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Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol

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To study the postnatal development of nociceptive synaptic inputs in the superficial dorsal horn of the neonatal rat spinal cord, we examined the effect of capsaicin and menthol on glutamatergic mEPSCs in postnatal day (P) 0–1, P5–6 and P9–11 slices of spinal cord. Capsaicin (100 nM to 2 μM) increased the mEPSC frequency in a concentration-dependent manner at all ages tested, with a significant enhancement of the effect between P5 and P10. This effect was sensitive to vanilloid receptor (VR) antagonists. The elevation in mEPSC frequency occurred at concentrations of capsaicin (100 nM) that did not alter the distribution of mEPSC amplitudes and was abolished by a dorsal rhizotomy, demonstrating that capsaicin acts via presynaptic VR1 receptors localized on primary afferents. Menthol significantly increased the mEPSC frequency with a similar developmental pattern to capsaicin without consistently affecting mEPSC amplitude. The increase in mEPSC frequency following capsaicin did not depend on transmembrane calcium influx since it persisted in zero $[Ca^{2+}]_o$. The facilitation of spontaneous glutamate release by capsaicin was sufficient to evoke action potentials in neonatal dorsal horn neurons but was accompanied by a block of EPSCs evoked by electrical stimulation of the dorsal root. These results indicate that VR1-expressing nociceptive primary afferents form functional synaptic connections in the superficial dorsal horn from birth and that activation of the VR1 receptor increases spontaneous glutamate release via an undetermined mechanism. In addition, the data suggest that immature primary afferents express functional menthol receptors that are capable of modulating transmitter release. These results have important functional implications for infant pain processing.

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Considerable interest in the measurement and treatment of pain in infancy has led to increasing research into the development of pain pathways and yet little is known of the postnatal development of nociceptive synaptic inputs to the mammalian spinal cord. Previous studies in the newborn rat spinal cord have documented the presence of synaptic terminals expressing tyrosine receptor kinase A (TrkA), isolectin B4 (IB4) and calcitonin gene-related peptide (CGRP), whose selective expression in small-diameter sensory neurons occurs from an embryonic age (Carroll *et al.* 1992; Bennett *et al.* 1996; Hall *et al.* 1997; Jackman & Fitzgerald, 2000). However, other evidence points to a delayed maturation of C-fibre synaptic connections in the substantia gelatinosa (SG) over the postnatal period. Extracellular single-unit recordings *in vivo* have demonstrated that while low-intensity electrical stimulation of A-fibres evokes action potentials in superficial dorsal horn neurons from postnatal day (P)3 (Jennings & Fitzgerald, 1998), long-latency C-fibre-evoked activity is not apparent until P10 (Fitzgerald, 1988; Jennings & Fitzgerald, 1998). Long-latency ventral root potentials are also not recorded before P10 (Hori & Watanabe, 1987; Fitzgerald *et al.* 1987). In addition, the C-fibre irritant mustard oil fails to induce a flexion reflex or c-fos expression in dorsal horn neurons until the second postnatal week, despite its ability to excite C-fibres in the newborn rat skin (Fitzgerald & Gibson, 1984; Williams *et al.* 1990; Soyguder *et al.* 1994). However, the lack of C-fibre-evoked spike activity between P0 and P10 does not necessarily mean that no synaptic contacts have been formed. Little is known about the developmental profile of nociceptive fibre-evoked excitatory-postsynaptic currents (EPSCs) in the dorsal horn during the first 10 postnatal days, which are potentially subthreshold and would thus escape detection via extracellular recording techniques.

One difficulty in studying developing afferent inputs is that rapid changes in axonal diameter and the degree of myelination in A-fibres during the first 2 postnatal weeks (Friede & Samorajski, 1968; Sima, 1974) result in a significant degree of overlap in the electrical stimulus parameters required to activate neonatal A- *vs.* C-fibres, preventing classification of synaptic responses evoked by dorsal root stimulation. To address this problem, we have

Journal of Physiology

used chemical stimuli to activate the central terminals of a subset of nociceptive primary afferent fibres directly and characterize the development of their synaptic inputs to the neonatal rat dorsal horn between P0 and P11. The identified capsaicin receptors (VR1), which are also sensitive to noxious heat (> 43 °C) and low pH (Caterina *et al.* 1997; Tominaga *et al.* 1998), have been localized to the synaptic terminals of small-diameter primary afferents in the superficial dorsal horn of the spinal cord (Guo *et al.* 1999) and capsaicin application increases the frequency of glutamatergic EPSCs in the adult dorsal horn via VR activation (Yang *et al.* 1998). Recent reports suggest that a significant fraction of DRG neurons express both VR1 and the recently cloned cold- and menthol-sensitive receptor CMR1 and can thus be classified as heat- and cold-sensitive nociceptors (McKemy *et al.* 2002). It is not yet known if CMR1 is also transported centrally and can modulate neurotransmitter release in the spinal cord.

The present study uses capsaicin and menthol as tools to excite nociceptive terminals in the neonatal dorsal horn and investigate the development of synaptic inputs originating from immature VR1-positive and menthol-sensitive primary afferents. Portions of this work have been published previously in abstract form (Baccei *et al.* 2002).

