

The development of the nociceptive responses in neurokinin-1 receptor knockout mice

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An important yet unanswered question is how neonates respond to painful stimuli, given the immaturity of their neural pathways. We examined the development of the neurokinin system using a novel approach, examining changes of this system by observing the pain responses of mice lacking the NK1 receptor at different stages of development. We show that the NK1 receptor is not involved in nociception to heat, mechanical or chemical stimuli, at 3 days. In contrast, the NK1 receptor is involved in nociceptive responses to high intensity

heat and mechanical stimuli, and mediates the second phase of the formalin response in 21-day-old mice. This indicates that nociception in neonates does not require the NK1 receptor and that the functional maturation of the NK1 receptor allows diversity in both the type of stimuli that activate the pain system and the types of responses elicited by nociceptive stimuli. *NeuroReport* 11:587–591 © 2000 Lippincott Williams & Wilkins.

Key words: Knockout mice; NK1; Ontogeny; Pain; Substance P; Transgenic mice

INTRODUCTION

Neonates are capable of responding to most forms of noxious stimulation, including thermal, chemical and mechanical stimuli, at or prior to birth [1–6]. However, these responses are immature, and show qualitative changes as the animal matures [1–6]. In rats, substance P fibers are found in the spinal cord, the dorsal root ganglion, the peripheral nerves, and skin at birth [7–9]. C-fibers reach their destination in the substantia gelatinosa 24–48 h after birth, and terminal arborization continues over the following week [6]. Substance P and NK1 receptors are both highly expressed in the spinal cord at birth, compared to adult animals, and also show maturational changes over the first 2 weeks of life [7–9]. Although neonatal rats respond to certain nociceptive stimuli [1–5], specific tests of C-fiber function indicate that these fibers are not mature until 10–11 days after birth. Application of mustard oil, a chemical irritant that specifically activates C-fibers, does not elicit the reflexive withdrawal response until postnatal days 10–11 [6], and does not induce c-Fos expression at 3 days of age [10]. In addition, neurogenic edema, an

inflammatory reaction mediated by C-fiber nociceptors, is not observed until 10–11 days after birth [6]. Further, NK1 receptors do not play a role in formalin-induced nociceptive behavior until the second week after birth in the rat [11]. However, whether the immature neurokinin system is involved in other pain responses in infants, or how these physiological maturational changes are involved in the changing behavioral responses to noxious input across development, are unknown. We examined this issue in a new way, by measuring baseline pain responses of mice lacking the NK1 receptor at different stages of C-fiber/NK1 receptor development, prior to when this system is putatively functional, at 3 days after birth, and after this system is functional, at 21 days of age. At each age, pain reactivity to a heat stimulus, a mechanical stimulus, and a chemical stimulus were measured.

MATERIALS AND METHODS

Subjects: Subjects were mice with a targeted mutation of the NK1 receptor on a 129/Sv background, or wildtype 129/Sv mice. The targeting vector consisted of a 9 kb

genomic fragment isolated from a 129/Sv genomic library. A PGK neo cassette was inserted at the ATG, deleting and replacing the first and second transmembrane domains of the NK1 receptor (unpublished data). ES cells heterozygous for the mutant allele were injected into 129/Sv blastocysts to derive chimeric animals. These were mated with 129/Sv mice to generate a mutant line on a pure 129/Sv background. Lack of NK1 receptor expression was confirmed by *in situ* hybridization, immunocytochemistry, and by ablation of the elevation of intracellular calcium in cultured dorsal horn neurons in response to substance P (unpublished data).

