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Tolerance and dependence following chronic intracerebroventricular infusions of Tyr-D-Arg^2-Phe-Sar^4 (TAPS)

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Abstract

The dermorphin-derived tetrapeptide Tyr-D-Arg^2-Phe-Sar^4 (TAPS) was tested for its ability to induce tolerance, cross-tolerance, withdrawal and its substitution properties in rats subjected to chronic intracerebroventricular (i.c.v.) infusions of μ-opiate receptor agonists. Tolerance and cross-tolerance were assessed by quantification of the thermally induced tail-flick response. Chronic intracerebroventricular infusion of TAPS resulted in antinociception at almost 1000-fold lower doses compared to morphine sulphate and [D-Ala^2, MePhe^{4}Gly(ol)^5]enkephalin (DAMGO). Tolerance to the antinociceptive effect of TAPS developed similar to DAMGO and morphine sulphate. Cross-tolerance to intracerebroventricular bolus injections of DAMGO, but not of TAPS, was evident in rats rendered tolerant to morphine sulphate and TAPS. Naloxone-induced withdrawal was equally pronounced in animals treated with morphine sulphate, DAMGO or TAPS. TAPS substituted for morphine sulphate and vice versa regarding the withdrawal syndrome in a cross-over experimental design. In contrast to DAMGO, TAPS retains its antinociceptive effect following bolus administration in rats rendered tolerant to μ-opioid receptor agonists.

Keywords: Dermorphin; TAPS (Tyr-D-Arg^2-Phe-Sar^4); Tetrapeptide; Antinociception; Tolerance; Withdrawal

1. Introduction

Since the advent of opiates in the treatment of acute or chronic pain, separation of the antinociceptive from undesired side effects, such as respiratory depression and sedation, has been an elusive goal. Goodman and Pasternak (1985) discovered that the supraspinal antinociceptive effect of morphine apparently was mediated through a subclass of μ-opioid binding sites, subsequently termed the μ_1-opioid receptor subtype, which was characterized by its differential sensitivity to the μ-opioid receptor antagonists naloxazone and naloxonazine. In contrast, the respiratory depressant effect of morphine may be mediated by an opioid-binding site of lower affinity, which was termed the μ_2-opioid receptor subtype (Pasternak and Wood, 1986). The hypothesis of multiple opioid receptor subtypes stimulated the search for subtype-selective compounds which would be instrumental to separate the desired μ_1-opioid receptor subtype-mediated antinociceptive effect from the undesired, μ_2-opioid receptor subtype-related side effects.

Dermorphin, a heptapeptide originally extracted from the skin of *Phyllomedusa sauvagei* (Montecucchi et al., 1981), is a potent μ-opioid receptor agonist which exerts an antinociceptive potency exceeding that of morphine by 752–2170 times (Broccardo et al., 1981; Stevens and Yaksh, 1986). It is a unique peptide due to the presence of a D-amino acid residue (D-Ala^2). This residue is essential for the binding of dermorphin to μ-opioid receptors (Giagnoni et al., 1987; Guglietta et al., 1987). The N-terminal tetrapeptide (*H*-Tyr-D-Ala-Phe-Gly) of dermorphin was shown to be the minimum sequence required to produce an antinociceptive effect (Broccardo et al., 1981). The efficacy of the N-terminal tetrapeptide is further enhanced by the substitution of the D-Ala^2 residue with D-Arg and Gly^4 with the synthetic amino acid analog sarcosine (Sar)

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(Sato et al., 1987). The resulting tetrapeptide Tyr-D-Arg^2-Phe-Sar^4 (TAPS) was shown to be three times more potent than dermorphin in antinociceptive tests (Sato et al., 1987). Moreover, TAPS is an effective antinociceptive compound both, at the supraspinal (Paakkari et al., 1993) and spinal (Vonhof et al., 2001) level. TAPS was shown in rats to elicit respiratory stimulation, which was blocked by naloxonazine, indicating an agonist action at the μ_1-opioid receptor binding site (Paakkari et al., 1993). In contrast, naloxonazine pretreatment caused an augmentation of the ventilatory depressant action of the parent peptide dermorphin (Paakkari et al., 1990). A partial agonist/antagonist action for TAPS at the μ_2-opioid receptor binding site was suggested by evidence, showing that pretreatment of the animals with naloxonazine and TAPS effectively blocked the ventilatory depressant action of dermorphin (Paakkari et al., 1993). Additionally, TAPS antagonized the fentanyl-induced reduction of cAMP formation in human SH-SY5Y neuroblastoma cells, which appears to be mediated via μ_2-opioid type receptors, further corroborating an antagonist property of TAPS at this opioid-binding site (Smart and Lambert, 1996). Correspondingly, receptor binding studies supported a potential partial agonist/antagonist mode of action at the μ_2-opioid receptor subtype (Vonhof et al., 2001).

