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An Animal Model to Assess Aversion to Intra-oral Capsaicin: Increased Threshold in Mice Lacking Substance P

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Abstract

Despite the widespread consumption of products containing chemicals that irritate the oral mucosa, little is known about the underlying neural mechanisms nor is there a corresponding animal model of oral irritation. We have developed a rodent model to assess aversion to capsaicin in drinking water, using a paired preference paradigm. This method was used to test the hypothesis that the neuromodulator substance P (SP) plays a role in the detection of intra-oral capsaicin. 'Knockout' (KO) mice completely lacking SP and neurokinin A due to a disruption of the preprotachykinin A gene and a matched population of wild-type (WT) mice had free access to two drinking bottles, one containing water and the other capsaicin at various concentrations. Both KO and WT mice showed a concentration-dependent aversion to capsaicin. KO mice consumed significantly more capsaicin than WT at a single near threshold (1.65 μM) concentration, indicating that SP plays a limited role in the detection and rejection of oral irritants.

Introduction

Preference for spicy food is learned and initial exposure of naïve humans to capsaicin, the pungent principal of chili peppers, is nearly always aversive (Rozin and Schiller, 1980; Rozin *et al.*, 1982). Rats (Hilker *et al.*, 1967; Rozin *et al.*, 1979; Galef, 1989; Dib, 1990) and chimpanzees (Rozin and Kennel, 1983) avoid food containing capsaicin, although anecdotal reports suggest that a preference for capsaicin can develop with considerable socialization (Rozin and Kennel, 1983; Galef, 1989). The aversiveness of capsaicin is not surprising, as it elicits irritation or pain when delivered to oral mucosa (Stevenson and Prescott, 1994; Dessirier *et al.*, 1997; Rentmeister-Bryant and Green, 1997; Green and Rentmeister-Bryant, 1998) or skin (Green, 1998; Simone *et al.*, 1998; Magnusson and Koskinen, 2000). Capsaicin binds to the recently cloned 'capsaicin' (VR-1) receptor (Caterina *et al.*, 1997) to depolarize nociceptor endings, which in turn transmit signals to the spinal cord (Carstens, 1997) or trigeminal subnucleus caudalis (Vc) (Carstens *et al.*, 1998) to activate ascending nociceptive pathways.

One aim of this study was to develop an animal model of oral irritation, using a paired-preference paradigm to assess if rodents reject drinking water containing known concentrations of capsaicin. Previous studies used unspecified (Hilker *et al.*, 1967; Rozin *et al.*, 1979; Galef, 1989) or high (0.025%) (Dib, 1990) capsaicin concentrations. Several human studies of oral irritation elicited by aqueous capsaicin have recently appeared (Stevens and Lawless, 1987; Stevenson and Prescott, 1994; Rentmeister-Bryant and Green, 1997) and we wished to develop a comparable rodent model.

Our second aim was to address the role of the nociceptive neurotransmitter/neuromodulator substance P (SP) (Millan, 1999) in oral irritation. SP is localized to the central and peripheral terminals of nociceptors (Jessell *et al.*, 1978; Cao *et al.*, 1998; Zimmer *et al.*, 1998; Millan, 1999; Li *et al.*, 2000). Noxious stimuli evoke SP release in superficial layers of the dorsal horn or Vc (Duggan *et al.*, 1995), where it binds preferentially to neurokinin 1 (NK-1) receptors (Millan, 1999) localized on neurons (Brown *et al.*, 1995; Mantyh *et al.*, 1997; De Felipe *et al.*, 1998; Li *et al.*, 1998) receiving synaptic input from SP-immunoreactive axons (Li *et al.*, 2000). Spinal SP induces a prolonged excitation of nociceptive neurons (Urbán and Randic, 1984; Millan, 1999) and nocifensive behavioral responses (Frenk *et al.*, 1988).

Destruction of spinal lamina I NK-1 receptor-expressing neurons by SP conjugated to saporin resulted in a significant reduction in hyperalgesia and allodynia resulting from inflammation or nerve damage, with lesser effects on nocifensive behavior elicited by acute noxious stimuli (Mantyh *et al.*, 1997; Nichols *et al.*, 1999). Mice with disruption of the gene encoding the NK-1 receptor show an absence of wind-up as well as loss of graded responses to stimuli of increasing noxious intensity (De Felipe *et al.*,

1998). Preprotachykinin A 'knockout' (KO) mice lacking SP and neurokinin A were less sensitive than wild-type (WT) mice to intense levels of thermal, mechanical and chemical noxious stimuli (Cao *et al.*, 1998). These data indicate that SP plays an important role in chronic pain and intense levels of acute pain, with a lesser role in mild or moderate levels of acute pain.