Nociceptive testing: Subjects (3–4 days old and 21–22 days old) were brought into the testing room and housed in an incubator maintained at 32°C for 3 h. Nociceptive responses were assessed using one of three test stimuli: thermal, mechanical or chemical. The thermal test had separate electrical resistors heated to 38, 42, 46 or 50°C. Each subject had the plantar surface of the forepaw, the hindpaw, and the ventral surface of the tail, placed on the resistor, and the latency for withdrawal was measured at each intensity to the nearest 0.01 s. The mechanical test measured the withdrawal latencies of the forepaw and the hindpaw to presentation of von Frey hairs to the dorsal surface of the paws across four intensities. The intensities used to measure both forepaw and hindpaw responses of 3-day-old mice were 0.172, 0.217, 0.445 and 0.745 g. Forepaw responses of 21-day old mice were measured to 4.19, 4.64, 5.16 and 7.37 g and hindpaw responses were measured to 12.5, 20.9, 46.54 and 84.96 g. The test order for both the heat and mechanical intensities was randomly assigned, and test order for the limbs was counterbalanced. A 20 s cutoff time was used for both the heat and mechanical tests. For the chemical test, 5 μ l of 0.5% or 2% formalin was injected s.c. into the hindpaw of 3-day-old mice, and 2% formalin was s.c. injected into the hindpaw of 21-day-old mice. Subjects were observed for 30 min after the formalin injection and their behavior was rated and recorded every minute using the 5-point scale described in the figure caption. Only one pup per litter was used for each test.

RESULTS

Three-day-old subjects: We observed the behavior of 3-day-old NK1^{-/-} and NK1^{+/+} mice to 0.5% or 2.0% formalin injected into the hindpaw. There were no differences in the responses of NK1^{+/+} and NK1^{-/-} mice at either concentration (Fig. 1a). We also measured fore- and hindlimb withdrawal latencies of 3-day-old NK1^{+/+} and NK1^{-/-} mice across four intensities of heat or mechanical stimuli. The NK1^{-/-} and NK1^{+/+} mice exhibited similar responses across all four intensities of the thermal stimuli, irrespective of the limb being tested (Fig. 2). For the mechanical stimuli, the 3-day-old subjects' forepaw responses did not differ between NK1^{+/+} and NK1^{-/-} mice across all four stimulus intensities (Fig. 3a). However, the hindpaws of the NK1^{-/-} mice were slightly less responsive to the second highest intensity than were NK1^{+/+} mice (Fig. 3b).

Twenty-one-day-old subjects: We rated the behavior of the 21-day-old mice after an injection of 2.0% formalin into

