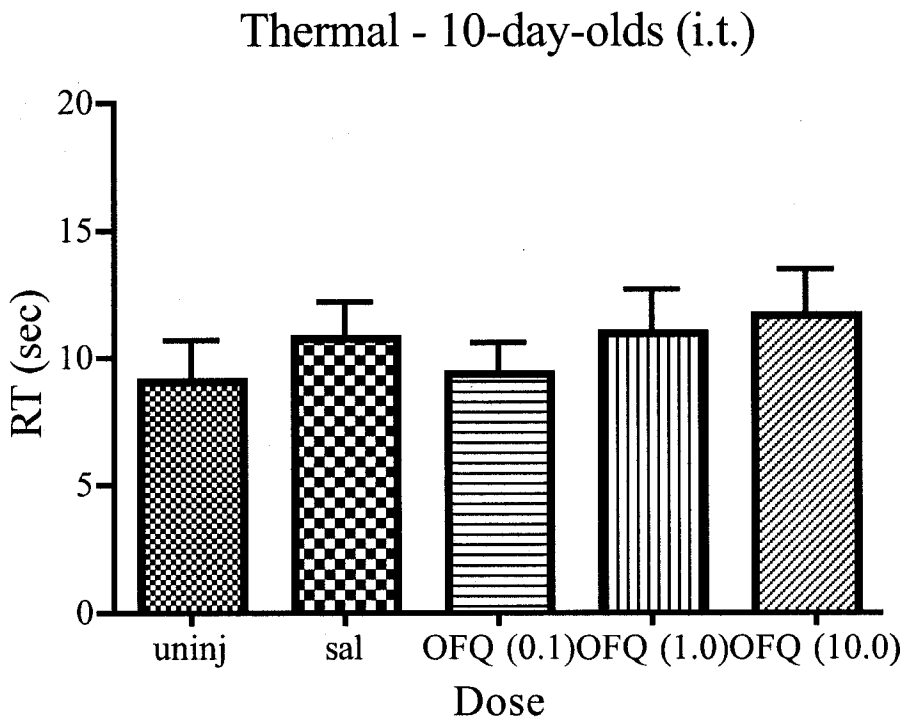

Figure 19: In 10-day-olds, differences were seen between the middle and high doses and uninjected controls, but not between the low dose and uninjected controls, in the thermal paradigm following spinal administration. Both the middle and high doses produced higher RTs, suggesting that higher doses are necessary to produce analgesia at this age.

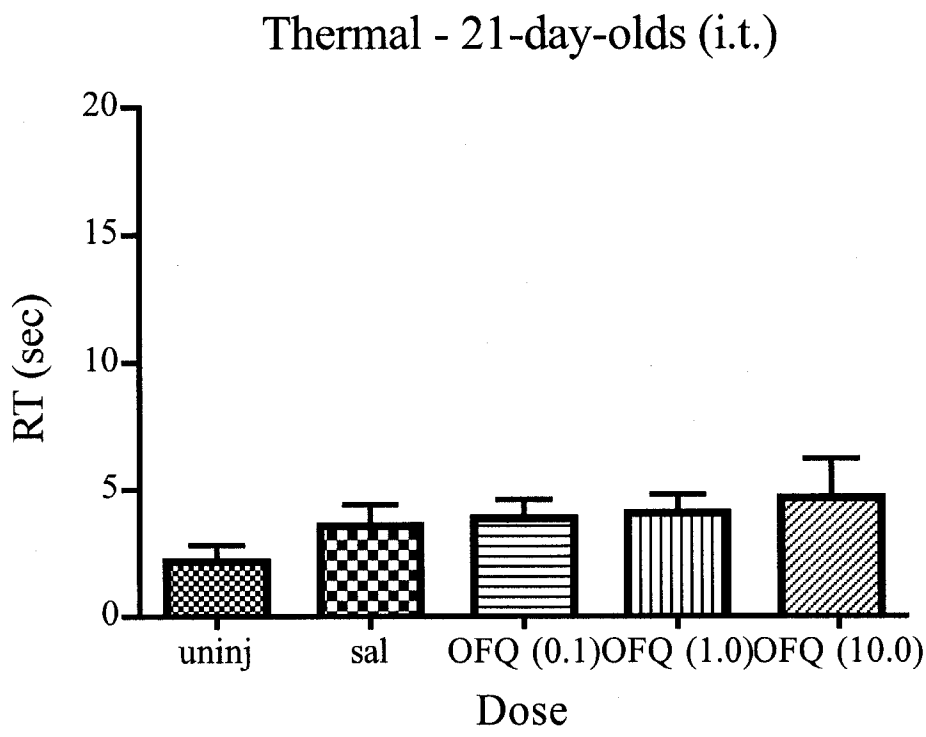

Figure 20: In the thermal paradigm, no significant dose-related effects were seen in 21-day-olds following spinal administration.

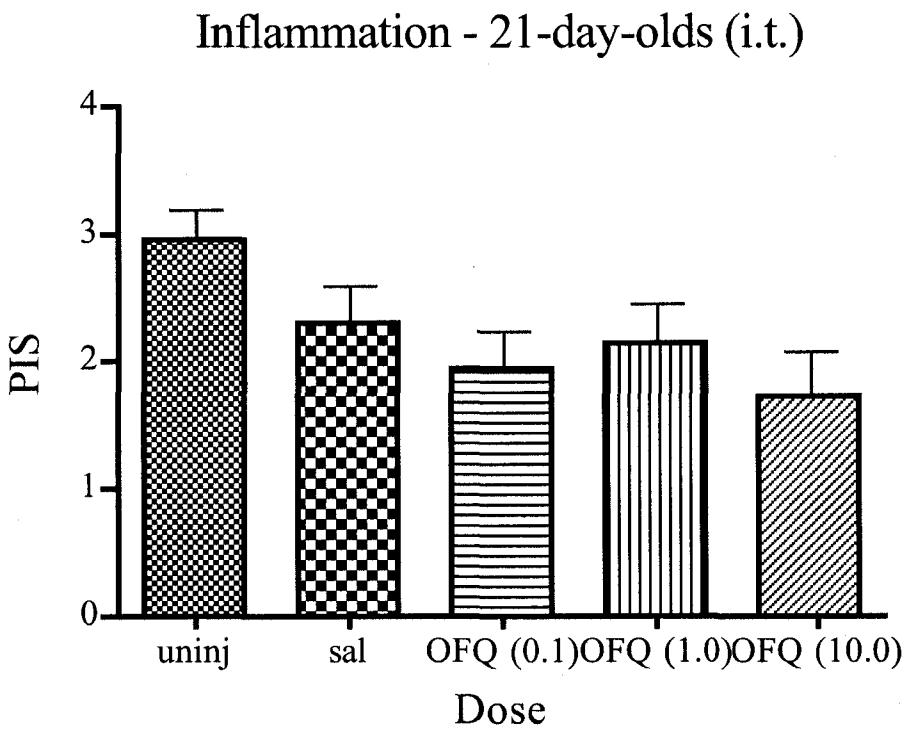

Figure 21: In the formalin test, differences were seen at 21-days of age between the OFQ-treated groups and uninjected controls following spinal administration. On the other hand, there were no differences between these OFQ-treated groups and the saline-treated controls: all injection groups demonstrated lower PIS values, suggesting no difference between saline-induced and OFQ-induced analgesia. However, there was a strong trend suggesting a difference between the high dose and the injected control, which would imply that high doses are necessary to produce analgesia in this paradigm.

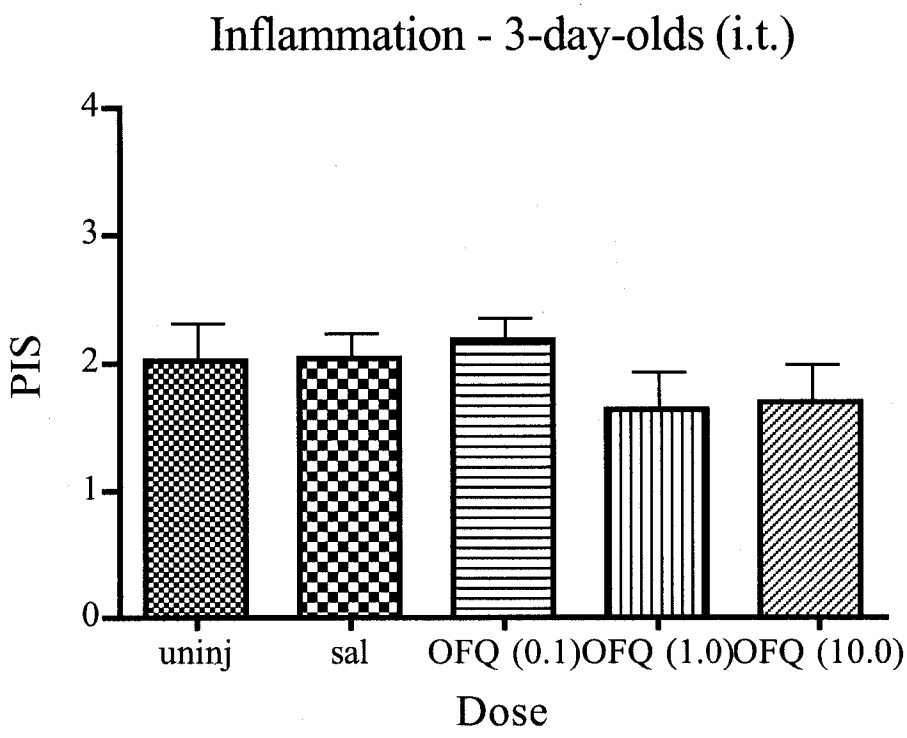

Figure 22: In the formalin test, no significant dose effects were seen in the 3-day-olds, suggesting that OFQ has little effect on inflammatory pain early in development when administered spinally.

