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Continuous Administration of the 5-Hydroxytryptamine$_{1A}$ Agonist (3-Chloro-4-fluoro-phenyl)-[4-fluoro-4-{[(5-methyl-pyridin-2-ylmethyl)-amino]-methyl}piperidin-1-yl]-methadone (F 13640) Attenuates Allodynia-Like Behavior in a Rat Model of Trigeminal Neuropathic Pain

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ABSTRACT

(3-Chloro-4-fluoro-phenyl)-[4-fluoro-4-{[(5-methyl-pyridin-2-yl-methyl)-amino]-methyl}piperidin-1-yl]-methadone (F 13640) is a recently discovered high-efficacy 5-hydroxytryptamine (HT)$_{1A}$ receptor agonist that produces central analgesia through the neuroadaptive mechanisms of inverse tolerance and cooperation. In a rat model of trigeminal neuropathic pain, the chronic constriction injury of the infraorbital nerve causes allodynia-like behavior that develops within 2 weeks and remains stable thereafter. We report that early after surgery, during which time allodynia develops, the continuous 2-week infusion of 0.63 mg/day F 13640 inhibited the allodynia-like behavior, whereas 5 mg/day morphine showed no significant effect. When F 13640 infusion was initiated late after surgery, when allodynia was well established, it produced an antiallodynic effect that was apparent during the entire infusion period. In contrast, morphine infusion caused an initially marked antiallodynic effect to which tolerance developed within the 2-week infusion period. The GABA-B receptor agonist baclofen (1.06 mg/day) that has a recognized usefulness in the treatment of trigeminal neuralgia, demonstrated effectiveness in both conditions. The data are consistent with a theory of nociceptive signal transduction, as well as with previous data, in demonstrating the neuroadaptive mechanisms of inverse tolerance and cooperation. That is, in contrast with morphine, the antiallodynic effect induced by 5-HT$_{1A}$ receptor activation does not decay, but, if anything, grows with chronicity. Also, 5-HT$_{1A}$ receptor activation seemed to cooperate with nociceptive stimulation in, paradoxically, inducing an antiallodynic effect. The data presented here suggest that F 13640 may perhaps offer a lasting treatment of trigeminal neuralgia.

The 5-HT$_{1A}$ receptor agonist F 13640 has been shown (Colpaert et al., 2002) to display a unique pattern of actions that is best understood in terms of a new theory of the mechanisms of pain and analgesia. This concept of signal transduction in nociceptive systems (Colpaert, 1978, 1996; Colpaert and Frégnac, 2001) specifies that any input to such systems causes not a single effect but two effects that are bidirectional, or opposite in sign. Thus, morphine produces both analgesia as a so-called first order effect, and also hyperalgesia as a second order effect. Upon chronic exposure to morphine, the second order hyperalgesia grows and neutralizes the first order analgesia (i.e., development of tolerance); the concept thus provides an account of the neuroadaptive actions that dynamically ensue upon μ-opioid receptor activation (Colpaert, 1996). Also, according to this concept, nociceptive stimulation should similarly produce dual, bidirectional effects that should amount to the mirror opposite of those produced by morphine.

We have recently discovered (Colpaert et al., 2002) that high-efficacy activation of 5-HT$_{1A}$ receptors constitutes a molecular mechanism that mimics the central actions of nociceptive stimulation. A single injection of the selective, high-efficacy 5-HT$_{1A}$ agonist F 13640 produced dual and bidirectional, hyper- and hypoalgesic effects on the vocalization threshold to mechanical stimulation in normal rats. These effects were shown to be related to the 5-HT$_{1A}$ receptor activation that F 13640 produces in a selective manner (Colpaert et al., 2002). Upon repeated injection, F 13640 produced an initial hyperalgesia that then decayed while the hypoalgesia became amplified, thus demonstrating the development of inverse tolerance. Continuous infusion of F 13640

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; IoN-CCI, chronic constriction injury of the infraorbital nerve; ANOVA, analysis of variance.

produced an antinociceptive effect in rodent models of chronic nociceptive pain and of chronic allodynia that surpassed that of morphine and of other mechanisms of central analgesia. These data thus identify large-amplitude 5-HT_{1A} receptor activation as a new molecular mechanism of analgesia, the neuroadaptive mechanisms of which consist, remarkably, of inverse tolerance and, also, of cooperation with nociceptive stimulation in producing analgesia (Colpaert et al., 2002). Indeed, inasmuch as F 13640 mimics the central effects of nociceptive stimulation, its effects should add to those of any such stimulation in producing analgesia (Colpaert, 1996). Thus, although 0.63 mg/kg F 13640 produced marked hyperalgesia in normal rats 15 min upon its i.p. injection, the compound produced profound analgesia in rats that were exposed to the severe, tonic nociception induced by the inoculation of 50 μl of 2.5% formaldehyde into the hind paw plantar surface (Colpaert et al., 2002).

The notion that 5-HT_{1A} receptor activation may offer a new approach to the treatment of pain is consistent with the long-standing recognition of serotonergic mechanisms in pain control (for review, see Cesselin et al., 1994; Hamon and Bourgoin, 1999). Both supraspinally and spinally projecting pathways are involved; importantly, pathways originating in the brainstem and projecting to the spinal cord dorsal horn are thought to mediate an endogenous, serotonergic pain-suppressing system (Villanueva and Le Bars, 1995). Although many different 5-HT receptors may be implicated (Millan, 1995; Barnes and Sharp, 1999), considerable evidence points to an important role of 5-HT_{1A} receptors (Hamon and Bourgoin, 1999). For example, 5-HT_{1A} receptors likely mediate the inhibitory effect, on spinal pain transmission, of the descending 5-HT pathway that originates in the nucleus raphe magnus (Zemlan et al., 1994) as well as the opioid-induced bulbospinal inhibition of spinal withdrawal reflexes (Clarke and Ward, 2000). Also, 5-HT_{1A} receptor activation applied directly onto the spinal cord inhibits the activity of spinal-wide dynamic range neurons after repeated electrical stimulation (Gjerstad et al., 1996).

In spite of the availability of opioids, reuptake inhibitors of noradrenaline and serotonin, and anticonvulsants, the treatment of neuropathic pains in patients, and of trigeminal neuralgia in particular, remains largely unsatisfactory (Ollat and Cesaro, 1995; Sindrup and Jensen, 1999, 2002; Attal, 2001). In a rat model of trigeminal neuropathic pain, in which a chronic constriction injury of the infraorbital nerve (IoN-CCI) causes von Frey hair stimulation of the skin area within the IoN territory to elicit allodynia-like behavior (Vos et al., 1994), it was shown that the GABA-B receptor agonist baclofen and the novel anticonvulsant gabapentin, but not the anticonvulsants carbamazepine or lamotrigine, morphine, or the tricylic antidepressants amitriptyline and clomipramine, partially attenuate the allodynia-like behavior (Idänpään-Heikkilä and Guilbaud, 1999; Christensen et al., 2001). The combination of the glycine/*N*-methyl-D-aspartate receptor antagonist (+)-1-hydroxy-3-aminopyrrolidine-2-one [(+)-HA966] and morphine was also found to dose dependently increase mechanical response thresholds (Christensen et al., 1999). A recent study reported that antimigraine 5-$HT_{1B/1D}$ receptor agonists also partially attenuate the allodynia-like behavior (Kayser et al., 2002). Importantly, the single i.p. injection of 0.63 mg/kg F 13640 fully normalized allodynic hyper-responsiveness in this model (Deseure et al., 2002). The magnitude of F 13640's effects was similar to that of 5 mg/kg baclofen, whereas as high a dose as 10 mg/kg morphine was required to achieve an equivalent antiallodynic effect. A 4-fold higher dose of baclofen, but not of F 13640, depressed response scores in a nonspecific manner (i.e., below prelesion levels, both ipsi- and contralaterally; Deseure et al., 2002). These data suggest that F 13640 may perhaps offer an effective treatment of trigeminal neuralgia.

