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Opioid receptor desensitization contributes to thermal hyperalgesia in infant rats

Jing-Gen Liu[a], Cynthia R. Rovnaghi[a], Sarita Garg[a], K.J.S. Anand[a,b,*]

[a] *Pain Neurobiology Laboratory, Arkansas Children's Hospital Research Institute, 1120 Marshall Street, Little Rock, AR 72202, USA*
[b] *University of Arkansas for Medical Sciences, USA*

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Abstract

Central nociceptive processing includes spinal and supraspinal neurons, but the supraspinal mechanisms mediating changes in pain threshold remain unclear. We investigated the role of forebrain neurons in capsaicin-induced hyperalgesia. Long–Evans rat pups at 21 days were randomized to undisturbed control group, or to receive tactile stimulation, saline injection (0.9% w/v) or capsaicin injection (0.01% w/v) applied to each paw at hourly intervals. Thermal paw withdrawal latency was measured 1 h later, forebrains were removed and purified forebrain neuronal membranes were assayed for adenylyl cyclase activity and opioid receptor function. Capsaicin-injected rats had decreased thermal latency ($P<0.0001$) compared to the other groups. Neuronal membranes showed increased basal ($P=0.0003$) and forskolin-stimulated ($P=0.0002$) adenylyl cyclase activity in the capsaicin group compared to other groups. The selective μ-opioid receptor agonist, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO) was less effective in inhibiting adenylyl cyclase activity in the capsaicin group ($P<0.001$) compared to other groups. These effects were naloxone-reversible and pertussis toxin-sensitive ($P<0.01$) in the control, tactile stimulation and saline injection groups but not in the capsaicin group. Binding capacity and affinity for μ-opioid receptors were similar in all four groups, suggesting that receptor downregulation was not involved. Exposure to DAMGO increased [^{35}S]GTPγS binding to neuronal membranes from the control, tactile and saline groups ($P<0.001$) in a naloxone-reversible and pertussis toxin-sensitive manner ($P<0.01$) but not in the capsaicin group, suggesting μ-opioid receptor desensitization. Dose responses to systemic morphine were also reduced in the capsaicin group compared to the tactile group ($P<0.05$). Capsaicin-induced hyperalgesia in 21-day-old rats was associated with an uncoupling of μ-opioid receptors in the forebrain. Opioid receptor desensitization in the forebrain may reduce opioidergic inputs to the descending inhibitory controls, associated with behavioral hyperalgesia and reduced responsiveness to morphine analgesia in capsaicin-injected young rats.

Keywords: Pain; Development; Morphine; Analgesia; Uncoupling; Neuron

1. Introduction

Noxious inputs are transmitted via peripheral nociceptors to the spinal cord or trigeminal nuclei, and then carried by ascending tracts to the thalamus and somatosensory cortex. Supraspinal centers (located in the brainstem, thalamus and forebrain) serve an important function in processing nociceptive information (Porro et al., 1999; Wei et al., 2001). Despite the correlation of painful stimuli with spinal processing and broad assemblies of supraspinal neurons, the underlying mechanisms coupling these stimuli with behavioral responses are not completely understood. Nociceptive input is integrated and modulated at multiple levels in these neural pathways and conditions that alter the synaptic and cellular interactions at any of these sites will potentially lead to abnormalities in nociceptive processing, manifesting as hyperalgesia, allodynia or atypical referral of pain.

Hyperalgesia (enhanced nociceptive responses to noxious stimuli) and allodynia (nociceptive responses to non-noxious stimuli) are associated with a variety of pathological conditions (i.e., tissue injury, inflammation, nerve damage). Capsaicin injection leads to mechanical allodynia, primary and secondary hyperalgesia in humans

* Corresponding author. Arkansas Children's Hospital, S-431, 800 Marshall Street, Little Rock, AR 72202, USA. Tel.: +1-501-320-1008; fax: +1-501-320-3188.
E-mail address: anandsunny@exchange.uams.edu (K.J.S. Anand).

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and animal models (Liu et al., 1998; Sethna et al., 1998). These changes are mediated by spinal or supraspinal mechanisms because neurophysiological studies show no changes in the sensitivity of C-fibers supplying the area of capsaicin-induced hyperalgesia (LaMotte et al., 1992; Torebjork et al., 1992). In the spinal cord neurons, capsaicin-induced hyperalgesia was associated with increased neuronal excitability, changes in receptive field size and increased responses to previously subthreshold stimuli (LaMotte et al., 1992; Torebjork et al., 1992), with limited understanding of the supraspinal mechanisms contributing to these neurophysiological changes.

Endogenous opioid systems at these supraspinal sites modulate noxious peripheral inputs by activating the descending inhibitory pathways and significantly change the pain behaviors associated with injury or inflammation (Kovelowski et al., 1999; Matthies and Franklin, 1992; Porro et al., 1999). Marsh and colleagues showed that capsaicin-induced hyperalgesia occurs in infant rats and was not reversed by epidural opioids (Marsh et al., 1999a,b). This evidence further suggests a role for supraspinal mechanisms in mediating capsaicin-induced hyperalgesia.

Several studies have reported hyperalgesia in association with opioid tolerance and withdrawal (Liu and Anand, 2001). Hyperalgesic responses to noxious stimuli occurred in infant rats undergoing naloxone-precipitated opioid withdrawal (Bederson et al., 1990; Marsh et al., 1999a) or inflammatory pain (Marsh et al., 1999b). These lines of evidence further suggest a role for opioid receptors in the mechanisms leading to hyperalgesia. Hyperalgesia induced by capsaicin injection involves activation of adenylyl cyclase (Sluka and Willis, 1997). Modulation of adenylyl cyclase activity by opioids has an important role in the mechanisms of analgesia, hyperalgesia and tolerance (Liu and Anand, 2001).