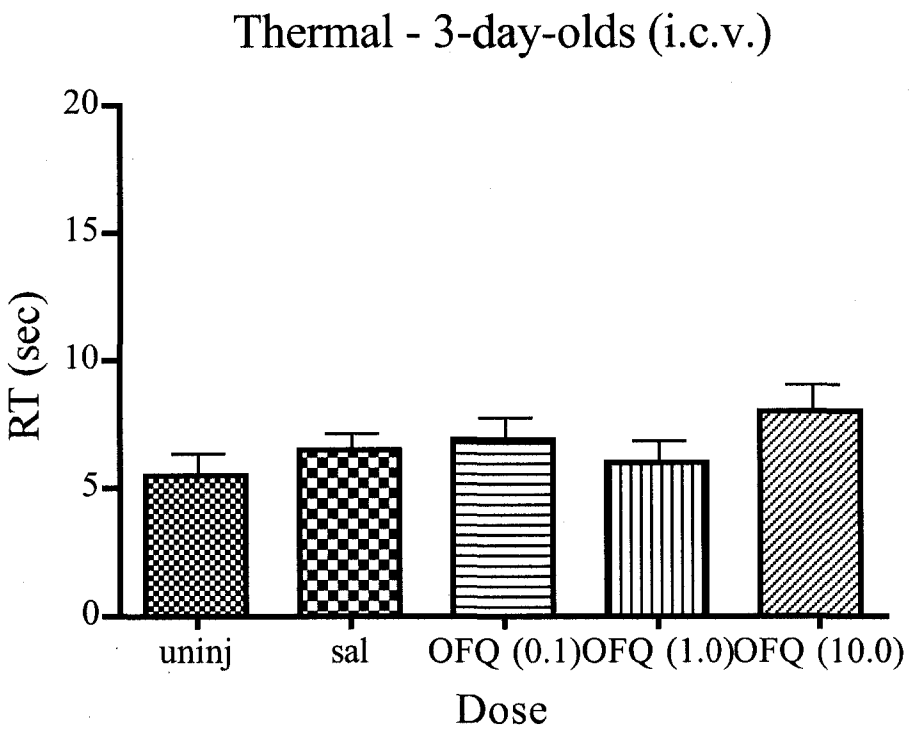

Figure 23: In the thermal paradigm, differences were seen between the high dose and un-injected, but not injected, controls in 3-day-olds following supraspinal administration, with the high dose demonstrating higher RTs, suggesting that a high OFQ dose can produce analgesia during early development in this paradigm, but that this analgesia may not differ from that produced by saline injection. However, there were strong trends suggesting differences between the high dose and the injected control and low dose, which would imply that the analgesia seen is distinct from that produced by saline injection.

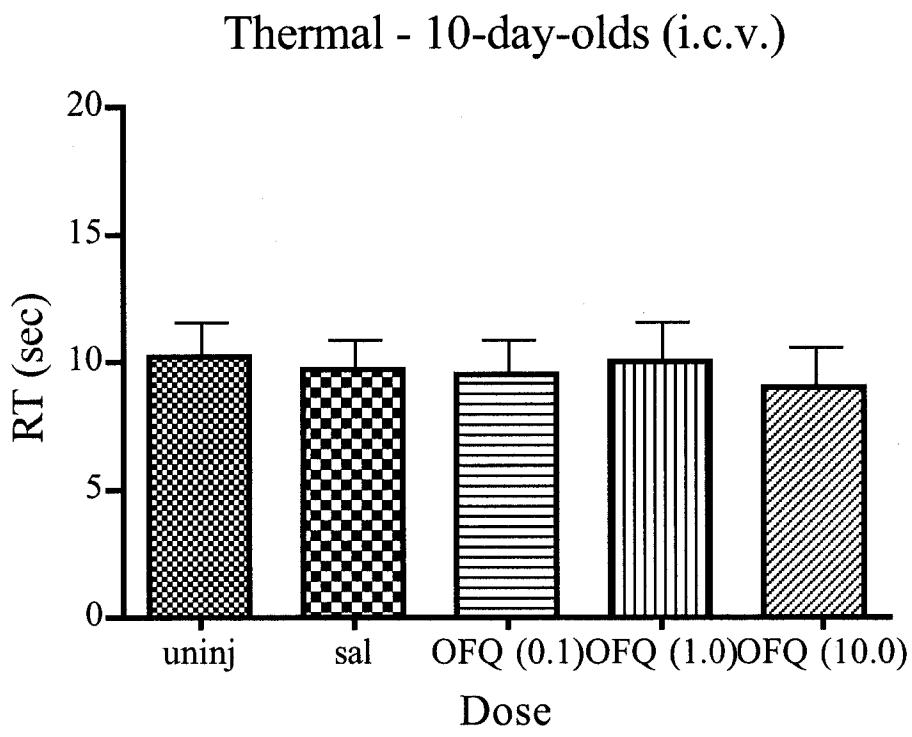

Figure 24: Following supraspinal administration, no significant dose-related effects were seen in 10-day-olds in the thermal paradigm.

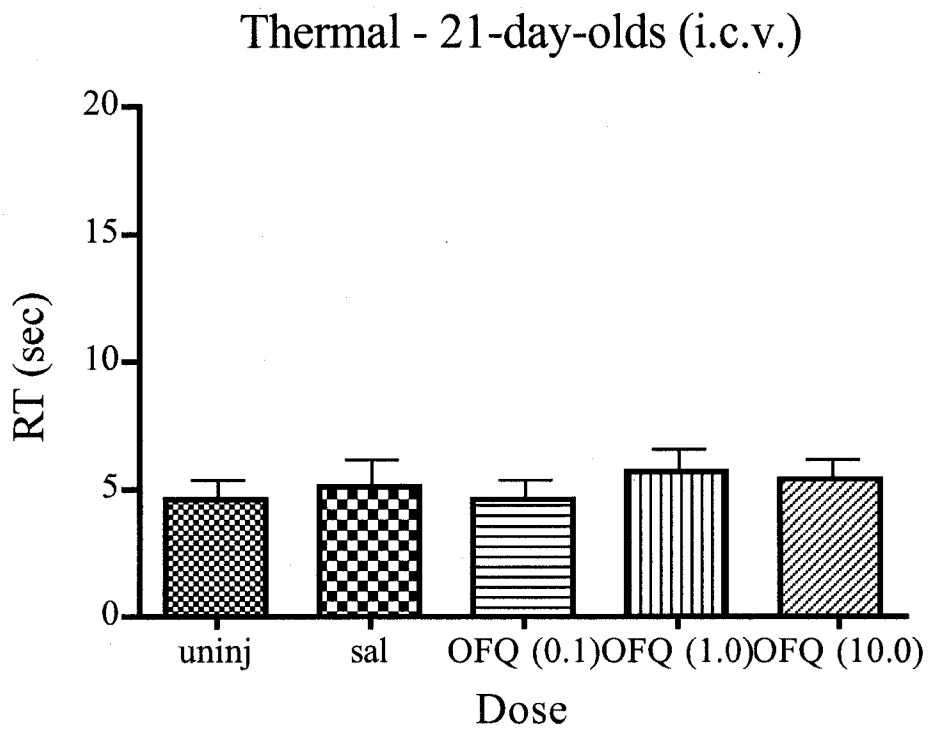

Figure 25: Following supraspinal administration, no significant dose-related effects were seen in 21-day-olds in the thermal paradigm.

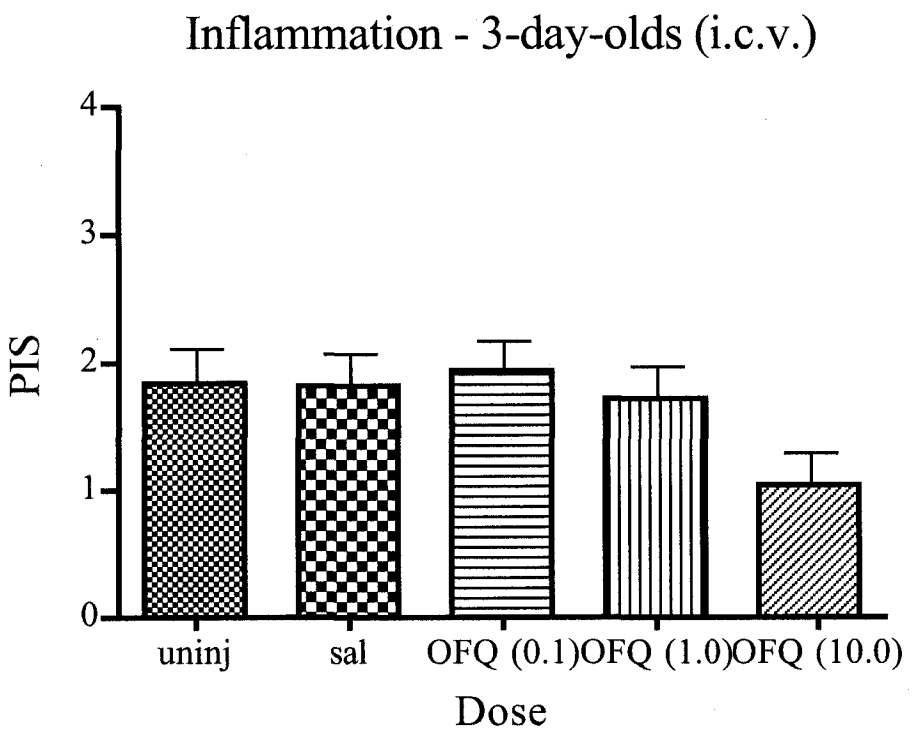

Figure 26: In the thermal paradigm, differences were seen between the high dose and both controls, as well as between the high dose and the low and middle doses, in the 3-day-olds following supraspinal administration, with the high dose demonstrating lower PISs. No differences were seen between the low and middle doses, or between the low and middle doses and the injected control, implying that a high dose of OFQ is capable of producing a distinct analgesia during development.

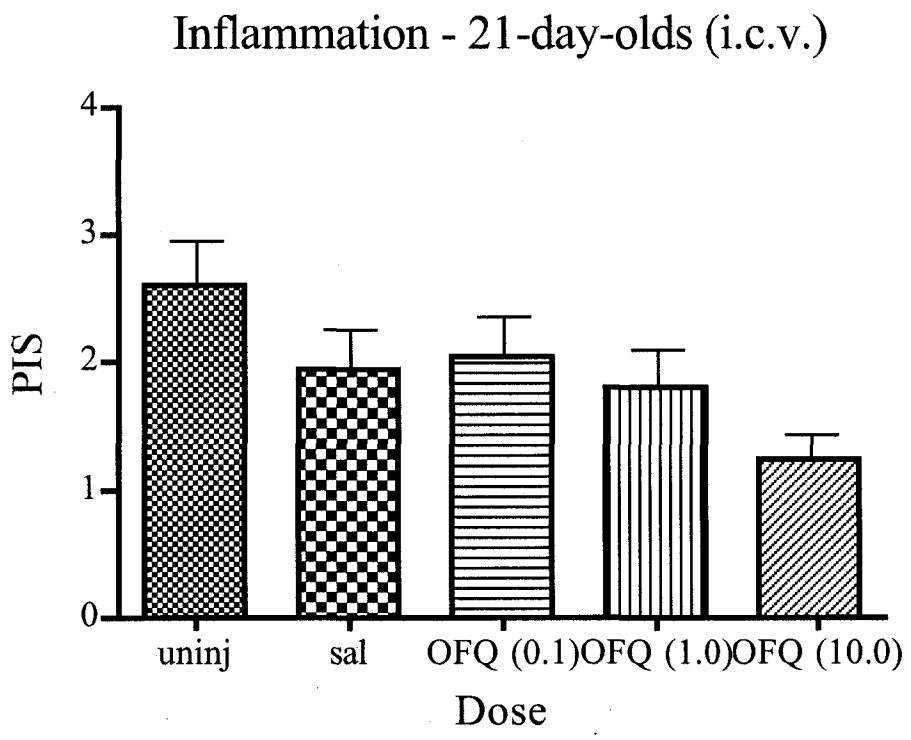

Figure 27: Like the 3-day-old data, differences were seen between the high dose and both controls, as well as between the high dose and the low and middle doses, in the 21-day-olds following supraspinal administration in the thermal paradigm, with the high dose demonstrating lower PISs. No differences were seen between the low and middle doses, or between the low and middle doses and the injected control, again suggesting that a high dose of OFQ is capable of producing a distinct analgesia during development.