METHODS

All experimental procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986 and the European Community Council directive 86/609/EEC.

Preparation of spinal cord slices

Neonatal Sprague-Dawley pups (P0–P11) were anaesthetized with halothane (5 % in medical oxygen) and exsanguinated. The spinal column was quickly removed and placed in an ice-cold dissection solution consisting of (mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 6 $MgCl_2$, 0.5 $CaCl_2$, 25 glucose and continuously bubbled with 95 % O_2–5 % CO_2. After a ventral laminectomy was performed, the spinal cord was removed and immersed in low melting point agarose (3 % in above dissection solution; GibcoBRL, Paisley, UK) at 37 °C. Following cooling, the block of agarose was glued to the chamber of a Vibroslice tissue slicer (HA-752; Campden Instruments, Leicester, UK) and 400 μm sagittal slices were cut. For experiments involving the recording of evoked EPSCs from slices with attached dorsal roots (5–8 mm in length), a similar procedure was used except that thick (500–700 μm) transverse sections were obtained using a modified Vibroslice in order to preserve the dorsal root entry zone. The slices were transferred to a chamber filled with oxygenated dissection solution and allowed to recover for 1.5–2 h at room temperature.

Dorsal rhizotomy procedure

Four pups (at P3) were anaesthetized with halothane (5 % in medical oxygen), a portion of the lumbar spinal cord was exposed, and the L3–L5 dorsal roots were transected unilaterally. Approximately 5–8 days following the surgery, the pups were again anaesthetized with halothane, exsanguinated, and subsequently slices of lumbar spinal cord were obtained for electrophysiological recording as described above.

Patch clamp recordings

Following recovery, slices were transferred to a submersion-type recording chamber (RC-22; Warner Instruments) and mounted on the stage of an upright microscope (Zeiss Axioskop 2, Welwyn Garden City, UK). The slices were continually perfused at room temperature with oxygenated aCSF solution containing (mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 25 glucose at a rate of 1–3 ml min^{-1}.

Patch electrodes were fashioned from thin-walled single-filamented borosilicate glass (1.5 mm o.d.; World Precision Instruments, Hertfordshire, UK) using a two-stage vertical micro-electrode puller (PP-830; Narishige, London, UK). Pipette resistances ranged from 5 to 7 MΩ, seal resistances were > 1 GΩ. For voltage-clamp experiments, electrodes were filled with a solution containing (mM): 130 caesium gluconate, 10 CsCl, 10 Hepes, 11 EGTA, 1.0 $CaCl_2$, 2.0 MgATP. Current clamp experiments used an electrode solution of (mM): 130 potassium gluconate, 10 KCl, 10 Hepes, 1.0 EGTA, 0.1 $CaCl_2$, 2.0 MgATP. Dorsal horn neurons were visualized with infrared differential interference contrast (IR-DIC) and whole-cell patch-clamp recordings were obtained using a Multiclamp 700A amplifier (Axon Instruments, Union City, CA, USA). EPSCs were recorded from a holding potential of −70 mV, thus minimizing the contribution of $GABA_A$- and glycine-mediated currents (Yoshimura & Nishi, 1993). Currents were filtered at 4–6 kHz through a −3 dB, 4-pole low-pass Bessell filter, then digitally sampled at 20 kHz and stored on a personal computer (Viglen, Middlesex, UK) using a commercially available data acquisition system (Digidata 1322A with pCLAMP8.0 software, Axon Instruments).

Drugs

Capsaicin was dissolved at 5 mg ml^{-1} in a mixture of 80 % aCSF, 10 % ethanol and 10 % Tween-80 and diluted on the day of the experiment to obtain a final concentration of 10 nM to 2 μM. Capsaicin, Ruthenium Red, dantrolene sodium, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP), oligomycin, caffeine, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetomoxymethyl ester) (BAPTA AM), bicuculline methiodide and strychnine were purchased from Sigma/RBI (Dorset, UK). Capsazepine, tetrodotoxin (TTX) and 1,2,3,4-tetra-hydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide (NBQX) were purchased from Tocris (Bristol, UK). All drugs were bath applied at 1–3 ml min^{-1}.

Data analysis and statistics

Data were analysed via visual inspection using Mini Analysis (v.5.4.1; Synaptosoft, Inc., Decatur, GA, USA) and Clampfit (Axon Instruments) software. The threshold for mEPSC detection was set at twice the mean amplitude of the background noise. mEPSC frequency was generally measured in the 90 s period before capsaicin application (in the presence of TTX), and compared with the mEPSC frequency observed 10–90 s after the end of the capsaicin application. Unless otherwise stated, one-way analysis of variance (ANOVA) was used to test for levels of significant difference between groups. Data are expressed as means ± S.E.M.

RESULTS

Patch-clamp recordings were obtained from 161 neurons in the neonatal rat dorsal horn from the following three age groups: P0–P1 (n = 32), P5–P6 (n = 26) and P8–P11

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Journal of Physiology

($n = 103$). Unless otherwise stated, the neurons were located in the superficial dorsal horn as judged by visual inspection.