With trigeminal neuralgia being a chronic pathology, it is essential that potential treatments be evaluated while being implemented in a chronic manner to determine their long-term efficacy. This is all the more pertinent because the cooperation between nociceptive stimulation and 5-HT_{1A} receptor activation in producing analgesia must be expected (Colpaert, 1996) to be both duration- and intensity-dependent. This duration dependence of F 13640's antiallodynic effect was studied here by monitoring responsiveness to von Frey hair stimulation in IoN-CCI rats throughout a 2-week period. The intensity dependence was studied by comparing F 13640's effects with those of another 5-HT_{1A} agonist, F 13714 (Koek et al., 2001), which activates 5-HT_{1A} receptors to a smaller extent than that achieved with F 13640 (Colpaert et al., 2002). Note that both F 13714 (Koek et al., 2001) and, in particular, F 13640 are highly selective for 5-HT_{1A} receptors; F 13640 binds to rat and human 5-HT_{1A} receptors with a pK_i value of 9.1 and 9.5, respectively, whereas its IC_{50} value exceeds 1000 nM with the 46 other receptors and ion channels for which its activity has been determined (Colpaert et al., 2002). The latter included various 5-HT receptor types and subtypes (i.e., 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_4, h5-ht_6, and 5-HT_7 receptors), as well as various dopaminergic, noradrenergic, GABA-ergic, and opioid receptors. These compounds were compared with two reference treatments, i.e., morphine and baclofen, which are effective upon acute administration in the IoN-CCI model, but have not been studied upon chronic administration (Deseure et al., 2002). Finally, allodynic hyper-responsiveness in the IoN-CCI model develops progressively over the first 2 weeks after the nerve injury, to reach an asymptote that persists for at least 7 weeks (Vos et al., 1994). Drug effects here were examined early after surgery, at the time when allodynia developed, as well as late after surgery, after it had reached asymptote. For the reasons outlined above, the transduction concept would suggest that the analgesic effects of both morphine and of 5-HT_{1A} agonists should be more prominent at a time when persistent allodynia had been established then when this allodynia had not yet fully developed.

Materials and Methods

Subjects. Male Sprague-Dawley rats (n = 208, weighing 220–240 g on arrival; Charles River, Brussels, Belgium) were used. Rats were housed in solid bottom polycarbonate cages in a colony room (38 ± 9% R.H.; 21 ± 1°C). Water and food were available ad libitum. Rats were kept under a reversed 12:12-h dark/light cycle (lights on at 8:00 PM) and were allowed to acclimate to the housing facilities for at least 12 days before preoperative testing. Animals were treated and cared for according to the guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain (1983).

IoN-CCI Model: Study Design. Two experiments were conducted. In both experiments, rats were implanted with osmotic pumps to examine the effects of chronic administration of four dif-

ferent drugs (see "Drugs and Doses") on allodynia-like behavior after IoN-CCI. Pumps contained one of four different compounds or saline and released their content at a constant rate (i.e., 0.12 ml/day) during a period of 14 days.

In the first experiment (n = 84 in total; n = 12 in each of the seven experimental groups), we examined the effects of the drug treatments on the development of allodynia during the first 2 weeks after surgery. Rats were implanted with osmotic pumps immediately after IoN-CCI surgery, and treatment effects were studied on days 4, 6, 8, 11, 13, and 15 after surgery. To evaluate the effect of IoN-CCI surgery, saline-treated rats were compared with sham-operated rats, which were also implanted with pumps containing saline. To examine the persistence of the effects of the drug treatments, rats were also tested 6 days after the removal of osmotic pumps (i.e., on day 21).

In the second experiment (n = 68 in total; n = 9–12 in each of the six experimental groups), we examined the effects of the drug treatments on allodynia that was already established, i.e., 3 to 5 weeks after IoN-CCI ligation. First, the effect of IoN-CCI surgery was evaluated by comparing the presurgery data with postsurgery data; rats were tested 1 day before surgery and 6, 11, 16, 21, and 24 days thereafter. After testing on day 24, rats were implanted with the pumps. During the following 2 weeks, treatment effects were examined on days 27, 29, 31, 34, 36, and 38 after surgery.

Surgery. The unilateral ligation of the IoN was performed as described previously (Vos et al., 1994). Rats were anesthetized with pentobarbital (60 mg/kg i.p.) and treated with atropine (0.1 mg/kg i.p.). Surgery was performed under direct visual control using an operation microscope (×10^{-25}; Carl Zeiss, Jena, Germany). The head of the rat was fixed in a stereotaxic frame and a mid-line scalp incision was made, exposing skull and nasal bone. The infraorbital part of the IoN was exposed using a surgical procedure similar to that described previously (Gregg, 1973; Jacquin and Zeigler, 1983). The edge of the orbit, formed by the maxillary, frontal, lacrimal, and zygomatic bones, was dissected free. To give access to the IoN, the orbital contents were gently deflected with a cotton-tipped wooden rod. The IoN was dissected free at its most rostral extent in the orbital cavity, just caudal to the infraorbital foramen. Two chromic catgut ligatures (5-0, Ethicon; Johnson and Johnson, Brussels, Belgium) were loosely tied around the IoN (2 mm apart). To obtain the desired degree of constriction, a criterion proposed by Bennet and Xie (1988) was applied: the ligatures reduced the diameter of the nerve by a just noticeable amount and slowed but did not interrupt the circulation through the superficial vasculature. The scalp incision was closed using polyester sutures (4-0, Ethicon; Johnson and Johnson). In sham-operated rats, the IoN was exposed on one side using the exact same procedure, but the exposed IoN was not ligated.

Implantation of Alzet osmotic pumps (model 2ML2, nominal pump rate, 5 μl/h; Alza, Palo Alto, CA) was also performed under anesthesia. In experiment 1, pumps were implanted immediately after IoN-CCI and while the rats were still anesthetized. In experiment 2, rats were implanted with the pumps 24 days after surgery. They were placed in a cage under 4% halothane (Fluothane; Zeneca, Brussels, Belgium). When the rats were fully anesthetized (after ca. 3–4 min), they were taken out of the cage, shaved, and placed under a mask with 2.5% halothane. The shaved dorsal area was disinfected with Hibitane (0.5% chlorhexidine in alcohol, 70°C). An incision of approximately 2 cm was made in the skin, a subcutaneous pocket was created with a hemostatic forceps, and 0.1 ml of antibiotic (Pentrexyl, Na ampicillin, 500 mg in 5 ml of saline; S.A. Bristol-Myers Squibb, Brussels, Belgium) was released into the pocket with a sterile syringe (1-ml luer). The pump was inserted subcutaneously with the opening toward the head of the rat. Finally, the incision was closed with four or five stainless steel staples (disposable skin stapler with autorelease Appose 35 Regular, Sherwood-Davis and Geck, Adliswil, Switzerland) and sprayed with Aluspray (Vétoquinol, Lure, France). Removal of the osmotic pumps was performed under halothane anesthesia as described above. The staples were removed from the skin using a hemostatic forceps and an incision of approximately 2 cm was made in the skin, the pump was removed from the subcutaneous pocket, and the incision was again closed with four or five staples and sprayed with Aluspray.