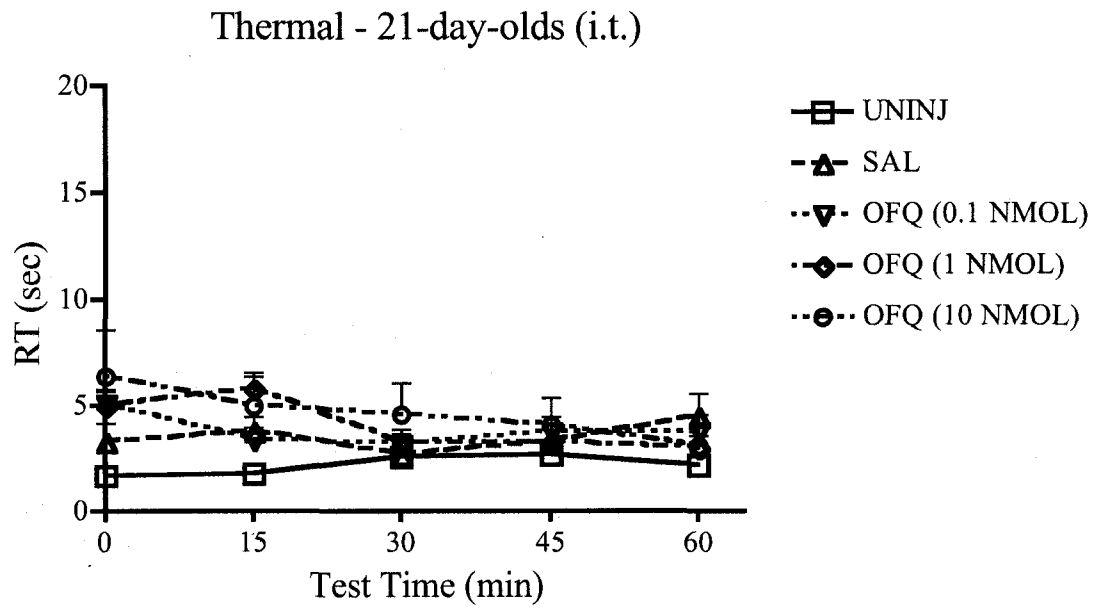

Figure 28: In the thermal paradigm, significant dose × time interactions were demonstrated in 21-day-olds following spinal administration. These intereactions were characterized by an increase in RTs in a dose-dependent manner during the first 30 minutes of testing, suggesting a quick onset and relatively long duration of effect.

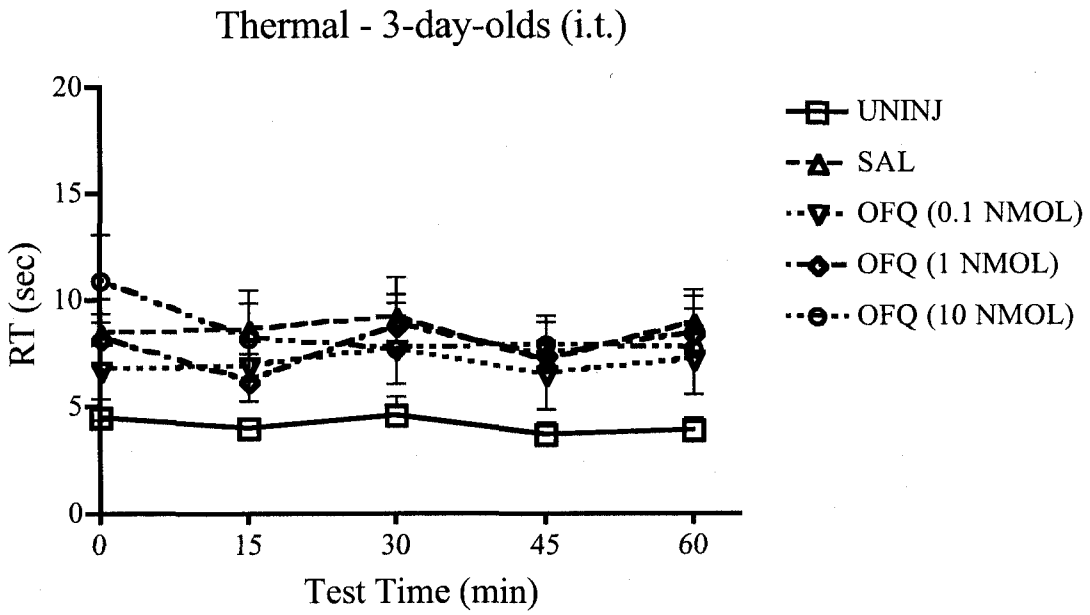

Figure 29: In the thermal paradigm, no significant dose × bin interactions were seen in 3-day-olds following spinal administration.

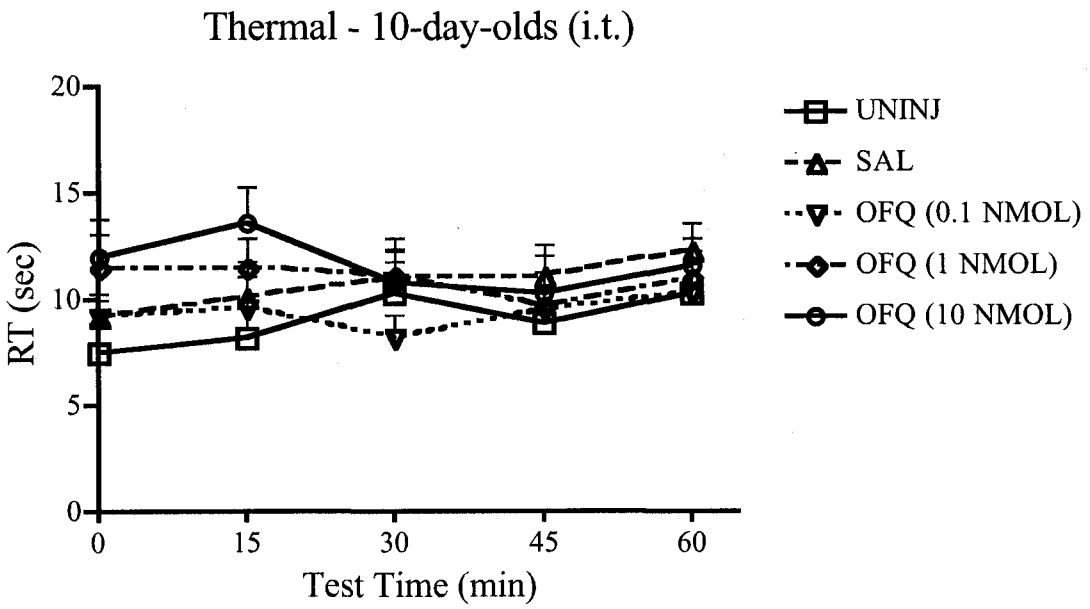

Figure 30: In the thermal paradigm, no significant dose × bin interactions were seen in 10-day-olds following spinal administration.

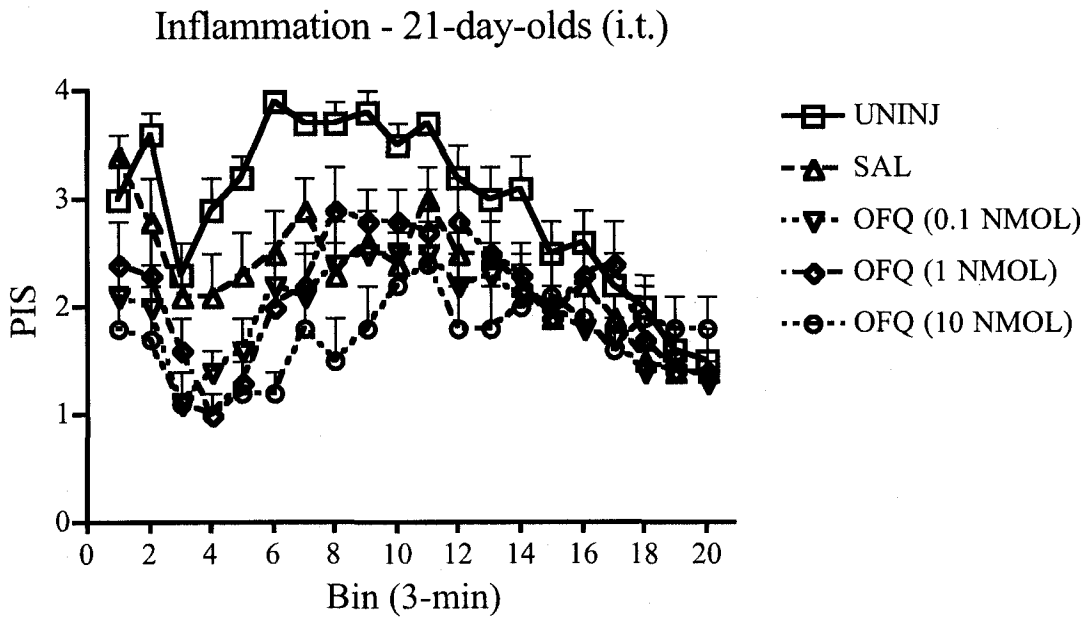

Figure 31: In the formalin test, significant dose × bin interactions were demonstrated in 21-day-olds following spinal administration. The 21-day-olds showed a dose-dependent decrease in PIS values that demonstrated later onsets and shorter durations (with a maximum period of 24 minutes for the high dose, beginning at bin 2), implying that OFQ administration is capable of producing analgesia in both phases of the formalin response.

