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Thrombin inhibits NMDA-mediated nociceptive activity in the mouse: possible mediation by endothelin

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The CNS expresses many components of an extracellular protease signalling system, including the protease-activated receptor-1 (PAR-1) whose tethered ligand is generated by thrombin. Activation of PAR-1 potentiates NMDA receptor activity in hippocampal neurons. Because NMDA activity mediates hyperalgesia, we tested the hypothesis that PAR-1 receptors also regulate pain processing. In contrast to the potentiating effect of thrombin in the hippocampus, NMDA-induced behaviours and the transient mechanical hyperalgesia (von Frey fibres) induced by intrathecally injected NMDA in mice were inhibited by thrombin in a dose-related fashion. This anti-hyperalgesic effect was mimicked by SFLLRN, the natural ligand at PAR-1 binding sites, but not SLIGRL-amide, a PAR-2 agonist. The effects of SFLLRN were less potent and shorter in duration than that of thrombin, consistent with its more transient effect on PAR-1 sites. Both thrombin and SFLLRN inhibited acetic acid-induced abdominal stretch (writhing) behaviours, which were also sensitive to NMDA antagonism, but not hot plate or tail flick latencies, which were insensitive to NMDA antagonists. TFLLR-amide, a selective ligand for PAR-1 sites, mimicked the effects of thrombin while RLLFT-amide, an inactive, reverse peptide sequence, did not. In addition, the effect of TFLLR-amide was prevented by RWJ-56110, a PAR-1 antagonist. Thrombin and TFLLR-amide produced no oedema (Evans Blue extravasation) in the spinal cord that would account for these effects. Based on the reported ability of thrombin to mobilize endothelin-1 from astrocytes, we tested the role of this compound in thrombin's activity. BQ123, an endothelin A receptor antagonist, prevented thrombin's inhibition of writhing and NMDA-induced behaviours while BQ788, an endothelin B receptor antagonist, did not. Thus, activation of PAR-1 sites by thrombin in the CNS appears to inhibit NMDA-mediated nociception by a pathway involving endothelin type A receptors.

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The central nervous system (CNS) expresses many serine proteases as well as their corresponding serpins, or serine protease inhibitors. The G-protein-coupled thrombin receptor, protease-activated receptor-1 (PAR-1), is present in the developing and mature CNS, the peripheral nervous system and dorsal root ganglia (DRG) (Niclou *et al.* 1994; Weinstein *et al.* 1995; Niclou *et al.* 1998). Prothrombin, the thrombin precursor, and Factor Xa, the protein that converts prothrombin to thrombin, are both expressed in CNS tissue (Dihanich *et al.* 1991; Yamada & Nagai, 1996). This distribution of synthetic machinery makes the formation of thrombin possible within the CNS, where it is poised to play a physiological role in areas expressing PAR-1.

Circulating PAR-1 activators also enter brain tissue during penetrating head wounds, haemorrhagic stroke, rupture of cerebral vasculature or mast cell-induced increases in permeability (Nagy *et al.* 1998) with a distribution similar to that of albumin (Laursen *et al.* 1993). Prothrombin, whose concentration in plasma is greater than 1 μM, may be converted to its active form in areas expressing Factor Xa. The presence of thrombin and other blood proteases in the brain under pathological conditions raises the possibility that activation of central protease receptors may also be responsible for events related to tissue damage.

PAR activity is associated with tissue damage in the periphery where PAR-1-activating peptides increase vascular permeability and oedema by neurogenic inflammation (de Garavilla *et al.* 2001), probably involving mast cells associated with substance P-containing primary afferent C-fibres (Kawabata *et al.* 1999). Activation of PAR-2 receptors by trypsin or tryptase also induces inflammation in the periphery by a neurogenic mechanism (Steinhoff *et al.* 2000). Because mRNAs for PAR-1 (Niclou *et al.* 1998) and PAR-2 (Steinhoff *et al.* 2000) are abundantly expressed in DRG, an influx or upregulation of enzymes that orchestrate the activation of PAR in areas surrounding these distally projecting neurons may be important in inflammatory events. Activation of PAR also modulates sensory activity along afferent fibres as thermal and mechanical hyperalgesia are associated with PAR-2 activity peripherally (Vergnolle *et al.* 2001*a*) while PAR-1 activity in the periphery attenuates nociception in normal and inflammatory conditions (Asfaha *et al.* 2002).

One of several effects of thrombin, via activation of PAR-1, is potentiation of NMDA receptor activity in the hippocampus (Gingrich *et al.* 2000). This potentiation was attenuated in mice lacking PAR-1 and mimicked by the peptide SFLLRN, an agonist whose amino acid sequence reflects the tether portion on human PAR-1. Based on the distribution of both PAR-1 and NMDA sites in the spinal cord, thrombin-induced modulation of spinal NMDA receptors may also occur during sensory transmission. NMDA receptors on the central projections of primary afferent C-fibres (Liu *et al.* 1994) are believed to be important in hyperalgesia (Yaksh *et al.* 1999), promoting release of nociceptive transmitters (Liu *et al.* 1997). Although PAR activity in the periphery is associated with pain and inflammation, it is not known whether PAR activity on central projections of afferent fibres influences pain transmission.

Based on the positive modulatory effect of PAR-1 on NMDA activity in brain, we tested the hypothesis that NMDA activity along spinal nociceptive pathways may be similarly potentiated by PAR-1, thereby enhancing nociceptive activity. To restrict the distribution of drugs to the CNS and optimize their concentrations in spinal areas, we administered compounds intrathecally (I.T.). The effect of PAR-1 activity on spinal NMDA receptor activity *in vivo* was assessed by the influence of the peptide corresponding to the tethered ligand of PAR-1 (SFLLRN) and of thrombin on behaviours induced by NMDA. Because SFLLRN can activate PAR-2 as well as PAR-1, we also tested TFLLR-amide for its greater selectivity for PAR-1 compared with SLIGRL-amide, for its selectivity for PAR-2 (Vergnolle *et al.* 2001*b*). The influence of PAR activity on nociception was measured by changes in NMDA-induced mechanical hyperalgesia (von Frey fibres), the latencies of hot plate and tail flick responses, and acetic acid-induced writhing behaviours.

Our data indicate an antinociceptive rather than a hyperalgesic effect of I.T. administered thrombin. We therefore explored the possibility that these antinociceptive effects are mediated indirectly by the release of an endogenous, antinociceptive compound. Endothelin-1 is a potent vasoconstrictor (reviewed by Stoltz *et al.* 1999) that is pronociceptive when applied to peripheral tissue. However, endothelin-1 is antinociceptive in mice following either an I.T. (Kamei *et al.* 1993) or intracerebroventricular (I.C.V.) injection (Nikolov *et al.* 1993). Because thrombin applied to astrocytic cultures elicits the release of endothelin-1 (Ehrenreich *et al.* 1993), we tested the additional hypothesis that the antinociceptive effects of spinally administered thrombin are mediated by activity at endothelin A or B receptors.

Some of the results were published previously in abstract form (Fang *et al.* 2001).

METHODS

Animals

Adult male Swiss–Webster mice weighing 20–30 g (Charles River, Omaha, NE and Harlan, Indianapolis, IN, USA) were housed four per cage and allowed to acclimate for at least 24 h prior to use. Mice were allowed free access to food and water, and housed in a room with a constant temperature of 20 °C on a 12 h light:12 h dark cycle. Animals were used strictly in accordance with the Guidelines of IASP and the University of Minnesota Animal Care and Use Committee. Mice were killed at the end of each experiment by an overdose of ether or pentobarbital.

Drugs

α-Thrombin (103 NIH units (mg protein)$^{-1}$, i.e. a solution of 200 units ml^{-1}, is approximately 60 μM) and D-Phe-Pro-Arg-chloromethylketone (PPACK), an irreversible inhibitor of thrombin (Tapparelli *et al.* 1993) were purchased from Calbiochem (La Jolla, CA). The PAR-1 agonist peptide, SFLLRN (Ser-Phe-Leu-Leu-Arg-Asp), the PAR-2 agonist peptide, SLIGRL-amide (Ser-Leu-Ile-Gly-Arg-Leu-NH$_2$), BQ123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)), an endothelin receptor A antagonist (Ihara *et al.* 1992), and BQ788 (*N*-cis-2,6-dimethylpiperidinocarbonyl-β-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH), an endothelin receptor B antagonist (Ishikawa *et al.* 1994), were obtained from Bachem California Inc. (Torrance, CA, USA). *N*-Methyl-D-aspartic acid (NMDA), DL-2-amino-5-phosphonovaleric acid (APV), an NMDA antagonist, actinomycin D and cycloheximide, protein synthesis inhibitors, were purchased from Sigma Chemical Company (St Louis, MO). 3-((*RS*)-2-Carboxypipecazin-4-yl)-propyl-1-phosphonic acid (CPP), an NMDA antagonist, and TFLLR-amide (Thr-Phe-Leu-Leu-Arg-NH$_2$), a PAR-1 agonist (Asfaha *et al.* 2002), were purchased from Tocris Cookson Inc. (Ellisville, MO, USA). RLLFT-amide (Arg-Leu-Leu-Phe-Thr-NH$_2$), a control peptide for TFLLR-amide that is inactive at the PAR-1 site (Asfaha *et al.* 2002), was synthesized at the University of Minnesota Microchemical Facilities (Minneapolis, MN, USA). Acetic acid was purchased from Fisher Scientific (Hampton, NH, USA). RWJ-56110, a PAR-1 antagonist (Andrade-Gordon *et al.* 1999), was a generous gift from Dr Patricia Andrade-Gordon and Dr Bruce Maryanoff of Johnson & Johnson Pharmaceutical Research & Development L.L.C. (Spring House, PA, USA).