Physical dependence, withdrawal symptoms and respiratory depression may be related to stimulation of the proposed μ_2-opioid receptors (Pasternak, 2001; Pasternak and Wood, 1986; Ling et al., 1984). Thus, on the basis of a partial agonist/antagonist action at these sites, TAPS administration may result in a reduced expression of these undesired effects. In previous studies on the development of tolerance and cross-tolerance of TAPS systemic administration of the compound has been utilized (Nakata et al., 1986; Sakurada et al., 1993), resulting in an action on both, supraspinal and spinal binding sites. When antinociceptive tests are performed in this experimental setup, the effects of the systemically administered peptide at the spinal site may interfere with its effects at the supraspinal site. In order to investigate the effect of chronic infusions of TAPS on tolerance, withdrawal and cross-tolerance at the supraspinal level, we applied a chronic intracerebroventricular (i.c.v.) infusion paradigm in rats. For comparison, the effects of morphine sulphate and [D-Ala^2, MePhe^{4}Gly(ol)^5] enkephalin (DAMGO), two prototypic μ-opioid receptor agonists, were studied. The specific aims of the study were (1) to characterize the development of tolerance associated with chronic intracerebroventricular infusion of TAPS, DAMGO and morphine sulphate, (2) to determine the severity of naloxone-precipitated withdrawal and (3) to examine whether cross-tolerance to the effects of TAPS and DAMGO would develop during chronic intracerebroventricular infusion of morphine sulphate or TAPS. Additionally, the ability of substitution regarding withdrawal symptoms was tested in TAPS- and morphine sulphate-treated animals.

2. Materials and methods

2.1. Animals

Conscious male Sprague–Dawley rats (250–390 g, Taconic farms, Germantown, NY) were used. The rats were kept at 22 °C and a 12-h light/dark cycle, and were fed standard rat pellets and water ad libitum. The experiments were conducted according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Medicine, National Research Council, DHEW Publication no. (NIH) 85-23, 1985.

2.2. Surgical procedures

The rats were anesthetized using halothane (4% in 100% oxygen), and their heads mounted in a stereotactic instrument (David Kopf Instruments, Tujunga, CA). A midline sagittal scalp incision was made extending to the dorsal neck. A subcutaneous pocket in the midscapular area of the rat's back was prepared in order to receive the osmotic mini pump.

For chronic administration of opioid peptides, the ALZET mini pump (model 2001, Alza, Palo Alto, CA) in combination with a brain infusion kit (3–5 mm, Alza) was used according to the instructions of the manufacturer. The brain infusion kit was assembled by cutting the catheter tubing to a length of 6 cm and one end of the tubing was attached to the infusion cannula of the infusion kit. The catheter tubing and cannula were filled with opioid solution or vehicle. The brain infusion kit was than attached to the flow moderator of the mini pump. The filled infusion assembly was incubated with the attached osmotic mini pump in sterile saline at 37 °C for 3–6 h or at room temperature over a period of 24 h.

The bregma and lambda were used as reference points. A hole of 1-mm diameter was drilled through the skull over the right lateral ventricle (a.p. −0.8 mm, r.l. +1.22 mm from bregma) for insertion of the infusion cannula. The osmotic pump was inserted into the subcutaneous pocket. The cannula and anchoring screw were covered with cement (KERR laboratories, Michigan, USA.). Following the surgical procedure, the animals were allowed to recover for 20–24 h, after which antinociceptive testing was performed daily. The chronic intracerebroventricular treatment with TAPS, morphine sulphate or DAMGO commenced immediately on implantation of the brain infusion assembly. At the end of the experiment, the rats were sacrificed with an intraperitoneal (i.p.) overdose of pentobarbital sodium. The placement of the intracerebroventricular cannula was verified by injection of 10 μl methylene blue dye, followed by the dissection of the brain.

In the experiments carried out to study cross-tolerance, the same procedure as above was used. Additionally, a second hole was drilled over the left lateral ventricle (a.p. −0.8 mm and l.l. +3.00 mm from bregma) for the

placement of a stainless steel guide cannula for intracerebroventricular bolus injections. At the end of this set of experiments, the correct placement of the intracerebroventricular infusion cannula was confirmed by fast green dye, while that for the intracerebroventricular bolus cannula was verified by methylene blue.

For intracerebroventricular bolus injections, a 30-gauge cannula attached to a Hamilton microliter syringe with a polyethylene tubing (PE-20) was inserted into the previously implanted guide cannula. Ten microliters of drug solution were injected over a period of 60 s.