In the present study we reasoned that oral irritation induced by capsaicin represents a persistent noxious stimulus that might induce the release of SP from the central terminals of oral nociceptors projecting to Vc. We therefore hypothesized that animals lacking SP should exhibit a reduction in behavioral sensitivity to capsaicin. We used the paired preference paradigm to assess differences between SP KO and WT mice in aversion to different concentrations of capsaicin. An abstract of this work has appeared (Simons *et al.*, 2000).

Materials and methods

Animals

C57BL/6 WT mice and KO mice, in which the preprotachykinin A gene was deleted, were a generous gift from A.I. Basbaum at the University of California–San Francisco; generation of the gene deletion is described elsewhere (Cao *et al.*, 1998). Nine WT and nine KO mice were used. The animals were housed one per cage in a room maintained on a 12 h/12 h light/dark cycle at an ambient temperature of ~21°C. Food and water were available *ad libitum*. The animals were acclimated to the testing conditions for 10 days prior to any experimental evaluation. All studies were approved and completed in accordance with the University of California–Davis Animal Use and Care Advisory Committee.

Chemicals

A 3.30 mM stock solution of capsaicin was prepared by dissolving crystalline capsaicin (Sigma) in 30% ethanol. Serial dilutions of the stock capsaicin produced working solutions of the following concentrations: 0.33, 0.83, 1.65, 3.30, 6.60, 16.50, 99.00 and 330.00 μM.

Behavioral assessment

Each capsaicin concentration was tested using the paired-preference paradigm. Two 50 ml plastic centrifuge tubes (Sarstedt) containing a stopper and sipper tube were suspended side-by-side at a height of 6 cm on the front of each cage by wire loops. One bottle contained capsaicin at a given concentration and the other contained distilled water to which ethanol was added to match the concentration found in the capsaicin solution. At the highest concentrations of capsaicin tested (99.0 and 330.0 μM) matching concentrations of ethanol (0.9 and 3%, respectively) were not added to the distilled water to avoid intoxication. The mice had *ad libitum* access to both bottles. Consumption volumes were measured on alternate days over a 10 day period by weighing the bottles; bottles were always weighed between 2 and 4 p.m. Fresh solutions were added to the bottles every second weighing or as needed. To negate a possible position preference, bottle positions were reversed at each weighing such that each solution was located on the left or right side of the cage an equal number of days. This paradigm was chosen over a deprivation/restricted access paradigm for several reasons. First, because the daily water consumption of a single mouse is of the order of ~7 ml, it is likely that too little water would be consumed during the restricted access period to be reliably measured. Second, we were interested in testing near-threshold levels of capsaicin. We wished to avoid the possibility that the mice consumed higher concentrations of capsaicin than they would accept normally because of an increased motivation to quench the thirst that might occur in a water restriction paradigm. Finally, water deprivation induces stress, which can lead to an analgesic state (Przewocki *et al.*, 1983; Konecka *et al.*, 1985), thus shifting aversion thresholds to higher concentrations.

Following the completion of a 10 day testing period, the bottles and stoppers were cleaned with water and ethanol. Between testing periods rats had free access to two bottles, both of which were filled with water. At least 3 days were allowed between sessions. Capsaicin solutions were presented in the following pseudo-ascending order: 0, 0.33, 3.30, 0.83, 1.65, 6.60, 16.50, 99.00 and 330.00 μM.

An ethanol control was performed to assess the degree to which the mice avoided solutions of 0.9 or 3% ethanol using the same paradigm as described above.