Drug administration

Except where noted, all injections were made intrathecally (I.T.) in mice at approximately the L5–L6 intravertebral space using a 30 gauge, 0.5 inch disposable needle on a 50 μl Luer tip Hamilton syringe in lightly restrained, unanaesthetized mice (Hylden & Wilcox, 1981). To accomplish this, the rostral portion of each mouse was placed under a towel and the hips held firmly using the thumb and forefinger. Except where indicated, a volume of 5 μl was used for all injections as this volume was found to be large enough to be easily measured yet small enough to have no influence on behavioural responses. Drugs were routinely dissolved in saline. Control groups were injected with an equivalent volume of saline.

NMDA-induced biting and scratching behaviours

Immediately after the injection of NMDA I.T., mice were placed in a large glass cylinder containing approximately 2 cm of bedding. The total number of caudally directed bites and scratches that occurred over a 1 or 2 min interval were recorded. Because most of the behavioural responses occurred within 1 min of the injection of NMDA, no difference was found between effects of drugs on measurements taken over 2 or 1 min intervals.

NMDA-induced hyperalgesia

Mechanical sensitivities to von Frey fibres of three sizes (#2.83, 0.663 mN; #4.08, 11.8 mN; #5.07, 115 mN) were measured in mice prior to I.T. injection and also 5 min after the administration of NMDA. The response to each von Frey fibre size prior to injection did not differ amongst groups. After injection, mice were placed on a wire mesh grating under a glass 6 ounce (177 ml) custard cup, which prevents escape but allows movement of all four limbs and head. Von Frey fibres were applied consecutively, beginning with the smallest size tested (#2.83), then #4.08 and finally #5.07. Each fibre was applied five times to the plantar surface of each hind paw, to the point of bending, for a total of 10 applications. A positive response was defined as a brisk shaking or licking of the paw. The number of positive responses out of 10 was recorded as the mechanical sensitivity. We selected a spectrum of three fibre sizes that evoke the smallest to largest responses that can still be potentiated by NMDA and that cover the spectrum of responses from allodynic (light touch perceived as nociceptive) to hyperalgesic (enhanced nociception). Fibre sizes 4.08 and greater are probably nociceptive as responses to this size were inhibited 30 min after injection of 10 mg kg^{-1} of morphine (mean ± S.E.M., 0 ± 0, $n = 6$) compared with 5.2 ± 0.9 before injection. In addition, responses to a fibre size of 5.07 averaged 8.2 ± 0.6 responses before injection and only 1.7 ± 0.5 after morphine ($n = 6$). Allodynia is often interpreted as activity along Aδ primary afferent fibres, a parameter not monitored in the present study. Therefore, we refer to the effect of NMDA on von Frey fibre responses as 'hyperalgesic', but do not exclude the possibility that it may also reflect allodynia at a fibre size of 2.83.

Abdominal stretch (writhing) assay

Mice were injected intraperitoneally (I.P.) with 0.3 ml of 1.0 % acetic acid while manually restrained. Immediately after injection, mice were placed in a large glass cylinder containing approximately 2 cm of bedding. The number of abdominal stretches in a 5-min interval was counted beginning 5 min after injection. All values shown for writhing experiments reflect a separate group of mice as each animal was tested only once and killed immediately after testing. Treatments that produced a significant decrease in the number of abdominal stretches compared with that of vehicle-injected control mice tested on the same day were considered to be antinociceptive.

Hot plate assay

Mice were placed on a hot plate maintained at 53 °C. The time transpired until the first avoidance response of either hind limb, such as licking or rapid shaking, was recorded as the latency of response. A cut-off value of 60 s was used to avoid tissue damage.

Tail flick assay

Animals were manually restrained and the tail submerged to a distance of 1 cm from the base of the tail in a water bath maintained at 53 °C. The withdrawal latency was defined as the time for the animal to withdraw its tail from the water. To avoid tissue damage, cut-off times of 15 s were utilized.

Behavioural data analysis

Mean values (± S.E.M.) are presented throughout the figures, tables and text. Statistical analysis of the results was performed using Student's unpaired *t* test or ANOVA followed by *post hoc* Newman-Keuls test for comparison between groups, as indicated. For clarity of presentation, in some cases the effects of drugs over a wide range of doses are depicted with respect to a single control value (continuous or dotted line) that represents the average of several control groups. However, the statistics were performed prior to pooling of the control values such that indications of significance reflect comparisons between individual groups tested on the same day.

Evans Blue extravasation

Because its distribution in normal tissue is restricted to the vasculature, Evans Blue dye was used as a marker of tissue oedema and plasma extravasation in the spinal cord (Saria & Lundberg, 1983). Mice were anaesthetized in a chamber equipped with an isoflurane drip. Drug was allowed to flow into the chamber until the righting and pain reflexes disappeared. Mice were then injected, by cardiac puncture, with 50 mg kg^{-1} of Evans Blue dye in a 10 mg ml^{-1} solution delivered using a 30 gauge needle attached to a PE10 tubing. Mice were allowed to recover from the anaesthesia for 30 min and then anaesthetized using a high dose (60 mg kg^{-1}) of sodium pentobarbital injected intraperitoneally (I.P.). Mice were perfused with 20 ml of ice-cold 0.32 M sucrose : PBS (1 : 1). Spinal cords were removed, weighed and placed in 0.5 ml of formamide (60 °C) and maintained in total darkness for 24 h. The concentration of Evans Blue dye extracted into formamide from each sample was measured using a spectrophotometer at 620 nm and the average concentration per wet weight of tissue compared between groups. Statistical analysis of the results was performed using ANOVA followed by *post hoc* Newman-Keuls test for comparison between groups.

RESULTS

Thrombin, SFLLRN and TFLLR-amide inhibit NMDA-induced behaviours

NMDA injected I.T. induced dose-related, caudally directed scratching and biting behaviours, as previously reported (Aanonsen & Wilcox, 1986; Velázquez *et al.* 1997; Laughlin *et al.* 1999) (Fig. 1*A*). Pretreatment (1 h) with thrombin dramatically inhibited behaviours in a dose-related fashion (Fig. 1*B*). The inhibitory effect of thrombin did not appear to result from desensitization to the effect of NMDA as increasing the concentration of NMDA from 20 to 40 μM overcame, rather than potentiated, the inhibitory effect of 3 pM thrombin (Fig. 1*B*). Behaviours induced by 8 μM NMDA were attenuated for an interval up to at least 2 h with recovery by 16 h after injection of 30 nM thrombin (Fig. 1*C*).

Recovery from the inhibitory effect of thrombin at 16 h was not impaired by pretreatment I.P. (30 min prior to thrombin) with either 1 mg kg^{-1} of actinomycin (34.7 ± 6.8 behaviours, $n = 6$) or 30 mg kg^{-1} of cycloheximide (38.3 ± 6.8 behaviours, $n = 6$) compared with that in mice pretreated with saline prior to thrombin (39.3 ± 6.4 behaviours, $n = 6$). Doses of actinomycin and cycloheximide were selected based on their ability to inhibit protein synthesis when injected I.P. in mice (Rosenblum *et al.* 1995). NMDA-induced behaviours were also not influenced by actinomycin (40.8 ± 6.4 behaviours, $n = 6$) or cycloheximide (36.7 ± 6.2 behaviours, $n = 6$) in the absence of thrombin when compared with control mice pretreated with saline only (35.7 ± 7.7 behaviours, $n = 6$). These data suggest that recovery from thrombin-induced inhibition does not depend on transcription or translation of protein.

Co-administration of SFLLRN, the natural ligand of PAR-1 receptors, with NMDA also suppressed NMDA-induced behaviours in a dose-related fashion whereas SLIGRL-amide, a PAR-2 selective agonist, did not (Fig. 2*A*). Similarly, pretreatment I.T. (5 min) with TFLLR-amide, a more selective PAR-1 agonist, produced a dose-related inhibition of NMDA-induced behaviours while RLLFT-amide, its inactive sequence, had no effect (Fig. 2*B*).