We postulated that capsaicin-induced hyperalgesia in young rats may result from opioid-mediated changes in central inhibitory controls from the forebrain. We tested this hypothesis by studying forebrain opioid receptor function in 21-day-old rats that were either undisturbed or received tactile stimulation, or saline injection (0.9%), or capsaicin injection (0.01%) sequentially to each paw at hourly intervals.

2. Materials and methods

2.1. Animals and treatments

Protocols for these experiments were approved by the local Animal Care and Use Committee and animal care was provided under the supervision of a veterinarian. Timed-pregnant Long Evans rats were housed individually, with a 12-h light/dark cycle (7 a.m./7 p.m.) and had free access to food and water. Rat pups were randomly cross-fostered on the day after birth (P0) and distributed amongst nursing dams with a litter size of 10–12 pups. Groups of rats received four different treatments at 21 days of age (P21): (a) *control group* rats were left undisturbed in their home cage until sacrifice ($N=17$); (b) *tactile stimulation group* rats were removed from their home cage and received four strokes on each paw with a cotton swab ($N=15$); (c) *saline injection group* rats received the same physical handling, with saline (0.9% w/v, 5 μl volume) injected into the dorsum of each paw ($N=15$); and (d) *capsaicin injection group* rats received the same physical handling with capsaicin (0.01%, 5 μl volume) injected into the dorsum of each paw ($N=17$). Each stimulus in the tactile, saline and capsaicin groups was applied over 10–15 s; separate paws were stimulated at hourly intervals in a fixed sequence (right forepaw, right hindpaw, left forepaw and left hindpaw) for all groups. Bleeding from the needle puncture site was controlled in the saline and capsaicin groups, and all young rats were returned to their respective home cages in the intervals between the hourly stimuli.

2.2. Hot plate test for pain threshold

Observers who were blinded to the group assignment measured pain thresholds using an Omnitech Electronic Algesimeter (Columbus, OH) at 1 h after the last stimulus as described previously (Anand et al., 1999; Hu et al., 1997, #1000). This is the period when maximal behavioral changes occur in adult rats due to capsaicin-induced hyperalgesia (Gilchrist et al., 1996; Sluka and Willis, 1997). The apparatus consist of a 25×25-cm metal hot plate surface (set at 52 °C) with a foot-switch operated timer. The young rats used in this experiment were exposed to three training sessions performed prior to their group assignment. Pain thresholds were measured with three testing sessions performed at intervals of 5 min between each exposure; for testing, the right hindpaw was placed on the 52 °C hot plate and the left hindpaw was placed on a contiguous wooden surface at room temperature. The averaged latency to withdrawal from the hot plate was taken as a measure of pain threshold in these rats, as validated in previous studies (Anand et al., 1999; Hu et al., 1997).

2.3. Sacrifice procedures

Immediately after Hot Plate testing animals were sacrificed by placement in a CO_2 chamber and decapitation. Their brains were rapidly removed, frozen by immersion in isopentane/ethanol (1:1 mixture (v/v) maintained on dry ice) and then stored at −70 °C. The intervals between removal from the home cage and sacrifice were less than 3 min, in order to minimize any neurochemical effects of the sacrifice procedures.

2.4. Membrane preparations

Tissues were thawed and forebrains (excluding the olfactory lobe and pituitary gland) were separated from the midbrain and hindbrain; forebrains were isolated from 12 animals in each treatment group. For the opioid receptor binding assays ($N=6$ in each group), the forebrain of each animal was homogenized separately in 40 ml of 50 mM Tris–HCl buffer (Tris-[hydroxymethyl] aminomethane buffered with hydrochloric acid to pH 7.4) with a polytron (PowerGen 125, Fisher Scientific, St. Louis, MO). The homogenate was centrifuged for 30 min with 27,200 × *g* at 4 °C; the pellet was re-suspended in the same volume of buffer and re-centrifuged for another 30 min with 27,200 × *g* at 4 °C. The final pellet was re-suspended in a sufficient amount of Tris–HCl buffer (50 mM, pH 7.4) to give a protein concentration of 2–4 mg/ml and stored at − 70 °C.

For the [^{35}S]GTPγS binding and adenylyl cyclase activity assays ($N=6$ in each group), the forebrain of each animal was homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM ethylene glycol-bis(β-aminoethyl ether) tetra-acetic acid (EGTA) and 1 mM dithiothreitol. The homogenate was centrifuged for 20 min with 40,000 × *g* at 4 °C. The pellet was re-suspended in the same buffer and incubated for 30 min at 37 °C and re-centrifuged for 20 min with 40,000 × *g* at 4 °C. The final pellet was re-suspended in the same buffer to give a protein concentration of 0.2–0.4 mg/ml and stored at − 70 °C.

Protein concentrations from both types of membrane preparations were determined by Bradford's method of using bovine serum albumin as standard (Bradford, 1976). Aliquots from the re-suspended membrane fractions were used for assays detailed below.

