Chronic forced swim stress inhibits ultra-low dose morphine-induced hyperalgesia in rats

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Ultra-low doses of morphine (UL-morphine) induce hyperalgesia, which is assumed to be mediated by stimulatory G proteins ($G_{\alpha s}$) signaling pathway. $G_{\alpha s}$ pathway inhibition and chronic stress both attenuate development of tolerance to analgesic effect of morphine. This study evaluated the effect of chronic stress on UL-morphine-induced hyperalgesia to find out if chronic stress interacts with the $G_{\alpha s}$ signaling pathway. Repeated daily forced swim stress was applied to induce chronic stress. UL-morphine (1 μg/kg, intraperitoneal)-induced hyperalgesia was assessed using the tail-flick test on day 6, in male rats that during days 1–5 received different treatments of swim stress, dexamethasone, swim stress following adrenalectomy (ADX) or swim stress after sham operation. Chronic stress by itself induced hyperalgesia in control and sham-operated rats but inhibited UL-morphine-induced hyperalgesia. In ADX animals, chronic stress did not produce hyperalgesia and could not inhibit UL-morphine-induced hyperalgesia. Chronic dexamethasone produced hyperalgesia but did not change the UL-morphine-induced hyperalgesia. Inhibition of UL-morphine hyperalgesia by chronic stress suggests that chronic stress interacts with the $G_{\alpha s}$ signaling pathway, which is responsible for UL-morphine-induced hyperalgesia. The absence of this effect in the ADX-rats or after repetitive dexamethasone administration demonstrates that hypothalamic–pituitary–adrenal (HPA) axis activation is necessary for controlling UL-morphine-induced hyperalgesia. Finally, the interaction of stress with the $G_{\alpha s}$ signaling pathway may provide an explanation for the inhibitory effect of stress on development of tolerance to the analgesic effect of morphine. *Behavioural Pharmacology* 18:667–672 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Morphine has a dual effect on pain perception. It is usually used as an analgesic, but a growing body of evidence has shown that morphine at ultra-low doses [e.g. 1 μg/kg, intraperitoneal (i.p.)] (UL-morphine) can elicit hyperalgesia (Crain and Shen, 2001a, 2004; Ruscheweyh and Sandkuhler, 2005; Galeotti *et al.*, 2006).

Opioid receptors contain seven transmembrane domains and are coupled to GTP-binding proteins. Morphine analgesia is mediated by opioid receptors coupled to inhibitory G-proteins ($G_{i/o}$) and consequent inhibition of adenylyl cyclase, activation of K^+ channels and inhibition of calcium conductance (Childers, 1991; Williams *et al.*, 2001; Powell *et al.*, 2002; Nestler, 2004).

The hyperalgesic effect of morphine is mediated by opioid receptors coupled to excitatory G-proteins ($G_{\alpha s}$). Blocking $G_{\alpha s}$ signaling using cholera toxin B subunit or oseltamivir blocks and reverses the hyperalgesic effect of UL-morphine (Crain and Shen, 1996, 1998, 2001b, 2004).

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Chronic administration of morphine produces analgesic tolerance. A variety of mechanisms, such as internalization and downregulation of opioid receptors, increased activity of some protein kinases (PKA, PKC, CaMKIIα, GRKs) (Williams *et al.*, 2001; Nestler, 2004), and changes in the expression of different G-protein subunits and G protein regulatory proteins (RGS), are believed to be involved in development of morphine tolerance (Ingi *et al.*, 1998; Garnier *et al.*, 2003; Clark *et al.*, 2004; Javan *et al.*, 2005). Some of these events may result from repetitive activation of the $G_{\alpha s}$ signaling pathway during chronic morphine administration. Consistent with earlier reports (Shen and Crain, 1989; Crain and Shen, 2000, 2001b) we have also reported that inhibition of $G_{\alpha s}$ signaling using oseltamivir prevents the development of tolerance to the analgesic effect of morphine (Movahedi *et al.*, 2006).

Stressors such as pain (Melzack, 1990; Solan and Melzack, 1999; Javan *et al.*, 2005), psychological stress (Takahashi *et al.*, 1988, 1992; Tokuyama *et al.*, 1989; Takahashi and Kaneto, 1991) and forced swim stress (Ghiafeh-Davoodi *et al.*, 2005) prevent morphine tolerance.