For the assessment of antinociception, the radiant tail-flick method was utilized with a commercial tail-flick apparatus (Socrel, Milano, Italy). Three consecutive readings were taken to establish the animals' baseline latencies. After the implantation of the osmotic mini pump, the reaction times were measured every day. Following intracerebroventricular bolus injections, the tail-flick latencies were measured at 15, 30, 60, 90 and 120 min. A maximal cut-off time of 12 s was chosen to limit tissue damage. The measure of the tail-flick latency time is expressed as percentage of the maximal possible effect (%MPE), i.e. %MPE=(postdrug response latency − predrug response latency)/(cut-off time − predrug response latency).

2.3. Development of tolerance

This experiment was carried out over a 6-day period. In each group, a minimum of four rats were used. The osmotic mini pumps were prefilled with artificial cerebrospinal fluid (aCSF, 1 μl/h), morphine sulphate (10 and 30 nmol/μl/h), TAPS (3, 10 or 30 pmol/μl/h) or DAMGO (0.3 and 1.0 nmol/μl/h). The doses for morphine sulphate and DAMGO were chosen according to experiments on chronic intrathecal infusions (Stevens and Yaksh, 1989). The animals' body weights were measured on the day before the surgical procedures and on each day after the onset of the chronic infusion. Additionally, baseline antinociception was measured before the osmotic mini pumps were implanted and on the following five consecutive days during the experiment.

2.4. Naloxone-induced withdrawal

On day 6 of the chronic infusion, the animals were injected with an intraperitoneal bolus of naloxone (20 mg/kg) and the severity of withdrawal was assessed for a period of 1 h at 15-min intervals along a standard rating scale including the following behavioral parameters according to Frederickson and Smits (1973): teeth chattering, salivation, diarrhea, defecation, wet-dog shakes, escape attempts, exploratory activity, rhinorrhea, urination, swallowing, tremor, hunchback posture, piloerection, reaction to poking, jumping, digging, ptosis, ear blanching, exophthalmus, self-stimulation, writhing, excessive cleaning/grooming.

2.5. Determination of cross-tolerance

The duration of the experiment was 9 days. Prior to surgery, the body weights of the animals and the baseline tail-flick latencies were determined. The osmotic mini pumps were prefilled with either morphine sulphate (30 nmol/μl/h), aCSF (vehicle, 1 μl/h) or TAPS (30 pmol/μl/h), and the infusion was started immediately following the implantation of the pump. Body weight and antinociception were determined before the surgical procedures and after 1, 2, 5 and 7 days during chronic intracerebroventricular infusion. A bolus intracerebroventricular injection of 10 μl of either DAMGO (30, 100 or 300 pmol) or TAPS (0.6, 6 or 30 pmol) was administered on the third day of the experiment.

2.6. Substitution experiments

The osmotic mini pumps were replaced on day 4 with a new device, which was prefilled with either morphine sulphate, DAMGO or TAPS. The substitution-elicited withdrawal was assessed for 60 min on day 6, since 1–2 days after pump change is required to overcome the dead space in the catheter and the intracerebroventricular cannula. Signs of withdrawal were assessed as described above.

2.7. Statistical analysis

The data are presented as arithmetical means ± S.E. for the indicated number n of animals per group. Analysis of variance was used to perform statistical analysis of normally distributed data. The Kruskal–Wallis test followed by the Mann–Whitney *U*-test was used for the evaluation of non-parametric values. A *p*-value of less than 0.05 was considered statistically significant. The statistical calculations were done using the CSS Software package (StatSoft, Tulsa, OK).

2.8. Drugs

DAMGO and TAPS were obtained from Peninsula, Belmont, CA. Naloxone was generous gift from DuPont Phamaceuticals, Wilmington, DE.

3. Results

3.1. Antinociception and development of tolerance following chronic intracerebroventricular infusion of opioids or vehicle

Chronic intracerebroventricular infusion of TAPS dose-dependently induced an increase in the tail-flick latencies with a maximum on days 1 and 2 following the onset of the chronic infusion (Fig. 1). Statistical analysis using repeated measures ANOVA revealed significant differences concerning the dose and time variables ($p < 0.001$, $F = 3.728$). Infusion of morphine sulphate resulted in a