2.5. Adenylyl cyclase activity assay

Membranes (10–20 μg) were incubated for 10 min at 30 °C with assay buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA, pH 7.4) containing 1 mM isobutyl-methylxanthine, 10 μm Forskolin, 10 μm GTP (guanosine-5′-triphosphate), 1 mM ATP (adenosine-5′ -triphosphate), 10 mM creatine phosphate, 10 IU of creatine phosphokinase, with various drug additions in a total volume of 250 μl. Enzyme blanks consisted of identical tubes in which the membranes had been immersed in boiling water for 2 min before addition of substrate. The enzymatic reaction was terminated by placing the samples in a 90–100 °C water bath for 3 min, then the tubes were cooled on ice for 10 min, followed by sedimentation at 3000 × *g* for 10 min. The supernatant was assayed for cAMP using enzymatic immunoassay kits (Amersham, Piscataway, NJ). For pertussis toxin treatment, membranes were pre-incubated with activated pertussis toxin for 1 h at 25 °C (pertussis toxin 100 ng/ml was activated with 1 mM dithiothreitol for 4 h at 25 °C), according to the method of Childers (1988).

2.6. Opioid receptor binding

Membranes were incubated with 50 mM Tris–HCl buffer (pH 7.4) at 22 °C for 30 min to remove endogenous opioid peptides. Membranes (300–500 μg/ml) in 50 mM Tris–HCl (pH 7.4) were incubated for 60 min at 22 °C in the presence of 50 μl of [^{3}H][D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO) at a final volume of 1 ml. Saturation assays were conducted using a variety of concentrations of [^{3}H]DAMGO from 0.1 to 10 nM. Nonspecific binding was determined in the presence of 10 μM morphine. Incubation was terminated by rapid filtration through GF/B glass fiber filter (Brandel, Gaithersburg, MD) under vacuum pressure using a cell membrane harvester (Brandel). Filters were washed three times with 3 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4), then dried at room temperature for 2 h and counted by liquid scintillation spectrophotometry (Beckman, Irvine, CA).

2.7. [^{35}S]GTPγS binding assay

For [^{35}S]GTPγS binding, forebrain membranes were prepared as described for adenylyl cyclase assays. Tubes contained 10–20 μg of protein, 30 μM GDP (guanosine-5′ -diphosphate) and 0.05 nM [^{35}S]GTPγS, all in 50 mM Tris–HCl buffer (pH 7.4) containing 3 mM $MgCl_2$, 1 mM EGTA and 100 mM NaCl in a final volume of 1 ml. Tubes were incubated for 1 h at 30 °C with or without drugs added to the above mixture. Pertussis toxin (100 ng/ml) was activated with 1 mM dithiothreitol for 4 h at 25 °C, before using it for pretreatment of forebrain membranes for 1 h at 25 °C. Basal binding was determined in the absence of agonist and nonspecific binding was determined in the presence of 10 μM nonradioactive GTPγS. The incubation was terminated by filtration under vacuum through GF/B glass fiber filter (Brandel), followed by three washes with 3 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry. Data were averaged from duplicate tubes and the percentage of stimulated [^{35}S]GTPγS binding was calculated by the formula:

$$100 \times (\mathrm{cpm}_{\mathrm{sample}} - \mathrm{cpm}_{\mathrm{nonspecific}})/(\mathrm{cpm}_{\mathrm{basal}} - \mathrm{cpm}_{\mathrm{nonspecific}})$$

2.8. Responsiveness to morphine analgesia

To confirm these receptor studies, additional P21 rats were randomized to the capsaicin injection group ($N=9$) and the tactile stimulation group ($N=9$) to measure their responses to morphine analgesia. The tactile group was selected as controls because they underwent similar physical handling as the capsaicin group but were not exposed to any noxious stimulation. Paw withdrawal latency to hot plate at 52 °C was determined as noted above and then was repeated after cumulative doses of morphine (1 and 2 mg/kg injected

subcutaneously in the interscapular area). Effects of morphine analgesia were calculated as a percentage of the maximal possible effect at baseline (%MPE) using the following equation:

$$\%\text{MPE} = (\text{Test reaction time} - \text{baseline latency}/15\ \text{sec} - \text{baseline latency}) \times 100$$

2.9. Statistical analyses

Cellular data are reported as mean ± standard deviation or mean and 95% confidence intervals from assays in four to seven separate experiments, each assay was performed in duplicate. If significant differences between groups were found by repeated-measures analysis of variance (ANOVA), these analyses were followed by the Tukey–Kramer post-hoc tests ($P<0.05$) to look for specific differences between groups. Data from [^{3}H]DAMGO and [^{35}S]GTPγS binding were analyzed by nonlinear regression using a curve fitting program in GraphPad Prism (GraphPad Software®, San Diego, CA, USA). Depending on the presence or absence of a normal distribution, behavioral data were analyzed either by parametric ANOVA (followed by post-hoc Tukey–Kramer tests) or Kruskal–Wallis ANOVA (followed by post-hoc Dunn's test).

3. Results

3.1. Capsaicin injections induce hyperalgesia in young rats

Hyperalgesia was noted by significant decreases in the hot plate paw withdrawal latency for capsaicin-injected P21 rats (Kruskal–Wallis ANOVA $P=0.0001$; Fig. 1) as compared to the control group ($P<0.001$), tactile stimulation group ($P<0.001$) and the saline injection group ($P<0.01$). Paw withdrawal latencies in these animals (Long–Evans hooded rats) were lower than those reported previously in Sprague–Dawley rats (Anand et al., 1999; Hu et al., 1997), confirming similar data from adult rats.