As both chronic stress and inhibition of the $G_{\alpha s}$ signaling pathway could prevent tolerance to morphine analgesic, there is a possibility for the hyperalgesic effect of morphine, mediated by the $G_{\alpha s}$ signaling pathway, to be inhibited by stressors. In this study we examined the possible effect of chronic forced swim stress on the hyperalgesic effect of morphine.

Methods

Subjects

All experiments were carried out on adult male Wistar rats weighing 200–250 g. Animals were housed four per cage under a 12 h light/12 h dark cycle in a room with average ambient temperature 22 ± 1°C. Food and tap water were freely available. Adrenalectomized (ADX) rats received saline instead of tap water. Animals were handled daily for 5 days before the experimental day, to minimize nonspecific stress responses. Experiments followed the guidelines for animal care approved by the committee for scientific ethics, Tarbiat Modares University.

Forced swim stress

Rats were subjected to a 5-day forced swim procedure (5 min/day) in a cylindrical plastic container (diameter = 30 cm, height = 50 cm) filled with 20 ± 1°C water to a depth of 35 cm. Animals with sham-swimming experience were allowed to wade in the same cylinder containing 2–3 cm of water. After the swimming sessions, rats were immediately dried using a warm container with a towel-covered floor (Mogil *et al.*, 1996; Quintero *et al.*, 2003).

Corticosterone assay

Plasma corticosterone level was measured to evaluate the potency of forced swimming as a stress to activate the hypothalamic–pituitary–adrenal (HPA) axis. The measurement was done in control and experimental groups 15 min after the first, third and fifth swimming sessions. Measurement was also done 24 h after the fifth swimming session. Rats were killed by decapitation between 09.00 and 10.00 h and trunk blood was collected in tubes containing 5% ethylenediaminetetraacetic acid. Plasma samples were separated using centrifugation of blood at 2500 rpm for 10 min. Samples were immediately stored at −20°C until the time of corticosterone assay, which was carried out using a radioimmunoassay kit for rat [^{125}I]-corticosterone (DRG International Inc., USA). The sensitivity of the assay was 0.25 ng/ml (100% reaction to corticosterone, 0.34% cross-reaction with desoxycorticosterone, and less than 0.10% cross-reaction with other steroids).

Evaluation of nociceptive threshold

Antinociception was assessed by tail-flick test (D'Amour and Smith, 1941) before and 30 min after drug injection. The tail-flick latency for each data point was determined using three consequent measures (1/min). Intensity of the light beam was adjusted to have a baseline reaction time equal to 4 and 5 s. A cut-off time of 15 s was used to avoid damage to the tail tissue.

The nociceptive threshold was assessed 30 min, 1, 2 and 3 h after saline or UL (1 μg/kg, i.p.) morphine hydrochloride (Temad, Tehra, Tehran, Iran). All the injections were given in the volume of 1 ml/kg (i.p.).

Adrenalectomy

To evaluate the role of the HPA axis in the inhibitory effect of chronic stress on UL-morphine-induced hyperalgesia, the adrenal gland was removed bilaterally. Animals were anesthetized by i.p. administration of ketamine (50 mg/kg) and xylazine (5 mg/kg). The adrenal glands were removed bilaterally via dorsal incisions. The sham operation was performed by making a bilateral dorsal incision and simply locating and exposing the adrenals. The animals were tested 5 days after the operation (Esmaeili-Mahani *et al.*, 2005). Change in nociceptive threshold was assessed 5 days after operation and/or 24 h after the fifth stress session in ADX-stressed animals.

Evaluation of morphine hyperalgesia after chronic administration of dexamethasone

To evaluate the role of the HPA axis and its glucocorticoid products in the effect of chronic stress on UL-morphine-induced hyperalgesia, dexamethasone, a synthetic corticosterone (Javan *et al.*, 2006), was used. Two mg/kg of dexamethasone was injected i.p. for 5 days and the hyperalgesic effect of UL-morphine was assessed on the sixth day.