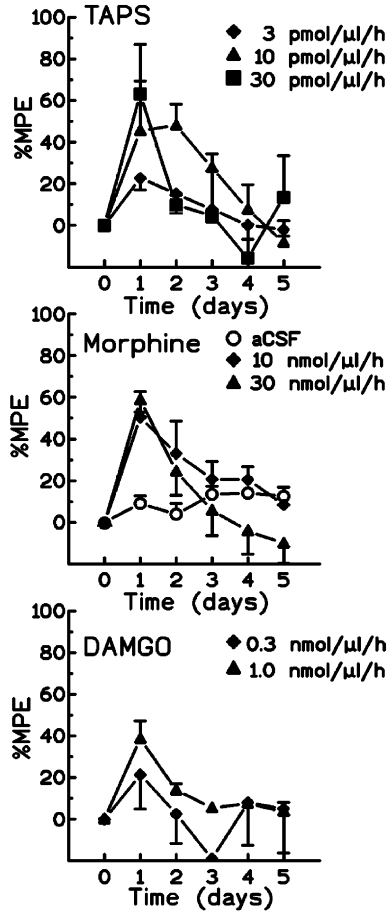

Fig. 1. Time course of the development of tolerance to the antinociceptive effect of TAPS, morphine sulphate and DAMGO. The %MPE was calculated from the tail-flick latencies of rats treated with TAPS (3, 10 or 30 pmol/µl/h, upper panel), morphine sulphate (10 or 30 nmol/µl/h) or aCSF (control, 1 µl/h, middle panel) and DAMGO (0.3 or 1.0 nmol/µl/h, lower panel) in the morning before pump implantation and for 5 days thereafter. Data represent means ± S.E., $n=4$ in all groups.

transient antinociceptive effect at 10 and 30 nmol/µl/h, respectively ($p<0.001$, $F=9.977$). Following a maximal effect on day 1 of the experiment, a gradual decline of antinociception was noted, until the effect was not different from the vehicle controls on day 5 at the end of the experiment. Intracerebroventricular infusion of 0.3 and 1.0 nmol/µl/h of DAMGO produced similar, albeit less pronounced antinociceptive effects as compared to morphine sulphate ($p<0.01$, $F=2.789$). Intracerebroventricular infusion of the vehicle (1 µl/h of aCSF) had no effect on tail-flick latencies during the infusion period over 5 days.

3.2. Development of cross-tolerance

3.2.1. Antinociception after intracerebroventricular bolus injections of TAPS during chronic intracerebroventricular infusion of vehicle, morphine sulphate or TAPS (day 3)

Intracerebroventricular bolus injections of TAPS (0.6, 6, 30 pmol/10 µl) in vehicle-treated rats produced a lasting, dose-related antinociceptive effect (Fig. 2). While 0.6 pmol TAPS i.c.v. had no significant effect on tail-flick latencies, the two higher doses induced a significant antinociceptive response, which lasted over 120 min and exceeded the observation period. During continuous intracerebroventricular infusion of 30 nmol/µl/h morphine sulphate i.c.v., bolus injections of TAPS produced a dose-related antinociceptive effect. The magnitude of this effect was not significantly different from the responses to TAPS during vehicle infusion. When TAPS was injected intracerebroventricularly in animals, which were chronically infused with 30 pmol/µl/h of TAPS i.c.v., it continued to exert a dose-dependent antinociception. There were no significant differences of the effects of the intracerebroventricularly administered TAPS bolus injection in the TAPS-pretreated vs. the vehicle-pretreated rats. Log-transformation of the maximum effects in the treatment groups correspondingly lacked a rightward shift as it was observed when bolus doses of DAMGO were injected intracerebroventricularly. Least square linear regression resulted in ED_{50} values per rat of

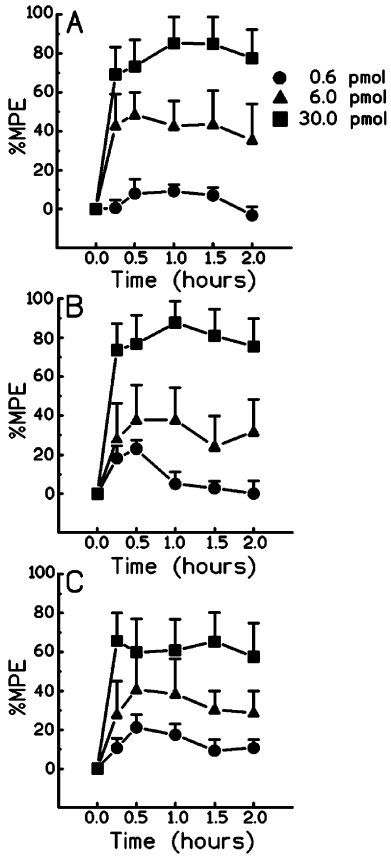

Fig. 2. Antinociceptive effect of bolus doses of TAPS (0.6, 6 or 30 pmol/10 µl, i.c.v.) during intracerebroventricular infusions of aCSF (control, 1 µl/h, panel A), morphine sulphate (30 nmol/µl/h, panel B) or TAPS (30 pmol/µl/h, panel C) on day 3. Data (means ± S.E.) are shown as %MPE. Three-way ANOVA with repeated measures demonstrated no significant difference between infusion paradigms ($p=0.871$) but showed a statistically significant interaction for dose ($p<0.001$) and time ($p<0.001$) in all groups.