Thrombin, SFLLRN and TFLLR-amide inhibit NMDA-induced hyperalgesia

For several minutes following termination of its characteristic biting and scratching behaviours, I.T. administered NMDA induced mechanical hyperalgesia. This was manifested as an increased sensitivity to von Frey fibres (Fig. 3) 5 min after NMDA compare with 5 min after saline. The hyperalgesia was most apparent when tested using a von Frey fibre of intermediate size (#4.08, equivalent to a force of 11.8 mN) (Fig. 3*B*). Pretreatment with thrombin reversed this NMDA-induced increase in mechanical hyperalgesia. Injection of thrombin itself did not produce any effect on von Frey fibre responses at any time over this 2 h interval. Co-administration of SFLLRN had a similar effect on NMDA-induced hyperalgesia, inhibiting hyperalgesia in a dose-dependent fashion (Fig. 4), without influencing the sensitivity to von Frey fibres when injected alone. Similar to the effects of

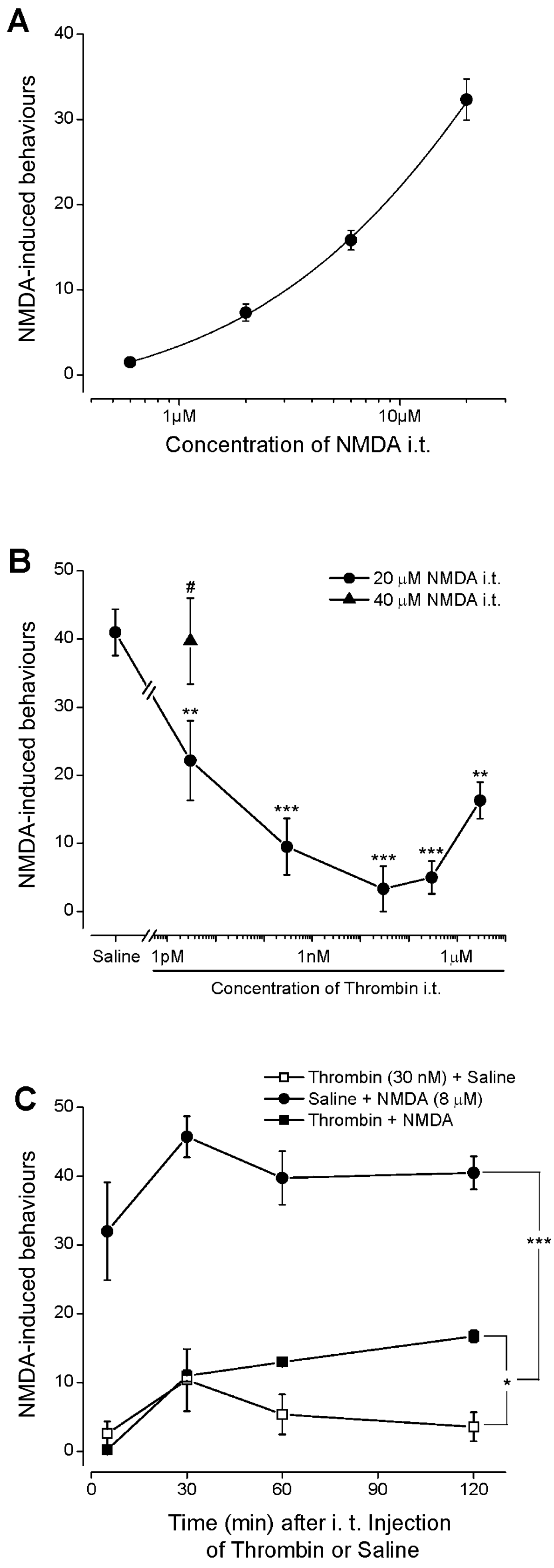

Figure 1. Effect of thrombin on NMDA-induced behaviours

A, dose-related increase in the number of biting and scratching behaviours induced by increasing concentrations of NMDA injected I.T.; *B*, dose-related inhibition of NMDA-induced behaviours (5 μl of a 20 μM solution; i.e. a 100 pmol dose) by I.T. injections of concentrations ranging from 3 pM to 30 nM thrombin (60 min pretreatment), and the ability of a higher dose of NMDA (40 μM) to overcome this inhibitory effect; *C*, the behavioural response to NMDA (8 μM) or saline I.T., 5, 30, 60 or 120 min after an I.T. injection of thrombin (30 nM) or saline. Caudally directed biting and scratching behaviours were counted for exactly 1 min (*A*) or 2 min (*B* and *C*) beginning immediately after administration of NMDA. Each point represents the mean (± S.E.M.) number of behaviours obtained from an independent group containing four to nine mice as follows: *A*, $n = 5$–6; *B*, $n = 4$–9; *C*, $n = 6$. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, *versus* the corresponding saline-injected control group (*B*) or treatment groups, as reflected by values obtained between 5 and 120 min after injection (*C*), as analysed by ANOVA followed by Newman-Keuls multiple comparisons. # indicates $P < 0.05$ *versus* 20 μM NMDA plus 3 pM thrombin group, as analysed by Student's *t* test.

thrombin and SFLLRN, pretreatment with TFLLR-amide prevented NMDA-induced hyperalgesia while RLLFT-amide did not (Fig. 5).

Acetic acid-induced abdominal stretching behaviours are mediated by the NMDA receptor

The acetic acid-induced nociceptive assay is believed to reflect mild abdominal pain as stretching behaviours are readily inhibited by non-narcotic analgesics (Singh *et al.* 1987). Endogenously released excitatory amino acids acting on NMDA receptors appear to play an important role in the generation of acetic acid-induced stretching (writhing) behaviours as, in contrast to many nociceptive assays, writhing is uniquely sensitive to NMDA antagonists (Bjorkman, 1995) and inhibition of nitric oxide synthase (Larson *et al.* 2000). To confirm mediation of this nociceptive assay by the NMDA receptor complex, APV and CPP, two widely used competitive antagonists of NMDA receptor activity, were injected I.T. at doses that decrease NMDA-induced biting and scratching behaviours (Giovengo *et al.* 1999). At doses that effectively inhibited the action of NMDA *in vivo*, as indicated by decreased behavioural responses to exogenously administered NMDA (Fig. 6*A*), both antagonists also inhibited acetic acid-induced stretching behaviours. In contrast, these drugs had little or no effect on thermal nociception, as indicated by their minimal influence on hot plate latencies (Fig. 6*A*).

Thrombin, SFLLRN and TFLLR-amide inhibit acetic acid-induced stretching behaviours

At doses and times that inhibited NMDA-induced behaviour and that attenuated NMDA-induced mechanical hyperalgesia, thrombin (30 nM, 1 h) (Figs 6*B* and 7*B*), SFLLRN (100 μM, 10 min) (Figs 6*B* and 7*C* and *D*) and TFLLR-amide (1–100 μM, 5 min) (Fig. 7*D*) greatly attenuated acetic acid-induced stretching behaviours whereas RLLFT-amide, the inactive peptide, did not (Fig. 7*D*). Treatment with thrombin or SFLLRN was without effect on thermal nociception, including the tail flick and hot plate assays (Fig. 6*B*). Pretreatment with 3 nM thrombin that had been boiled for 1 h prior to injection failed to inhibit writhing behaviours (17.4 ± 0.7 writhes, $n = 5$) compared with saline-injected control mice (17.2 ± 2.0 writhes, $n = 2$), indicating that the effect of thrombin is heat labile. Consistent with the generation of a tethered ligand by an enzymatic action, time course studies revealed that pretreatment with thrombin was inhibitory for 8 h in the abdominal stretch assay, with recovery by 16 h. In contrast, SFLLRN inhibited stretching behaviours for only 2 h with recovery by 4 h (Fig. 7*A* and *C*). Administration of SLIGRL-amide, the PAR-2 agonist, at a dose (100 μM) that was optimally effective for SFLLRN, failed to influence abdominal stretching behaviours (15.7 ± 2.7, $n = 6$) compared with that in saline-pretreated control mice (15.2 ± 2.1, $n = 6$).

Inhibition of abdominal stretching behaviours by thrombin and SFLLRN was dose dependent (Fig. 7*B* and *D*), but with quite different potencies, such that SFLLRN was approximately 1000 times less potent than thrombin. On the other hand, SFLLRN was more efficacious than the enzyme thrombin as SFLLRN inhibited abdominal stretching behaviours almost completely at the highest