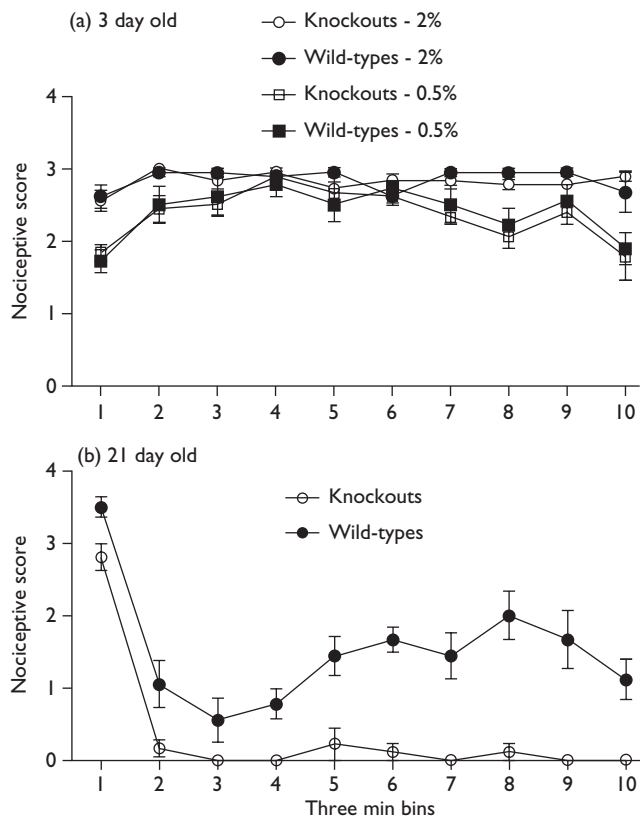


Fig. 1. Pain responses to subcutaneous injection of 5 μ l formalin into the plantar surface of the hindpaw. Behavior was rated according to a 5 point scale: 0 = no difference from uninjected paw; 1 = paw favored; 2 = paw lifted; 3 = paw shaken; 4 = paw licked. Data from the 30 min observation period were collapsed into ten 3 min bins. (a) Three-day-old responses to 0.5% and 2.0% formalin; NK1^{-/-} mice ($n=6$ per concentration) and NK1^{+/+} mice ($n=6$ per concentration) exhibited equivalent responses. (b) Twenty-one-day old responses to 2.0% formalin; both NK1^{+/+} ($n=6$) and NK1^{-/-} ($n=6$) mice responded during the first phase, however NK1^{-/-} mice failed to respond during the second phase of the response ($p < 0.05$). Data are mean \pm s.e.m. and were analyzed using an analysis of variance (ANOVA) with one between factor (mouse genotype) and one repeated factor (observation bin).

the hindpaw (Fig. 1b). During the first phase of the formalin test, both NK1^{+/+} and NK1^{-/-} mice responded robustly; however NK1^{-/-} mice did not respond at all during the second phase. We also measured limb withdrawal latencies of 21-day-old mice across four intensities of heat or mechanical stimuli. The 21-day NK1^{-/-} mice were less responsive to the higher intensities of heat (Fig. 4) and mechanical (Fig. 5) stimuli than were the NK1^{+/+} mice.

DISCUSSION

These data show that 3-day-old mice are capable of responding to noxious stimuli but that NK1 receptors do not play a significant role in nociceptive responding to thermal, mechanical or chemical stimuli at this age. This is the first direct evidence that the NK1 receptor is not involved in pain processing of a variety of stimuli in the neonate. They are consistent with rat studies showing that fetal and infant rats exhibit vigorous behavioral responses and c-fos expression after a for-