The frequency of spontaneous and miniature EPSCs in the dorsal horn increases with postnatal age

The frequency of spontaneous EPSCs (sEPSCs) recorded in aCSF increased over the first 10 days of postnatal life (Fig. 1*A*). At P0–P1, sEPSCs occurred at a frequency of 0.60 ± 0.18 Hz ($n = 31$) and increased significantly to 1.05 ± 0.27 Hz ($n = 21$) by P5–P6 ($P < 0.05$; one-way ANOVA) and 3.44 ± 0.45 Hz by P9–P10 ($n = 69$; $P < 0.001$). At a holding potential of −70 mV, inward EPSCs were abolished by NBQX (data not shown). This suggests that the currents observed in the present study

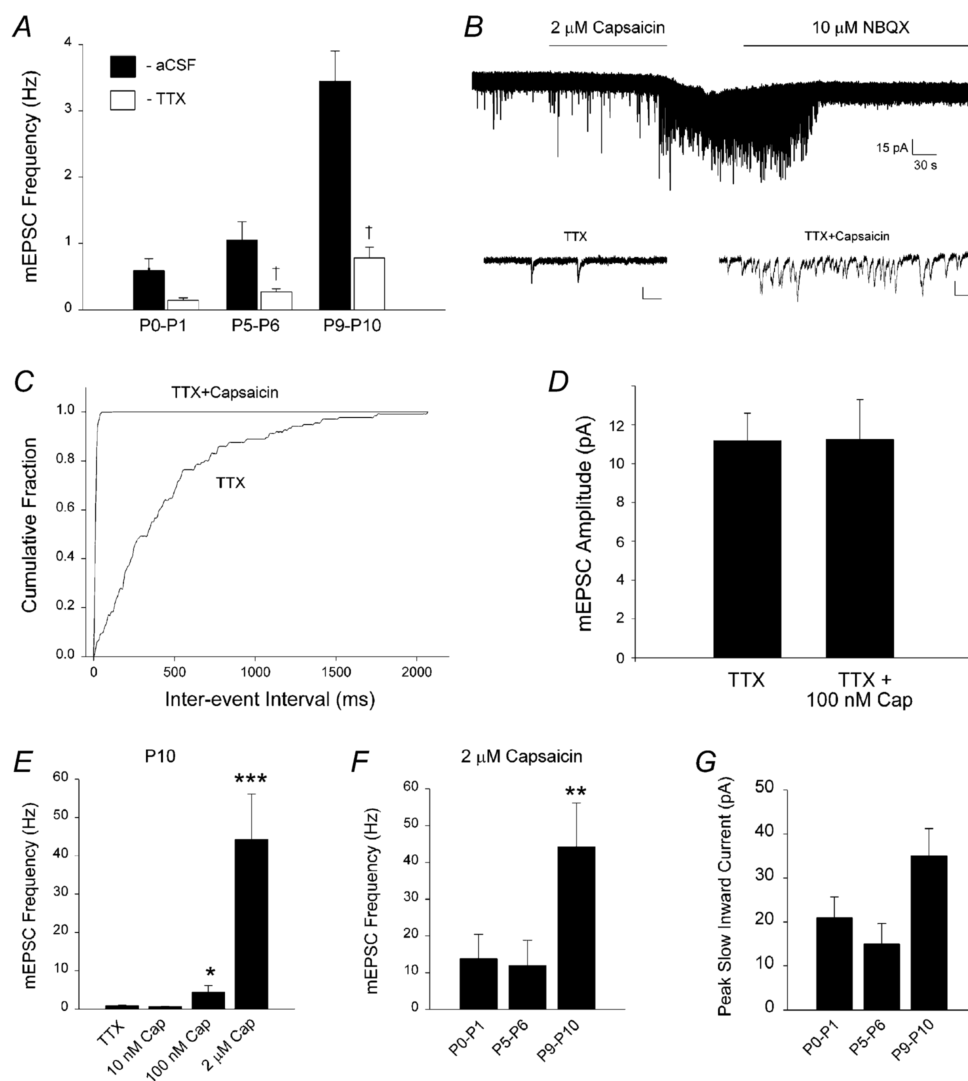

Figure 1. Capsaicin increases the frequency of mEPSCs in neonatal dorsal horn neurons

A, mean frequency of spontaneous EPSCs (recorded in aCSF) and miniature EPSCs (recorded in 500 nM TTX) increase with age over the first 10 postnatal days. † Significant difference between the frequency of sEPSCs and mEPSCs at a given age ($P < 0.0001$, t test). *B*, top panel, example of recording from $V_h = -70$ mV demonstrating the slow inward current and large increase in mEPSC frequency that result from the bath application of 2 μM capsaicin in a P10 neuron in the presence of 10 μM bicuculline and 0.5 μM strychnine. Subsequent perfusion with 10 μM NBQX abolished the mEPSCs. *B*, bottom panel, different time scale illustrating the kinetics of mEPSCs before and after 2 μM capsaicin in another P10 cell (scale bars: 20 pA, 50 ms). *C*, cumulative probability function showing an example of the altered distribution of mEPSC inter-event intervals after capsaicin treatment. *D*, lower concentrations of capsaicin (100 nM), which significantly increase mEPSC frequency, did not change the mean mEPSC amplitude in P9–P10 neurons ($n = 8$). *E*, dose dependence of capsaicin effect on mEPSC frequency. * Significant differences in mEPSC frequency compared to baseline conditions (* $P < 0.05$; *** $P < 0.001$; one-way ANOVA). *F*, the average frequency of mEPSCs following 2 μM capsaicin was significantly greater in the P9–P10 group compared to other age groups (** $P < 0.01$; ANOVA). *G*, the peak amplitude of the slow inward current evoked by 2 μM capsaicin did not significantly vary according to postnatal age.