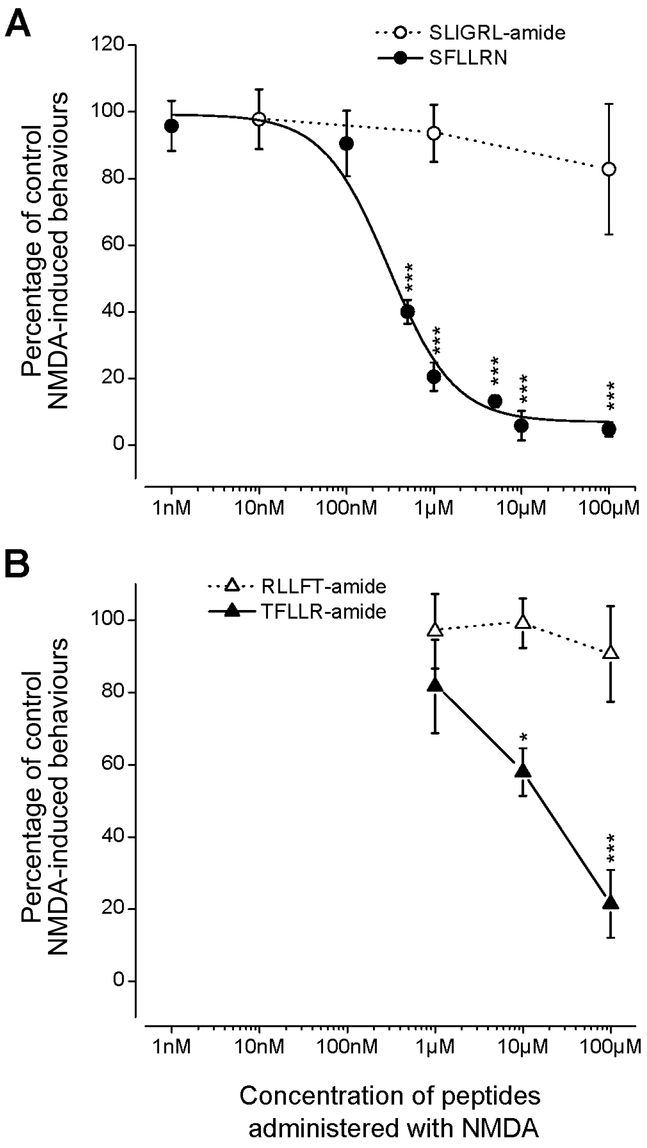

Figure 2. Inhibition of NMDA-induced behaviours by SFLLRN

NMDA (40 pmol) was either co-administered with different concentrations of SFLLRN or SLIGRL-amide (*A*) or injected 5 min after pretreatment with TFLLR-amide or RLLFT-amide (*B*). Each value represents the average number of biting and scratching behaviours, expressed as a percentage of control (± S.E.M.), for an independent group composed of the following number of mice: SLIGRL-amide and RLLFT-amide, $n = 5$; SFLLRN, $n = 4$; and TFLLR-amide, $n = 5$–6. Statistical analysis prior to transformation of data revealed differences as indicated by $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with their corresponding control groups, as analysed by ANOVA, followed by Newman-Keuls multiple comparisons.

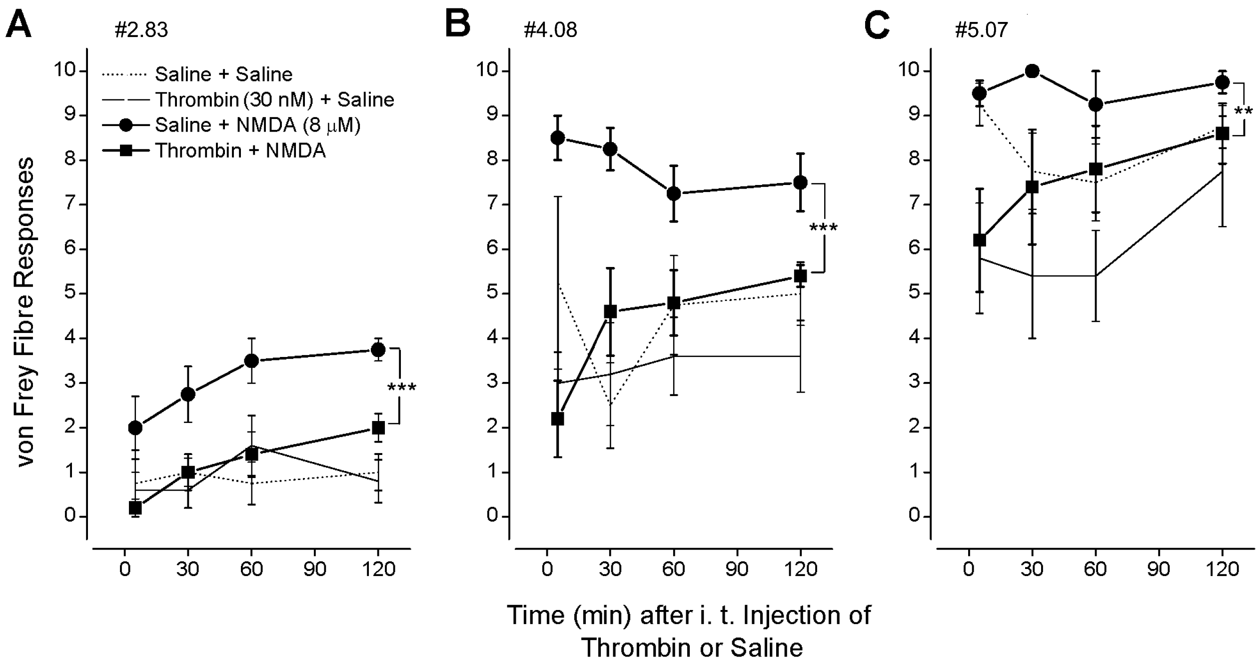

Figure 3. Effect of pretreatment with thrombin on NMDA-induced mechanical hyperalgesia

Von Frey tests with fibres of different sizes (*A*, #2.83; *B*, #4.08; *C*, #5.07) were performed at the times indicated relative to thrombin or saline and 5 min after the I.T. injection of NMDA or saline. Values represent the mean (± S.E.M.) number of positive responses out of a total of 10 trials on the plantar surface of both hind paws. Each group represents a value obtained from a group of five mice. **$P < 0.01$ and ***$P < 0.001$ reflect differences between treatment groups (over 5–120 min), as analysed by ANOVA followed by Newman-Keuls multiple comparisons.

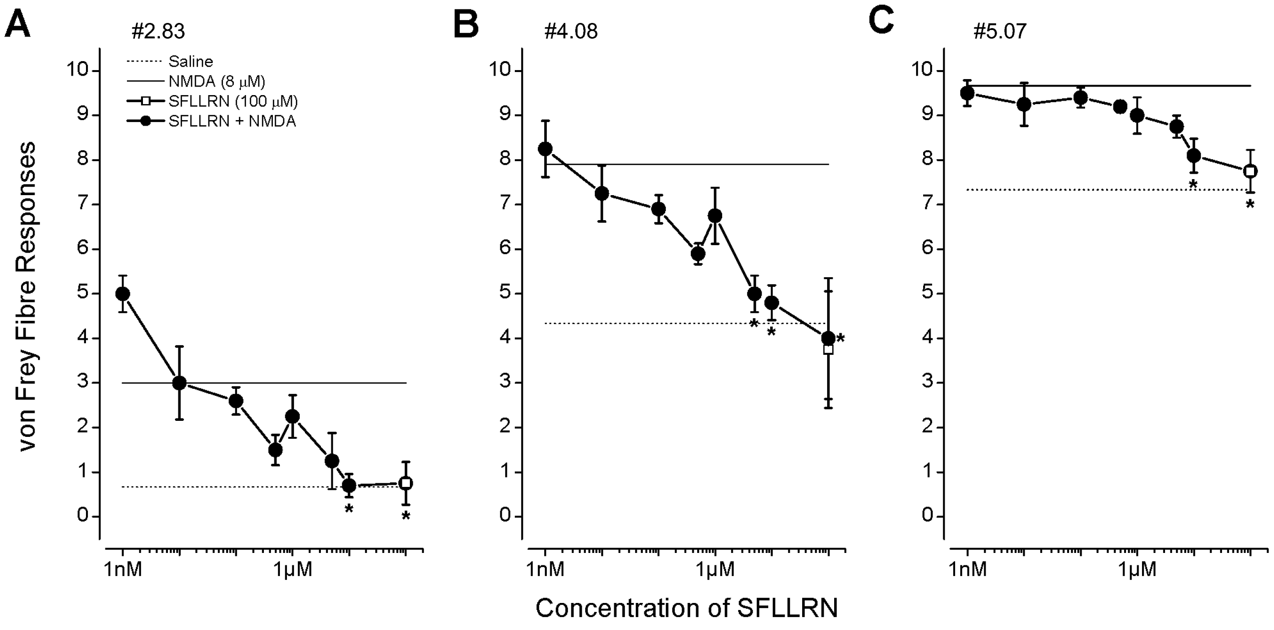

Figure 4. Inhibition of NMDA-induced mechanical hyperalgesia by SFLLRN

Von Frey tests with fibres of different sizes (*A*, #2.83; *B*, #4.08; *C*, #5.07) were performed 5 min after co-administration of NMDA with different concentrations of SFLLRN. Values represent the mean (± S.E.M.) number of responses to von Frey fibres in a group composed of five mice (100 μM SFLLRN) and all others in groups of four mice. The value for SFLLRN (100 μM) alone (□) is indistinguishable from that of SFLLRN + NMDA (●) due to overlapping means. Inhibition of NMDA-induced hyperalgesia by SFLLRN, compared with saline-injected control groups, is indicated by *$P < 0.05$ as analysed by ANOVA, followed by Newman-Keuls multiple comparisons.

doses examined, whereas thrombin only reduced the intensity of behaviours to about 40% at its most effective dose. Higher doses of thrombin were not inhibitory, resulting in a U-shaped dose–response curve. The effect of 1.5 μM thrombin (15.9 ± 1.2, $n = 6$) was not significantly less than that of saline (14.0 ± 1.2, $n = 6$). However, co-administration of thrombin together with 100 μM SFLLRN inhibited acetic acid-induced behaviours (5.7 ± 1.7, $n = 6$, $P < 0.001$ *vs.* either of the two groups) to a value similar to that following injection of SFLLRN alone, suggesting that PAR-1 sites are still functional after high doses of thrombin.