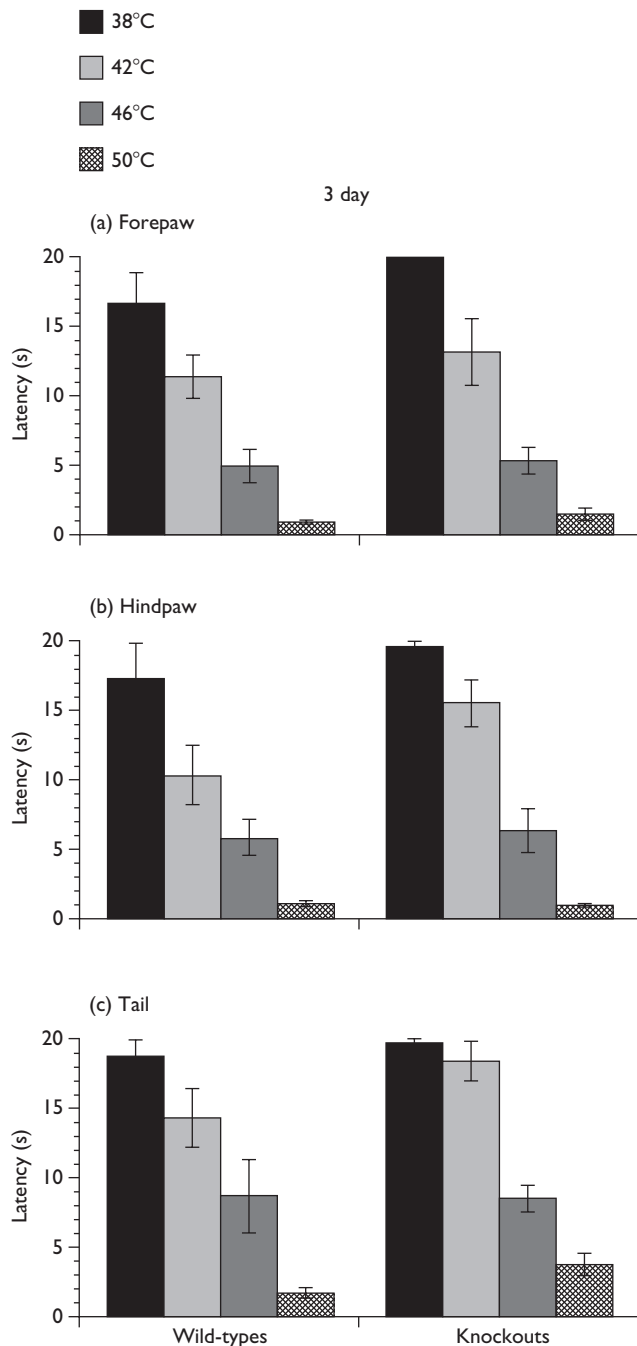


Fig. 2. Limb withdrawal latencies of 3-day-old subjects to each of 4 thermal stimulus intensities. Forepaw (a), hindpaw (b), and tail (c) withdrawal latencies were measured for each subject. $NK1^{-/-}$ ($n=6$) and $NK1^{+/+}$ ($n=6$) showed similar withdrawal latencies for all limbs across all heat intensities. Data are mean \pm s.e.m. Data for each limb were analyzed separately using an ANOVA with one between factor (mouse genotype) and one repeated factor (stimulus intensity).

malin injection [3–5,11], and that NK1 receptors do not play a role in 3-day-old subjects' responses to formalin [11]. These data also imply that substance P, normally active via NK1 receptors, is not involved in nociception in neonatal animals. It is unknown whether this is due to the functional immaturity of the presynaptic compo-