Effect of oseltamivir on ultra-low doses of morphine and chronic stress-induced hyperalgesia

To examine the involvement of the $G_{\alpha s}$ signaling pathway in UL-morphine-induced hyperalgesia and in the effect of chronic stress or dexamethasone, oseltamivir (1 mg/kg), which blocks $G_{\alpha s}$ signaling (Crain and Shen, 2004), was applied before daily swimming sessions for 5 days. Hyperalgesia was assessed on the sixth day, 24 h after the last oseltamivir and stress session.

Statistical analysis

Results are expressed as mean ± SEM. Differences between means were compared by one-way or two-way analysis of variance followed by the Newman–Keuls post hoc test. P less than 0.05 was considered significant.

Results

Plasma corticosterone level in stressed and adrenalectomized animals

Stress increased the level of corticosterone as measured 15 min after the first, third and fifth swimming sessions, and also 24 h after the fifth swimming session. Different groups were used for each data point (n = 6–8). The

Table 1 Plasma corticosterone level in rats after different sessions of forced swim stress and adrenalectomy

Groups	Corticosterone (ng/ml)
Sham	194.2 ± 15.09
ADX	9.7 ± 1.4
Sham + stress (first session)	836.99 ± 60.53***
Sham + stress (third session)	967.81 ± 42.97***
Sham + stress (fifth session)	924.74 ± 92.87***
Sham + stress (fifth session) 24 h later	352.9 ± 54.054**

Values represent mean ± SEM ($n \geq 6$).
$F(5,34) = 91.98$, $^{**}P < 0.01$, $^{***}P < 0.001$.

measured level of corticosterone in ADX animals confirmed the effectiveness of the ADX operation (Table 1).

Effect of chronic stress on nociceptive threshold and morphine hyperalgesia

As shown in Fig. 1a, an UL-morphine (1 μg/kg)-induced hyperalgesia, which persisted for 3 h [$F(4,30) = 17.98$, $P < 0.001$]. Chronic stress inhibited this hyperalgesic effect of UL-morphine and interestingly, UL-morphine produced analgesia in the group of animals that also were exposed to chronic stress for 5 days [$F(4,30) = 8.68$, $P < 0.01$].

Saline (1 ml/kg, i.p.) had no effect on nociceptive threshold in control animals and also 24 h after the fifth session of swim stress. Comparing the baseline latency times in intact and stress-treated groups showed that chronic stress by itself produced hyperalgesia 24 h after the last swim-stress session [$F(3,27) = 30.49$, $P < 0.001$] (Fig. 1a) ($n = 6$–8).

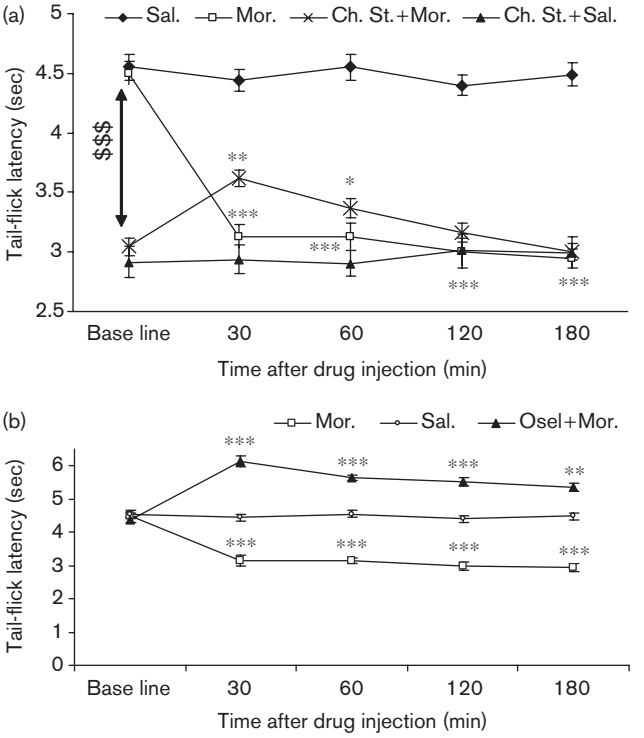
The effect of chronic dexamethasone on nociceptive threshold and morphine hyperalgesia

Like chronic stress, chronic administration of dexamethasone (2 mg/kg)-induced hyperalgesia 24 h after the fifth treatment [$F(2,21) = 86.33$, $P < 0.001$] (Fig. 2a). Twenty-four hours after the fifth dexamethasone treatment, UL-morphine produced hyperalgesia, [$F(4,32) = 3.735$, $P < 0.05$] (Fig. 2b) ($n = 6$–8).