PPACK prevents the inhibitory effect of thrombin

Co-administration of PPACK, a non-reversible inhibitor of serine protease activity, prevented the inhibition by thrombin of acetic acid-induced stretching behaviour (Fig. 8*A*). PPACK also attenuated the inhibitory effect of thrombin on NMDA-induced biting and scratching behaviours, but to a lesser extent (Fig. 8*A*). The dose of PPACK was selected based on its ability to inhibit thrombin when present at this ratio with thrombin in CNS

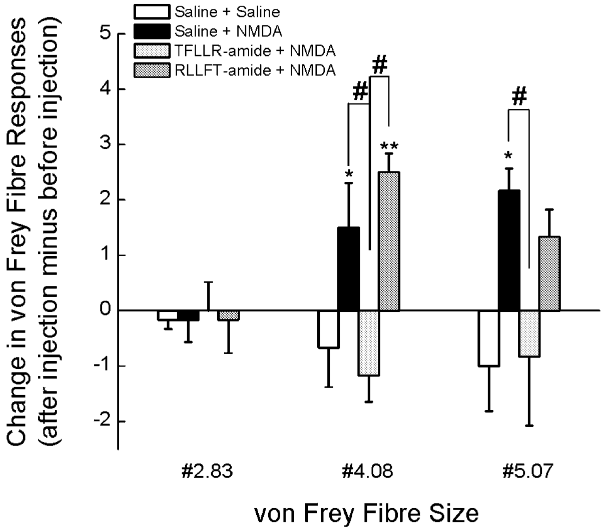

Figure 5. TFLLR-amide, but not RLLFT-amide, negates the hyperalgesic effect of NMDA

Von Frey tests with fibres of different sizes (*A*, #2.83; *B*, #4.08; *C*, #5.07) were performed before and 5 min after injections of either NMDA (8 μM) and saline, TFLLR-amide (100 μM) and NMDA (8 μM), or RLLFT-amide (100 μM) and NMDA (8 μM). Values represent the mean difference (± S.E.M.) in the number of responses to von Frey fibres obtained 5 min after injection compared with immediately prior to injection, such that increases reflect increased nociceptive responses or hyperalgesia. Each value represents a group of six mice. Statistically significant differences in these values compared with those obtained in saline-injected control mice (open bars) are indicated by $^*P < 0.05$ and $^{**}P < 0.01$, as analysed by ANOVA followed by Newman-Keuls multiple comparisons. # indicates a significant difference ($P < 0.05$) between the groups indicated, as analysed by ANOVA and followed by Newman-Keuls multiple comparisons.

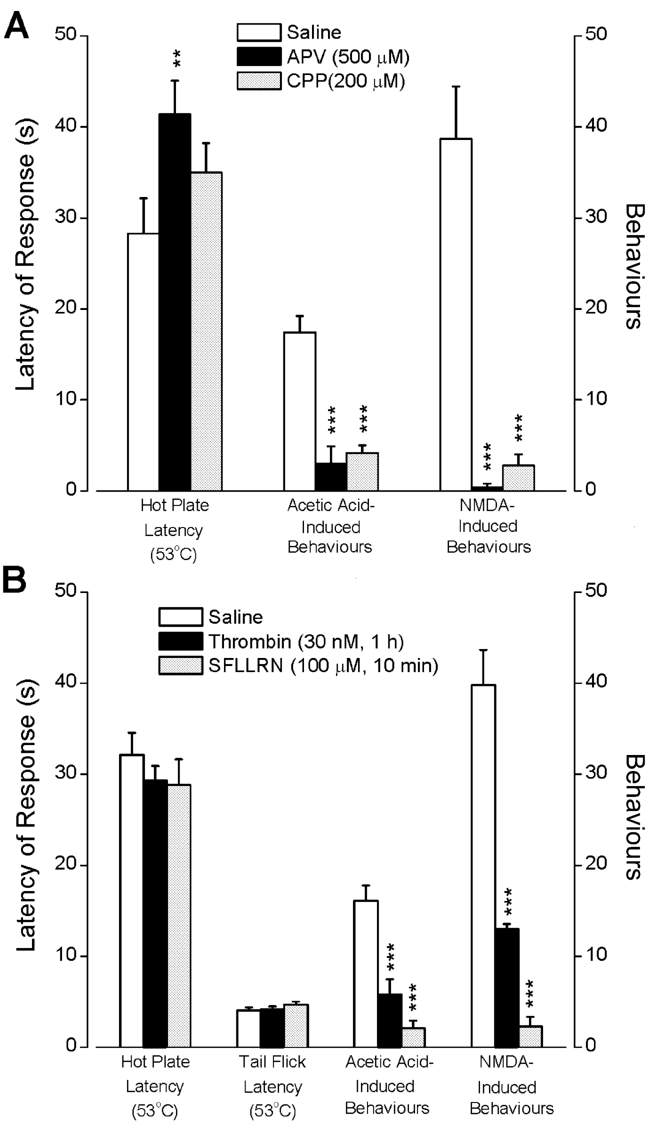

Figure 6. Effects of APV, CPP, thrombin or SFLLRN on nociceptive and NMDA-induced behaviours

Bars represent either the mean (± S.E.M.) number of behaviours produced by an I.P. injection of acetic acid (abdominal contractions or 'stretching' behaviours) or an I.T. injection of NMDA (biting and scratching behaviours), or the mean (± S.E.M.) latency of response in the hot plate (53 °C) or tail flick assay (53 °C) obtained from independent groups of five to eight mice. *A*, nociceptive assays were performed 5–15 min after the I.T. injection of either APV or CPP, two competitive antagonists at NMDA receptors, or saline. *B*, nociception was measured 5–15 min after the I.T. injection of either thrombin or SFLLRN. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, compared with its corresponding saline control group, as analysed by ANOVA and followed by Newman-Keuls multiple comparisons.

tissue slices (Gingrich *et al.* 2000). When administered alone, this dose of PPACK had no effect on either of these behavioural responses when compared with values after saline pretreatment.

RWJ-56110 attenuates the inhibitory effect of TFLLRN-amide

To ensure that the action of these peptides is via a PAR-1 receptor, we tested the effect of RWJ-56110, a PAR-1 antagonist, on the inhibition of behaviours induced by NMDA or acetic acid by TFLLR-amide (Fig. 8*B*). Pretreatment (5 min) with RWJ-56110 had no effect on the intensity of the behavioural response to either NMDA or acetic acid. However, this PAR-1 antagonist completely reversed the inhibitory effect of 100 μM TFLLR-amide on NMDA-induced biting and scratching behaviours as well as on acetic acid-induced abdominal stretches.

BQ123, but not BQ788, inhibits the behavioural effects of thrombin

Based on the reported ability of thrombin to induce release of endothelin from astrocytes (Ehrenreich *et al.* 1993), we