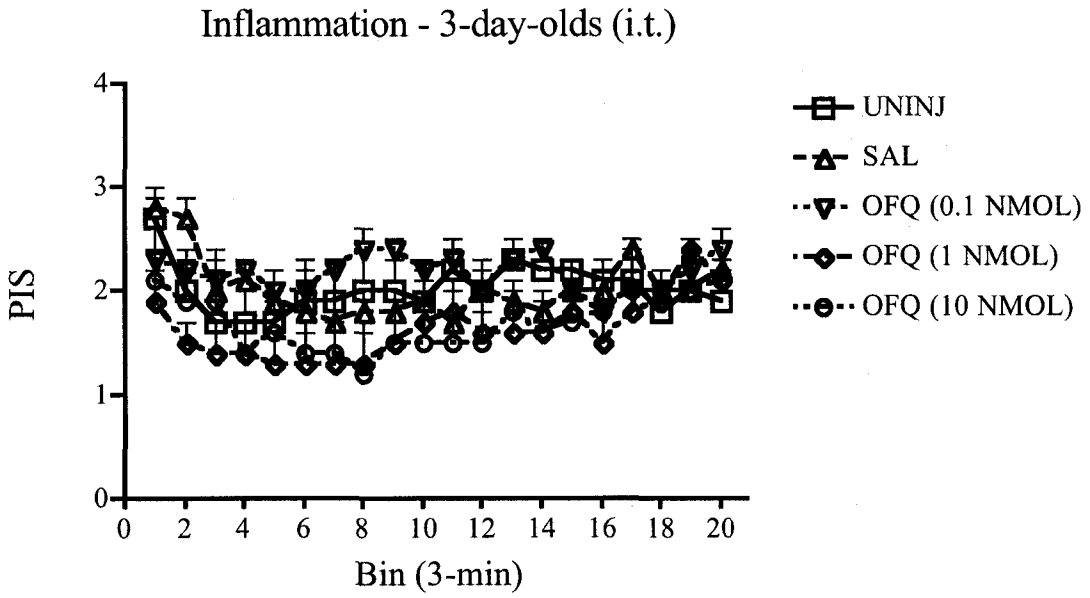

Figure 32: In the formalin test, no significant dose × bin interactions were seen following spinal administration in 3-day-olds, again suggesting that OFQ has no effect on inflammatory pain early in development when administered spinally.

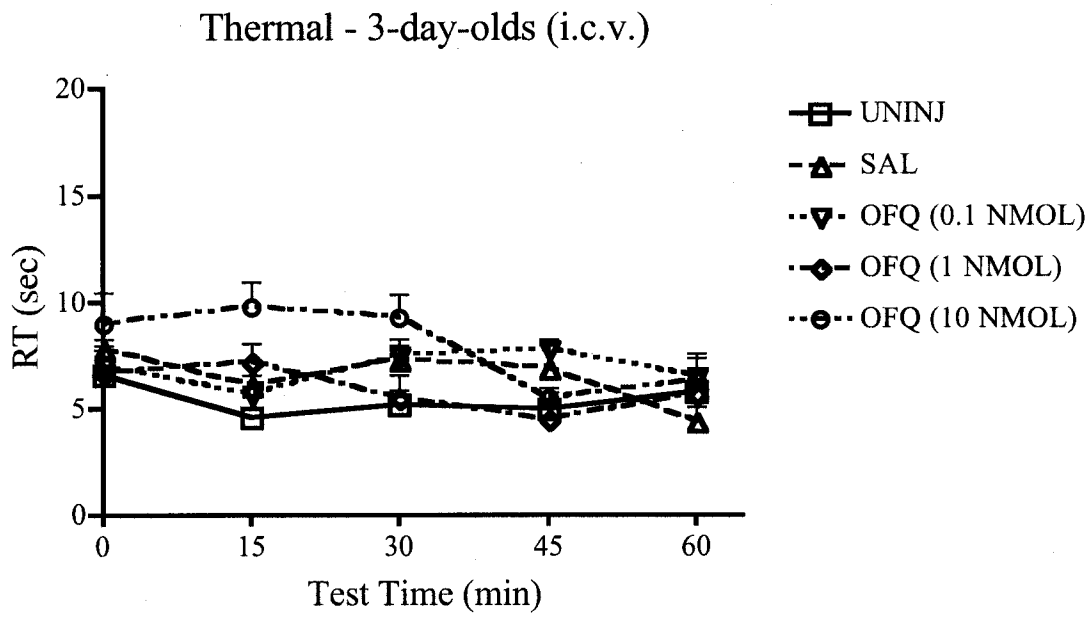

Figure 33: In the thermal paradigm, significant dose × time interactions were demonstrated in 3-day-olds following supraspinal administration. The 3-day-olds showed an increase in RTs between the high dose and controls at the 35 minute test time, implying a quick-acting and long duration analgesic effect of OFQ.

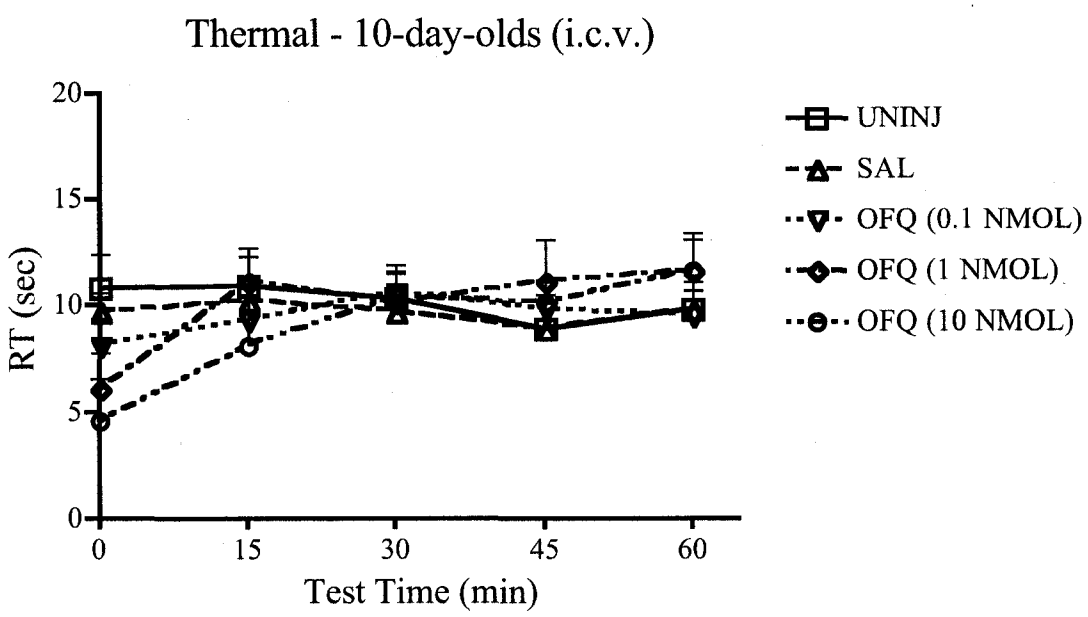

Figure 34: In the thermal paradigm, significant dose × time interactions were demonstrated in 10-day-olds following supraspinal administration. The 10-day-olds showed a decrease in RTs during the first 15 minutes of testing between the two high doses and controls, implying a quick-acting and short duration hyperalgesic effect of OFQ. These results were unexpected and may reflect an irregularity in the thermal paradigm.

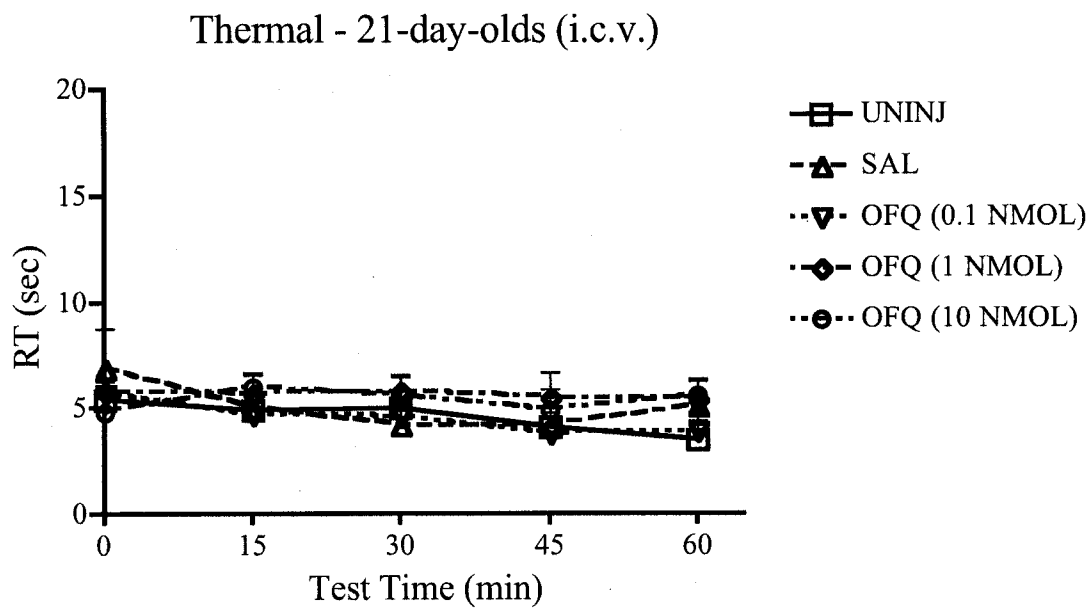

Figure 35: In the thermal paradigm, no significant dose × bin interactions were seen in 21-day-olds following supraspinal administration.

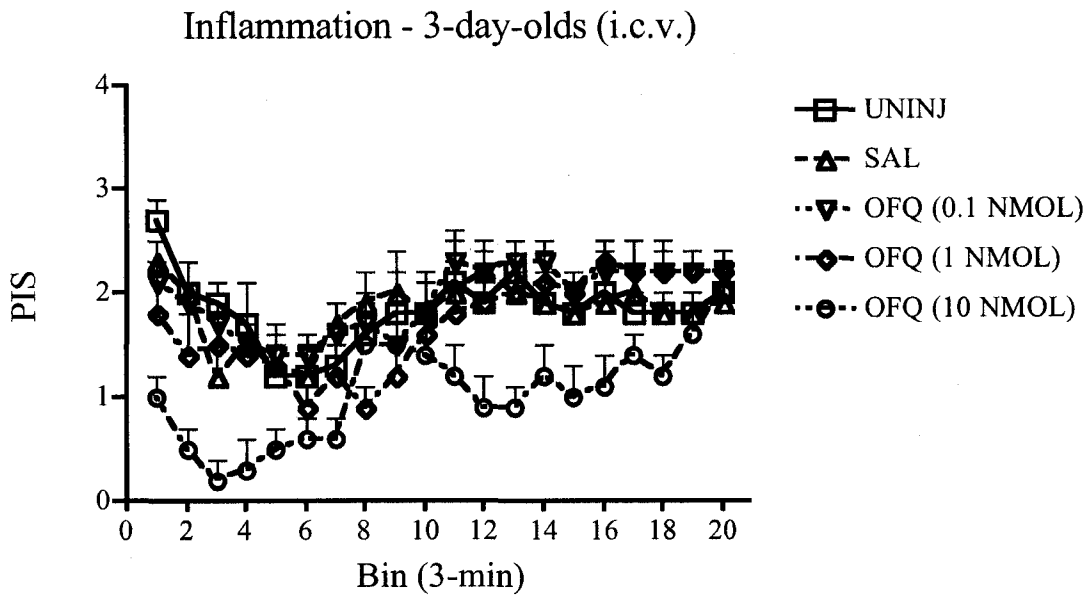

Figure 36: In the formalin test, significant dose × bin interactions were demonstrated in 3-day-olds following supraspinal administration. The 3-day-olds showed a decrease in PIS values between the high dose and controls during the first 12 minutes of testing, suggesting that OFQ is only capable of influencing the first phase of the formalin response at this age.