Effect of oseltamivir on ultra-low doses of morphine, chronic stress and dexamethasone-induced hyperalgesia

Acute administration of oseltamivir (1 mg/kg) before UL-morphine treatment inhibited the morphine-induced hyperalgesia (Fig. 1b) [$F(4,30) = 14.64$, $P < 0.001$]. Chronic administration of oseltamivir (1 mg/kg) before daily swimming sessions failed to reverse stress-induced hyperalgesia on sixth day (Fig. 3a). As shown in Fig. 3b, after chronic administration of oseltamivir and stress, UL-morphine produced analgesia. Single-dose administration of oseltamivir 24 h after the fifth stress session did not inhibit stress-induced hyperalgesia (Fig. 3c). A single dose of oseltamivir failed to reverse the hyperalgesia induced by chronically injected dexamethasone (Fig. 3d) ($n = 6$–8).

Fig. 1



(a) The effects of chronic forced swim stress on morphine [1 μg/kg, intraperitoneal (i.p.)] (UL-morphine)-induced hyperalgesia. The baseline for the stress groups is the tail-flick latency after chronic stress. (b) Replication of the effect of the oseltamivir (1 mg/kg, i.p.), the $G_{\alpha s}$ signaling pathway blocker, on UL-morphine hyperalgesia. Values represent mean ± SEM (=6–8). $^{\$\$\$}P < 0.001$ versus baseline of saline-treated group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus baseline. Ch. St. + Mor., chronic stress (5 days) + UL-morphine on day 6; Ch. St. + Sal., chronic stress (5 days) + saline on day 6; Mor., UL-morphine; Osel + mor., Oseltamivir (1 mg/kg, i.p.) 15 min before UL-morphine; Sal., saline; UL-morphine, ultra-low doses of morphine.

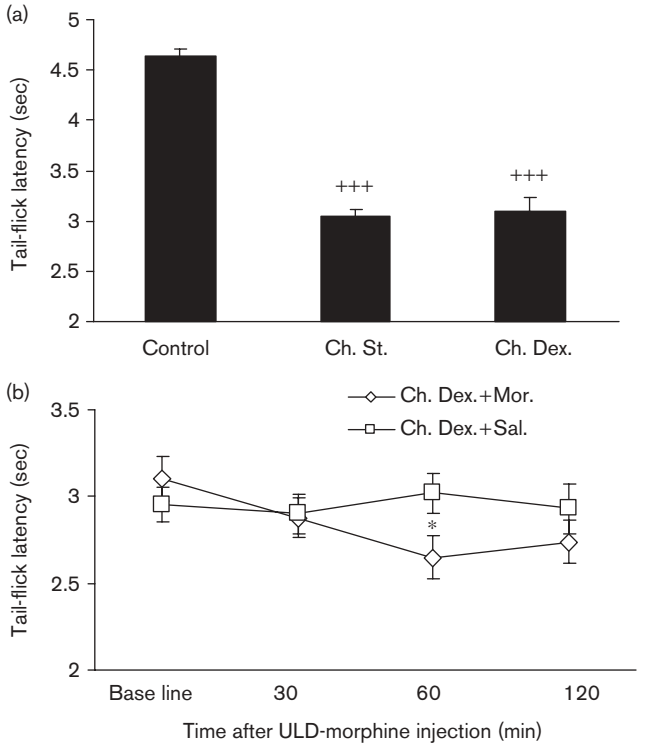
Effect of chronic stress on nociceptive threshold and morphine hyperalgesia in adrenalectomized rats

Figure 4 shows that chronic stress did not produce hyperalgesia in ADX animals but produced hyperalgesia in sham-operated animals {Fig. 4a [$F(3,34) = 36.03$, $P < 0.001$]}. UL-morphine-induced hyperalgesia in ADX animals {Fig. 4b, [$F(4,30) = 36.006$, $P < 0.01$]}. Chronic stress in sham-operated animals prevented the hyperalgesic effect of UL-morphine. In fact, UL-morphine produced analgesia in this group of animals [$F(4,30) = 14.42$, ($P < 0.05$)] (Fig. 4b) ($n = 6$–8).