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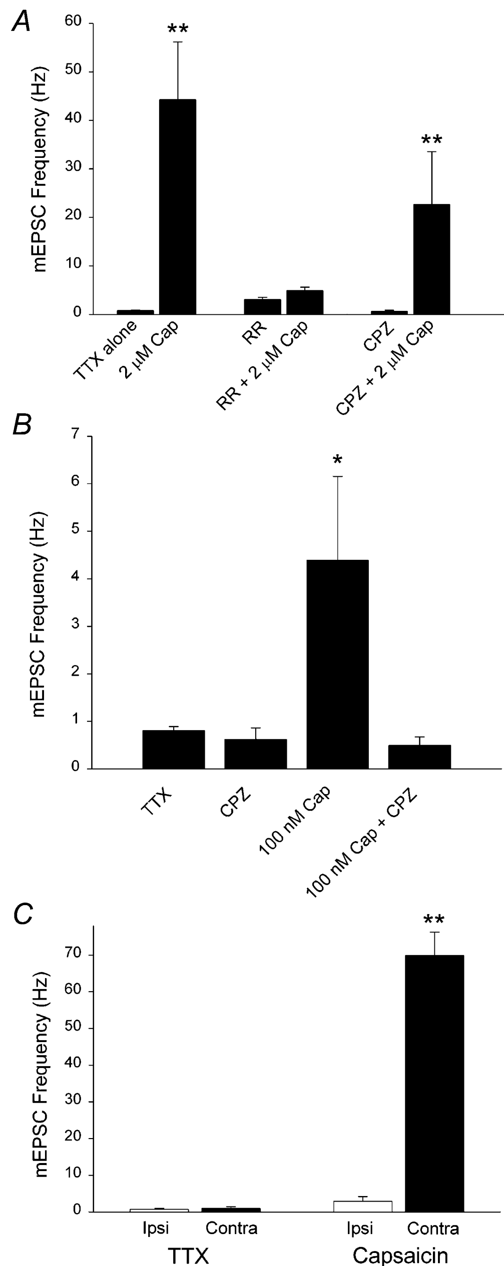

Figure 2. VR1 receptors on primary afferents mediate capsaicin-evoked increase in mEPSC frequency

A, the effect of 2 μM capsaicin on mEPSC frequency was abolished by prior application of the non-competitive VR1 antagonist Ruthenium Red (RR; 10 μM; $n = 4$) but persisted in the presence of the competitive antagonist capsazepine (CPZ; 10 μM; $n = 5$; ** $P < 0.001$). For purposes of comparison, the modulation of mEPSC frequency by 2 μM capsaicin in the absence of antagonists is illustrated to the left ($n = 7$; $P < 0.001$). *B*, the facilitation of mEPSC frequency observed with 100 nM capsaicin (*$P < 0.05$) was completely blocked by CPZ ($n = 5$). *C*, capsaicin (2 μM) had no significant effect on mEPSC frequency in P8–P11 dorsal horn neurons located ipsilateral to a previous dorsal rhizotomy ($n = 7$), while a dramatic elevation was witnessed on the contralateral side ($n = 5$; ** $P < 0.001$).

were mediated by the AMPA/kainate subtype of glutamate receptor, as has been reported in many previous studies of spontaneous synaptic transmission in the dorsal horn (Yoshimura & Nishi, 1993; Yang *et al.* 1998).

The frequency of miniature EPSCs (mEPSCs), isolated via the bath application of TTX (500 nM) for approximately 5 min, also depended on postnatal age (Fig. 1*A*). P0–P1 neurons exhibited mEPSCs at a rate of 0.14 ± 0.04 Hz ($n = 28$), which was significantly lower than the frequency observed at P5–P6 (0.26 ± 0.05 Hz; $n = 26$; $P < 0.05$). The mEPSC frequency at P9–P10 was significantly higher than both younger groups (0.78 ± 0.16 Hz; $n = 44$; $P < 0.001$). In contrast, mEPSC size did not significantly increase with age, with mean amplitudes of 12.11 ± 1.28 pA ($n = 27$) at P0–P1, 12.54 ± 1.31 pA at P5–P6 ($n = 26$) and 12.90 ± 0.92 pA at P9–P10 ($n = 44$; $P > 0.05$). The frequency of EPSCs recorded in TTX was significantly lower than the sEPSC frequency (in aCSF) in both the P5–P6 and P9–P10 groups (Fig. 1*A*; $P < 0.0001$; Student's two-tailed *t* test), suggesting that the generation of spontaneous action potentials contributes to sEPSC frequency in the neonatal dorsal horn *in vitro*.