Behavioral Testing. Rats were habituated to the test procedure on preoperative days −7, −5, and −3 in the first experiment and on preoperative days −7 and −5 in the second experiment. Baseline data were obtained 1 day before surgery. Habituation and testing were conducted in a darkened room (light provided by a 60-W red light bulb suspended 1 m above the test area) with a 45-dB background noise, by an observer that was blind to the drugs that were used. Rats were place in a small transparent cage (24 × 14 × 17 cm; length × width × height). As elsewhere (Vos et al., 1994), a graded series of five von Frey hairs (Pressure Aesthesiometer, Stoelting Co., Chicago, IL) were used. The force required to bend the hairs was 0.015, 0.127, 0.217, 0.745, and 2.150 g, respectively. The stimuli were applied within the IoN territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. This area was stimulated unilaterally before surgery, and on both sides of the face after surgery, i.e., ipsilateral and contralateral to the side of surgery. Stimuli were applied in an ascending order of intensity. The ipsilateral and contralateral sides were stimulated in a randomized order for each stimulus intensity, within each subject. The scoring system described by Vos et al. (1994) was used to evaluate the response of the rats to the stimulation. The response was observed to belong to one of the following response categories: score 0, no response; score 1, detection = the rat turns the head toward the stimulating object and the stimulus object is then explored; score 2, withdrawal reaction = the rat turns the head slowly away or pulls it briskly backward when the stimulation is applied, sometimes a single face wipe ipsilateral to the stimulated area occurs; score 3, escape/attack = the rat avoids further contact with the stimulus object, either passively by moving its body away from the stimulating object to assume a crouching position against the cage wall, or actively by attacking the stimulus object, making biting and grabbing movements; and score 4, asymmetric face grooming = the rat displays an uninterrupted series of at least three face-wash strokes directed toward the stimulated facial area.

Drugs and Doses. The following drugs were used: (3-chloro-4-fluoro-phenyl)-[4-fluoro-4-{[(5-methyl-pyridin-2-ylmethyl)-amino]-methyl}piperidin-1-yl]-methadone (F 13640; 0.63 mg/day) and 3-chloro-4-fluorophenyl-(4-fluoro-4-[[(5-methyl-6-methylamino-pyridin-2-ylmethyl)-amino]-methyl]-piperidin-1-yl-methanone) (0.16 mg/day; F 13714 glycolate) (Pierre Fabre, Castres, France); morphine hydrochloride (5 mg/day) (Belgapio, Louvain-La-Neuve, Belgium); and baclofen (1.06 mg/day) (Sigma-Aldrich, St. Louis, MO). All drugs were dissolved in sterile water and administered subcutaneously by osmotic pumps (cf. supra). F 13640, F 13714, and morphine were administered through one pump. The doses of F 13640 and morphine where the same as those used in previous studies; these doses were the highest at which the compounds were water-soluble, chemically stable, and sufficiently tolerated (Bruins Slot et al., 2002; Colpaert et al., 2002); the 0.63-mg/day dose is also that at which F 13640 produced full antinociceptive effects in the arthritic rat model (Colpaert et al., 2002); the dose of F 13714 was four times smaller than that of F 13640, in accordance with its 4 to 16 times greater potency on bolus injection (Colpaert et al., 2002; Deseure et al., 2002). Because of limitations in solubility, baclofen was administered through two pumps, and rats treated with baclofen were compared with rats implanted with two saline pumps. The baclofen dose corresponds to the largest concentration at which baclofen was soluble and remained stable at 37°C over 2 weeks (E. Carilla and F. C. Colpaert, unpublished observations). Sham-operated rats were implanted with one saline pump and compared with IoN-CCI rats that were also implanted with one saline pump. Doses refer to the free base.

Drug Effects in Normal Rats. All treatment conditions that were studied in the IoN-CCI model were tested beforehand for their

tolerability in normal rats. Housing conditions were as described previously (Bruins Slot et al., 2002). Rats were implanted on day 0 either with one pump releasing 0.63 mg/day F 13640, 0.16 mg/day F 13714, 5 mg/day morphine, or saline, or with two pumps releasing 1.06 mg/day baclofen or saline; each drug treatment was studied in parallel with its own (saline) control group (n = 7/group).

Measurements were made of rectal body temperature (to the nearest 0.1°C) by means of a thermal probe (Ellat model RM6; Carrieri Instruments, Paris, France) and of body weight (to the nearest gram). Measurements were made immediately before pump implantation as well as 30 min, 60 min, 2 h, 4 h, and 8 h after pump implantation. Further measurements were taken once daily (at 9:00 AM) on postimplantation days 1, 2, 3, 4, and 7. At all of these times, an observation was also made of the rat's behavior to assess any behavioral anomaly.

Statistical Analysis. The response scores to mechanical stimulation that were obtained in the present studies constitute an ordinal variable; accordingly, the data should be analyzed nonparametrically and represented graphically by nonparametric measures of central tendency and of variation. However, here as in a previous study (Deseure et al., 2002), nonparametric representation of the data using medians reflected the statistical outcome of the nonparametric analyses quite poorly. The use of median scores failed to appropriately visualize the nonetheless highly significant effects, which both the IoN injury and the agents produced. This mismatch results from the limited variation that the scoring system allows.

Therefore, a parametric data analysis was done of which the statistical outcome demonstrated an excellent concordance with that of the nonparametric analysis. Of the 64 comparisons that the present studies called for, only six provided significance ($p < 0.05$) in the parametric analysis while not doing so in the nonparametric analysis (nonparametric p values were 0.05, 0.07, 0.11, 0.11, 0.16, and 0.18); and in one instance, the nonparametric analysis reached significance (i.e., $p = 0.027$), whereas the parametric analysis did not. Furthermore, the graphical representation of the data by means of the average and S.E.M. provided a far more satisfactory reflection of both analyses. Thus, here as elsewhere (Deseure et al., 2002), data are both analyzed and represented in a parametric manner.

For each rat and at every designated time, the mean of the response scores to the five von Frey hairs was determined. Postoperative changes of baseline values before drug treatment were analyzed by means of repeated measures ANOVA with day as within-subjects factor and followed by an unpaired t test per time point. This ANOVA and those described hereafter were performed separately on data obtained after ipsilateral and after contralateral stimulation. Mechanical stimulation data obtained during drug treatment were also analyzed by means of repeated measures ANOVA with day as within-subjects factor and followed by a unifactorial ANOVA per time point. Post hoc comparisons were carried out using Dunnett's test for comparing means with a control mean. Mechanical stimulation data obtained 6 days after drug treatment were analyzed by means of a unifactorial ANOVA followed by post hoc comparisons carried out using Dunnett's test for comparing means with a control mean. Data obtained during and after drug treatments were analyzed separately for rats implanted with one pump and rats implanted with two pumps.