Data processing and analysis

Raw consumption weights for each bottle were converted to a percentage of total volume consumed. An 'aversion index', which measures the degree to which a solution was avoided, was calculated by subtracting the percentage of capsaicin consumed from the percentage of water consumed. The aversion index, when expressed as a decimal, ranges from –1 to 1. A score of –1 indicates that the capsaicin is completely preferred to the water, a score of 0 indicates that capsaicin and water are preferred equally and a score of 1 indicates complete rejection of the capsaicin solution (i.e. only water was consumed). Within-group differences between water and capsaicin consumption were assessed using paired *t*-tests. To determine whether within-group capsaicin consumption decreased with increasing concentration, two-way analyses of variance with *post hoc* LSD tests were performed. The occurrence of desensitization (or lack thereof) was evaluated at each capsaicin concentration using ANOVA. To compare the magnitude of the aversion index between WT and KO mice the goodness-of-fit crossover test was applied as derived in Andersen *et al.* (Andersen *et al.*, 1992) and employed by Romanovsky *et al.* (Romanovsky *et al.*, 1998). Briefly, the

number of times the KO curve crossed the WT curve was assessed and the probability of this occurrence was determined by summing the tails of a binomial distribution. *Post hoc* unpaired *t*-tests were then used to determine at which capsaicin concentrations the aversion index differed significantly between the WT and KO mice. All values are reported as means ± SE.

Results

Both WT and KO mice showed a concentration-dependent decrease in capsaicin consumption ($P < 0.001$ and $P < 0.001$, respectively). However, the WT mice began to consume significantly ($P < 0.01$) more water than capsaicin at 1.65 μM (Figure 1A), whereas the KO mice did not begin to consume significantly ($P < 0.001$) more water until the capsaicin was at a substantially higher concentration of 6.60 μM (Figure 1B). At higher capsaicin concentrations both groups rejected the capsaicin to approximately the same extent (Figure 1A,B). Indeed, both WT and KO mice consumed significantly ($P < 0.001$) more water than capsaicin at all concentrations >6.60 μM.

Figure 2 plots the aversion index as a function of capsaicin concentration. The goodness-of-fit crossover test reveals a significant difference between the WT and KO groups ($P < 0.05$). Specifically, as indicated by the *post hoc* *t*-tests, WT mice had significantly higher aversion index scores at the threshold (1.65 μM, $P < 0.01$) as well as at the highest capsaicin concentration tested (330 μM, $P < 0.05$).

Both WT and KO mice exhibited a significant preference for water over either the 0.9 ($P < 0.001$) or 3% ($P < 0.001$) ethanol solutions. Interestingly, however, the KO mice consumed significantly more of the 3% ethanol than the WT mice (9 versus 5% of total volume consumed respectively, $P < 0.05$). Thus, at the highest capsaicin concentration the presence of ethanol as the capsaicin solvent may have contributed to the aversiveness of the solution.

We were concerned that a build-up of desensitization to the irritant effect of capsaicin may have been a confounding factor. When consumption was assessed during the 10 day treatment block with 3.30 μM capsaicin it was observed that the KO mice consumed more of the capsaicin solution during days 6–10 as compared with days 1–5 (46 versus 33%, respectively, $P < 0.022$), suggesting that a limited amount of desensitization to capsaicin may have occurred. However, even during days 1–5 the KO mice consumed more capsaicin than did the WT mice (33 versus 30%), suggesting that the KO mice were less sensitive to the irritant qualities of this chemical. Moreover, no such increasing trend in capsaicin consumption across days was observed at any other capsaicin concentration, in either the WT or KO groups. Therefore, we believe that the difference between WT and KO mice at the threshold capsaicin concentration reflects a difference in sensitivity rather than a confounding influence of desensitization.

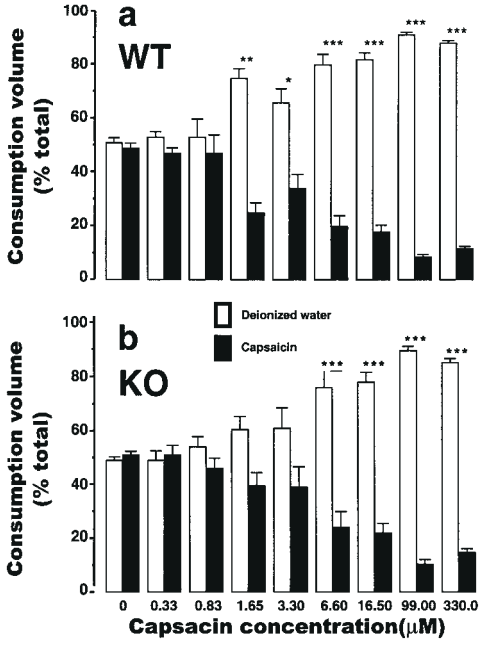

Figure 1 Concentration-dependent reduction in capsaicin consumption. **(A)** WT. The graph plots water (open bars) and capsaicin (filled bars) consumption, expressed as a percentage of total volume consumption during the 10 day exposure period at each capsaicin concentration. **(B)** KO. Graph format as in (A). Error bars, SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