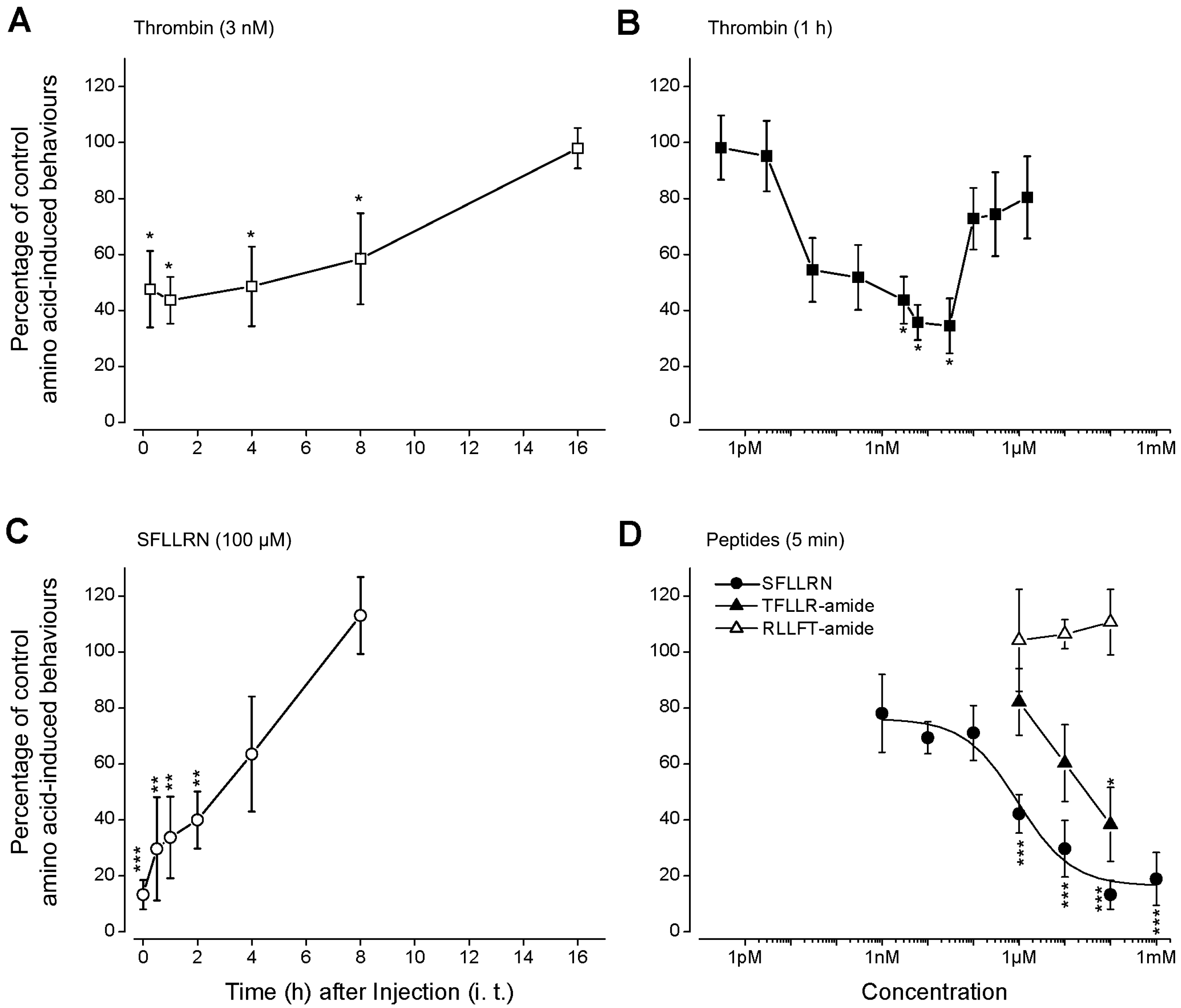

Figure 7. Effects of thrombin and SFLLRN on acetic acid-induced behaviours

Each value represents the mean number of acetic acid-induced stretching behaviours, expressed as a percentage (± S.E.M.) of control from an independent group of mice as follows (in ascending order of concentration): *A*, $n = 6–8$; *B*, $n = 4–10$; *C*, $n = 4–8$; *D*, $n = 6$ for TFLLR-amide and RLLFT-amide, $n = 8–9$ for SFLLRN. *A* and *C*, time course of responses following thrombin (3 nM) and SFLLRN (100 μM), respectively. *B* and *D*, dose–response relationships produced by the injection of thrombin (1 h), SFLLRN (5 min), TFLLR-amide (5 min) or RLLFT-amide (5 min), respectively. Inhibition of behaviours compared with corresponding saline-injected control groups (100 %), calculated prior to transformation, are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively, as analysed by ANOVA and followed by Newman-Keuls multiple comparisons.

Table 1. Effect of thrombin on Evans Blue dye in spinal tissue

	Percentage of saline-injected control concentrations	
Treatment (10 µl I.T.)	1 h	24 h
Saline	100 ± 8 (*n*=9)	100 ± 25 (*n*=5)
3 nM thrombin	79 ± 12 (*n*=5)	Not measured
30 nM thrombin	88 ± 16 (*n*=6)	90 ± 8 (*n*=3)
3 µM thrombin	89 ± 16 (*n*=5)	88 ± 11 (*n*=6)
30 µM thrombin	80 ± 19 (*n*=5)	101 ± 35 (*n*=4)

Data are presented as mean ± S.E.M. No significant differences were observed between groups.

tested the hypothesis that endothelin receptor A or B activity mediates the effect of thrombin. Mice were pretreated with 10 µl of a 1, 5, 10 or 20 µM solution of either BQ123, an endothelin receptor A antagonist, or BQ788, an endothelin receptor B antagonist, immediately prior to either 30 nM thrombin or saline. These doses were based on concentration ranges that have been previously found to be effective in astrocyte cultures (Hasselblatt *et al.* 1998). Ten minutes later, the inhibitory effect of 30 nM thrombin on NMDA-induced biting and scratching behaviours was absent in mice pretreated with 10 or 20 µM BQ123 but unaffected in mice pretreated with 20 µM BQ788 (Fig. 9*A*). In a similar fashion, the inhibitory effect of 30 nM thrombin on acetic acid-induced writhing behaviours was completely reversed by as little as 1 µM BQ123 but unaffected by 20 µM BQ788 (Fig. 9*B*). When administered prior to saline in lieu of thrombin, BQ123 (20 µM) alone did not influence NMDA-induced behaviours (41.6 ± 3.1, *n* = 5) compared with saline-pretreated controls (42.6 ± 2.8, *n* = 5). Neither did BQ123 alone alter acetic acid-induced behaviours (16.4 ± 1.4, *n* = 5) compared with writhing in saline-injected control mice (17.2 ± 2.0, *n* = 5).

Thrombin does not induce spinal oedema

Changes in plasma extravasation were measured by the concentration of Evans Blue dye in the spinal cord 60 min and 24 h after injection of either thrombin or saline. Compared with saline-injected control mice, the concentration of Evans Blue in spinal cord tissue was not influenced by doses of thrombin (3 and 30 nM) that inhibited acetic acid-induced writhing (Table 1). The concentration of Evans Blue in spinal cord tissue was also not influenced by higher doses of thrombin (300 nM to 30 µM) that reflect the upper portion of the U-shaped dose–response curve in the writhing assay (Fig. 7*B*). Neither was plasma permeability in the spinal cord influenced by an antinociceptive dose (100 µM, 30 min pretreatment) of TFLLR-amide (121.7 ± 15.8 %, *n* = 5) or its inactive control peptide RLLFT-amide (134.1 ± 22.8 %, *n* = 8) compared with non-injected control mice (100 ± 25.2 %, *n* = 4) analysed that day.

DISCUSSION

Our studies demonstrate inhibition of acetic acid-induced nociception, NMDA-induced mechanical hyperalgesia and of NMDA-induced biting and scratching behaviours by injection of thrombin. The inhibitory effect of thrombin was attenuated by PPACK, a drug that inhibits

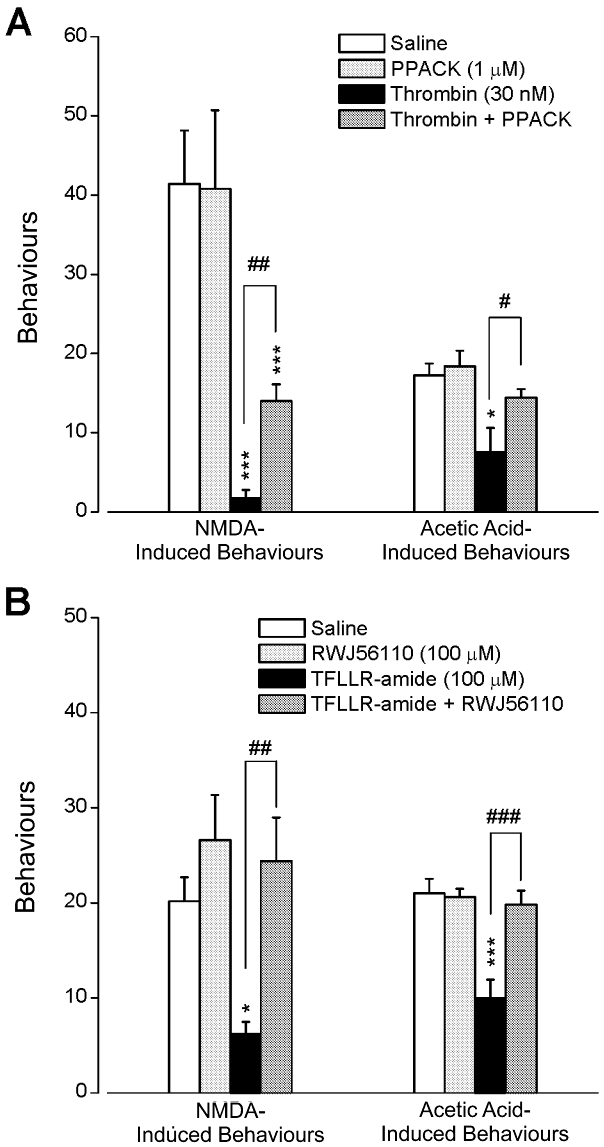

Figure 8. Prevention of thrombin-induced effects by PPACK and PAR-1-induced effects by RWJ-56110

A, acetic acid- or NMDA-induced behaviours were examined 1 h after pretreatment with PPACK, thrombin or saline (co-administration). *B*, acetic acid- or NMDA-induced behaviours were also examined after pretreatment with RWJ-56110 (5 min), TFLLR-amide (5 min) or saline. Bars represent the mean (± S.E.M.) intensity of behaviours from groups of five to eight mice (*A*) and five to six mice (*B*). * and *** indicate $P < 0.05$ and $P < 0.001$, respectively, compared with the corresponding saline control group; #, ## and ### indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, between the groups indicated, as analysed by ANOVA and followed by Newman-Keuls multiple comparisons.