Results

Effects Early after Surgery When Allodynia Develops. Responsiveness to ipsilateral mechanical stimulation in IoN-CCI rats was significantly different from that in sham-operated rats [group × time interaction: $F(5,110) = 37.45$, $p < 0.001$] (Fig. 1). Post hoc comparisons (unpaired t tests) showed significant differences on days 4, 8, 11, 13, and 15 ($p < 0.05$), but not on day 6 ($p = 0.34$). Responsiveness to contralateral mechanical stimulation was not significantly

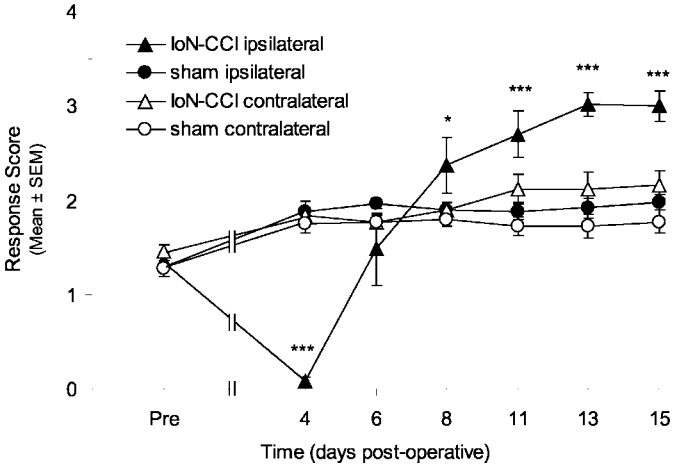

Fig. 1. Time course of the effects of IoN-CCI ligation on responsiveness to mechanical stimulation. Data points represent the mean (±S.E.M.) response score to von Frey hair stimulation of the territory of the ligated nerve (closed symbols) and of the contralateral side (open symbols) of IoN-CCI rats (n = 12; triangles) and sham-operated rats (n = 12; circles) 1 day before (Pre) and 4 to 15 days after surgery. Asterisks indicate a significant difference compared to sham-operated control rats (unpaired t test, *, $p < 0.05$; ***, $p < 0.001$).

different between these groups [groups × time interaction: $F(5,110) = 2.27$, $p > 0.05$]. Also, no significant differences were found between IoN-CCI rats treated with one or two saline pumps in responsiveness to ipsilateral [not shown; group × time interaction: $F(5,110) = 1.56$, $p > 0.05$] or contralateral [not shown; group × time interaction: $F(5,110) = 1.04$, $p > 0.05$] stimulation.

Mechanical stimulation data shown in Fig. 2, A to C, were obtained in rats implanted with one pump and were analyzed together. The effects of the drug treatments varied with time in a manner that was significantly different among the drugs [treatment × time interaction: $F(15,220) = 3.66$, $p < 0.001$]. Because the effects of surgery changed over time (i.e., from an initial hyporesponsiveness, through an apparent lack of effect, to hyper-responsiveness; Fig. 1), possible drug effects were analyzed at each of the time points. Unifactorial ANOVA per time point, with treatments as between-subjects factor, showed significant differences between the treatments on days 11, 13, and 15 [$F(3,44) \geq 4.35$, $p \leq 0.009$], but not on days 4, 6, and 8 [$F(3,44) \leq 1.74$, $p \geq 0.08$]. As indicated with asterisks in the respective panels in Fig. 2, post hoc comparisons between the different treatments and their respective vehicle showed significant drug effects of F 13714 on days 13 and 15; F 13640 was effective on days 11, 13, and 15; no significant drug effects were found for morphine. The effects of F 13640 and F 13714 significantly changed over time [treatment × time interaction: $F(5,110) \geq 5.73$, $p < 0.001$]; in contrast, the effects of morphine, if any, did not significantly change over time [treatment × time interaction: $F(5,110) = 1.29$, $p > 0.05$].

Mechanical stimulation data shown in Fig. 2D were obtained in rats that were implanted with two osmotic pumps. No significant overall difference was found between baclofen- and saline-treated rats, although the p value fell only narrowly short of significance [$F(1,22) = 4.15$, $p = 0.053$]. Because the effect of the surgery changed over time (see above), further analyses were conducted to detect potential differences between baclofen and saline on one or more of the

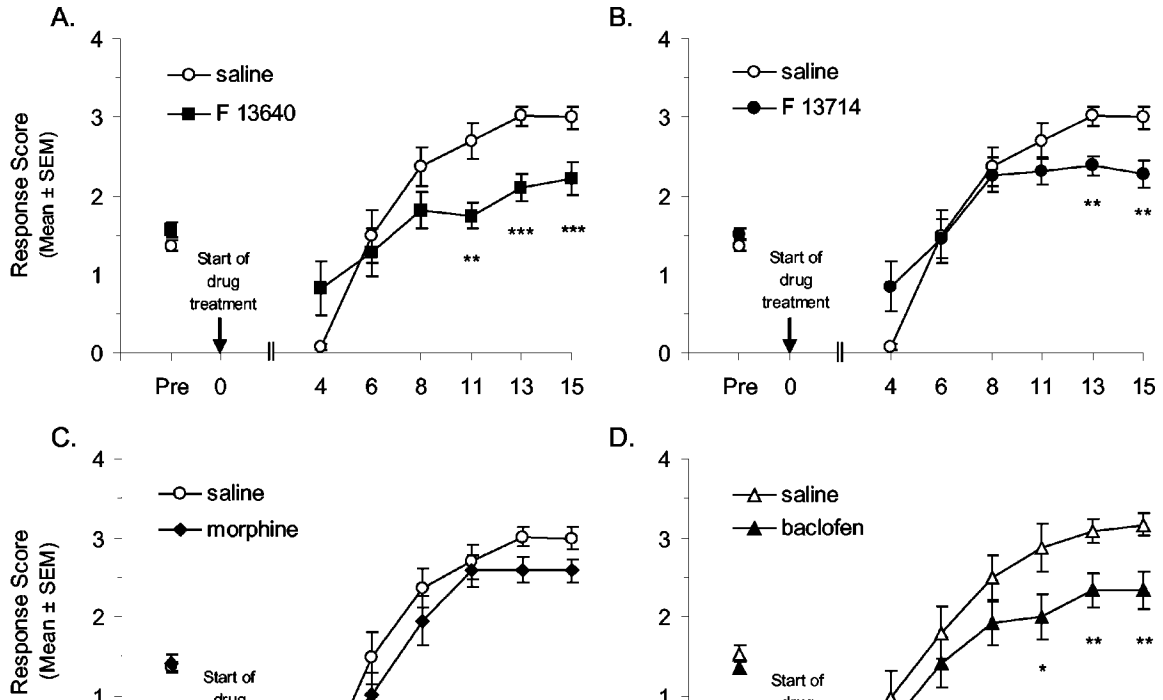

Fig. 2. Effects of F 13640, F 13714, morphine, and baclofen on responsiveness to mechanical stimulation early after IoN-CCI ligation when allodynia develops. Data points represent the mean (±S.E.M.) response score to von Frey hair stimulation of the territory of the ipsilateral, ligated nerve of rats treated with F 13640 (0.63 mg/day; $n = 12$; A), F 13714 (0.16 mg/day; $n = 12$; B), morphine (5 mg/day; $n = 12$; C), baclofen (1.06 mg/day; $n = 12$; D) or their respective vehicle ($n = 12$) one day before (Pre) and 4 to 15 days after IoN-CCI ligation. Mechanical stimulation data in A, B, and C were obtained in rats implanted with one pump; data shown in D were obtained in rats implanted with two pumps. Drug or vehicle infusion via osmotic pumps started immediately after IoN-CCI ligation on day 0. Asterisks indicate a significant difference compared with vehicle control rats (Dunnett's test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

individual time points. Post hoc comparisons between baclofen- and saline-treated rats showed significant differences on days 11, 13, and 15 (Fig. 2D). Despite this apparent increase in the effects of baclofen, repeated measures ANOVA did not yield a significant change in the effects of baclofen over time [treatment × time interaction: $F(5,110) = 0.92$, $p > 0.05$].

Because no significant differences were found between IoN-CCI rats and sham-operated rats for responsiveness to contralateral mechanical stimulation, no analysis of possible drug effects was performed for this variable.