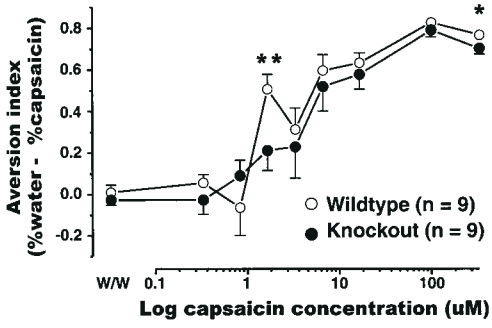

Figure 2 Concentration-related aversion to capsaicin in WT and KO mice. The graph plots 'aversion index' versus concentration of capsaicin. Error bars, SEM. *$P < 0.05$; **$P < 0.01$. W/W, dH_2O in both bottles.

We also examined the data to determine whether sensitization or a learning effect may have caused the animals to decrease their capsaicin consumption over time. At higher capsaicin concentrations (16.5, 99.0 and 330.0 μM) both WT and KO mice consumed significantly less capsaicin over days 6–10 as compared with days 1–5, with the decline in the WT mice more pronounced than in the KO mice. For instance, at 16.50 μM capsaicin consumption by WT mice decreased from 23% of the total volume during days 1–5 to 11% during days 6–10. Comparable values for the KO mice were 23% during days 1–5 and 17% during days 6–10. While a time-dependent reduction in consumption of high capsaicin concentrations might be explained by sensitization,

we believe that it is more readily explained by a learning effect in which the detection of specific cues enhanced discrimination between the two solutions (see Discussion).

Discussion

The present study employed a paired-preference paradigm to assess aversion to aqueous capsaicin by mice as a model of oral irritation. KO mice lacking the tachykinins SP and neurokinin A were less sensitive than WT mice at detecting and rejecting a threshold concentration of oral capsaicin. At supra-threshold capsaicin concentrations KO mice rejected capsaicin to nearly the same extent as WT mice except at the highest concentration, where KO mice consumed significantly more (Figure 2). However, the latter effect may reflect the small variance rather than a real sensory difference.

Methodological considerations

Although previous studies have confirmed the aversive nature of capsaicin (see Introduction), the method described here allows a more quantitative assessment of intra-oral detection of soluble irritant chemicals. The paired-preference paradigm appears to be a sensitive measure of the aversiveness of capsaicin over a broad range of concentrations and provides an estimate of the capsaicin aversion threshold in WT mice (1.65 μM), which is somewhat higher than the capsaicin detection threshold reported for humans (0.66 μM) (Szolcsànyi, 1990). Animals might actually detect even lower concentrations of capsaicin; the presently measured aversion threshold presumably reflects the concentration at which the innate aversiveness to capsaicin is overcome by positive hedonic factors associated with drinking. Above this threshold level the degree of rejection of capsaicin increased in a concentration-dependent manner, consistent with human studies showing that increasing concentrations of capsaicin or other irritants elicit higher intensity ratings for irritation (Lawless, 1984; Green and Rentmeister-Bryant, 1998). At the highest capsaicin concentrations tested the curve relating capsaicin aversion to concentration reached a plateau (Figure 2). This ceiling effect might be explained if the strategy by which animals sample the two solutions has attained its maximal efficiency.

Another advantage of the present method is that the animals remained in their home cages for the duration of the experiment and were not deprived of water. The data are thus less likely to have been influenced by stress-induced analgesia (Przewocki *et al.*, 1983; Konecka *et al.*, 1985; Allen *et al.*, 1986).