9.6 pmol for TAPS during aCSF infusion, 10.0 pmol during morphine sulphate infusion and 14.5 pmol during TAPS infusion (Fig. 4).

3.2.2. Antinociception after intracerebroventricular bolus injections of DAMGO during chronic infusion of vehicle (aCSF), morphine sulphate or TAPS (day 3)

Intracerebroventricular bolus injections of DAMGO produced a dose-related antinociceptive effect (Fig. 3) in animals infused with aCSF intracerebroventricularly. Fifteen to thirty minutes following intracerebroventricular administration of 30, 100 and 300 pmol (10 µl volume) DAMGO, the antinociceptive effect was maximal, reaching 40%, 90% and 85% of the MPE, respectively. After the 300 pmol bolus, the antinociceptive effect remained significantly increased for up to 60 min following the injection.

During chronic infusion of 30 nmol/µl/h of morphine i.c.v., bolus injections of DAMGO produced an attenuated response, which was significantly reduced, compared to corresponding values during vehicle infusion ($p<0.05$). Only the highest bolus dose tested, 300 pmol, produced a significant antinociceptive effect with about 60% of the MPE, which was achieved 15 min after drug injection and lasted for 30 min after drug infusion. The corresponding log dose–response curve for the maximum effects of DAMGO intracerebroventricular bolus injections in animals which were treated with a continuous intracerebroventricular infusion of morphine sulphate showed a rightward shift compared to animals receiving an infusion of aCSF (Fig. 4). The ED_{50} values per rat of DAMGO were 19.2 pmol during aCSF infusion and 179 pmol during chronic morphine sulphate infusion.

During chronic intracerebroventricular infusion of 30 pmol/µl/h of TAPS, intracerebroventricular bolus injections of DAMGO produced a dose-related antinociceptive effect. The effect of the 100 pmol dose of DAMGO was reduced as compared to the corresponding response during vehicle infusion. The highest dose, however, induced an antinociceptive response of similar magnitude compared to vehicle infusion. Log transformation of the maximum effect resulted in a similar shift to the right compared to the chronic morphine sulphate infusion. The calculated ED_{50} value of DAMGO during TAPS infusion was 114 pmol/rat.

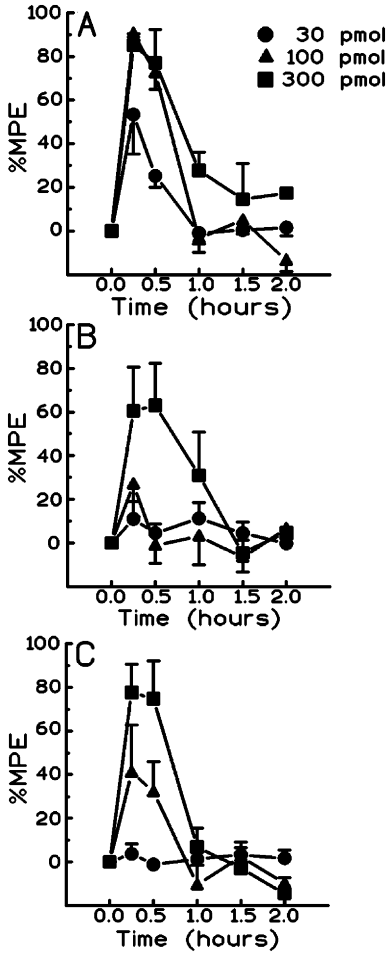

Fig. 3. Antinociceptive effect of bolus doses of DAMGO (30, 100 or 300 pmol/10 µl, i.c.v.) during intracerebroventricular infusions of aCSF (control, 1 µl/h, panel A), morphine sulphate (30 nmol/µl/h, panel B) or TAPS (30 pmol/µl/h, panel C) on day 3. Data represent mean ± S.E. and are shown as %MPE. Three-way ANOVA with repeated measures revealed a statistically significant difference between infusion paradigm ($p<0.05$), bolus dose ($p<0.001$) and time ($p<0.001$), $n=4$ in all groups.