In a previous study (Deseure et al., 2002), each of the compounds examined here induced behavioral effects upon acute intraperitoneal injection in IoN-CCI-lesioned rats. Signs of the so-called 5-HT syndrome were not observed in the course of von Frey testing (i.e., starting 3 days after pump implantation) in any of the rats treated with F 13640 or F 13714. Also, no signs of immobility or akinesia were observed in rats treated with baclofen or morphine, respectively.

Effects on Allodynia 6 Days after Removal of Osmotic Pumps. Unifactorial ANOVA on day 21, i.e., 6 days after the removal of the osmotic pumps, showed significant differences between drug treatments for rats treated with one pump [$F(3,35) = 6.23$, $p < 0.01$]; no significant difference

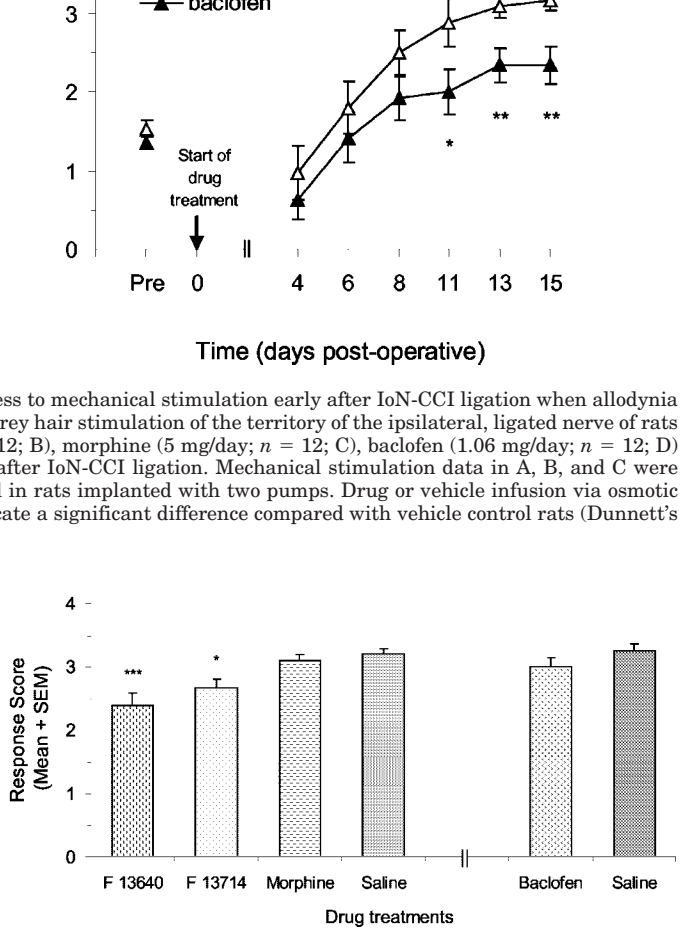

Fig. 3. Effects of F 13640, F 13714, morphine, and baclofen on hyperresponsiveness to mechanical stimulation 6 days after removal of the osmotic pumps releasing these agents. Data points represent the mean (±SEM) response score to von Frey hair stimulation of the territory of the ipsilateral, ligated nerve of rats that had been infused with F 13714 ($n = 12$), F 13640 ($n = 12$), morphine ($n = 12$), baclofen ($n = 12$), or their respective vehicle ($n = 12$) during the first 2 weeks after IoN-CCI ligation (see legend to Fig. 2). Mechanical stimulation data shown on the left were obtained in rats implanted with one pump; data shown on the right were obtained in rats implanted with two pumps. Drug or vehicle infusion via osmotic pumps started immediately after IoN-CCI ligation on day 0 and was terminated on day 15 when the pumps were removed. Rats were subsequently tested on day 21. Asterisks indicate a significant difference compared with vehicle control rats (Dunnett's test; *, $p < 0.05$; ***, $p < 0.001$).

was found between rats treated with two pumps [$F(1,18) = 1.77$, $p = 0.20$] (Fig. 3). Post hoc comparisons showed that in rats that had been treated with either F 13640 or F 13714 during the first 2 weeks after IoN-CCI surgery, hyper-responsiveness to mechanical stimulation was still significantly reduced on day 21.

Effects Late after Surgery When Allodynia Is Established. After IoN ligation, rats demonstrated significant changes in responsiveness to mechanical stimulation of the territory of the ligated ipsilateral nerve [$F(5,310) = 142.34$, $p < 0.001$] (Fig. 4). An initial decrease in ipsilateral response scores on day 6 ($p < 0.001$) was followed by a marked increase on days 11 to 24 ($p < 0.001$). The rats also demonstrated a small but significant increase in responsiveness to mechanical stimulation of the territory of the nonligated contralateral IoN [$F(5,310) = 39.92$, $p < 0.001$]. Post hoc comparisons showed significant differences from days 6 to 24 ($p < 0.001$).

Mechanical stimulation data shown in Fig. 5, A to C, were obtained in rats implanted with one pump and were analyzed together. Unifactorial ANOVA per time point, with treatments as between-subjects factor, showed significant differences between the treatments on all six time points [$F(3,46) \geq 5.06$, $p < 0.01$]. The effects of the drug treatments varied with time in a manner that was significantly different among the drugs [treatment × time interaction: $F(15,230) = 5.76$, $p < 0.001$]. Post hoc comparisons between F 13640 and saline showed that F 13640 significantly reduced the hyper-responsiveness to mechanical stimulation at all time points; its effects did not significantly change over time [treatment × time interaction: $F(5,120) = 0.61$, $p > 0.05$]. Significant effects were found for F 13714 on days 34, 36, and 38; morphine was effective on all days except day 38. The ability of F 13714 to reduce the hyper-responsive behavior significantly increased over time [treatment × time interaction: $F(5,120) = 4.57$, $p < 0.001$]; in contrast, the effects of morphine significantly decreased over time [treatment × time interaction: $F(5,120) = 5.67$, $p < 0.001$].

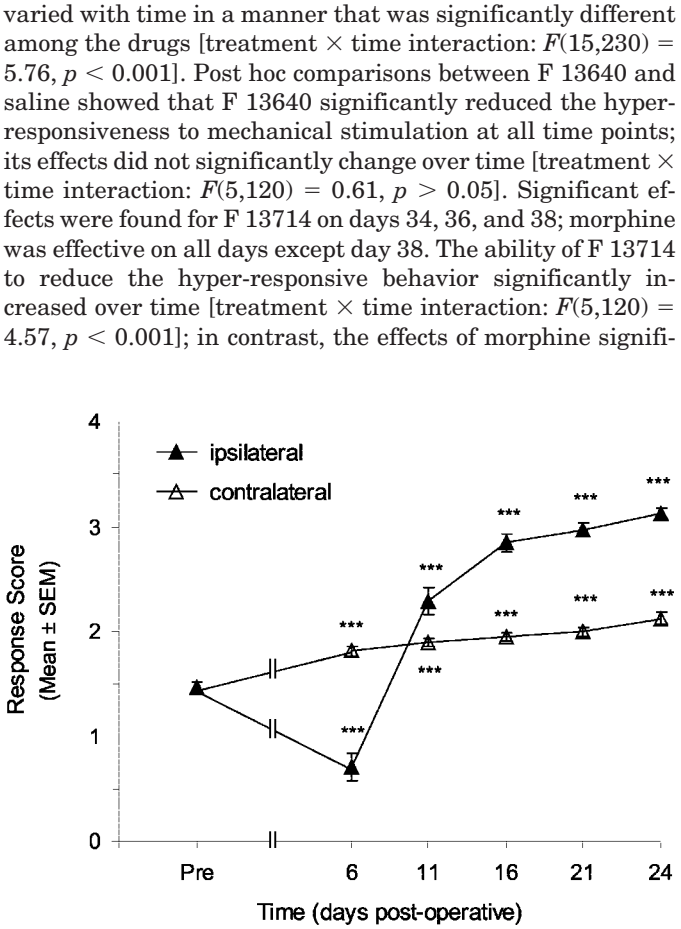

Fig. 4. Time course of the effects of IoN-CCI ligation on responsiveness to mechanical stimulation. Data points represent the mean (±S.E.M.) response score to von Frey hair stimulation of the territory of the ligated nerve (ipsilateral) and of the contralateral side (contralateral) 1 day before (Pre) and 6 to 24 days after surgery ($n = 68$; these animals were implanted with pumps immediately after mechanical stimulation testing on day 24; see Fig. 5). Asterisks indicate a significant difference compared with the respective preoperative values (Dunnett's test; ***, $p < 0.001$). Note the similarity of these data to those presented in Fig. 1.