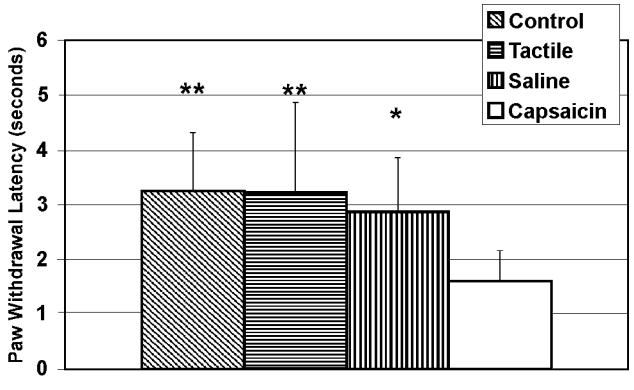

Fig. 1. Hot plate latencies following repetitive stimulation in P21 rats. Observers blinded to group assignment for P21 rats measured paw withdrawal latencies at 1 h post-stimulation using a hot plate set at 52C. Data represents the hot plate latencies as mean ± standard deviation control ($n=17$), tactile ($n=15$), saline ($n=15$), capsaicin ($n=17$). Kruskal–Wallis ANOVA $P=0.0001$, post-hoc Dunn's test: control vs. capsaicin $P<0.001$, tactile vs. capsaicin $P<0.001$, saline vs. capsaicin $P<0.01$.

Table 1
Adenylyl cyclase activity in forebrain membrane preparations from infant rats

	Adenylyl cyclase activity	
	Basal	Stimulated
	pmol/min/mg protein	
Control	36 ± 4.7[a]	268 ± 24
Tactile	29 ± 5.2[a,b]	287 ± 32
Saline	41 ± 7.8[b]	275 ± 33
Capsaicin	51 ± 6.6[a]	376 ± 36[c]

Membranes were incubated for 10 min at 30 °C in the absence (basal) or presence (stimulated) of 10 μM forskolin. Values listed are mean ± S.D. of five separate experiments performed in duplicate.
Basal AC activity: ANOVA: $P=0.0003$, Tukey–Kramer post-tests show significant differences between the capsaicin group and control ($P<0.01$)[a] and tactile ($P<0.001$)[a] groups, and between the tactile and saline groups ($P<0.05$)[b].
Forskolin-stimulated AC activity: ANOVA: $P=0.0002$, Tukey–Kramer post-tests shows significant differences of the capsaicin group[c] from the control ($P<0.001$), tactile ($P<0.01$) and saline ($P<0.001$) groups.

3.2. Forebrain adenylyl cyclase activity increases with hyperalgesia

To determine if adenylyl cyclase activity is implicated in capsaicin-induced hyperalgesia in young rats, we assayed basal and forskolin-stimulated cAMP production in the forebrain neuronal membranes from all animals. Basal (ANOVA: $P=0.0003$) and forskolin-stimulated (ANOVA: $P=0.0002$) adenylyl cyclase activity was significantly increased in the capsaicin group (Table 1) as compared to the other groups ($P<0.01$ to $P<0.001$), whereas a significant difference was also noted in the basal adenylyl cyclase

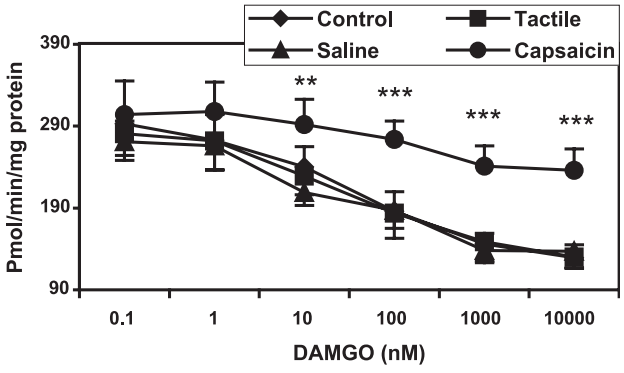

Fig. 2. Effects of DAMGO on forskolin-stimulated adenylyl cyclase activity in forebrain membranes. Membranes were incubated with various concentrations of DAMGO (0.1–10,000 nM) in the presence of 10 μM forskolin for 10 min at 30 °C. (Mean ± standard deviation from four to six separate experiments performed in duplicate. **$P<0.001$, ***$P<0.0001$).

Table 2
Inhibition of adenylyl cyclase activity by DAMGO in forebrain membrane preparations

Groups	Adenylyl cyclase	
	IC_{50}	cAMP (pmol/min/mg protein)
Control	36.2 (19.9, 65.9)	129 ± 16
Tactile	32.7 (9.8, 108.9)	130 ± 13
Saline	20.6 (0.25, 170.4)	137 ± 21
Capsaicin	118.2 (14.2, 980.5)[a]	236 ± 2 6[a]

Values for IC_{50} (nM) are listed as mean (95% confidence intervals) and for cAMP production at maximal concentrations of DAMGO (10,000 nM) as mean ± S.D. from five to seven experiments performed in duplicate.

[a] ANOVA $P<0.0001$, Tukey–Kramer multiple comparison post-tests show significant differences between the capsaicin group and all other groups ($P<0.001$).

activity between the tactile stimulation and saline-injected groups ($P<0.05$).

3.3. Diminished opioid inhibition of adenylyl cyclase activity in capsaicin group

To investigate whether the adenylyl cyclase activity in forebrain membranes is modulated by μ-opioid receptor agonists, the effect of DAMGO (0.1–10,000 nM) on forskolin-stimulated adenylyl cyclase activity was assayed. DAMGO dose-dependently inhibited adenylyl cyclase activity in the control, tactile or saline-injected groups, but inhibition of adenylyl cyclase activity was significantly reduced in the capsaicin-injected rats (ANOVA: $P<0.0001$) as compared to all other groups ($P<0.001$; see Fig. 2 and Table 2).