Capsaicin increases mEPSC frequency in P0–P10 dorsal horn neurons

The addition of capsaicin (2 μM) to the bath resulted in a large increase in mEPSC frequency, which generally was superimposed upon an inward current displaying slow kinetics (Fig. 1*B*). The miniature postsynaptic currents (mPSCs) elicited by capsaicin were verified to be glutamatergic (AMPA/kainate-mediated) mEPSCs, with no significant contamination of the recordings by mIPSCs, by the following observations. (a) Pretreatment with NBQX (10 μM) prevented the mPSCs during a subsequent capsaicin application ($n = 3$; data not shown). (b) The inclusion of bicuculline (10 μM) and strychnine (0.5 μM) in the extracellular solution did not affect the elevation in mPSC frequency by capsaicin ($n = 5$), although subsequent perfusion with NBQX abolished the mPSCs (see Fig. 1*B*), and (c) capsaicin (2 μM) did not increase the frequency of mIPSCs isolated at a holding potential of 0 mV ($n = 6$; data not shown).

An example of the significant shift in the distribution of mEPSC inter-event intervals after capsaicin treatment is shown in Fig. 1*C* for a representative neuron at P10 ($P < 0.0001$; Kolmogorov-Smirnov two-sample test). The elevation of mEPSC frequency over baseline occurred for a prolonged duration, often remaining > 20 min after the cessation of capsaicin application (data not shown). The magnitude of the capsaicin response decreased with subsequent applications, thus all measurements of mEPSC properties were made after the primary application of capsaicin and slices were discarded after first exposure to the drug. The effect was concentration dependent (Fig. 1*E*), as the mEPSC frequency at P9–P10 was elevated from

Journal of Physiology

0.78 ± 0.02 Hz ($n = 15$) in TTX alone to 4.39 ± 1.77 Hz after addition of 100 nM capsaicin ($n = 8$; $P < 0.05$), and 44.26 ± 11.92 Hz after addition of 2 μM capsaicin ($n = 7$; $P < 0.001$). The application of vehicle solution alone did not significantly alter the mEPSC frequency (data not shown). Although 2 μM capsaicin significantly augmented mEPSC frequency in all neonatal dorsal horn neurons examined ($P < 0.05$; Kolmogorov-Smirnov test; $n = 20$), the magnitude of the effect varied with postnatal age. As illustrated in Fig. 1*F*, the P0–P1 and P5–P6 groups showed similar mEPSC frequencies after exposure to 2 μM capsaicin (P0–P1: 13.76 ± 2.70 Hz, $n = 6$; P5–P6: 11.90 ± 2.82 Hz, $n = 6$). However, the ability of capsaicin to facilitate mEPSC frequency was rapidly enhanced over the next few days, as demonstrated by the comparatively high rate of mEPSCs seen at P9–P10 (Fig. 1*E* and *F*).

After application of 2 μM capsaicin at P9–P10, the overlap of mEPSCs was extensive, which may compromise the accurate measurement of mEPSC amplitudes. As a result, in order to evaluate the effect of capsaicin on the amplitude of mEPSCs, we chose a lower concentration of capsaicin (100 nM) that significantly enhanced the mEPSC frequency (Fig. 1*E*) without causing the same degree of mEPSC superimposition. At this concentration, capsaicin failed to significantly alter the amplitude of mEPSCs in P9–P10 dorsal horn neurons ($n = 8$; Fig. 1*D*), suggesting it acts presynaptically to increase the spontaneous release of glutamate in the neonatal dorsal horn.

Finally, the peak amplitude of the slow inward current evoked by capsaicin was not clearly related to postnatal age (Fig. 1*G*), as the peak amplitude measured 20.94 ± 4.71 pA at P0–P1 ($n = 6$), 14.96 ± 4.76 pA at P5–P6 ($n = 6$), and 34.99 ± 6.15 pA at P9–P10 ($n = 12$; $P > 0.05$; one-way ANOVA). The mechanism underlying this slow conductance was not investigated in detail.

Role of VR1 receptors on primary afferent terminals

As seen in Fig. 2*A*, exposure to the non-competitive VR1 antagonist Ruthenium Red (RR; 10 μM) significantly increased the mEPSC frequency from 0.60 ± 0.17 Hz in TTX to 3.07 ± 0.45 Hz, which is consistent with previous work indicating that RR induces transmitter release via Ca^{2+}-independent mechanisms by binding to external sites on the presynaptic membrane (Trudeau *et al.* 1996). More importantly, application of RR prevented a significant enhancement in mEPSC frequency with subsequent perfusion with 2 μM capsaicin (4.90 ± 0.71 Hz; $n = 4$) at P9–P10. Bath application of the competitive VR1 antagonist capsazepine (CPZ; 10 μM) did not alter the mEPSC frequency (TTX: 0.76 ± 0.27 Hz; CPZ: 0.62 ± 0.24 Hz; $n = 10$), and failed to block the increase in mEPSC frequency seen with 2 μM capsaicin (22.65 ± 10.90 Hz; $n = 5$; $P < 0.001$). However, capsazepine abolished the facilitating effects of 100 nM capsaicin on mEPSC frequency at P9–P10 (Fig. 2*B*).