Mechanical stimulation data shown in Fig. 5D were obtained in rats that were implanted with two pumps. A significant overall difference was found between baclofen-and saline-treated rats [$F(1,16) = 5.17$, $p < 0.05$]; the effects of baclofen treatment did not significantly interact with time [treatment × time interaction: $F(5,80) = 1.07$, $p > 0.05$]. Post hoc comparisons between baclofen- and saline-treated rats showed significant differences on days 29 through 38.

No significant differences were found for responsiveness to contralateral mechanical stimulation [not shown; $F(15,230) \leq 1.52$, $p \geq 0.10$]. Signs of the 5-HT syndrome were again not observed in the course of von Frey testing (i.e., starting 3 days after pump implantation) in any of the rats treated with F 13640 or F 13714. Also, no signs of immobility or akinesia were observed in rats treated with baclofen or morphine, respectively.

Drug Effects in Normal Rats. None of the drug treatments exerted a significant effect on body weight (not shown). All treatments, however, lowered body temperature (Fig. 6). Repeated measures ANOVA of the F 13640 data indicated a significant effect of treatment [$F(1,12) = 30.07$, $p < 0.001$], of time [$F(10,120) = 12.35$, $p < 0.001$], and of their interaction [$F(10,120) = 25.80$, $p < 0.001$]. F 13640 's hypothermic effect was apparent for up to 2 h after pump implantation (Fig. 6). F 13714 produced a similar effect for up to 4 h (F values: 19.21, 5.32, and 8.02 for the respective effects; $p < 0.001$ in each case). With morphine, no significant effect of treatment [$F(1,12) = 0.00$, $p > 0.05$] was found, but the effect of time [$F(10,120) = 9.28$, $p < 0.001$] and that of the treatment × time interaction [$F(10,120) = 2.68$, $p < 0.01$] were significant; morphine's hypothermic effect was apparent 30 min after pump implantation, but not at any later time interval (Fig. 6). With baclofen, the effects of treatment [$F(1,12) = 11.64$, $p < 0.01$] and of time [$F(10,120) = 10.73$, $p < 0.001$] were significant, but not that of their interaction [$F(10,120) = 0.77$, $p > 0.05$]; baclofen's hypothermic effect was apparent for up to 60 min after pump implantation (Fig. 6).

F 13640 induced lower lip retraction, flat body posture, and forepaw treading for a few hours, and at most, 1 day after pump implantation; similar signs of the 5-HT syndrome were observed with F 13714 (Fig. 6). No grossly observable behavioral anomalies were found with morphine or baclofen.

Discussion

In IoN-CCI rats that were implanted immediately after the surgery with either one or two subcutaneous osmotic pumps releasing saline, hyper-responsiveness to mechanical von Frey hair stimulation developed in a manner similar to that described previously (Vos et al., 1994; Deseure et al., 2002); this suggests that the pump implantation did not noticeably interfere with the development of allodynia. That is, the injury initially caused an almost complete loss of ipsilateral responsiveness that was followed, from day 8 onward, by hyper-responsiveness (Fig. 1). The 5-HT_{1A} receptor agonist F 13640 significantly reduced the hyper-responsiveness at the time that the latter developed (Fig. 2); this was also found with the other 5-HT_{1A} receptor agonist F 13714, although its effects required two more days to achieve statistical signifi-

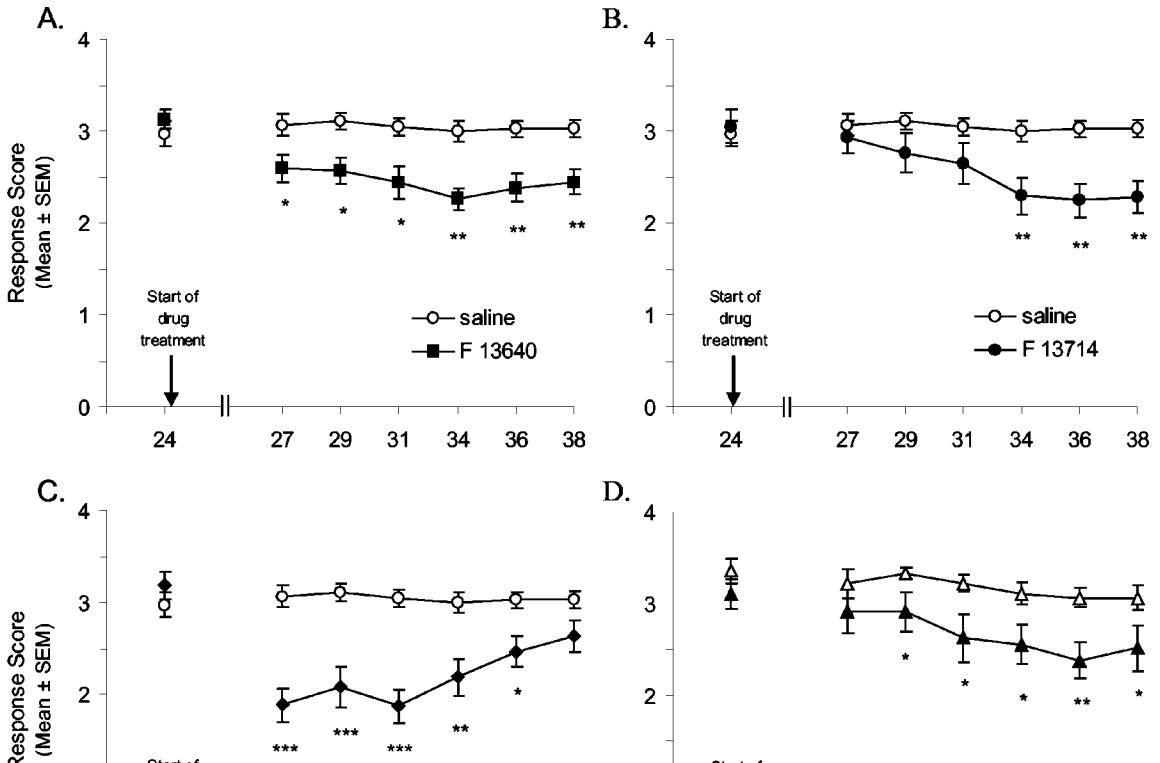

Fig. 5. Effects of F 13640, F 13714, morphine, and baclofen on hyper-responsiveness to mechanical stimulation late after IoN-CCI ligation when allodynia is well established. Data points represent the mean (±S.E.M.) response score to von Frey hair stimulation of the territory of the ipsilateral, ligated nerve of rats that were infused with F 13640 (0.63 mg/day; $n = 12$; A), F 13714 (0.16 mg/day; $n = 12$; B), morphine (5 mg/day; $n = 12$; C), baclofen (1.06 mg/day; $n = 9$; D), or their respective vehicle ($n = 14$ for A–C; $n = 9$ for D) from days 24 to 38 after IoN-CCI ligation. Mechanical stimulation data in A, B, and C were obtained in rats implanted with one pump; data shown in D were obtained in rats implanted with two pumps. Drug or vehicle infusion via osmotic pumps started immediately after mechanical stimulation testing on day 24. Asterisks indicate a significant difference compared with vehicle control rats (Dunnett's test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