In a subsequent experiment, forebrain membranes were incubated with the maximum concentration of DAMGO (10,000 nM) in the presence of increasing concentrations of naloxone (0.1–10,000 nM). Naloxone dose-dependently blocked the DAMGO inhibition of forskolin-stimulated

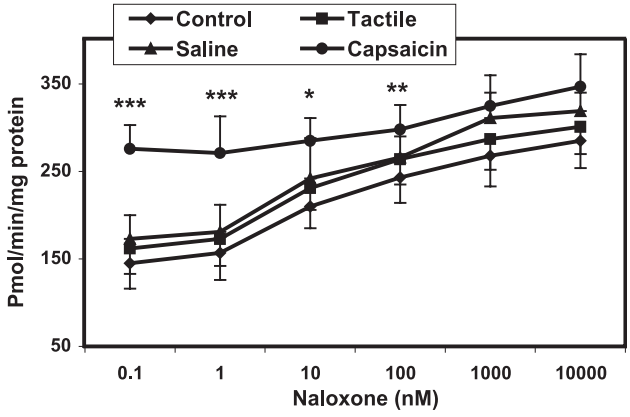

Fig. 3. Influence of naloxone on DAMGO inhibition of forskolin-stimulated adenylyl cyclase activity in forebrain membrane preparations. Membranes were incubated with DAMGO (10,000 nM) in the presence of various concentrations of naloxone (0.1–10,000 nM) for 10 min at 30 °C. (Mean ± standard deviation from four to six separate experiments performed in duplicate. *$P<0.01$, **$P<0.001$, ***$P<0.0001$).

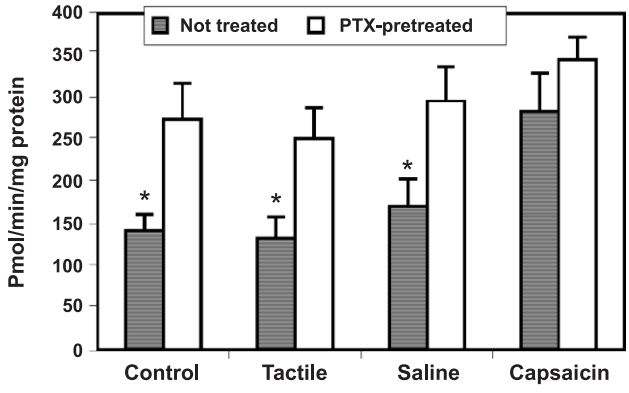

Fig. 4. Influence of pertussis toxin-pretreatment on DAMGO inhibition of forskolin-stimulated adenylyl cyclase activity in forebrain membranes. Membranes were incubated with DAMGO (10,000 nM) with (light bars) or without (dark bars) pertussis toxin-pretreatment (100 ng/ml medium) for 1 h at 30 °C. Results are expressed as the mean ± standard deviation of three separate experiments, each performed in duplicate. *$P<0.01$ (unpaired *t*-test) between nontreated vs. pertussis toxin-pretreated membranes.

adenylyl cyclase activity, with an IC_{50} (concentration producing 50% inhibition) of 45.1, 46.7 and 66.9 nM in the control, tactile and saline-injected groups, respectively. In the capsaicin-injected young rats, naloxone showed no dose-dependent effects (IC_{50} of >1000 nM) on the DAMGO inhibition of adenylyl cyclase activity (Fig. 3). These results suggest that the DAMGO inhibition of adenylyl cyclase activity was specifically mediated via μ-opioid receptors located on forebrain neuronal membranes.

Opioid receptors inhibit cAMP production through the activation of pertussis toxin-sensitive inhibitory G-proteins. To confirm that forskolin-stimulated adenylyl cyclase activity was inhibited via G protein-coupled opioid receptors, forebrain membranes were pre-incubated with activated pertussis toxin (100 ng/ml), after which DAMGO (10,000 nM) inhibition of forskolin-stimulated cAMP production was assayed. Pre-incubation with pertussis toxin significantly attenuated the DAMGO inhibition of adenylyl cyclase activity in membranes prepared from control, tactile and saline-injected rats ($P<0.01$, unpaired t test), but pertussis toxin pretreat-

Table 3
Binding of [^{3}H]DAMGO to forebrain membranes in infant rats

	B_{max} (fmol/mg protein)	K_d (nM)
Control	127.5 ± 8.8	0.99 ± 0.17
Tactile	116.8 ± 6.5[a]	1.08 ± 0.27
Saline	137.8 ± 15.7[a]	0.84 ± 0.33
Capsaicin	132.7 ± 6.5	1.14 ± 0.25

Membranes were incubated with eight concentrations of radiolabeled ligand varying from 0.1 to 10 nM using 10 μM morphine to measure nonspecific binding. Values represent mean ± S.D. of six separate experiments performed in duplicate.

B_{max} data: ANOVA $P=0.0118$, Tukey–Kramer multiple comparison post-tests show a significant difference only between the tactile and saline ($P<0.01$)[a] groups. K_d data: ANOVA $P=0.2462$.

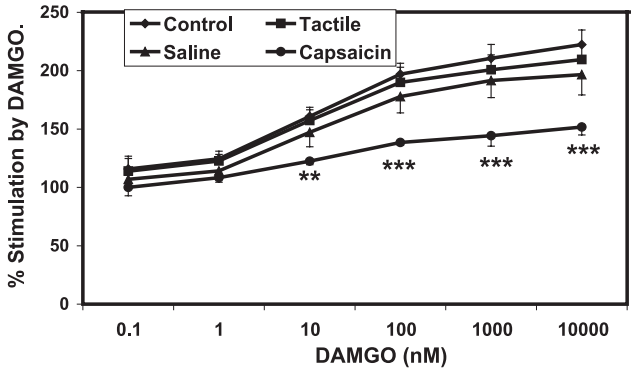

Fig. 5. Effect of DAMGO on [^{35}S]GTPγS binding in forebrain membranes. Membranes were incubated with 0.05 nM [^{35}S]GTPγS in the presence of increasing concentrations of DAMGO (0.1–10,000 nM) for 1 h at 30 °C. Data are expressed as percentage of stimulation above basal [^{35}S]GTPγS-binding in the absence of DAMGO. (Mean ± standard deviation of six independent experiments performed in duplicate. **$P<0.001$, ***$P<0.0001$).