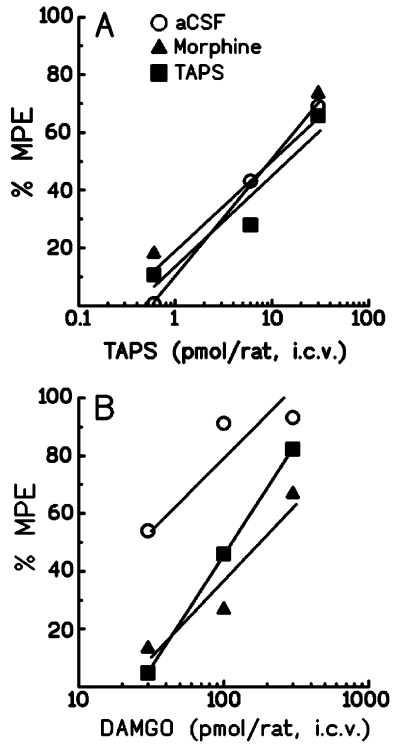

Fig. 4. Log dose–response curves for intracerebroventricular bolus injections of TAPS (panel A) and DAMGO (panel B) during chronic infusion of aCSF, morphine sulphate and TAPS. The maximum response after each bolus dose was used to construct log dose–response curves which were analyzed with least square linear regression to determine ED_{50} values ($n=4–6$).

3.3. Development of physical dependence and withdrawal

3.3.1. Naloxone-induced withdrawal after intracerebroventricular infusion of morphine sulphate, DAMGO or TAPS (day 6)

Naloxone injection (20 mg/kg, i.p.) produced potent withdrawal signs in the TAPS-treated animals (Fig. 5). In both groups, DAMGO and TAPS treatment, there was a negative dose–response, in that the animals continuously infused with higher opioid doses showed less withdrawal symptoms than with the lower doses. In TAPS-treated animals, the animals which were infused with the highest dose of TAPS (30 pmol/μl/h) exhibited only minimal withdrawal symptoms which were equal in magnitude to the vehicle-treated group. The total withdrawal score was significantly enhanced, when naloxone was injected during morphine sulphate (30 nmol/μl/h) infusion compared to the group of animals receiving a continuous intracerebroventricular infusion of aCSF ($p<0.05$). Similarly, the animals receiving 0.3 and 1.0 nmol/μl/h of DAMGO exhibited significantly more withdrawal symptoms after naloxone injection. Naloxone itself produced no significant behavioral changes in vehicle-treated rats.

3.3.2. Withdrawal symptoms during substitution

In the substitution experiments baseline conditions for the presence of withdrawal symptoms were established at the time (Table 1), when the continuous infusion of aCSF was changed to infusion of morphine sulphate (30 nmol/μl/h) and TAPS (30 pmol/μl/h). The body weights remained constant following the change to morphine or TAPS infusion. When morphine sulphate was substituted with aCSF, however, the total as well as single (e.g. wet-dog shakes, irritability) withdrawal scores significantly increased. Correspondingly, the body weight dropped significantly by

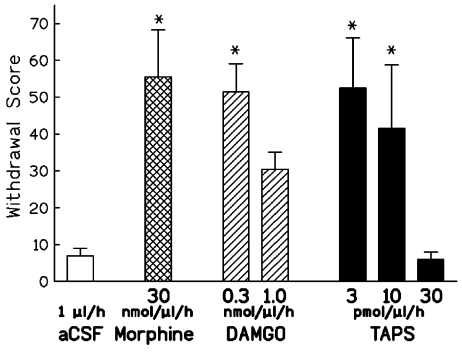

Fig. 5. Naloxone (20 mg/kg, i.p.)-induced withdrawal on day 6 during intracerebroventricular infusion of aCSF (l μl/h), morphine sulphate (30 nmol/μl/h), DAMGO (0.3 or 1.0 nmol/μl/h) and TAPS (3, 10 or 30 pmol/μl/h). Data (means ± S.E.) are presented as total scores of withdrawal symptoms which were observed during a 60-min monitoring period following naloxone administration ($n=4$ in all groups). Kruskal–Wallis nonparametric ANOVA followed by Mann–Whitney *U*-test was used to analyze statistical differences between the groups. Asterisks indicate a statistically significant difference compared to controls receiving aCSF intracerebroventricularly ($p<0.05$).

Table 1
Withdrawal symptoms in substitution paradigms

Change of treatment	Total score	Wet-dog shakes	Irritability
aCSF to morphine ($n=9$)	57 ± 12	10 ± 7	4 ± 2
aCSF to TAPS ($n=6$)	46 ± 11	4 ± 3	7 ± 3
Morphine to aCSF ($n=9$)	93 ± 17***	28 ± 9***	11 ± 2***
Morphine to TAPS ($n=5$)	87 ± 30	11 ± 9	13 ± 5
TAPS to aCSF ($n=6$)	124 ± 24***	45 ± 14***	12 ± 5
TAPS to morphine ($n=7$)	49 ± 14*	13 ± 6*	3 ± 1
TAPS to TAPS ($n=4$)	41 ± 7**	3 ± 2*	1 ± 1*

Data represent means ± S.E. Chronic intracerebroventricular infusions were performed at a flow rate of 1 μl/h. The animals were treated with either morphine sulphate (30 nmol/μl/h) or TAPS (30 pmol/μl/h). The treatment was changed to TAPS, morphine sulphate or aCSF at identical concentrations and flow rate on day 4. The numbers of rats in each group are given in parentheses. Statistical evaluation was done using the Kruskal–Wallis nonparametric ANOVA followed by Mann–Whitney *U*-test.