cance. The effects of both agents are consistent with the duration-dependent cooperation that is expected (Colpaert, 1996; Colpaert et al., 2002) to develop between 5-HT$_{1A}$ receptor activation and nociceptive stimulation; the antiallodynic effect with either agent became more robust as the duration of coexposure to both 5-HT$_{1A}$ receptor activation and IoN-CCI-induced nociception grew longer. This increase in effectiveness is not likely due to drug accumulation; circulating plasma levels of F 13640 in rats similarly implanted with a pump releasing 0.63 mg/day reach asymptote some 24 h after pump implantation and then remain stable (at about 80 ng/ml) throughout the further 2-week period (E. Carilla and F. C. Colpaert, unpublished observations). Baclofen exerted a significant antiallodynic effect from day 11 onward (Fig. 2). Finally, response scores with morphine also were lower than with saline, but this difference was not significant. Morphine's inability to produce any significant effect here may be surprising in that acutely injected morphine in this model does produce a significant antiallodynic effect (DeMulder et al., 1994; Deseure et al., 2002). However, in normal rats implanted with a morphine pump similarly releasing 5 mg/day, tolerance to morphine's analgesic action in the Randall-Selitto assay (Randall and Selitto, 1957) developed within 24 h after pump implantation (Colpaert et al., 2002). Here, the first postpump implantation recordings of allodynic behavior were made 4 days after morphine infusion was initiated. It is therefore possible that the morphine pump implantation induced an antiallodynic effect, but that tolerance had developed by the time that the first recordings were made and hyper-responsiveness was established.

When pumps were implanted at a time that allodynia had been well established (i.e., 24 days after the IoN-CCI injury; Fig. 4), F 13640 caused an antiallodynic effect that now was statistically significant at all postimplantation times (Fig. 5); the effects of F 13714 required a longer duration of exposure for them to reach significance. This difference between the two 5-HT$_{1A}$ ligands is consistent with the expected (Colpaert, 1996; Colpaert et al., 2002) intensity dependence of cooperation; F 13640 exerts a greater activation of 5-HT$_{1A}$ receptors than F 13714 (Colpaert et al., 2002), so that its cooperation with nociception in inducing analgesia should develop more effectively. In stark contrast with its apparent ineffectiveness early after the injury (Fig. 2) as well as in other models of neuropathic pain (Colpaert et al., 2002), morphine was

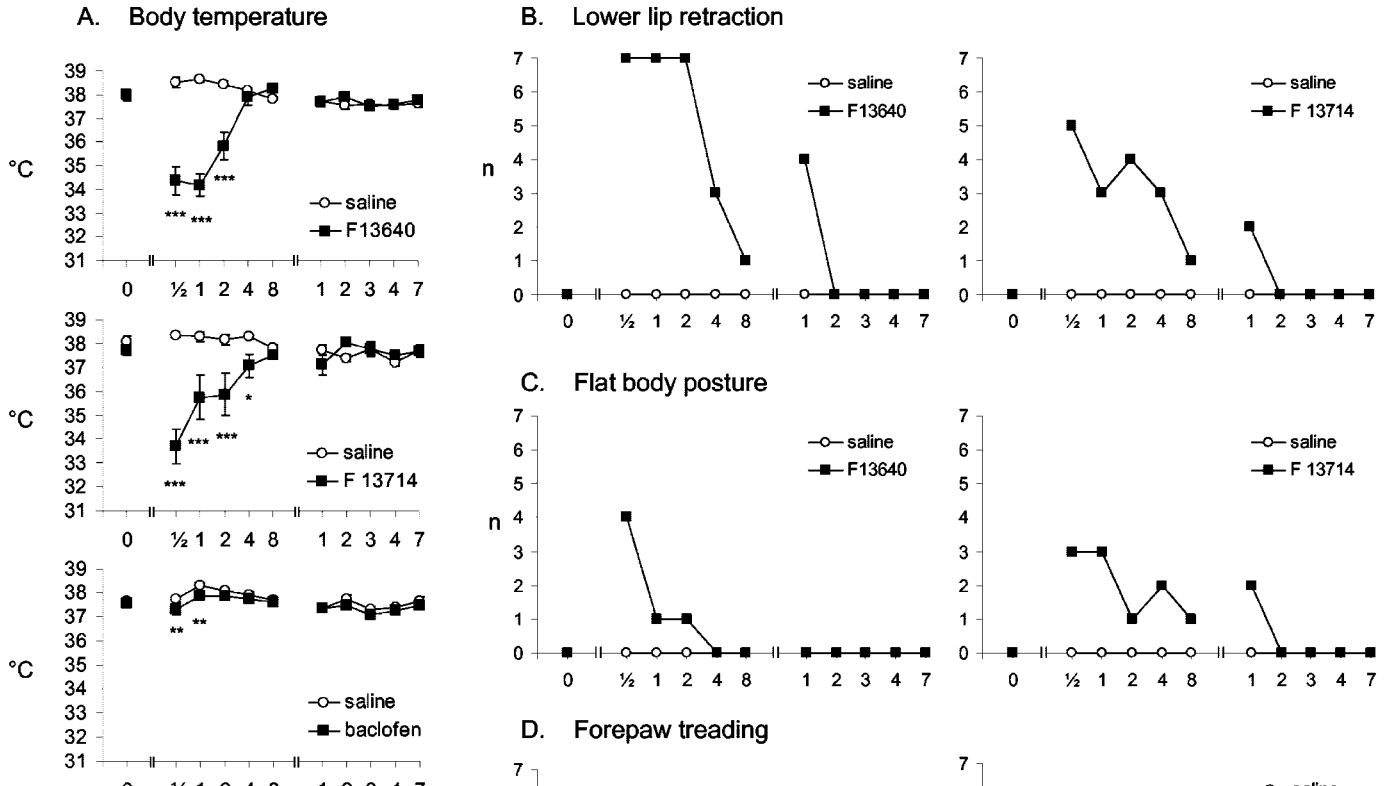

Fig. 6. Drug effects in normal rats. Data points in A represent the mean (±S.E.M.) rectal temperature immediately before and at the indicated hours and days after normal rats were implanted with pumps releasing F 13640 (0.63 mg/day), F 13714 (0.16 mg/day), morphine (5 mg/day), baclofen (1.06 mg/day), or saline (n = 7/group). Asterisks indicate a significant difference compared with control animals (Dunnett's test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data points in B, C, and D represent the number of rats (n = 7) demonstrating lower lip retraction, flat body posture, or forepaw treading, respectively.

clearly effective in the IoN-CCI model at this later stage (Fig. 5). However, tolerance to morphine developed, and the compound no longer produced a significant effect 2 weeks after its continuous infusion had been initiated. At the later stage, baclofen also was effective (Fig. 5).