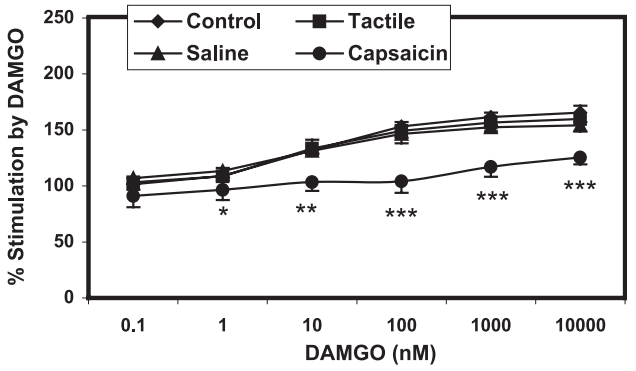

Fig. 6. Influence of naloxone on DAMGO-stimulated [^{35}S]GTPγS binding in forebrain membranes. Membranes were incubated with 0.05 nM [^{35}S]GTPγS and naloxone (1000 nM) in the presence increasing concentrations of DAMGO (0.1–10,000 nM) for 1 h at 30°C. Data are expressed as percentage of stimulation above basal [^{35}S]GTPγS-binding in the absence of DAMGO. (Mean ± standard deviation of six independent experiments performed in duplicate. *$P<0.01$, **$P<0.001$, ***$P<0.0001$).

ment showed no effects in the capsaicin group (Fig. 4). These results indicate impaired opioid receptor function in the capsaicin-injected rats, associated with thermal hyperalgesia.

3.4. No change in μ-opioid receptor binding properties

We hypothesized that receptor downregulation or changes in binding affinity may be associated with decreased opioid receptor function. Using [^{3}H]DAMGO binding to investigate opioid receptor binding capacity and binding affinity in forebrain membranes, we found no significant differences between the capsaicin rats and other groups (Table 3). Small differences in receptor binding capacity occurred between the tactile and saline groups ($P<0.01$) but their biological importance is unclear.

Table 4
Stimulation of [^{35}S]GTPγS binding by DAMGO in forebrain membrane preparations

	[^{35}S]GTPγS binding		[^{35}S]GTPγS binding with naloxone	
	EC_{50}	Maximal stimulation	EC_{50}	Maximal stimulation
Control	10.2 (4.1, 25.2)	222.4 ± 12.5	11.3 (6.8, 18.5)	165.6 ± 6.2
Tactile	13.2 (3.9, 45.0)	209.5 ± 12.8	11.4 (4.4, 29.5)	159.9 ± 2.7
Saline	31.9 (6.2, 163.2)	196.7 ± 17.4	13.2 (3.0, 58.6)	154.1 ± 5.3
Capsaicin	>1000	151.8 ± 6.9[a]	>1000	124.6 ± 6.2[a]

Values for EC_{50} are listed as mean (95% confidence intervals) and for maximal effect (at 10,000 nM DAMGO) as mean ± S.D. from six experiments performed in duplicate.

[a] ANOVA P <0.0001, Tukey–Kramer multiple comparison post-tests show significant differences between the capsaicin group and all other groups (P < 0.001).

3.5. Uncoupling of opioid receptors from G-proteins

Uncoupling of opioid receptors from the underlying G-proteins leads to dis-inhibition of adenylyl cyclase activity, thus contributing to the mechanisms of hyperalgesia and opioid tolerance (Liu and Anand, 2001). DAMGO-stimulated binding of [^{35}S]GTPγS is a standard method for measuring the μ-opioid receptor activation of G-protein. Increasing concentrations of DAMGO (0.1–10,000 nM) stimulated the binding of [^{35}S]GTPγS (0.05 nM) to membranes from the control, tactile, or saline-injected rats, whereas DAMGO-stimulated binding of [^{35}S]GTPγS was significantly reduced in forebrain membranes from the capsaicin-injected rats (ANOVA: $P<0.0001$) (Fig. 5 and Table 4).

To test whether the DAMGO-stimulated [^{35}S]GTPγS binding was a specific μ-opioid receptor effect, we incubated the membranes with different concentrations of

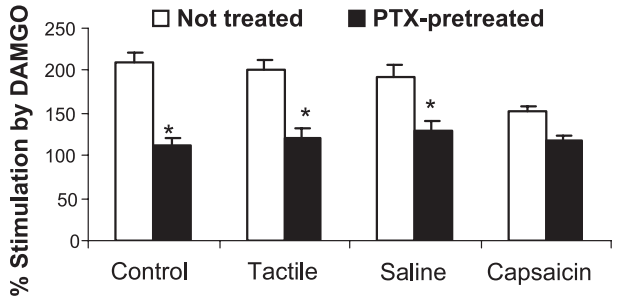

Fig. 7. Influence of pertussis toxin-pretreatment on DAMGO-stimulated [^{35}S]GTPgS binding in forebrain membranes. Membranes were incubated with DAMGO (10,000 nM) with (light bars) or without (dark bars) pertussis toxin-pretreatment (100 ng/ml medium) for 1 h at 30 °C. Data are expressed as percentage of stimulation above basal [^{35}S]GTPgS binding. (Mean ± standard deviation of three separate experiments performed in duplicate. *$P<0.01$ unpaired t-test between nontreated vs. pertussis toxin-pretreated membranes).