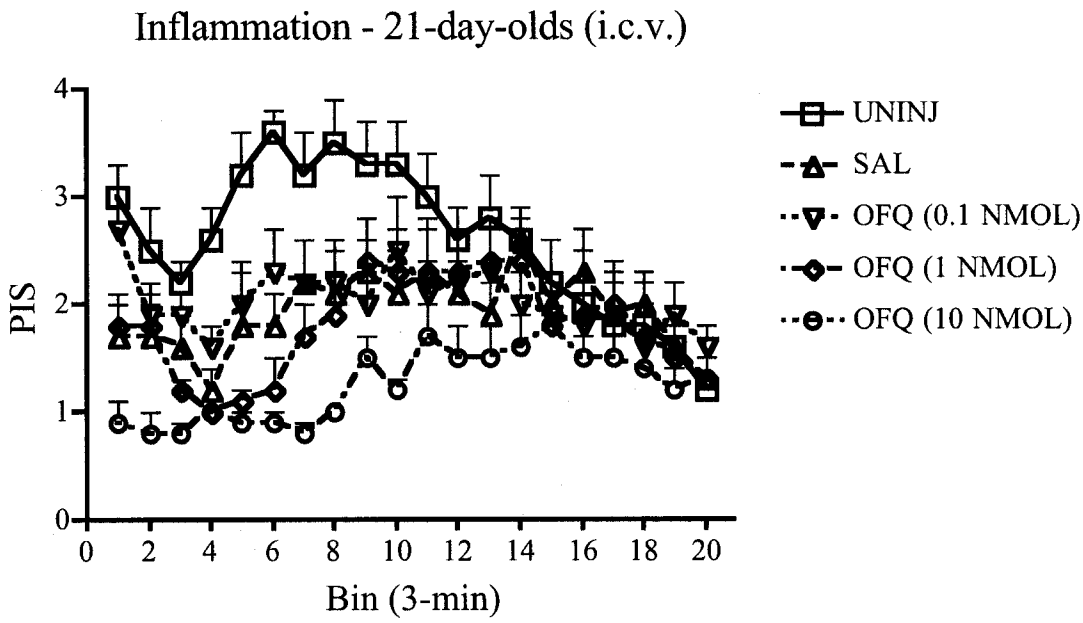

Figure 37: In the formalin test, significant dose × bin interactions were demonstrated in 21-day-olds following supraspinal administration. The 21-day-olds showed a dose-dependent decrease in PIS values that demonstrated an earlier onset and longer duration as doses increased (reaching a maximum period of the first 33 minutes of testing with the high dose), implying a greater influence of OFQ that affects both phases of the formalin response.

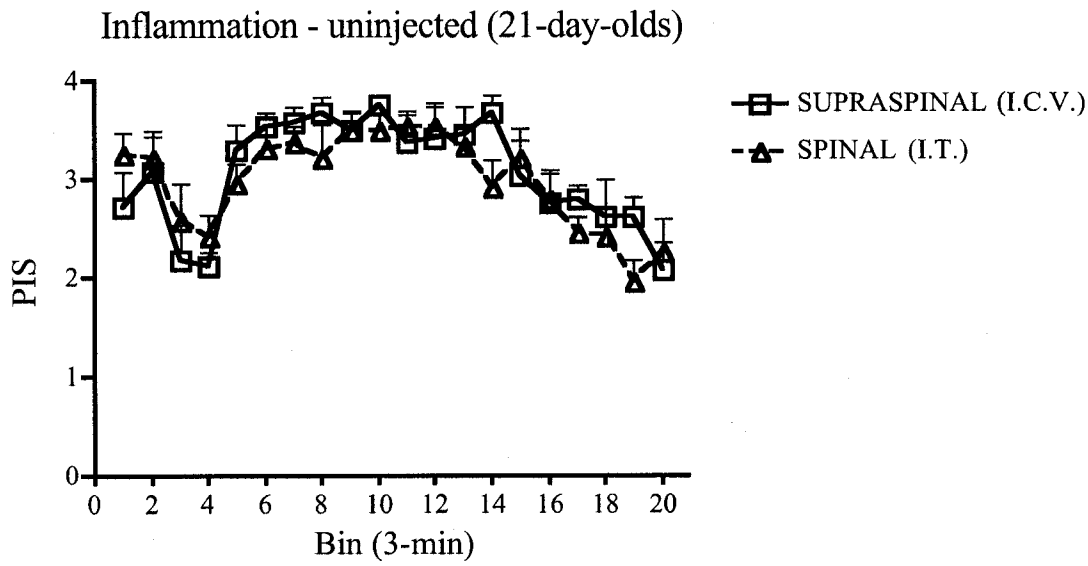

Figure 38: In the antagonist experiments, no differences were seen between 21-day-old uninjected controls assigned to the supraspinal and spinal administration groups in the formalin test.

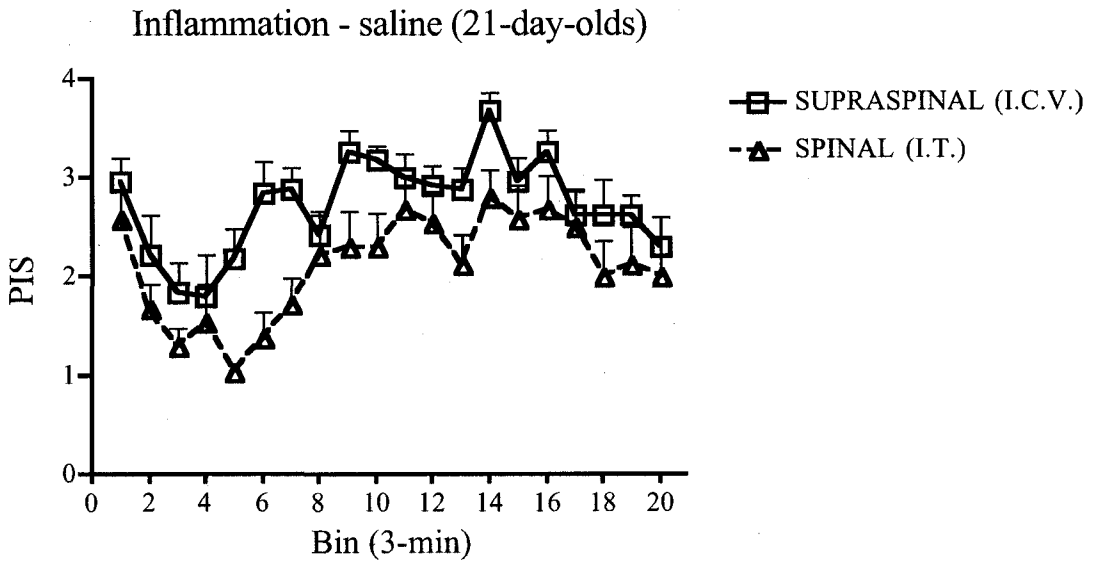

Figure 39: Overall, no differences were seen between routes of injection in saline-treated controls at 21-days of age in the formalin test.

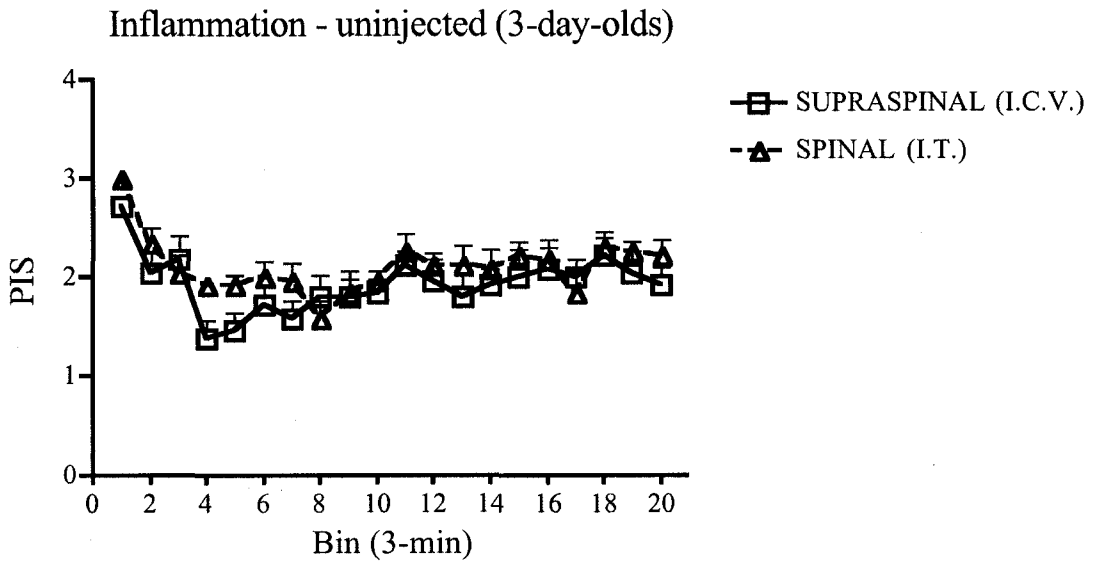

Figure 40: In the formalin test, no differences were seen between 3-day-old uninjected controls assigned to the supraspinal and spinal administration groups in the antagonist experiments.