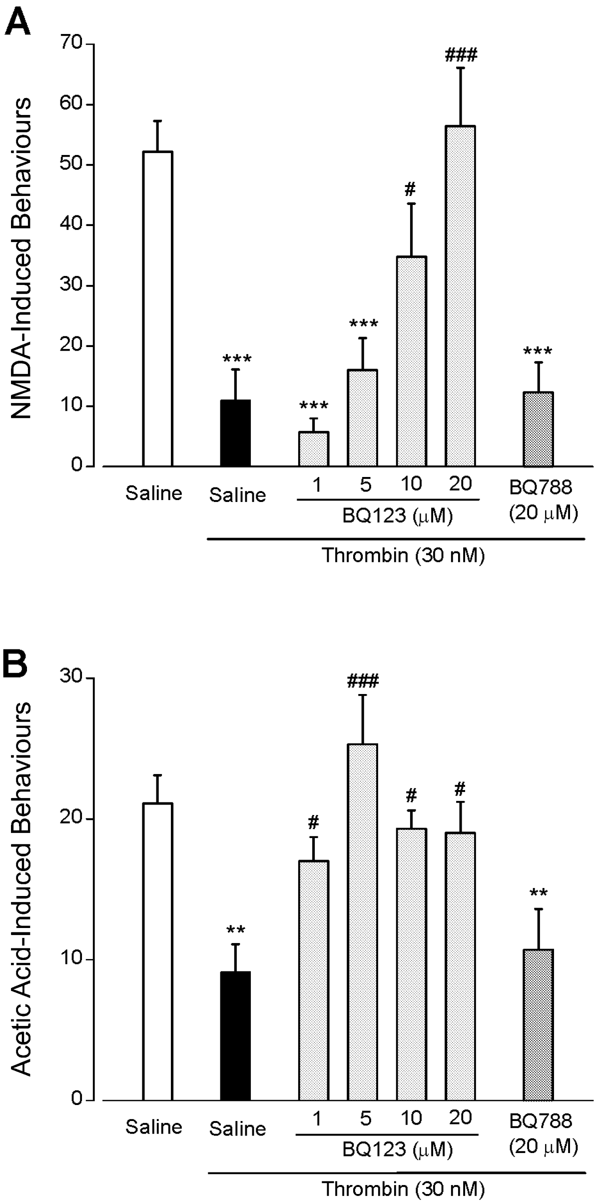

Figure 9. BQ123, but not BQ788, inhibits behavioural effects of thrombin

The number of NMDA-induced biting and scratching behaviours (*A*) or acetic acid-induced writhing behaviours (*B*) was measured in mice pretreated (10 min) with either saline or thrombin (30 nM). The effect of pretreatment (immediately prior to thrombin) with either BQ123 (10 μl of the solutions indicated), an endothelin receptor A antagonist, or BQ788 (10 μl of a 20 μM solution), an endothelin receptor B antagonist, on the inhibitory effect of thrombin was compared with saline-pretreated control mice. Each value represents the mean number of behaviours from an independent group of mice as follows: *A*, $n = 4$–8; *B*, $n = 4$–12. Inhibition of behaviours compared with corresponding saline-injected control groups (white bars) are indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively, as analysed by ANOVA and followed by Newman-Keuls multiple comparisons; # and ### further indicate $P < 0.05$ and $P < 0.001$, respectively, between groups as indicated.

the enzymatic activity of thrombin. The effect of thrombin was mimicked by SFLLRN, the sequence of a naturally occurring PAR-1 ligand, by TFLLR-amide, a relatively more selective PAR-1 ligand, but not by either RLLFT-amide, the reverse sequence of TFLLR-amide that is inactive, or SLIGRL-amide, the PAR-2 ligand. Finally, the effect of TFLLR-amide was reversed by RWJ-56110, a PAR-1 antagonist, together suggesting that PAR-1 receptors are probable targets influencing nociception. Consistent with the previously reported ability of thrombin to mobilize endothelin from astrocytes (Ehrenreich *et al.* 1993), the anti-hyperalgesic effects of thrombin were also inhibited by drugs that inhibit endothelin A, but not endothelin B, receptors. Thus, the inhibitory effect of thrombin along nociceptive pathways *in vivo* probably results from mobilization of endothelins that are antinociceptive in the CNS (Kamei *et al.* 1993; Nikolov *et al.* 1993).

PAR-1 activity

The influence of thrombin, SFLLRN and TFLLR-amide on NMDA-induced behaviours was consistent with previously reported interactions with PAR-1 sites as (a) thrombin was more potent than either SFLLRN or TFLLR-amide; (b) the effect of thrombin lasted longer than that of SFLLRN; and (c) PPACK attenuated the action of thrombin. The actions of thrombin, SFLLRN and TFLLR-amide on nociception appear to be selective for PAR-1 as the PAR-2 agonist, SLIGRL-amide, did not mimic the effects of SFLLRN or TFLLR-amide at a dose that was optimal for inhibition of NMDA-induced behaviours (biting and scratching), NMDA-induced hyperalgesia (von Frey fibre assay) and abdominal nociception (acetic acid-induced stretching). An important caveat is that, even though PAR-2 is not involved, activation of additional protease receptors other than PAR-1 is possible (Hollenberg *et al.* 1997; Hollenberg, 1999).

The relatively greater potency of thrombin probably reflects its action as an enzyme, catalysing the generation of multiple tethered ligands covalently linked to PAR-1 sites. Rather than giving birth to tethered ligands, SFLLRN interacts directly with individual PAR-1 sites. In the absence of either a tether or the multiplicative action of an enzyme, SFLLRN and TFLLR-amide interact more randomly with PAR-1 and are, therefore, less potent and shorter acting than thrombin. The greater efficacy of SFLLRN than thrombin may result from the more complicated action of thrombin endogenously. Thrombin activity may result in the production of thrombin inhibitors, such as protease nexin 1, that are known to be expressed in the nervous system (Festoff *et al.* 1996). The greater efficacy of SFLLRN than TFLLR-amide may result from differences in the absorption, distribution or metabolism of these compounds *in vivo* that may not be reflected by their efficacy *in vitro*.

Responses to NMDA were restored rapidly on termination of drug action (SFLLRN, 4 h; thrombin, 16 h). A longer time is required for synthesis of new PAR-1 protein following desensitization (Hamilton *et al.* 1999), based on estimates of turnover (Shapiro *et al.* 1996). Because resensitization requires the synthesis of new receptor populations, restoration of PAR-1 receptor populations, such as those on rat astrocytes, is inhibited by protein synthesis inhibitors such as cycloheximide (Ubl *et al.* 2000). The failure of either cycloheximide or actinomycin D to prolong the inhibitory influence of thrombin in the present studies argues against desensitization of PAR-1 sites as the basis for the inhibitory effect of thrombin. The ability of PPACK, a serine protease inhibitor, to attenuate the effect of thrombin is also in keeping with an enzymatic action of thrombin.

Although possible, it is unlikely that the inhibition of NMDA activity by thrombin results from cleavage of NMDA receptor subunits (Gingrich *et al.* 2000) as SFLLRN and TFLLR-amide, which have no catalytic activity, each mimic the effect of thrombin. In addition, enzymatically cleaved NMDA subunits would necessitate the synthesis of new NMDA receptor subunits for recovery from such a lytic effect of thrombin. Protein synthesis inhibitors failed to prolong the inhibitory effect of thrombin, arguing against enzymatic cleavage of NMDA receptors.

Endothelin receptors

The distribution of [^{125}I]endothelin-1 binding sites, in the porcine spinal cord (laminae I–III and the intermediolateral nucleus) (Niwa *et al.* 1991) and on satellite cells of rat ganglia (Kar *et al.* 1991), suggests a neuromodulatory role along sensory pathways. Endothelin A receptor immunoreactivity is present in a subset of small sized peptidergic and non-peptidergic sensory neurons in DRG and their axons and to a lesser extent in a subset of medium sized sensory neurons (Pomonis *et al.* 2001). Endothelin B receptor immunoreactivity is localized on DRG satellite cells and non-myelinating ensheathing Schwann cells (Pomonis *et al.* 2001). Endothelins released in peripheral tissues probably act directly on endothelin A receptor-expressing sensory neurons and on endothelin B receptor-expressing non-myelinating Schwann cells. Consistent with this, subcutaneous administration of endothelin-1 to the rat plantar hind paw produces pain-like behaviour (flinching) by excitation of C- and Aδ nociceptive fibres via endothelin A-type receptors (Gokin *et al.* 2001). When injected I.P., endothelins produce abdominal constriction (writhing) in mice (Raffa & Jacoby, 1991). Endothelin-1 is also hyperalgesic, increasing nociception induced by capsaicin when both are injected subcutaneously into the mouse hind paw (Piovezan *et al.* 1998).