Repetitive exposure to capsaicin, as in the present study, may lead to a reduction in irritant sensations ('tachyphylaxis' or desensitization), as shown previously in human psychophysical studies (Lawless, 1984; Dessirier *et al.*, 1997; Green and Rentmeister-Bryant, 1998) and in electrophysiological studies of trigeminal ganglion neurons (Liu and Simon, 1996; Caterina *et al.*, 1997) and Vc neurons (Carstens *et al.*, 1998). We found very little evidence for a build-up of tachyphylaxis during exposure to capsaicin, since the mice did not exhibit a progressive increase over time in capsaicin consumption at any of the concentrations tested, except for one capsaicin concentration (3.30 μM) in the KO group. The opposite pattern (i.e. a progressive decrease in capsaicin consumption across days) might be observed if sensitization occurred (Green, 1998; Dessirier *et al.*, 1997) or if animals learned to identify sensory cues that aided their ability to distinguish between capsaicin and water. Such 'training' effects improve performance in human sensory discrimination tasks (Tedja *et al.*, 1994). Indeed, at the three highest capsaicin concentrations tested both WT and KO mice consumed less capsaicin during the last 5 days of the exposure period, even though the overall consumption for the 10 day test period was fairly low. While this pattern is consistent with sensitization, it is very unlikely that the mice received sufficient repetitive exposure to capsaicin to induce sensitization. Instead, we believe that the pattern of reduced capsaicin consumption over time is much more readily explained by a learning or training effect. Interestingly, the magnitude of this effect was larger in the WT than KO mice. One possible explanation is that if the KO mice were less sensitive to capsaicin they would consume more during the initial exposure period and thereby be more likely to become desensitized, further reducing their ability to detect capsaicin. However, as noted earlier, the KO mice did not exhibit a desensitizing pattern at most capsaicin concentrations. A second possibility is that the reduced training effect in the KO mice reflects a learning or memory deficit. SP has been implicated in learning [for a review see Huston and Hasenöhrl (Huston and Hasenöhrl, 1995)]. Because the paired-preference paradigm can be considered a spatial memory task in which the animal must remember the side with capsaicin, a learning deficit in the SP KO mice might contribute to poorer performance. While this may partly explain the poorer performance of the KO mice at high capsaicin concentrations, it does not as readily explain why the KO mice were less sensitive than WT mice at near-threshold capsaicin concentrations where no training effect was observed across days.

Such a learning effect may also partly explain the decreased aversion index between the 1.65 and 3.3 μM capsaicin concentrations observed for the WT mice (Figure 2). Both the WT and KO groups were first tested with 3.3 μM capsaicin, which was supra-threshold (Figure 2), followed by 0.83 μM (sub-threshold) and then 1.65 μM. The learning of discriminative cues from prior supra-threshold stimulation might aid the animals in detecting the near-threshold 1.68 μM concentration, an effect that was larger in the WT compared with KO animals.

Role of SP relative to other neurotransmitter systems in pain

As noted in the Introduction, SP is thought to play a role in pain and hyperalgesia. Animals are less sensitive to moderate or intense levels of acute noxious stimuli, or hyperalgesia, when the gene encoding SP (Cao *et al.*, 1998; Zimmer *et al.*, 1998) or its receptor (De Felipe *et al.*, 1998) are disrupted or when targeted neurotoxins destroy NK-1-expressing neurons in the superficial laminae of the dorsal horn (Mantyh *et al.*, 1997; Nichols *et al.*, 1999). Our present data, however, indicate that SP plays only a relatively minor role in the detection of threshold levels of oral irritation by capsaicin. We had reasoned that intra-oral capsaicin elicits a persistent irritant sensation that might be related to a prolonged action on trigeminal nociceptive pathways by SP released from the central terminals of oral nociceptors. The data suggest instead that oral irritation is a mild form of acute pain, rather than persisent pain, and thus should not be expected to be markedly reduced in the absence of SP.

The residual capacity of SP KO mice to detect capsaicin presumably reflects the normal operation of parallel nociceptive neurotransmitter systems and/or developmental changes that compensate for the absence of SP and neurokinin A. In addition to SP, both glutamate and calcitonin gene-related peptide (CGRP) have been implicated in nociception. Glutaminergic neurons have been identified in nociceptive systems at all levels (Millan, 1999) and glutamate is released over a wide range of noxious stimulus intensities (Marvizón *et al.*, 1997). Moreover, glutamate and SP are co-localized within the central terminals of afferent fibers thought to be nociceptive (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988). Similarly, CGRP, which co-localizes with SP (Samsam *et al.*, 2000), is a neuropeptide released peripherally in neurogenic inflammation (Kilo *et al.*, 1997; Kessler *et al.*, 1999; Kress *et al.*, 1999), as well as centrally (Millan, 1999). Since glutamate, CGRP and SP act cooperatively to excite central nociceptive neurons (Millan, 1999; Samsam *et al.*, 2000), the absence of SP would not be expected to result in a complete loss of sensitivity to noxious chemical stimuli. Indeed, some dorsal root ganglion neurons express VR-1 receptors (Caterina *et al.*, 1997) but not SP (Basbaum, 1999), suggesting that SP is not a requisite neurotransmitter/neuromodulator in capsaicin-sensitive nociceptors.