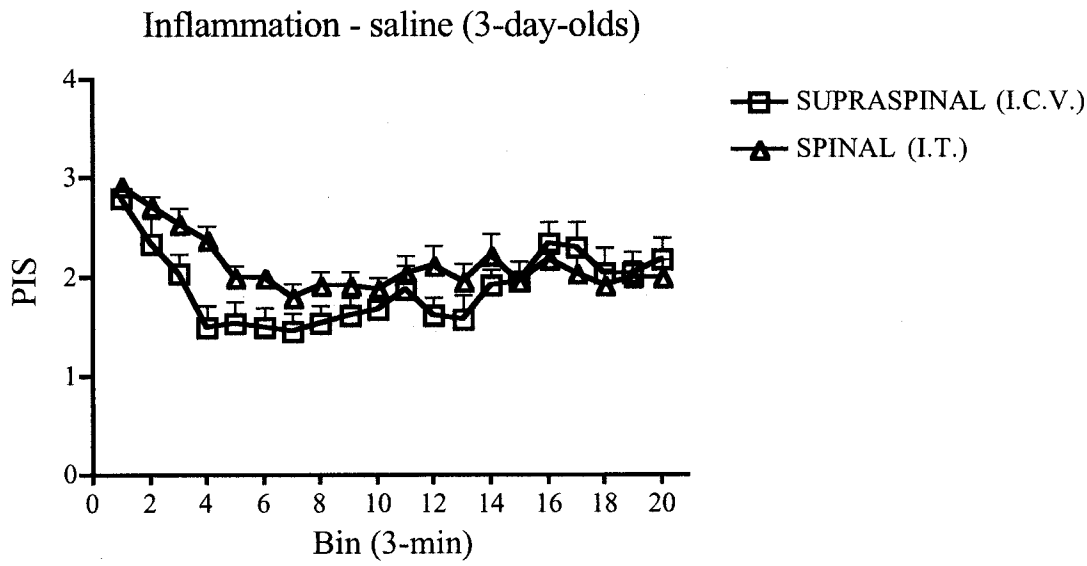

Figure 41: In the formalin test, differences were seen between injection routes in saline-treated controls at 3-days of age, with supraspinal administration resulting in lower PISs (from 9-18 min).

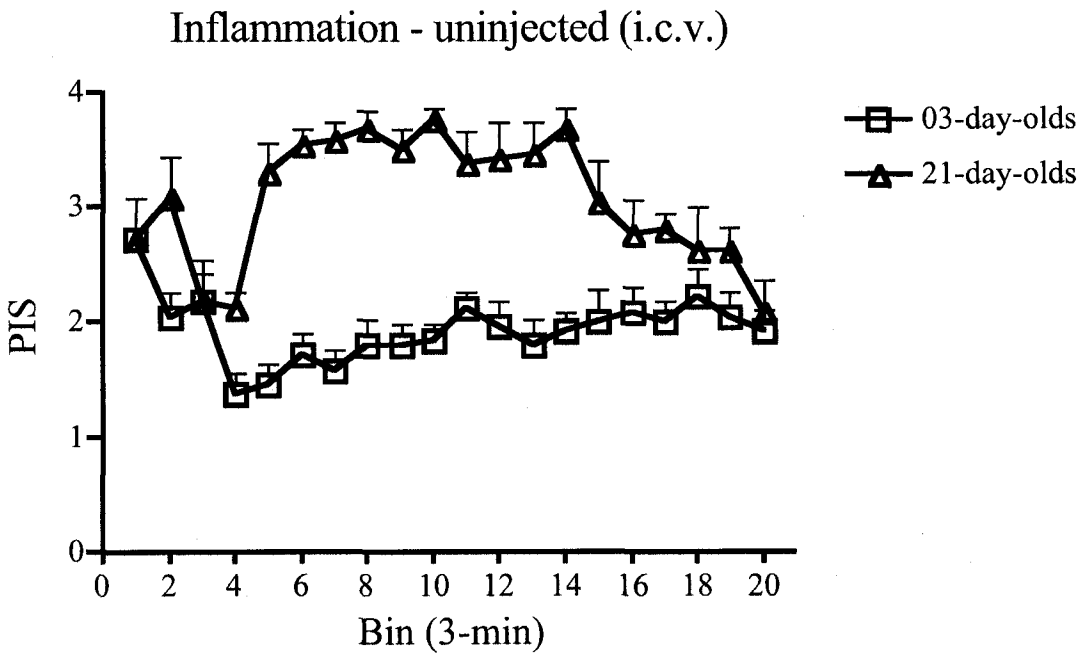

Figure 42: In uninjected controls, differences were seen between 3- and 21-day-olds assigned to the supraspinal administration groups in the formalin test, with 21-day-olds demonstrating much higher PISs than 3-day-olds during both the first and second phases of the formalin response. These results suggest differences in the mechanisms governing inflammatory response at these ages.

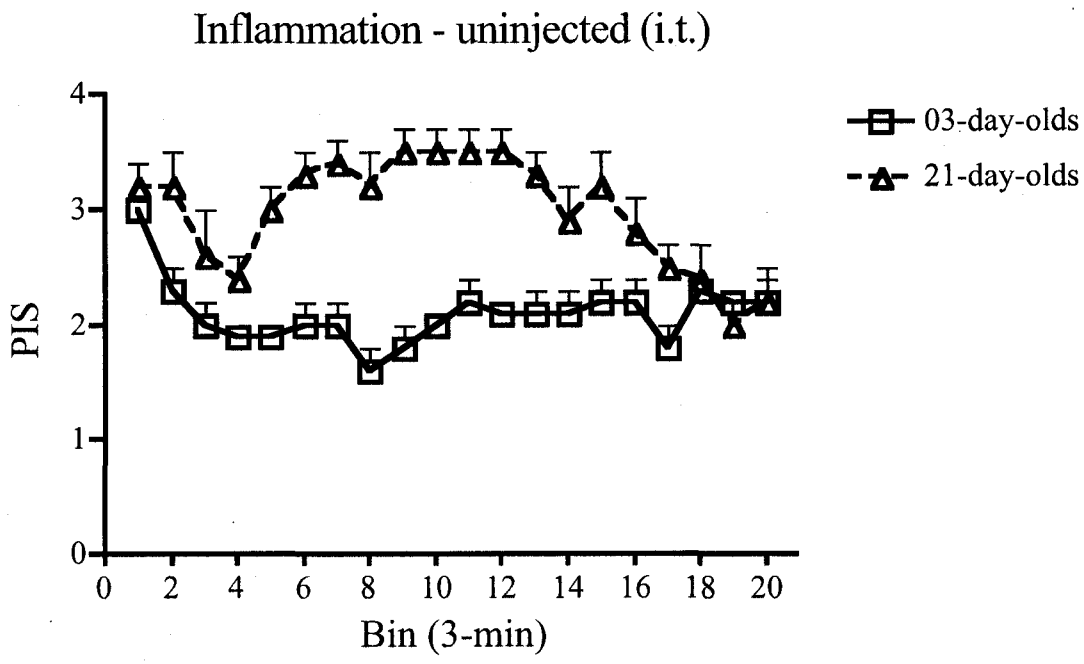

Figure 43: Differences were also seen between 3- and 21-day-old uninjected controls assigned to the spinal administration group in the formalin test, with 21-day-olds again demonstrating much higher PISs. Again, these results suggest differences in the mechanisms governing inflammatory response at these ages.

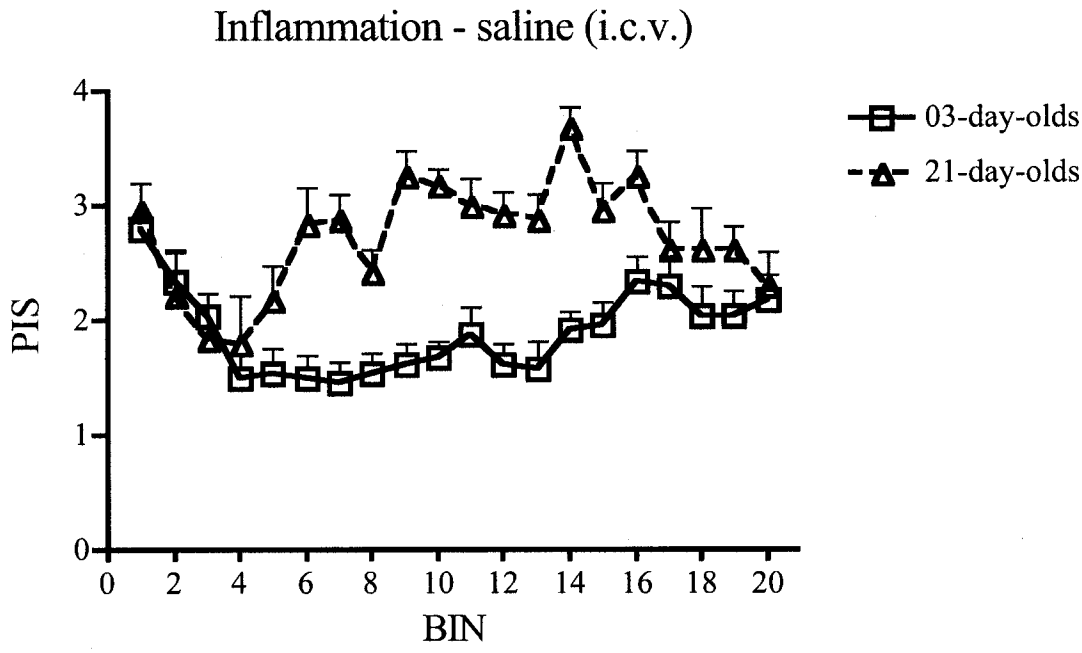

Figure 44: In the formalin test, differences between ages were seen in saline-treated controls following supraspinal administration, with 3-day-olds demonstrating lower PISs. The differences seen between injection routes in 3-day-olds suggest the presence of a stress-mediated analgesia as a result of spinal administration; however, the overall similarity between these results and those of the uninjected controls suggests that the differences between ages are mainly the result of maturational issues.