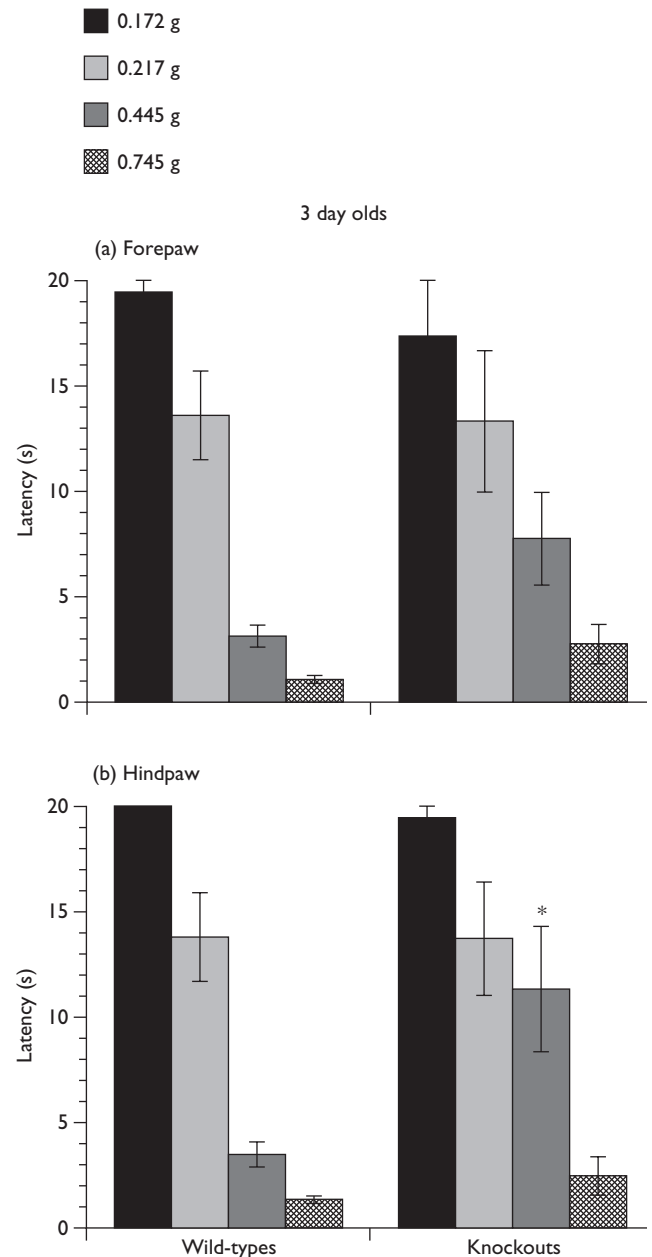


Fig. 3. Limb withdrawal latencies of 3-day-old subjects to four mechanical stimulus intensities. Forepaw (a) and hindpaw (b) withdrawal latencies were measured for each subject. $NK1^{-/-}$ ($n=6$) and $NK1^{+/+}$ ($n=6$) showed similar forepaw withdrawal latencies across all mechanical intensities. $NK1^{-/-}$ mice exhibited longer withdrawal latencies compared to $NK1^{+/+}$ mice only at the second highest intensity. Data are mean \pm s.e.m. Data for each limb were analyzed separately using an ANOVA with one between factor (mouse genotype) and one repeated factor (stimulus intensity). *Post-hoc* tests were conducted using the Tukey HSD test to determine differences between $NK1^{+/+}$ and $NK1^{-/-}$ mice at each intensity, *Significant differences, $p < 0.05$.

nent (i.e. substance P and C-fibers), the postsynaptic component (NK1 receptors or interneuron connections), or both.

In 21-day old subjects, NK1 receptors mediate the second (tonic) phase of the nociceptive response to formalin, associated with inflammatory pain [12], and