The binding of glutamate to AMPA receptors is the initial event that depolarizes nociceptive neurons. Co-release of SP activates NK-1 receptors mediating the more prolonged after-discharge (Urbán and Randic, 1984; Millan, 1999), a response that is lacking in SP KO mice (Martin *et al.*, 1999). That the KO mice exhibited a higher capsaicin aversion threshold suggests that the SP-induced slow after-discharge in central trigeminal nociceptive neurons may be an important neural signal to detect and reject oral irritants. At higher capsaicin concentrations the initial glutamate-evoked activation of central nociceptive neurons, and a possible contribution of CGRP, is apparently sufficient for the detection of capsaicin.

Altered peripheral responses may also contribute to the reduction in threshold capsaicin sensitivity observed in the KO mice. Ordinarily, neurogenic inflammation contributes to hyperalgesia via peripheral sensitization of nociceptors (Simone, 1992). However, in mice lacking SP the neurogenic inflammatory response is markedly reduced, with a significant reduction in hyperalgesia/allodynia (Cao *et al.*, 1998). However, it seems unlikely that such changes would affect the detection of threshold levels of capsaicin where sensitization does not appear to occur. Peripheral release of SP also causes hyperemia and plasma extravasation leading to local edema [for a review see Holzer (Holzer, 1998)]. Capsaicin-induced vascular permeability and edema were diminished in SP KO compared with WT mice (Cao *et al.,* 1998). However, we do not believe that these findings affect the present conclusions. Capsaicin-induced edema in the oral cavity of the WT mice would have the effect of reducing the concentration of capsaicin at the peripheral endings of intra-oral nociceptors, thereby rendering the capsaicin less aversive in WT compared with KO mice. The presence of edema should therefore reduce the aversion index of WTs at all supra-threshold capsaicin concentrations, thereby reducing any difference between the WT and KO groups.

Role of SP in taste

Even at concentrations below those shown to excite Vc neurons (Carstens *et al.*, 1998) ethanol is avoided (Petersen, 1983), presumably due to some aversive olfactory and/or taste component. In accordance, we found that mice consumed significantly more water than either 0.9 or 3% ethanol. However, the presence of ethanol did not contribute to the rejection of capsaicin since a matching concentration of ethanol was also added to the capsaicin-free water, with the exception of the two highest capsaicin concentrations (99.0 and 330.0 μM), to which ethanol was not added to avoid intoxication. We therefore cannot rule out the possibility that rejection of capsaicin at these highest concentrations may have been partly due to detection of ethanol.

Interestingly, in the vehicle control experiments there was a trend for the KO mice to consume more ethanol than the WT mice, implying that SP may be involved in gustatory and/or olfactory processing. SP and NK-1 immunoreactivity has been documented in the rostral aspect of the nucleus of the solitary tract (NST) (Davis and Kream, 1993; Maley, 1996), the first central taste relay, as well as the olfactory epithelium (Getchell *et al.*, 1989). Additionally, SP was reported to modulate NaCl-evoked responses of gustatory neurons in the NST (Davis and Smith, 1997) as well as to affect olfactory receptor cell function (Bouvet *et al.*, 1988). Further work is needed to delineate the exact role of SP in gustatory and olfactory chemoreception.

In conclusion, the two-bottle paired-preference paradigm appears to be a useful model to assess the aversiveness of oral capsaicin. SP appears to play a relatively modest role in detecting and avoiding threshold levels of capsaicin. This extends earlier results showing that SP is important in the detection of supra-threshold noxious stimuli (Mantyh *et al.*, 1997; Cao *et al.*, 1998; De Felipe *et al.*, 1998; Zimmer *et al.*, 1998; Nichols *et al.*, 1999). The observation that mice showed nearly normal aversion to higher concentrations of oral capsaicin indicates that other mechanisms (e.g. glutamate, CGRP) are in place to signal oral irritation.

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