Discussion

In this study, we used five sessions of daily forced swimming as a chronic stress. Our data show that forced swim stress elevated the plasma level of corticosterone

Fig. 2



(a) The effects of chronic forced swim stress and chronic dexamethasone (2 mg/kg, i.p.) on tail-flick latency, 24 h after the last treatment. (b) The effects of chronic dexamethasone on UL-morphine hyperalgesia. The baseline is the tail-flick latency after chronic administration of dexamethasone 24 h after the last treatment. Values represent mean ± SEM ($n=6$–8). $^{+++}P<0.001$ versus control. $^{*}P<0.05$ versus baseline. Ch. Dex, chronic dexamethasone (5 days); Ch. Dex. + Mor., chronic dexamethasone (5 days) + UL-morphine on day 6; Ch. Dex. + Sal., chronic dexamethasone (5 days) + saline on day 6; Ch. St., chronic stress (5 days); Mor., UL-morphine; Sal., saline; UL-morphine, ultra-low doses of morphine.

more than 400% compared with its plasma level in sham-stressed animals. An increase in plasma level of corticosterone was also present 24 h after the fifth stress session (at the same time as the behavioral tests). This finding confirmed the potency of swimming procedure as a model for chronic stress and an activator of the HPA axis (Table 1).

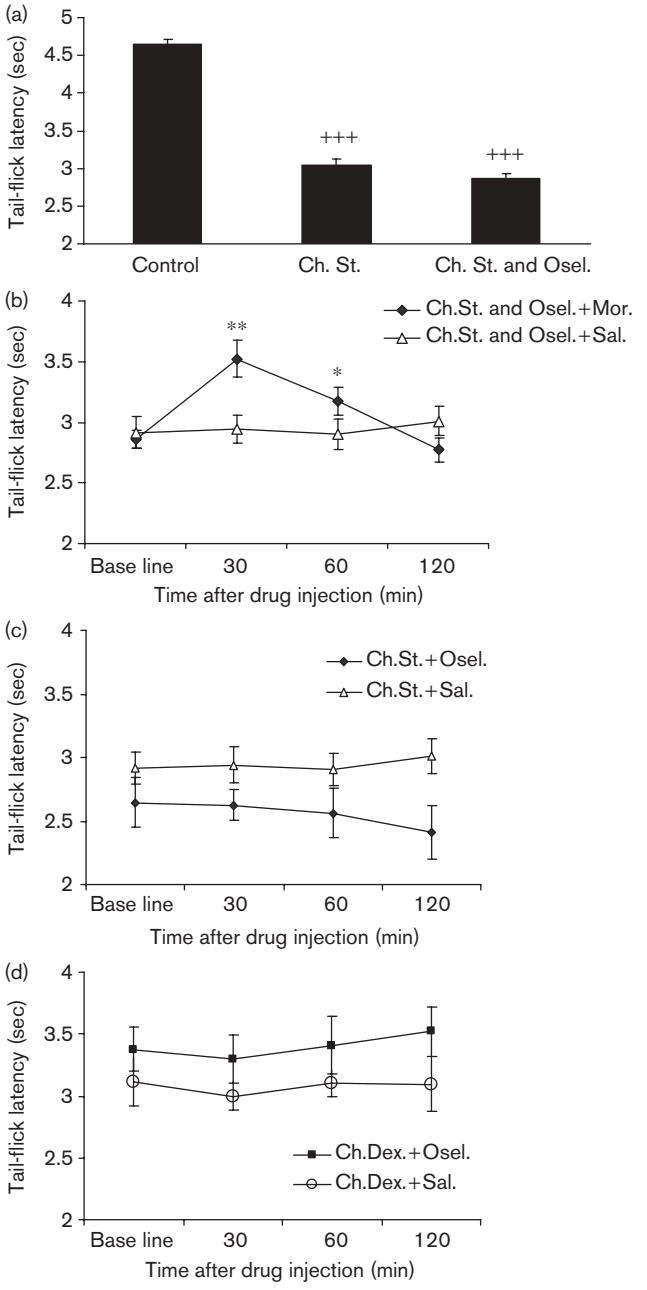
Morphine at analgesic doses activates both $G_{i/o}$ and $G_{\alpha s}$ signaling pathways (Shen and Crain, 1997; Crain and Shen, 2001a). Stress is reported to inhibit the development of morphine tolerance (Takahashi *et al.*, 1988, 1992; Ghiafeh-Davoodi *et al.*, 2005; Javan *et al.*, 2005). In this study, chronic stress, induced by daily forced swimming sessions, caused inhibition of UL-morphine-induced hyperalgesia (Fig. 1a) a phenomenon which is thought to be mediated by activation of stimulatory G proteins coupled to opioid receptors (Crain and Shen, 2004). Considering the earlier reports showing the inhibition of UL-morphine hyperalgesia and tolerance to morphine analgesic by blocking $G_{\alpha s}$ signaling pathway (Shen and Crain, 1989; Crain and Shen, 2000, 2001b, 2004; Movahedi *et al.*, 2006), and our present data showing the block of morphine UL hyperalgesia by chronic stress (Fig. 1), it can be suggested that the inhibitory effect of chronic stress on the development of analgesic tolerance could be at least partially mediated by inhibition of stimulatory G proteins signaling pathway.