DAMGO (0.1–10,000 nM) in the presence of naloxone (1000 nM). Naloxone reduced the DAMGO-mediated maximal stimulation of [^{35}S]GTPγS binding in all groups, but the maximal stimulation of [^{35}S]GTPγS in the capsaicin group was significantly lower than the three other groups (ANOVA: $P<0.0001$, post-hoc Tukey tests: $P<0.001$) (see Fig. 6 and Table 4). Pretreatment with pertussis toxin (100 ng/ml) substantially reduced the DAMGO (10,000 nM) stimulated binding of [^{35}S]GTPγS S in forebrain membranes from the control, tactile and saline-injected rats ($P<0.01$), with no effects in the capsaicin group (Fig. 7).

3.6. Opioid receptor uncoupling reduces the efficacy of morphine analgesia

The behavioral effects of opioid receptor uncoupling were confirmed by measuring dose–response curves to systemic morphine analgesia in P21 rats exposed to capsaicin injection or tactile stimulation. No differences occurred between the two groups at baseline ($P=0.0903$, unpaired t-test), with significant differences in hot plate latency following morphine doses of 1 mg/kg ($P=0.0286$) and 2 mg/kg ($P=0.0218$, unpaired t-test). Morphine dose–response curves were presented as a percentage of the maximum possible effect (%MPE) to control for baseline differences. Differences in the %MPE were significant between the tactile and capsaicin groups following morphine doses of 1 mg/kg ($P=0.0165$) and 2 mg/kg ($P=0.0353$, unpaired t-test with Welch correction) (Fig. 8).

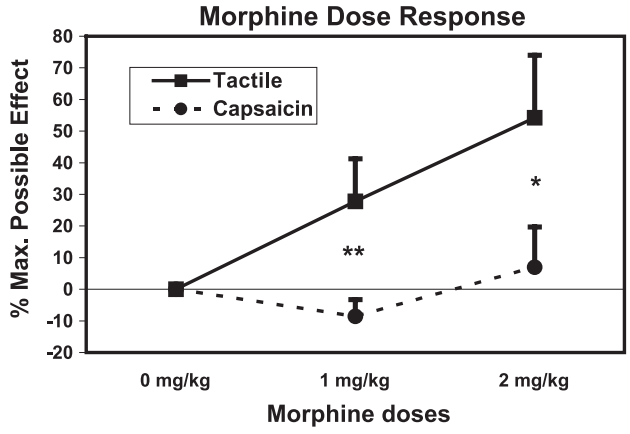

Fig. 8. Hot plate latencies following morphine analgesia in P21 rats from the tactile ($N=9$) and capsaicin ($N=9$) groups. Observers blinded to group assignment measured paw withdrawal latencies at baseline following subcutaneous injection of 1 and 2 mg/kg of morphine sulfate (mean ± standard deviation). Results are presented as a percentage of the maximum possible effect (%MPE) compared between the capsaicin and tactile stimulation groups (unpaired t-test, with Welch correction). Infant rats in the tactile group responded to morphine analgesia with significant differences from the capsaicin group at morphine doses of 1 mg/kg ($P=0.0165$) and 2 mg/kg ($P=0.0353$).

4. Discussion

Nociceptive inputs are integrated and modulated at multiple levels in the pain system, with a dynamic equilibrium maintained between supraspinal centers and the spinal cord. Most previous studies on hyperalgesia were focused on peripheral nociceptors, primary afferent neurons or dorsal horn spinal neurons, because of limited evidence for the putative role of supraspinal centers (Suh et al., 1995; Urban and Gebhart, 1999). There is considerable evidence that diffuse noxious inhibitory controls from supraspinal centers effectively modulate the sensation of pain by releasing monoamine (serotonin, norepinephrine, dopamine) and opioid neurotransmitters in the spinal cord (Aimone and Gebhart, 1987; Ossipov and Gebhart, 1986). The ventrolateral orbital cortex, anterior cingulate cortex and other cortical areas involved in pain modulation provide higher order processing within an endogenous analgesic system linked via the thalamic nucleus submedius and periaqueductal gray to the brainstem and spinal cord (Calejesan et al., 2000; Donahue et al., 2001; Huang et al., 2001). Opioidergic inputs from these forebrain areas indirectly activate OFF cells in the rostral ventromedial medulla (Hirakawa et al., 2000), or activate the locus coeruleus/subcoeruleus or nucleus raphe magnus (Wei et al., 1999), to produce tonic inhibition of nociceptive impulses in the dorsal horn.

Opioid receptors are distributed widely throughout the brain, developing most densely in the brainstem, thalamus, amygdala and cortex (Jensen, 1997; Rahman et al., 1998; Thornton et al., 1998). Opioid receptors are abundantly expressed in the supraspinal sites found close to the origin of diffuse noxious inhibitory controls (Matthies and Franklin, 1992; Ossipov et al., 1995) and current evidence supports their active role in pain processing and modulation (Budai and Fields, 1998; Budai et al., 1998; Calejesan et al., 2000; Hirakawa et al., 2000; Porro et al., 1999). Given the role of endogenous opioid systems in mediating descending inhibition and the involvement of supraspinal mechanisms in capsaicin-induced hyperalgesia, we tested the hypothesis that capsaicin-induced hyperalgesia in rats may be mediated by changes in central opioidergic control from the forebrain. We chose to study 21-day-old rats because descending inhibitory controls only innervate the cervical cord at P10–P12, but are completely developed and functional in P21 rats (Boucher et al., 1998; Ren et al., 1997). Moreover, nociceptive mechanisms in P21 rats have been the focus of intense research (Collins et al., 1998; Guy and Abbott, 1992; McDougall et al., 1997; McDougall et al., 1999) because of their importance for adult pain processing.