As seen in Fig. 2*C*, following unilateral L3–L5 dorsal rhizotomy at P3, capsaicin (2 μM) failed to significantly elevate mEPSC frequency in P8–P11 neurons ipsilateral to the rhizotomy (TTX: 0.76 ± 0.23 Hz; capsaicin: 2.94 ± 1.22 Hz; $n = 7$; $P > 0.05$), while significantly increasing mEPSC frequency on the contralateral side (TTX: 1.0 ± 0.43 Hz; capsaicin: 69.91 ± 6.37 Hz; $n = 5$; $P < 0.001$). This suggests that capsaicin acts via the VR1 receptor on the terminals of primary afferents to increase the release of glutamate in the P0–P11 dorsal horn.

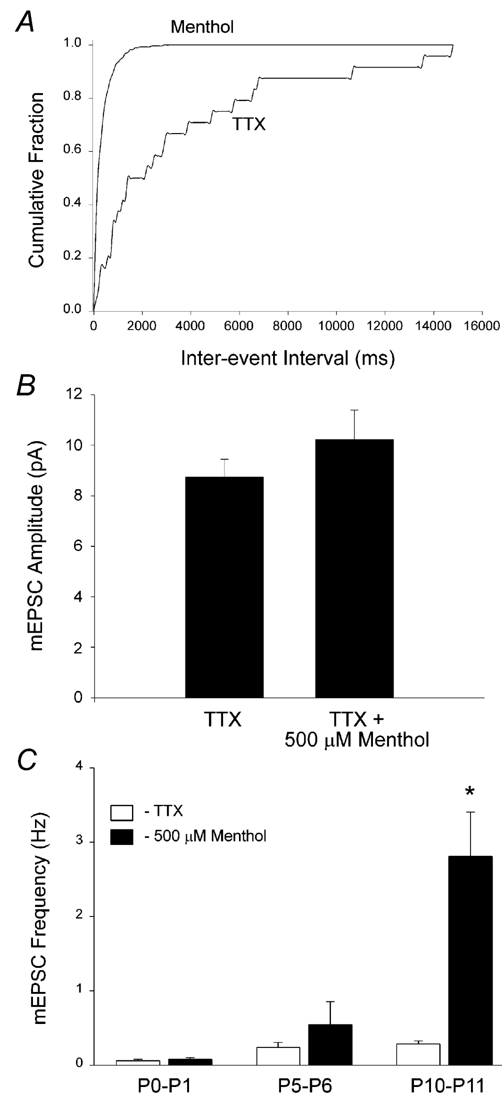

Figure 3. Menthol increases mEPSC frequency in P10–P11 neurons of the superficial dorsal horn

A, cumulative probability function describing the shift in mEPSC inter-event intervals seen after application of 500 μM (−)menthol in a P10 cell. The intervals were significantly reduced following menthol treatment ($P < 0.05$; Kolmogorov-Smirnov two-sample test) in all P10–P11 neurons examined ($n = 6$). *B*, menthol (500 μM) did not significantly affect mEPSC amplitudes in a population of P10–P11 neurons ($n = 6$; $P > 0.05$; paired *t* test). *C*, facilitation of mEPSC frequency by menthol is dependent on postnatal age (* $P < 0.01$; Student's unpaired *t* test).

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Modulation of glutamate release by menthol in neonatal dorsal horn

We wished to determine if CMR1 receptors were also transported centrally to the terminals of primary afferent fibres and could modulate glutamate release in the P0–P11 dorsal horn. At P0–P1, the frequency of mEPSCs immediately following perfusion with 500 μM (−)menthol (0.08 ± 0.02 Hz) was similar to the baseline frequency (0.06 ± 0.02; $n = 5$; Fig. 3*C*). Menthol also failed to have a significant effect on mEPSC frequency at P5–P6 (TTX: 0.24 ± 0.06 Hz; menthol: 0.54 ± 0.31 Hz; $n = 7$; $P > 0.05$; Student's paired t test). However, as demonstrated in Fig. 3*C*, by P10–P11 the application of menthol significantly increased the average mEPSC frequency from 0.29 ± 0.04 to 2.81 ± 0.60 Hz ($n = 6$; $P < 0.01$; Student's paired t test). In all six neurons tested at this age, 500 μM (−)menthol significantly altered the distribution of inter-event intervals ($P < 0.05$; Kolmogorov-Smirnov two-sample test) as illustrated for a representative neuron in Fig. 3*A*. The application of vehicle (ethanol) alone had no effect on mEPSC frequency (data not shown). In addition, the mean mEPSC amplitude was not significantly affected by menthol at P10–P11 ($n = 6$; $P > 0.05$; Student's paired t test) as summarized in Fig. 3*B*, suggesting a presynaptic site of action.