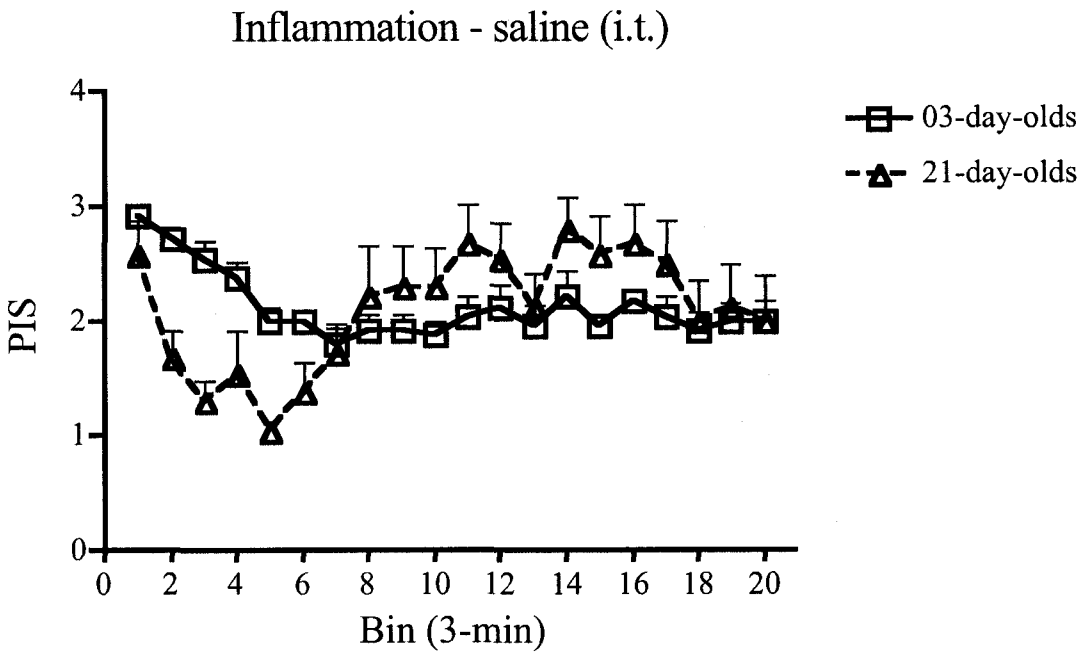

Figure 45: Differences between ages were also seen in saline-treated controls following spinal administration in the formalin test; however, unlike the results following supraspinal administration, 21-day-olds demonstrated lower PISs during the first phase of the formalin response. suggesting an acute stress-mediated analgesia resulting from spinal administration.

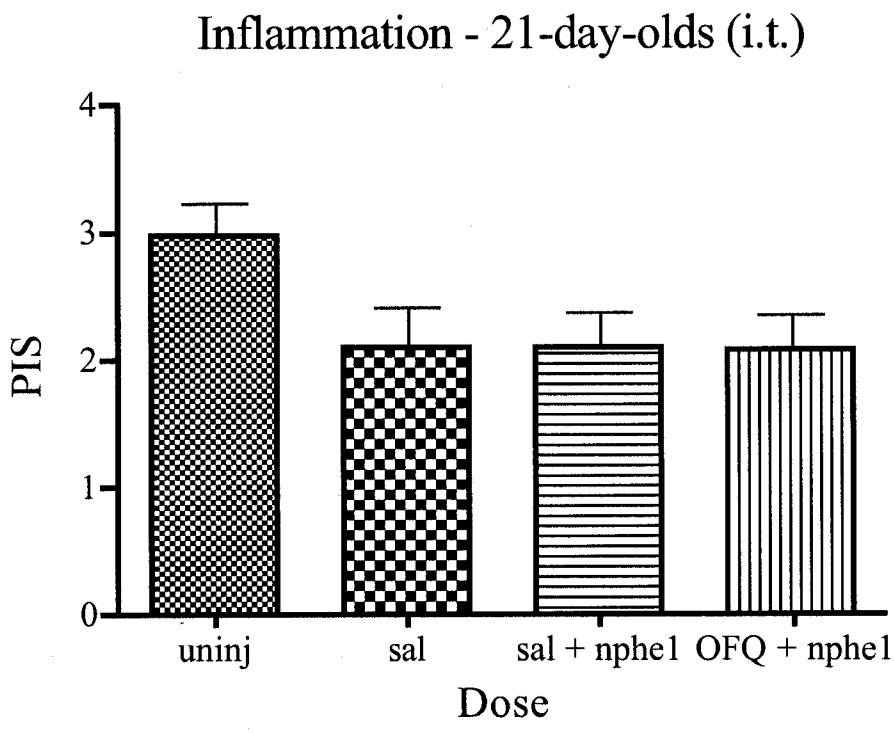

Figure 46: In the formalin test, differences were seen between Nphe1 co-administration groups and un-injected controls in 21-day-old pups, implying an analgesic effect. However, there were no differences between Nphe1 co-administration groups and the saline-treated controls: all injected pups demonstrated lower PIS values, suggesting that the observed analgesia is stress-mediated.

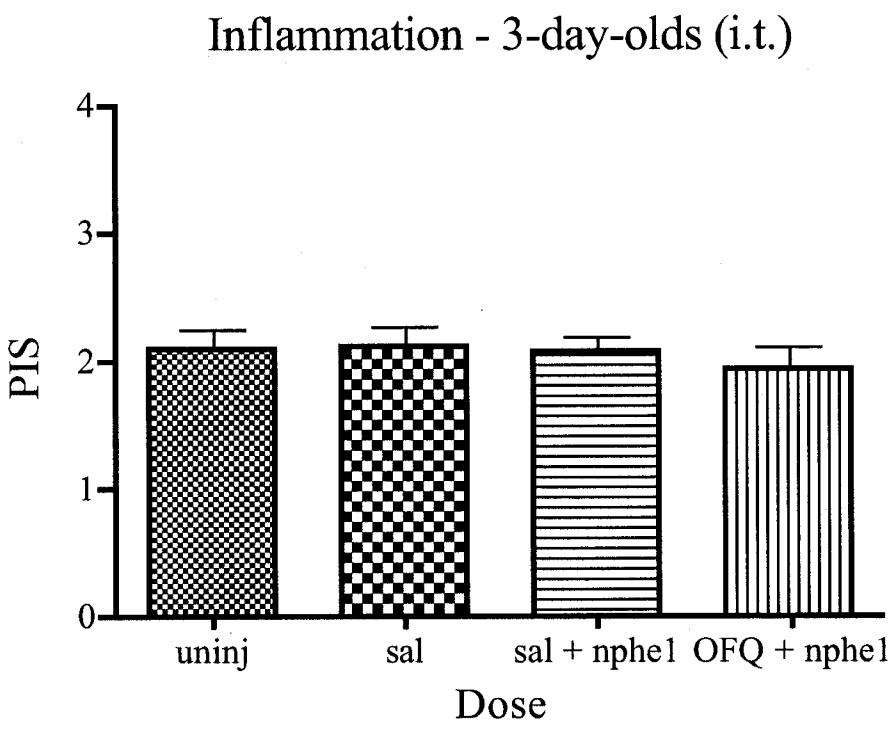

Figure 47: In the formalin test, no significant dose effects were seen in 3-day-olds following spinal administration.

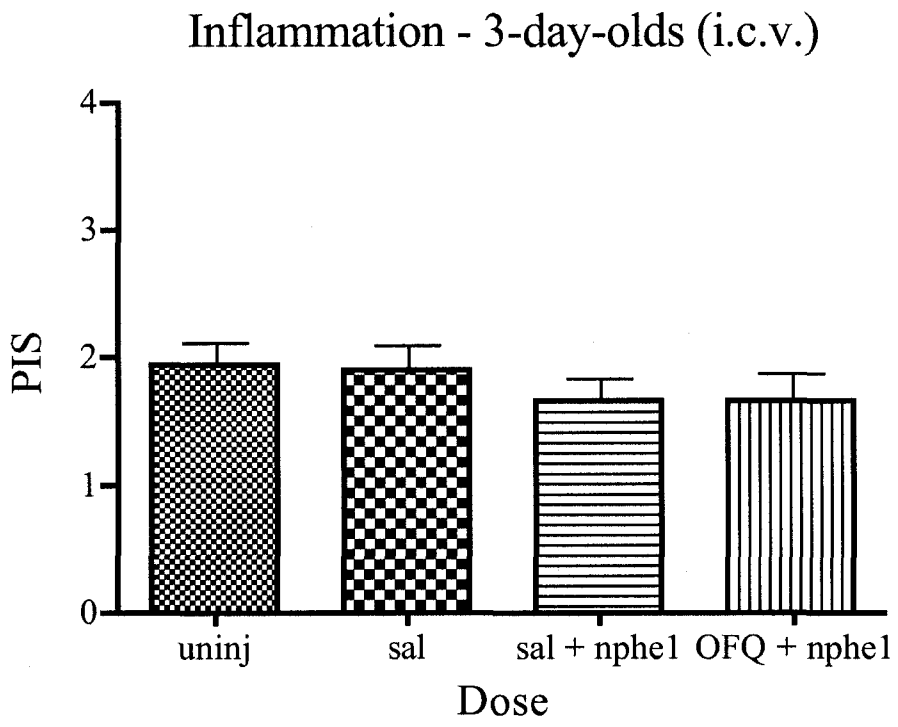

Figure 48: In 3-day-olds, significant differences were seen between the saline-treated control and the saline + Nphe1 co-administration group only. The saline + Nphe1 co-administration group demonstrated lower PISs, suggesting an analgesic nature of Nphe1 and hinting at its possible antagonism of OFQ. No differences were seen between controls, arguing against the presence of a SIA, or the two Nphe1 co-administration groups. The lack of differences in the co-administration groups could indicate that 1) the analgesia produced by Nphe1 is equal to that produced by OFQ or 2) the analgesia seen in both cases is mediated by Nphe1.

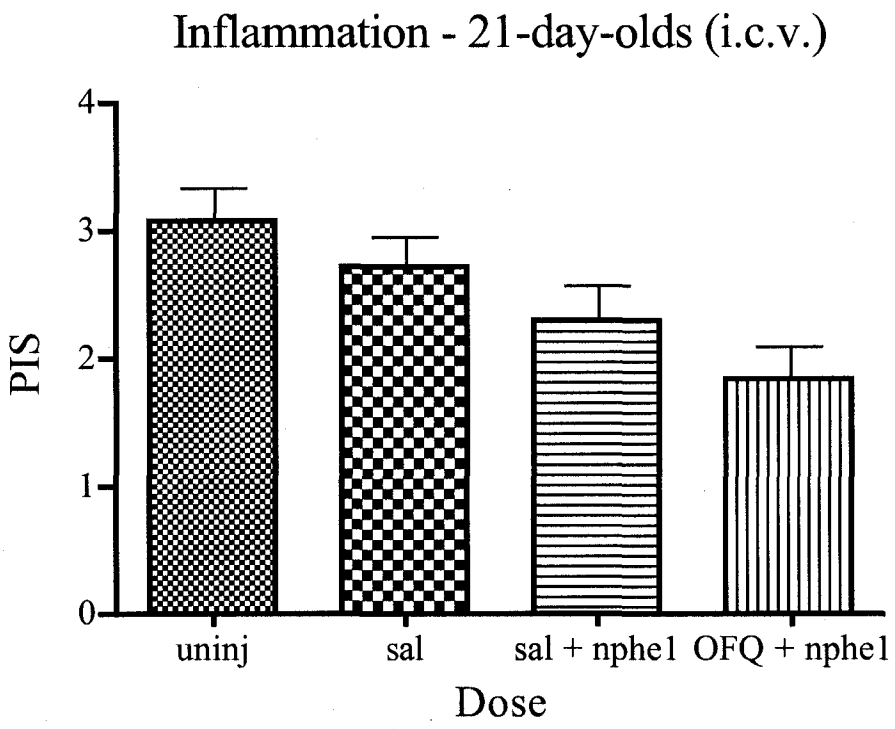

Figure 49: In 21-day-olds, differences were seen between the uninjected controls and Nphe1 co-administration groups in the formalin test, with the co-administration groups demonstrating lower PISs. These results again suggest an analgesic response to the co-administration groups. However, differences were seen between the saline-treated control and the OFQ + Nphe1 co-administration group only, with the OFQ + Nphe1 co-administration group also demonstrating lower PISs. Taken together, these two sets of results could imply that differences in the nature of the analgesia produced by each of the co-administration groups, with the saline + Nphe1-produced analgesia being the result of a stress-mediated analgesia and the OFQ + Nphe1-produced analgesia the result of OFQ activity.