The present study demonstrated that capsaicin injections in each paw were associated with hyperalgesia and significant increases in adenylyl cyclase activity in the forebrain membranes, indicating upregulation of the cAMP pathway in the capsaicin-injected young rats. Importantly, this study revealed that the increased adenylyl cyclase activity follow-

ing capsaicin injection was associated with desensitization of forebrain μ-opioid receptors. DAMGO, a selective μ-opioid receptor agonist, had significantly reduced effects on the inhibition of forskolin-stimulated cAMP production and the stimulation of [^{35}S]GTPγS binding in forebrain membranes prepared from capsaicin-injected young rats. Following capsaicin injection, desensitization of μ-opioid receptors in the forebrain is mediated by uncoupling from pertussis toxin-sensitive G proteins, with no changes in the μ-opioid receptor binding capacity or affinity. These results suggest that μ-opioid receptor desensitization in the forebrain is associated with capsaicin-induced hyperalgesia, resulting from activation of the cAMP signal transduction pathway.

Several lines of evidence show that activation of the cAMP messenger pathway is significantly implicated in the development of hyperalgesia. For example, Sluka (1997) demonstrated previously that activation of the cAMP transduction cascade contributes to hyperalgesia and allodynia induced by intradermal injection of capsaicin (Sluka, 1997). Peripheral injection of forskolin or analogues of cAMP also lead to mechanical hyperalgesia (Taiwo and Levine, 1991), whereas inhibitors of protein kinase A (PKA, a cAMP-dependent protein kinase) reduce capsaicin-induced mechanical hyperalgesia (Ouseph et al., 1995; Sluka, 1997; Taiwo et al., 1992). Our results are consistent with these studies, further supporting that hyperalgesia mediated by activation of the cAMP signal transduction cascade occurs in young rats. More importantly, we have found that capsaicin-induced hyperalgesia is associated with activation of the cAMP messenger pathway in the forebrain, resulting from a desensitization of forebrain μ-opioid receptors. Recent findings from Zhuo and colleagues show that activation of adenylyl cyclases highly expressed in the forebrain (e.g. anterior cingulate cortex) contribute to the behavioral hyperalgesia and allodynia caused by inflammatory pain (Donahue et al., 2001; Wei et al., 2002).

Interactions between NMDA and opioid receptors play a significant role in the development of opioid tolerance and hyperalgesia (Liu and Anand, 2001; Lutfy et al., 1996). For example, in neuronal cell cultures, NMDA-activation attenuates the opioid inhibition of cAMP production, associated with uncoupling of these opioid receptors from pertussis toxin-sensitive G-proteins (Cai et al., 1997; Fan et al., 1998). Dizocilpine maleate (MK801), an NMDA receptor antagonist, potently prevented the development of chronic morphine tolerance and thermal hyperalgesia in rats (Fan et al., 1998), indicating that NMDA receptor activation is essential for mediating the thermal hyperalgesia associated with morphine tolerance and withdrawal. Conversely, morphine given systemically also produced its analgesic effect by recruiting NMDA-mediated excitatory processes to activate the OFF cells within the rostral ventromedial medulla (Heinricher et al., 2001; Spinella et al., 1996).

Perhaps the desensitization of μ-opioid receptors, as shown in this study, was mediated via capsaicin-induced activation of NMDA receptors. NMDA receptor activation by capsaicin has been reported from primary afferent neurons and spinothalamic tract neurons and treatment with NMDA receptor antagonists prevents the capsaicin-induced sensitization of these neurons (Sakurada et al., 1998). On the other hand, opioids have inhibitory effects on NMDA receptor function (Ebert et al., 1998; Ma et al., 1998); therefore, a loss of the inhibitory modulation by opioids would result in increased NMDA receptor activity leading to windup and central sensitization in the spinal cord, mechanisms that are fundamental to the development of hyperalgesia.

Although we propose that opioid receptor uncoupling in the forebrain contributes to capsaicin-induced hyperalgesia, the converse is also possible, such that spinal hyperalgesia leads to an uncoupling of forebrain opioid receptors. The context in which pain occurs is likely to be transduced by the forebrain and may initiate these changes in pain threshold (Mitchell et al., 2000). A primary role for the forebrain in initiating these changes would be more consistent with the known effects of contextual factors on pain responsiveness in children (Fearon et al., 1996; Sweet et al., 1999). The uncoupling of forebrain opioid receptors may reduce inhibitory inputs to the brainstem and spinal cord, thus promoting an increased excitability in the dorsal horn, which manifests as hyperalgesia. Indirect evidence from neurophysiological recordings and brain imaging studies suggest similar mechanisms in adult rats (Casey, 1999; Jensen, 1997).

We conclude that capsaicin-induced hyperalgesia in 21-day-old rats involves forebrain areas, where an uncoupling of forebrain opioid receptors reduces opioidergic inputs to descending inhibitory controls, thus reducing nociceptive thresholds. Further research should be designed to identify the anatomical areas mediating these changes, the role of similar mechanisms during different periods of development, as well as the precise biochemical events that lead to the uncoupling of opioid receptors associated with capsaicin-induced hyperalgesia.

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