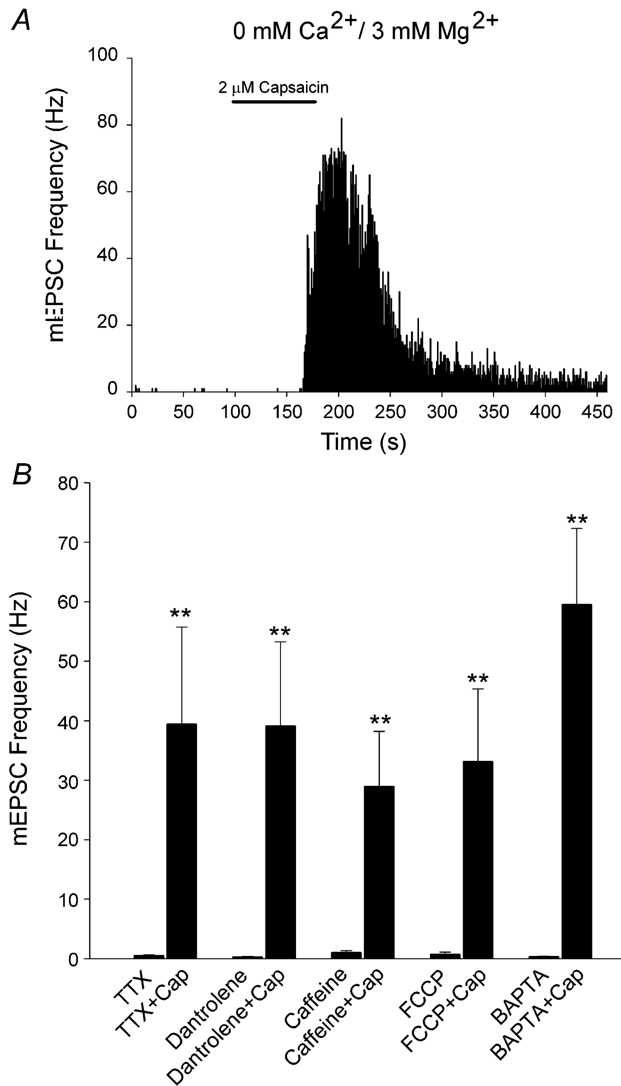

Figure 4. Effect of capsaicin on mEPSC frequency does not require external Ca^{2+}

A, plot of mEPSC frequency *vs.* time for a representative P10 neuron perfused in zero Ca^{2+} and 3 mM Mg^{2+} solution, illustrating a significant rise in mEPSC frequency after application of 2 μM capsaicin (bar). *B*, at P10, prior application of dantrolene sodium (30 μM; $n = 3$), caffeine (20–30 mM; $n = 8$) or FCCP (2 μM; $n = 5$) failed to prevent the significant increase in mEPSC frequency by capsaicin (2 μM) under zero-Ca^{2+} conditions (** $P < 0.001$; ANOVA). The capsaicin effect also persists despite pre-exposure of the slice to the membrane permeable Ca^{2+} chelator BAPTA AM (50 μM) for 10–15 min ($n = 6$; ** $P < 0.001$).

Capsaicin effects on mEPSC frequency are independent of external Ca^{2+}

Surprisingly, the removal of extracellular Ca^{2+} (in the presence of 3 mM Mg^{2+}) did not prevent the elevation in mEPSC frequency seen after 2 μM capsaicin treatment (Fig. 4*A*), although the EPSC evoked by electrical stimulation of the dorsal root was abolished under these conditions ($n = 4$; data not shown). In fact, the magnitude of the capsaicin effect was similar to that observed in the presence of 2 mM extracellular Ca^{2+}, as the mEPSC

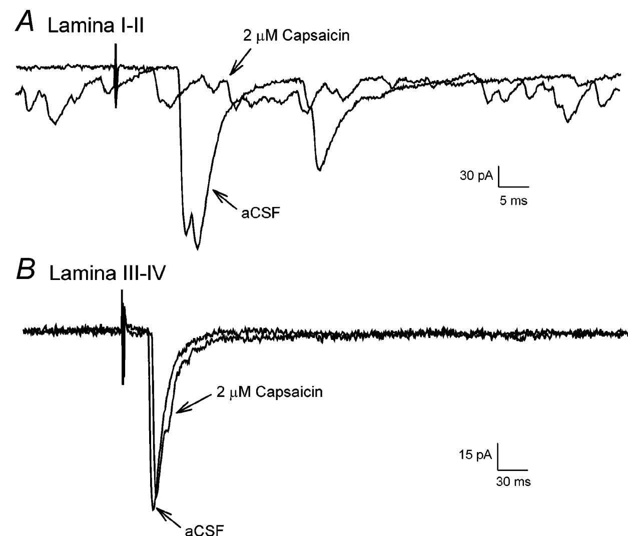

Figure 5. Capsaicin inhibits electrically evoked EPSCs in the superficial laminae of the neonatal dorsal horn

A, examples of evoked EPSCs (eEPSCs) recorded in a lamina I–II neuron at P10 in response to electrical stimulation of the attached dorsal root (threshold: 69 μA, 50 μs; latency: 10.5 ms). In this cell, a complete block of both the early and late components of the eEPSC accompanies the increased sEPSC frequency seen after capsaicin (2 μM). *B*, representative EPSCs evoked in a deep (lamina III–IV) dorsal horn neuron (threshold: 70 μA, 50 μs; latency: 3.4 ms) illustrating the lack of effect of capsaicin on eESPC amplitude.