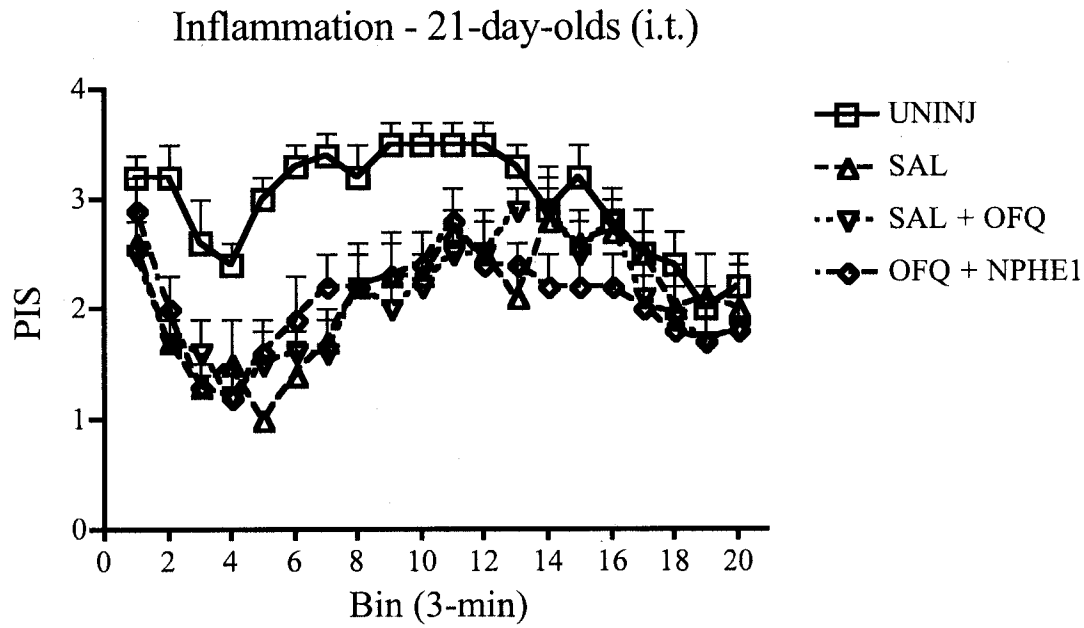

Figure 50: In the formalin test, significant dose x bin interactions were demonstrated in 21-day-olds following spinal administration, showing a decrease in PIS values characterized by early onsets and long durations (with a maximum period of 39 minutes), implying that spinal OFQ administration is capable of producing analgesia in both phases of the formalin response.

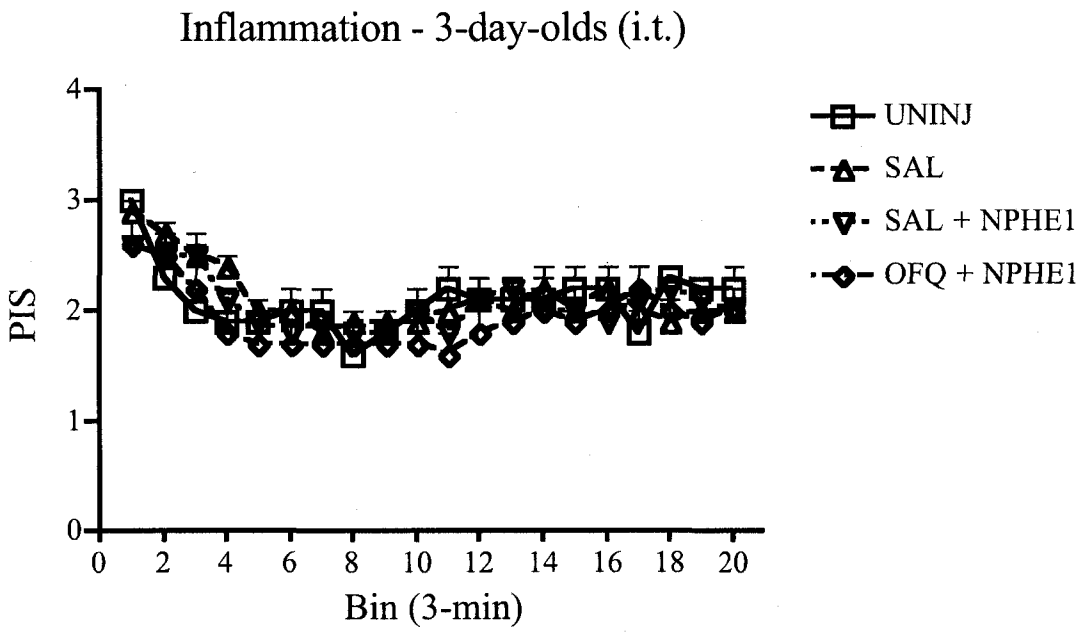

Figure 51: No significant dose × bin interactions were seen in 3-day-olds following spinal administration in the formalin test, suggesting that OFQ has no effect on inflammatory pain early in development when administered spinally.

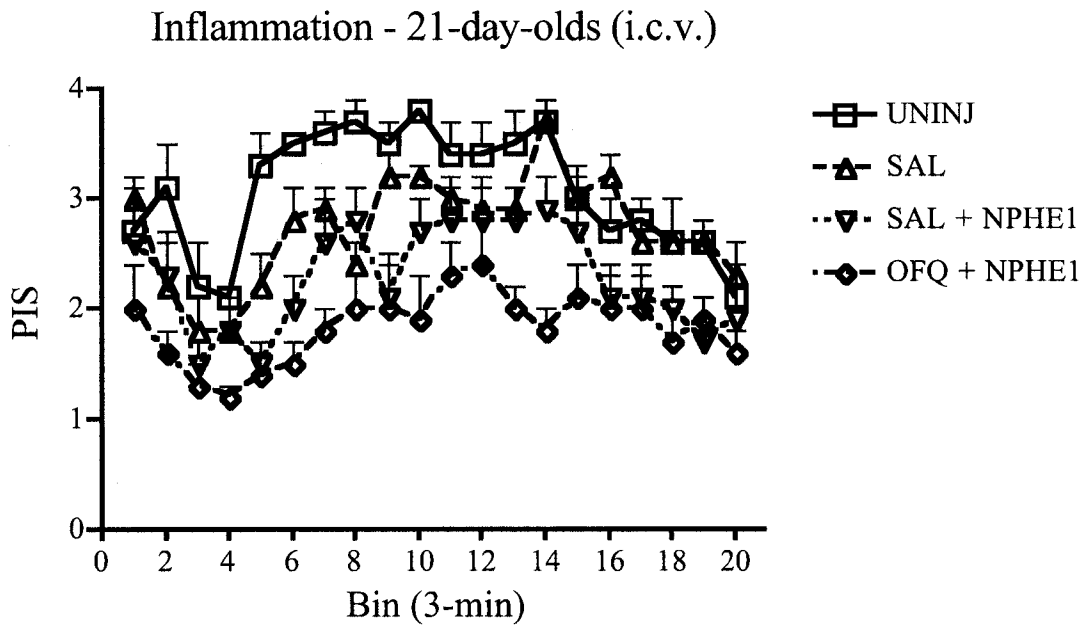

Figure 52: Significant dose × bin interactions were demonstrated in 21-day-olds following supraspinal administration in the formalin test. The 21-day-olds showed a decrease in PIS values that demonstrated a slightly later onset (except in the case of OFQ + Nphe1 co-administration) and longer duration (in the case of OFQ + Nphe1 co-administration, as long as 57 minutes), suggesting that supraspinal OFQ administration is also capable producing analgesia in both formalin phases.

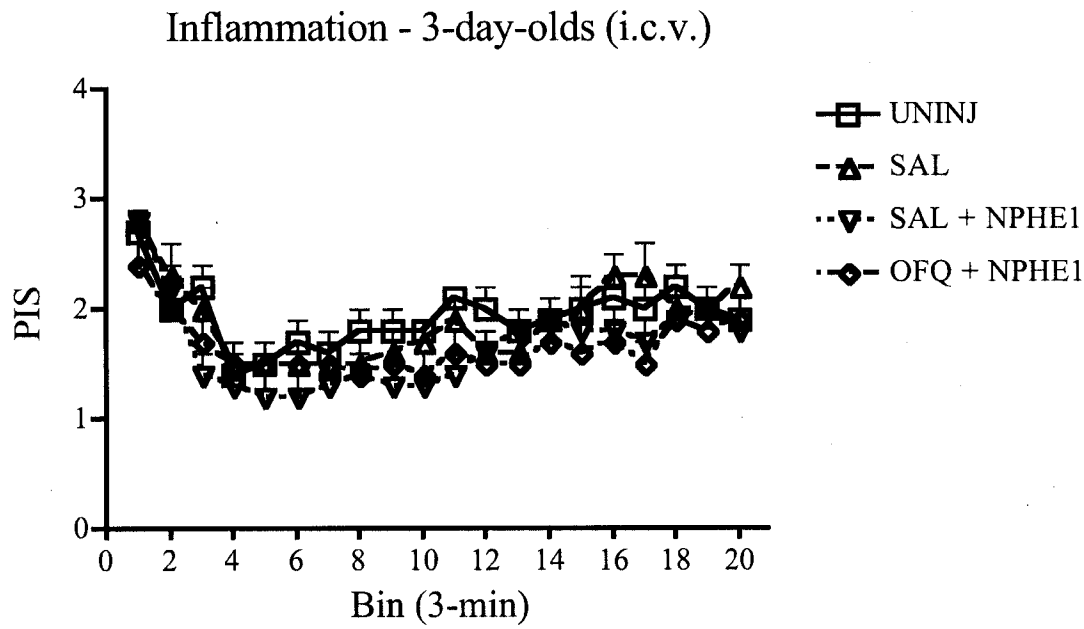

Figure 53: In the formalin test, no significant dose × bin interactions were seen in 3-day-olds following formalin injection, again implying a lack of effect by OFQ on inflammatory pain early in development.

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