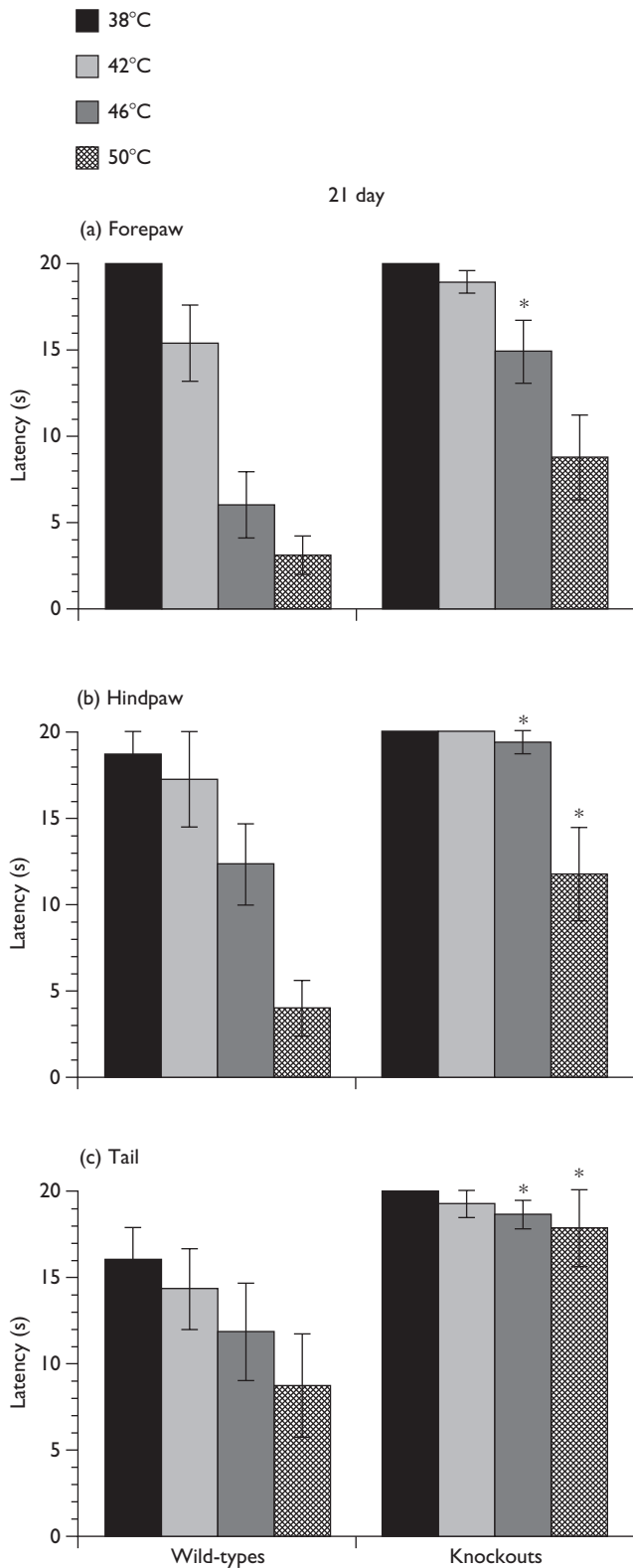


Fig. 4. Limb withdrawal latencies of 21-day-old subjects to four thermal stimulus intensities. Forepaw (a), hindpaw (b) and tail (c) withdrawal latencies were measured for each subject. $NK1^{-/-}$ mice ($n=6$) exhibited longer withdrawal latencies than $NK1^{+/+}$ mice ($n=6$). Further, increasing the intensity of the stimulus also increased the difference observed between $NK1^{+/+}$ and $NK1^{-/-}$ mice. Details are as in Fig. 3.