In contrast to its action peripherally, endothelin-1 inhibits nociception when administered centrally. Endothelin-1 increases hot plate and tail flick latencies and decreases acetic acid-induced writhing after I.C.V. injection in mice (Nikolov *et al.* 1993). Intrathecal administration of endothelin-1 also produces dose-dependent increases in tail flick latencies in mice (Kamei *et al.* 1993). The possibility that thrombin induces antinociception in the writhing assay and reverses hyperalgesia in the von Frey assay by an action mediated by endothelins is consistent with the central effects of endothelin. The ability of BQ123, but not BQ788, to reverse the antinociceptive effect of thrombin in the present studies suggests that endothelin released in response to thrombin is antinociceptive via activation of endothelin A receptors, which are densely located on primary afferent nociceptors.

Dual effects of thrombin

Thrombin produced a U-shaped inhibition of writhing responses, similar to its dual actions in other systems, including cultured cholinergic neurons (Debeir *et al.* 1996), tumour cells (Zain *et al.* 2000) and toxicity in the hippocampus (Striggow *et al.* 2000). The concentration ranges of thrombin (30 pM to 30 nM), SFLLRN (1 μM to 1 mM) and TFLLR-amide (1–100 μM) injected to achieve antinociception in the writhing assay were similar to those of thrombin (50 pM) and a synthetic thrombin receptor agonist (10 μM Ala-pFluoro-Phe-Arg-Cha-HomoArg-Tyr-NH$_2$) that were neuroprotective in the hippocampus *in vitro* (Striggow *et al.* 2000). The concentration of thrombin that was found to potentiate neurodegeneration (50–500 nM) also corresponds roughly with the dose range of thrombin (100 nM to 1 μM) over which its antinociceptive effect deteriorated in our studies. Although it is unclear what accounts for these U-shaped dose–response relationships, a balance between a potentiative effect of PAR-1 on NMDA receptor subunits (Gingrich *et al.* 2000), and the antinociceptive effect of endothelin (Kamei *et al.* 1993; Nikolov *et al.* 1993) might contribute to the dual effect of thrombin in the present study.

PAR-1 binding sites appear to remain functional even after treatment with a high dose of thrombin as co-administration of mice with both 1.5 μM thrombin and 100 μM SFLLRN inhibited writhing to a similar degree as in mice injected with SFLLRN only. Thus, PAR-1 binding does not appear to be completely activated or desensitized (by phosphorylation or internalization) by high doses of thrombin alone. Because of the higher efficacy of SFLLRN than thrombin, SFLLRN may interact with PAR-1 receptors with which thrombin did not, or in the novel fashion previously described by Hammes & Coughlin (1999). Alternatively, thrombin may cause removal of the tethered ligand in a fashion similar to the proteolytic action of thermolysin (Ubl *et al.* 2000), either directly, due to its lack of selectivity at high doses, or indirectly, via activation of other proteases (Molino *et al.* 1995; Resento *et al.* 1997). This process might leave the receptor intact and available for interaction with SFLLRN. Alternatively, SFLLRN has been found to interact with other receptors, including PAR-2 in endothelial cells (Molino *et al.* 1997; O'Brien *et al.* 2000). However, the failure of SLIGRL-amide to mimic the effects of thrombin, SFLLRN and TFLLR-amide in the present studies does not support this possibility.

NMDA activity

The inhibitory effect of PAR-1 on nociception and hyperalgesia appears to be selective for pathways mediated by NMDA. Competitive antagonists of the NMDA receptor (APV and CPP) suppressed acetic acid-induced stretching behaviours (Fig. 6*A*) at doses that inhibited NMDA-induced activity. PAR-1 activity inhibited abdominal stretching (writhing) behaviours (Fig. 7) and NMDA-induced mechanical hyperalgesia (Figs 3 and 4), both of which depend on NMDA activity. In contrast, PAR-1 activity had no effect on thermal nociception, which was relatively less sensitive to NMDA antagonists, consistent with our previous studies (Giovengo *et al.* 1999). It is not likely that thrombin is tonically active in the normal CNS. In support of this, RWJ-56110, the PAR-1 antagonist, did not potentiate either NMDA- or acetic acid-induced behaviours. In addition, PPACK only attenuated the effect of exogenously administered thrombin on NMDA-induced behaviours and on acetic acid-induced abdominal stretches. In the absence of injected thrombin, PPACK did not potentiate the magnitude of these responses, as would be predicted in the presence of endogenous thrombin. Thrombin, SFLLRN and TFLLR-amide also had no effect on von Frey responses in the absence of NMDA, suggesting that NMDA-induced activity is sensitive to PAR-1 proportionately to its ability to increase nociceptive input. It is, therefore, unlikely that PAR-1 antagonists would induce analgesia in healthy individuals in the absence of pain.

Variations in NMDA subunit composition and/or distribution may contribute to the differing effects of thrombin centrally. NMDA receptor complexes are heteromeric structures that can be reconstituted from two subunit types (Sprengel & Seeburg, 1993): NR1 (ζ1 in mice) plus one of four modulatory NR2 subunits (NR2A–NR2D) (ϵ1–ϵ4 in mice). NR2 subunits differ in anatomical distribution, functional properties, affinity for agonists and antagonists acting at glycine and glutamate sites, and affinities for Mg^{2+}. Because PAR-1 activity was found to potentiate recombinant NR1–NR2A (1.7-fold) and NR1–NR2B (1.4-fold), but not NR1–NR2C or NR1–NR2D receptor responses (Gingrich *et al.* 2000), NR2 subunits appear to determine the sensitivity of NMDA receptors to direct modulation by PAR-1. The relatively greater abundance of NR2D in the spinal cord of human (Sundstrom *et al.* 1997) and rat (Tölle *et al.* 1993) than in hippocampal tissue (Dunah *et al.* 1996), may lend selectivity to the effects of PAR-1 in this area.

The selective distribution of NR2B immunoreactivity in laminae I and II of the dorsal spinal cord suggests that this subtype is located on primary afferent fibres (Boyce *et al.* 1999), structures that also express endothelin A receptors (Pomonis *et al.* 2001). About 35 % of NR1-immunoreactive synapses in the superficial dorsal horn are presynaptic and the majority (> 70 %) of presynaptically localized NMDA receptors in the superficial dorsal horn are located on terminals immunopositive for glutamate, suggesting that they function as autoreceptors. These sites presumably increase release of neurotransmitter(s) involved in sensory processing (Liu *et al.* 1994). Consistent with their role in nociception, NR2B antagonists produce antinociception at doses that fail to influence rotorod performance, while non-selective NMDA antagonists, such as MK-80l, produce motor effects at doses that are antinociceptive (Boyce *et al.* 1999).

Because of their selective distribution on afferent fibres, activation of endothelin A receptors may modulate the action of the presynaptic pool of NMDA receptors. A slowly developing depolarization of presynaptic terminals can cause presynaptic inhibition of afferent input, similar to that associated with dorsal root potentials (Willis, 1999). In this fashion, endothelin released in response to PAR-1 activity might negate rather than potentiate NMDA activity at these sites. Although the hippocampus represents the most intensively NR2B-immunolabelled region within the telecephalon (Goebel & Poosch, 1999) with heavy staining in CA1 pyramidal neurons (Charton *et al.* 1999), the majority of these sites are believed to be postsynaptic (Liu *et al.* 1994). PAR activity would thus be expected to have an impact on presynaptic NR2B sites in the dorsal spinal cord differently from identical subunits located postsynaptically in the hippocampus.

Vascular effects of thrombin

We explored the possibility that thrombin alters nociceptive processing by its ability to increase vascular permeability, causing inflammation and physical constriction of spinal pathways. Thrombin is inflammatory when injected at high doses (approximately 1000 × the dose used in the present study) directly into brain tissue (Kawai *et al.* 2001). High doses of PAR-1 agonists injected directly into the rat paw produce oedema by a neurogenic mechanism (de Garavilla *et al.* 2001). High doses of endothelin (I.T.) also recapitulate the immediate vasoconstrictive and more delayed (3–24 h) oedematous effects of spinal injury (weight-drop injury) known to increase spinal endothelin concentrations over 4–24 h in the rat (Salzman *et al.* 1996). Endothelin is capable of decreasing blood flow and breaking down the blood–brain barrier (Westmark *et al.* 1995). In the current study, the failure of antinociceptive doses of thrombin or TFLLRN-amide to induce any change in Evans Blue extravasation in the spinal cord suggests that doses necessary for antinociception are lower than those required to increase vascular permeability. Because the neurogenic inflammatory effect of PAR-1 agonists appears to depend on mast cells (Kawabata *et al.* 1999), the absence of mast cells in normal spinal cord tissue, compared with the visceral and dermal tissue in which PAR-1 agonists induce inflammation, may preclude the development of inflammation in this area. Regardless of the mechanism, the antinociceptive and antihyperalgesic effects of thrombin probably do not result from simple oedema, although we cannot rule out microinflammatory effects that may be below the level of detection of the Evans Blue assay.

In summary, PAR-1 activity in the CNS, induced pharmacologically by the I.T. injection of thrombin, SFLLRN or TFLLR-amide, inhibits rather than potentiates nociception that is mediated by NMDA activity. The anti-hyperalgesic effect occurs at concentrations that have no effect on vascular permeability in the spinal cord and appears to be mediated by activation of the endothelin A receptor.

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