Throughout the 1st week after their infusion had been initiated, neither F 13640 nor morphine produced any reliable effect in the first study (Fig. 2), whereas at that same time both agents produced a significant and consistent antiallodynic effect in the second study (Fig. 5). These apparently larger analgesic effects of both F 13640 and morphine when allodynia was well established relative to those observed early after the IoN-CCI injury, suggest that the ongoing nociceptive stimulation was of higher intensity at that later stage. This is because the (initial) antinociceptive action of both 5-HT_{1A} and opioid receptor activation, must on theoretical grounds be expected (Colpaert, 1996) to be larger because ongoing nociception is more intense (Colpaert, 1978, 1979; Colpaert et al., 1980).

Six days after pump removal, and not surprisingly, the analgesic effects, if any, of both morphine and baclofen had disappeared (Fig. 3). Remarkably, however, the effects of both F 13640 and F 13714 persisted at this point of time. The persistence of this effect is not likely to be due to the prolonged presence of F 13640; although the 2-week infusion of 0.63 mg/kg generates a stable plasma level of about 80 ng/ml, the latter drops below the 0.1 ng/ml detection limit 3 days after pump explantation (E. Carilla and F. C. Colpaert, unpublished observations). This may suggest that mechanical allodynia did not develop to the same level in 5-HT_{1A}-treated rats compared with saline-treated rats. The development of neuropathic pain is considered to be due to neuronal hyperexcitability, followed by neuroplastic changes and reorganization in the nervous system (i.e., from a process of central sensitization: for review, see Attal and Bouhassira, 1999; Ji and Woolf, 2001; Zimmerman, 2001). It is possible that the intensity-dependent cooperation between 5-HT_{1A} receptor activation and nociceptive stimulation paradoxically attenu-

ated this process of central sensitization. On the other hand, as pointed out by Jensen (2002), the development of neuropathic pain is preceded by a loss of sensory function, of nociceptive input in particular. This loss is exemplified in the IoN-CCI model by the loss of ipsilateral responsiveness to mechanical stimulation early after the injury (Figs. 1 and 4; Deseure et al., 2002). According to the concept that guided the present research, F 13640 mimics the central effects of nociceptive input (Colpaert, 1996; Colpaert et al., 2002). It is therefore possible that F 13640 preempts the development of neuropathic allodynia in the IoN-CCI model by providing the input that lacks early after the somatosensory nerve injury.

Transient hypothermia and signs of the 5-HT syndrome were observed during the first hours after pump implantation in rats treated with F 13640 and F 13714 (Fig. 6). In view of the neuroprotective properties of hypothermia (Marsala et al., 1994; Gunn and Gunn, 1998) and of 5-HT$_{1A}$ receptor agonists (Uchiyama et al., 2001), it is conceivable that the antiallodynic effects of F 13640 and F 13714, 6 days after pump removal, to some extent reflect a reduced development of allodynia caused by the neuroprotection during the first hours after IoN-CCI surgery. However, for up to 8 days after pump implantation, response scores in F 13640- and F 13714-treated rats were similar to those in rats treated with morphine or baclofen (Fig. 2), in spite of the latter two agents producing a much smaller and shorter-lived hypothermia (Fig. 6). Furthermore, at the time that allodynia started to develop, i.e., from days 4 to 6 onward (Fig. 1), none of the agents any longer produced hypothermia. It is also unclear how these putative neuroprotective properties could account for the antiallodynic effects that were observed when treatment was started only at a time when allodynia was already well established. Still, it would be interesting to investigate whether experimentally controlled hypothermia during or immediately after IoN ligation can prevent or attenuate the development of mechanical allodynia in this model.

The present data may also contribute to resolve the much debated controversy concerning the ability of opioids to relieve neuropathic pain (Arnér and Meyerson, 1988; Portenoy et al., 1990; Xu et al., 1999). The acute injection of high morphine doses in particular induces an antiallodynic effect in the IoN-CCI model (DeMulder et al., 1994; Deseure et al., 2002) as well as in other animal models of neuropathic pain (Attal et al., 1991; Ossipov et al., 1999; Bulka et al., 2002). However, because morphine is administered chronically, both the magnitude of its (initial) analgesic action and the rate at which tolerance develops, depend on persistent, ongoing nociception (Colpaert, 1978, 1979, 1996; Colpaert et al., 1980). This explains why morphine, administered at a time when allodynic hyper-responsiveness was not or not fully established (Fig. 1), produced little, if any, antiallodynic effect (Fig. 2), presumably because, during the hyporesponsive phase, tolerance had developed; and why morphine, administered only at a time when allodynia was fully established, produced an initially powerful antiallodynic effect to which a tolerance developed that required 2 weeks for it to result in a complete loss of its effectiveness (Fig. 5). In comparison, and as mentioned above, in normal rats that are not exposed to any persistent nociception, complete tolerance develops to the same 5-mg/day dose of morphine within 24 h (Colpaert et al., 2002). Similar to the present data, Bulka et al. (2002) found that in mononeuropathic rats injected (s.c.) twice daily with 10 mg/kg morphine, mechanical hypersensitivity to von Frey hair stimulation of the hind paw was initially inhibited, but after 2 weeks morphine's effects had disappeared almost entirely. Thus, although the acute administration of high morphine doses in particular may effectively alleviate neurogenic allodynia and induce side effects that are characteristic of opioids (Deseure et al., 2002), during chronic administration, tolerance to opioids develops at a rate that depends on the magnitude of the persistent nociception that is associated with the neuronal injury. This predictably results in observations that morphine's ability to alleviate neuropathic allodynia is transient and of a variable duration.

It is useful to note that, at the doses used here, neither F 13640 nor F 13714 fully restored mechanical responsiveness to the preinjury level (Fig. 5). It is likely, however, that higher doses would produce a full effect; both these agents, as well as morphine and baclofen, produce a full antiallodynic effect in this model upon their acute injection at sufficiently high doses (at a postinjury time at which allodynia was well established; Deseure et al., 2002). Indeed, the issue addressed here concerned the neuroadaptive changes to these effects in a condition where the compounds are administered chronically. In this condition, the effect of morphine decayed and that of baclofen remained stable, whereas that of the 5-HT$_{1A}$ receptor agonists, if anything, increased (Fig. 5).

In conclusion, the present findings indicate that the continuous infusion of the high-efficacy 5-HT$_{1A}$ receptor agonist F 13640 produces definite antiallodynic effects in the IoN-CCI rat model of trigeminal neuropathic pain. Consistent with a concept of signal transduction in nociceptive systems, 5-HT$_{1A}$ receptor activation seemed to cooperate, in both a duration- and intensity-dependent manner, with persistent nociception in this model, to produce antiallodynic effects. In contrast to the tolerance that developed to morphine-induced antiallodynic effects, the effects produced by F 13640 did not decay. Equally, and in contrast to both morphine and baclofen, the antiallodynic effects of F 13640 persisted 6 days after its administration had been discontinued. Further investigations of F 13640's mechanisms and usefulness in the treatment of trigeminal neuropathic pain should address several issues. Inverse tolerance developed to some extent to F 13640's antiallodynic action over the 2-week period of continuous infusion; it would be of interest to determine whether the antiallodynic effect would continue to grow with longer infusion periods. The molecular and neuroadaptive mechanisms of these agents being very different, it would also be of interest to examine the effects of chronically administered F 13640 in association with baclofen and morphine. Finally, the persistent antiallodynic effect that F 13640 produced 6 days after its administration was discontinued, warrants further study; this possible preemptive action on the development of neuropathic allodynia is scientifically and potentially also clinically interesting.

Acknowledgments

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