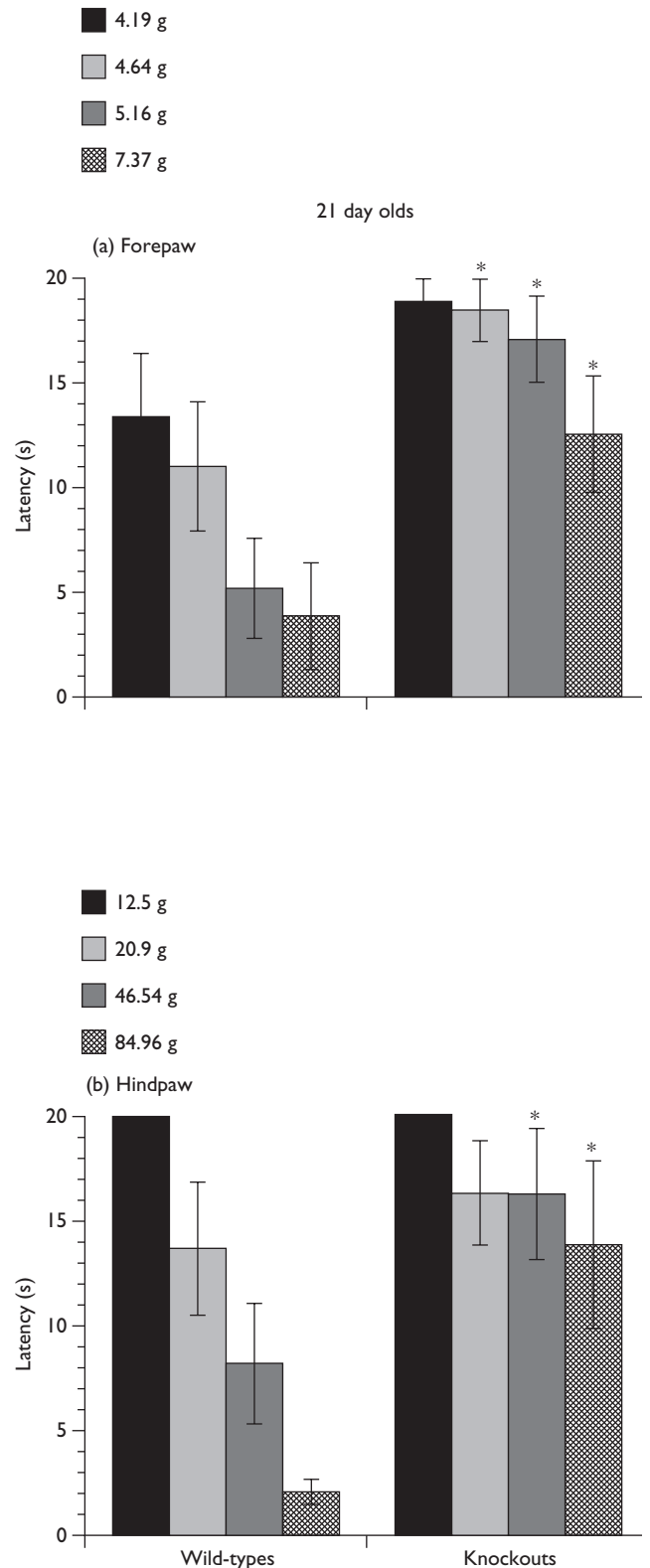


Fig. 5. Limb withdrawal latencies of 21-day-old subjects to four mechanical stimulus intensities. Forepaw (a) and hindpaw (b) withdrawal latencies were measured for each subject. $NK1^{-/-}$ mice ($n=6$) exhibited longer withdrawal latencies than $NK1^{+/+}$ mice ($n=6$). Further, increasing the intensity of the stimulus also increased the difference observed between $NK1^{+/+}$ and $NK1^{-/-}$ mice. Details are as in Fig. 3.

are involved in responses to high intensity thermal and mechanical stimuli. As in rats [3–5,11], 21-day-old mice exhibited different responses to the nociceptive stimuli than the 3-day-old mice. The older wildtype mice were less sensitive to mechanical stimuli, and had a biphasic response to the formalin injection. Furthermore, elimination of the NK1 receptor significantly reduced nociceptive responding to high intensity thermal and mechanical stimuli, and eliminated the second phase of the formalin response. These results indicate that the NK1 receptor plays a critical role in the inflammatory pain response in 21-day-old mice. This suggests that the development of this receptor increases the variety of nociceptive stimuli that elicit a response and broadens the range of responses available for reacting to noxious stimuli. It is unknown whether the differences in the nociceptive responses of NK1^{+/+} and NK1^{-/-} mice found at postnatal day 21 will change further in maturation. Others have shown reorganization of the primary afferent fibers during postnatal development that continues past 21 days after birth [13]. Recently, the role of NK1 receptors in baseline measures of nociception has been assessed in adult mice with single gene mutations that resulted in the elimination of the NK1 receptor [14]. In these mice, the second phase of the formalin response was attenuated, not eliminated, and baseline responses to thermal and mechanical stimuli were not altered. It is possible that the nociceptive responses observed in the adult are mediated by mechanisms, in addition to the NK1 receptor, not yet mature in 21-day-old mice. Maturation of other neural pathways in the nociceptive system may reduce, or eliminate the differences observed between the NK1^{-/-} and NK1^{+/+} mice 21 days after birth. Alternatively, the differences between our results, and those reported in the adult NK1^{-/-} mice, may be due to differences in testing procedures or genetic backgrounds [15,16]. Differences in baseline responses to heat and mechanical stimuli depend on the intensity of the stimulus. In adult rats, others have demonstrated that increasing the duration of the mechanical stimulus and the intensity of the thermal stimulus increased the number of NK1 receptors that were internalized [17]. Therefore, our findings that 21-day-old mice demonstrated attenuated responses to the highest intensities of the heat and thermal stimuli may be because

we measured responses across several intensities of heat and mechanical stimuli.

CONCLUSION

To our knowledge, this is one of the first studies to assess the effects of transgenic mutations on behavior across development, and to examine the impact of genetic mutations both before and after the system is functionally mature. Using this novel approach, we found that the NK1 receptor is not involved in pain responses to thermal, chemical, and mechanical stimuli in infant mice. This is the first direct evidence that NK1 receptors are not involved in these types of nociception in infants tested prior to the functional maturation of the C-fibers. In older animals, NK1 receptors mediate the second phase of the pain response to formalin, and are involved in nociceptive responses to both thermal and mechanical stimuli in an intensity dependent manner. This study, along with others discussed above [6,10,11], demonstrates that some of the mechanisms that mediate nociceptive responses of adult animals are not involved in the nociceptive responses of very young animals. Thus, the physiological processes underlying behavioral responses to painful stimuli change as the animal matures, allowing the animal to respond to a wider range of threatening stimuli with an increasing number of behavioral and physiological responses. It is unclear what physiological systems are involved in the neonatal nociceptive system.

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