In our study, swim stress induced analgesia for 2 h (data not shown) and then produced a significant hyperalgesia, as measured 24 h after the last session of chronic swimming stress (Fig. 2a). At the same time, it inhibited UL-morphine-induced hyperalgesia and caused the induction of analgesia by this dose of UL-morphine (Fig. 1a). Logically two different interpretations were possible for the lack of hyperalgesic effect of UL-morphine in stressed animals: first, stress which produces hyperalgesia (with the time course mentioned previously) is able to block UL-morphine hyperalgesia by itself; second, stress and UL-morphine use the same mechanisms to induce hyperalgesia. This mechanism could be saturated by stress alone, which would prevent a further hyperalgesic effect of UL-morphine. To rule out the second hypothesis, we carried out other experiments, as discussed below.

We evaluated the effect of both single-dose and chronic administration of dexamethasone (2 mg/kg), a synthetic corticosteroid, to mimic the effects of adrenal cortex secretions (Javan *et al.*, 2006) on nociceptive threshold and UL-morphine-induced hyperalgesia. Dexamethasone induced hyperalgesia both with single-dose (data not shown) and chronic administration (24 h after fifth treatment; Fig. 2a). The level of hyperalgesia was the same as that produced by chronic swim stress. Interestingly, unlike the chronic stress, UL-morphine, induced hyperalgesia 24 h after the fifth dexamethasone treatment (Fig. 2b). It might be concluded that different mechanisms are responsible for glucocorticoid and UL-morphine-induced hyperalgesia. Although dexamethasone is a glucocorticoid and can mimic stress-induced hyperalgesia, it did not inhibit UL-morphine-induced hyperalgesia. Maybe the products of the upper parts of HPA axis and/or its intact presence are required for the execution of the inhibitory effect of stress on morphine hyperalgesia, and thus on the $G_{\alpha s}$ signaling pathway.

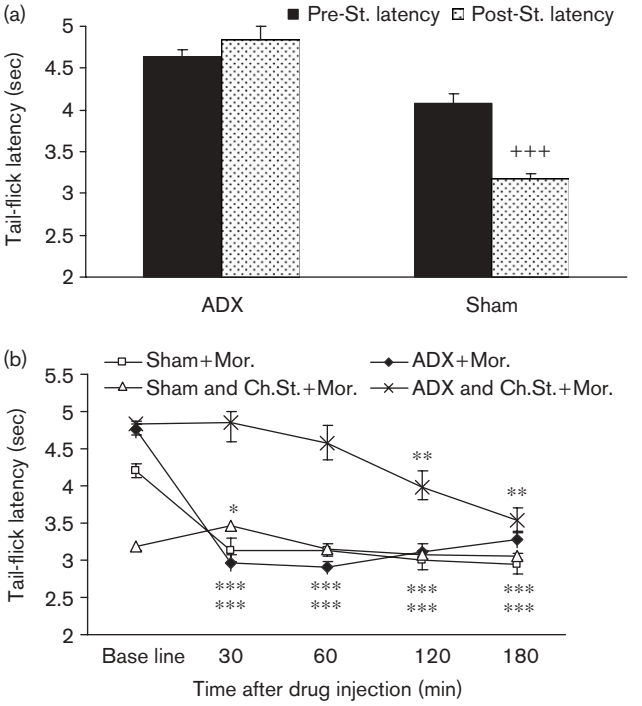
To examine the presence of a common mechanism for stress and UL-morphine-induced hyperalgesia, we used oseltamivir, a neuraminidase inhibitor that blocks GM1 ganglioside-mediated coupling of opioid receptors to stimulatory G proteins (Crain and Shen, 2004). Our data show that oseltamivir reversed the hyperalgesia induced by UL-morphine (Fig. 1b) but did not reverse the