*$p<0.05$, compared to "TAPS to aCSF".

**$p<0.01$, compared to "TAPS to aCSF".

***$p<0.05$, compared to "aCSF to Morphine" and "aCSF to TAPS".

4.3 ± 1% ($p<0.05$). When morphine sulphate treatment was switched to TAPS, there was an increase of the total withdrawal score, which was not statistically significant, when compared to the control groups, possibly due to an increased variability. The incidence of wet-dog shakes was not increased in contrast to the substitution of morphine infusion with aCSF. Further, no weight loss was observed, indicating that TAPS substituted for morphine sulphate in regards to the behavioral and physiologic test paradigms.

When the continuous intracerebroventricular infusion of TAPS was changed to aCSF, significant withdrawal symptoms occurred ($p<0.05$). Body weight dropped by 5.2 ± 0.3% ($p<0.05$), corroborating the onset of withdrawal. When morphine sulphate was infused following TAPS, the withdrawal symptoms were attenuated compared to the substitution with aCSF indicating that morphine substituted for TAPS. A third group of animals was established where the initial infusion of TAPS was replaced by a second continuous infusion of TAPS at the same dose serving as a control group. There was no difference in the total withdrawal score compared to the switch from TAPS to morphine treatment and the exchange of aCSF to TAPS or morphine treatment.

4. Discussion

In 1989, Lazarus et al. (1989) demonstrated that the affinity of several dimeric dermorphin-derived tetrapeptides to μ-opioid receptors was reduced, except for TAPS which exhibited an increased μ-opioid receptor affinity and decreased δ-opioid receptor affinity, resulting in an enhanced μ-opioid receptor selectivity compared to dermorphin. The potent and long lasting antinociceptive response to TAPS (Paakkari et al., 1993; Sasaki et al., 1984; Vonhof et al., 2001) in several antinociceptive test paradigms has been

attributed to its high affinity to μ-opioid receptors and its ability to inhibit enkephalin metabolizing enzymes (Sato et al., 1987). In the present study, continuous intracerebroventricular infusions of TAPS, in order to minimize interference of spinal antinociceptive mechanisms were performed to investigate the development of tolerance and withdrawal.

The continuous chronic intracerebroventricular infusions of TAPS produced antinociceptive effects which, in their extent, were comparable to morphine sulphate, but at up to 1000-folds increased potency. The time course of antinociception after infusion of morphine sulphate and TAPS showed a maximum on the first day following the onset of the experiment and tolerance developed within another 2–3 days (Fig. 1). In order to examine cross-tolerance, the antinociceptive effects of bolus injections of DAMGO or TAPS in controls (aCSF-treated) and in rats rendered tolerant to morphine sulphate or TAPS were compared. When a bolus of DAMGO was administered intracerebroventricularly, cross-tolerance was observed to the highest extent in animals receiving chronic morphine sulphate infusions with an increase of the ED_{50} by about 9–10-folds compared to control animals (Figs. 3 and 4). The ED_{50} for DAMGO to elicit an antinociceptive response in TAPS-pretreated animals was increased by 6.7-folds, indicating cross-tolerance also exists between TAPS and DAMGO. In contrast, when TAPS was administered as an intracerebroventricular bolus, the ED_{50} remained similar in aCSF-, morphine- and TAPS-pretreated animals (Figs. 2 and 4). Thus, there apparently exists an asymmetric cross-tolerance between DAMGO, TAPS and morphine with TAPS retaining its antinociceptive potential following bolus administration even in otherwise opiate-tolerant animals. Given the complexity of mechanisms involved in the development of tolerance in view of the potential involvement of multiple μ-opiate receptor subtypes, there obviously is no simplistic explanation for this intriguing observation. The development of tolerance and the acute antinociceptive effect of TAPS are likely to be mediated by different mechanisms. TAPS may act as full agonist at the μ_1-opioid receptor subtype (Vonhof et al., 2001) and consequently the development of tolerance in the chronic infusion paradigm may be related to down-regulation and/or desensitization of this opioid-binding site. On the other hand, intracerebroventricular bolus injections of TAPS in morphine sulphate- and TAPS-tolerant rats may still produce an antinociceptive effect via other opioid receptor subtypes such as the proposed μ_2-opioid binding site, by means of a partial agonist mode of action (Vonhof et al., 2001; Smart and Lambert, 1996).

Our second goal was to assess physical dependence following chronic treatment with TAPS, DAMGO or morphine sulphate. Signs of withdrawal were observed after TAPS, morphine sulphate and DAMGO treatment following the administration of naloxone, demonstrating that all three agents cause dependence and withdrawal can be induced with an unselective opiate antagonist (Fig. 5). In these experiments, a rather high dose (20 mg/kg) of naloxone was used, which may have resulted in an inhibition of behavioral responses. This may have masked withdrawal symptoms, which would otherwise be observed after lower doses of naloxone. An overall reduction of withdrawal scores compared to the experiments, when chronic opiate treatment was substituted with aCSF treatment can indeed be noted. However, significant dose-related differences remain evident. The scores following administration of naloxone in animals treated with 30 pmol/μl/h of TAPS were significantly reduced compared group of animals treated with 0.3 pmol/μl/h ($p < 0.05$). A similar trend was noted in the animals receiving chronic morphine infusions (Fig. 5). Following the administration of the highest dose of TAPS (30 pmol/μl/h), the overall withdrawal score was similar to the control group. This reversed dose response concerning the induction of withdrawal may be related to a concentration-dependent competitive antagonism at the involved receptor sites.

The development of withdrawal symptoms was further studied by abrupt cessation of opioid therapy and substitution with another opioid. When morphine sulphate or TAPS infusion was followed by infusion of aCSF, a withdrawal syndrome, including a significant decrease in body weight ensued. When TAPS was infused following morphine treatment, the development of withdrawal signs was suppressed, suggesting that TAPS substituted for morphine sulphate. Similarly, the occurrence of withdrawal in TAPS-pretreated animals was avoided when TAPS pretreatment was changed to morphine sulphate or a second infusion of TAPS (Table 1).

Previous studies on the development of physical dependence, tolerance and withdrawal using TAPS showed that naloxone-precipitated withdrawal resulted in less changes in some but not all behavioral aspects of the withdrawal syndrome in TAPS-dependent compared to morphine sulphate-dependent animals (Nakata et al., 1986). Additionally, cessation of treatment resulted in a less severe change in body weight in TAPS-treated animals compared to morphine sulphate treatment (Nakata et al., 1986). In these studies, TAPS was unable to substitute for morphine sulphate in terms of loss of body weight, food and water intake. In the experiments by Sakurada et al. (1993), investigating the development of tolerance and cross-tolerance to the antinociceptive effects of TAPS and morphine sulphate in a tail-flick and digit-pinching test paradigm, animals rendered tolerant to TAPS did not exhibit cross-tolerance to morphine sulphate. Vice versa, morphine sulphate-tolerant animals exhibited also cross-tolerance to TAPS treatment. These findings are in contrast to our results using chronic intracerebroventricular infusions and intracerebroventricular bolus injections in order to more selectively target supraspinal opioid-binding sites. Here, no cross-tolerance was observed when TAPS was administered as an intracerebroventricular bolus in morphine sulphate-tolerant rats. Thus, the route of administration, i.e. central (intracerebroventri-

cular) vs. systemic administration, appears to result in different response patterns regarding cross-tolerance.

The concept of μ-opioid receptor subtypes, as proposed by Pasternak (2001), Pasternak and Wood (1986) and Ling et al. (1984), relates the desired effects of morphine sulphate to μ_1-opioid receptors and its undesired actions (respiratory depression, physical dependence) to μ_2-opioid receptors. Within this concept, TAPS may represent a candidate antinociceptive compound for the separation of the desired opiate actions from at least part of the unwanted side effects. To date, however, definite proof for the existence of μ-opiate receptor subtypes remains elusive. No distinct genetic sequences encoding for subtypes of the μ-opiate receptor have been isolated so far. Recently, on the other hand, several variants of μ-opiate receptor mRNA resulting from differential splicing have been identified, which may be translated into distinct receptor subtype proteins mediating individual pharmacological responses (Pasternak, 2001). It is yet to be proven that these splice variants of the μ-opioid receptor protein are identical with the proposed μ_1- and μ_2-opioid receptor subtypes.

In conclusion, chronic intracerebroventricular infusion of TAPS is characterized by potent antinociception at almost 1000-fold lower doses compared to morphine sulphate and DAMGO. Development of tolerance to TAPS occurs with a similar time course as to DAMGO and morphine sulphate. In contrast to DAMGO, however, TAPS retains its antinociceptive effect following a bolus administration in rats rendered tolerant μ-opioid receptor agonists. This effect may be due to a differential action at μ-opioid receptor subtypes.

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