Fig. 3



(a) The effects of chronic forced swim stress and chronic forced swim stress and chronic oseltamivir (1 mg/kg, intraperitoneal) on tail-flick latency, 24 h after the last treatment. (b) The effects of chronical forced swim stress and chronic oseltamivir on UL-morphine hyperalgesia; the baseline is the tail-flick latency 24 h after the last treatment of swim stress and oseltamivir. (c) The effects of oseltamivir on nociceptive threshold after chronic forced swim stress; the baseline is the tail-flick patency 24 h after the last swim-stress session. (d) The effects of oseltamivir on nociceptive threshold in chronically dexamethasone (2 mg/kg, intraperitoneal)-treated animals; baseline is the tail-flick latency after the last dexamethasone treatment. Values represent mean ± SEM (n=6–8). $^{+++}P<0.001$ versus control. $^{*}P<0.05$, $^{**}P<0.01$ versus baseline. Ch. Dex. + Osel, chronic dexamethasone (5 days) + single dose of oseltamivir on day 6; Ch. St., chronic stress (5 days); Ch. St. and Osel, chronic stress and oseltamivir (5 days); Ch. St. + Osel, chronic stress (5 days) + single dose of oseltamivir on day 6; Mor., UL-morphine; Sal., saline; UL-morphine, ultra-low doses of morphine.

Fig. 4



(a) The effect of chronic forced swim stress on tail-flick latency in sham-operated (Sham) and in adrenalectomized (ADX) animals after a 5-day recovery period. (b) The effects of chronic stress (5 days) in sham-operated (Sham and Ch. St.) and ADX (ADX and Ch. St.) animals on UL-morphine hyperalgesia (Mor.) on day 6. Values represent mean ± SEM (n=6–8). $^{+++}P<0.001$ versus before stress; $^{**}P<0.01$, $^{***}P<0.001$ versus baseline. UL-morphine, ultra-low doses of morphine.

hyperalgesic effect of chronic stress (Fig. 3a and c). This can be interpreted as further support for the idea that the hyperalgesic effects of chronic stress and UL-morphine are mediated through different mechanisms. In another experiment, oseltamivir did not reverse the hyperalgesic effect of dexamethasone (Fig. 3d). Therefore, while the hyperalgesic effect of morphine is mediated by the $G_{\alpha s}$ signaling pathway, the hyperalgesic effect of stress seems to be independent of stimulatory G proteins. In addition, chronic stress seems to exert a negative control over the $G_{\alpha s}$ signaling pathway.

In ADX animals, chronic stress did not produce hyperalgesia and did not reverse UL-morphine-induced hyperalgesia (Fig. 4). In addition, chronic dexamethasone did not inhibit UL-morphine-induced hyperalgesia. It seems that an intact HPA axis is needed for inhibitory effect of stress on UL-morphine-induced hyperalgesia.

The HPA products after stress seems to interact both directly and indirectly with G proteins. For example, CRH activates CRH receptors and activates a Gs protein-signaling pathway in the pituitary; and corticosterone

interacts with the regulators of G protein signaling in hypothalamus and pituitary (Ni *et al.*, 1999). It may be finally concluded that chronic stress interacts negatively with stimulatory G protein signaling to inhibit UL-morphine-induced hyperalgesia. Inhibition of the $G_{\alpha s}$ signaling pathway by chronic stress may be considered as a mechanism for the inhibitory effect of stress on the development of morphine analgesic tolerance.

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