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frequency increased from 0.26 ± 0.11 Hz in TTX alone to 39.43 ± 16.11 Hz after capsaicin ($n = 5$; $P < 0.001$; Fig. 4*B*). The elevated mEPSC frequency with capsaicin also occurred when 1 mM EGTA was added to the external solution under zero-Ca^{2+} conditions (data not shown).

Although the modulation of neurotransmitter release by intracellular Ca^{2+} stores has not yet been described in the rat dorsal horn, it has been documented in other areas of the CNS (Reyes & Stanton, 1996; Brodin *et al.* 1999; Scotti *et al.* 1999). To determine if the capsaicin effect was dependent on Ca^{2+} release from ryanodine-sensitive stores that may exist in the terminals of primary afferents, we examined the effect of capsaicin on mEPSC frequency in slices that had been previously perfused for 5–10 min with either 20–30 mM caffeine or 30 μM dantrolene sodium (an antagonist at the ryanodine receptor). Caffeine did not significantly alter the baseline mEPSC frequency (TTX: 0.53 ± 0.17 Hz; caffeine: 1.04 ± 0.29 Hz; $n = 8$; $P > 0.05$) under zero-Ca^{2+} conditions and failed to occlude the capsaicin response (28.96 ± 9.23 Hz; $n = 8$; $P < 0.001$) as seen in Fig. 4*B*. Similarly, the elevation in mEPSC frequency by capsaicin persisted in the presence of dantrolene ($n = 3$). In an attempt to manipulate any existing presynaptic mitochondrial Ca^{2+} stores, we perfused P9–P10 slices with the protonophore FCCP (2 μM) prior to the capsaicin application under zero-Ca^{2+} conditions. The frequency of mEPSCs in FCCP (0.74 ± 0.34 Hz, $n = 5$) was not significantly different from that in TTX alone (0.31 ± 0.09 Hz; $n = 4$). In addition, in the presence of FCCP, capsaicin still increased the frequency of mEPSCs to 33.13 ± 12.2 Hz (Fig. 4*B*; $n = 5$; $P < 0.001$).

In addition, the membrane-permeable Ca^{2+} chelator BAPTA AM (50 μM) was applied to P9–P10 dorsal horn neurons for 10–15 min before exposure to 2 μM capsaicin in an external solution containing 0 mM Ca^{2+} and 3 mM Mg^{2+}. A similar application of BAPTA AM significantly reduced EPSCs evoked by dorsal root stimulation in the presence of 2 mM Ca^{2+} (18.9 ± 4.0 %; $n = 4$; data not shown), suggesting that the chelator gained access to the synaptic terminals within this time period. Despite the presence of BAPTA AM, capsaicin increased the mEPSC frequency from 0.33 ± 0.07 to 59.55 ± 12.77 Hz ($n = 6$; $P < 0.001$) as illustrated in Fig. 4*B*.

Capsaicin inhibits EPSCs evoked by dorsal root stimulation

The effect of VR1 activation on the EPSCs evoked by electrical stimulation of the attached dorsal root was also examined in P7–P10 dorsal horn neurons. In 7 out of 10 neurons located in the superficial dorsal horn (laminae I–II), the increased frequency of sEPSCs seen with the application of 2 μM capsaicin was accompanied by either a complete block or a significant reduction in the amplitude of the evoked EPSC (Fig. 5*A*). At room temperature, monosynaptic EPSCs recorded in this region showed a mean latency of 12.12 ± 2.14 ms ($n = 10$) and a highly variable recruitment threshold (data not shown). Evoked EPSCs recorded in neurons located deeper in the dorsal horn (laminae III–IV) exhibited shorter latencies (3.28 ± 0.18 ms; $n = 4$) and lower stimulus thresholds than the more superficial cells. However, in three of four deep neurons there was no apparent effect of capsaicin on both the sEPSC frequency and the amplitude of the evoked EPSC (Fig. 5*B*), consistent with a preferential action of capsaicin on nociceptive inputs to the spinal cord dorsal horn.

Capsaicin evokes action potentials in P1–P5 dorsal horn neurons

To determine if capsaicin application could evoke APs in dorsal horn neurons during the first postnatal week, we recorded the response of P1–P5 neurons to bath application of 2 μM capsaicin under current clamp conditions. Spontaneous APs rarely occurred in P1–P5 neurons at membrane potential (V_m) = −60 to −65 mV. At P1, capsaicin application produced a slow depolarization that was accompanied by an increase in the frequency of EPSPs in every cell tested ($n = 6$). In two out of six neurons, APs were observed after exposure to capsaicin (Fig. 6*A*). By P5, the depolarization evoked by capsaicin was sufficient to produce bursts of APs in all six neurons examined (Fig. 6*B*), with an average of 77.7 ± 38.6 spikes observed ($n = 6$).

DISCUSSION

The present study characterizes the development of nociceptive synaptic transmission in the superficial dorsal

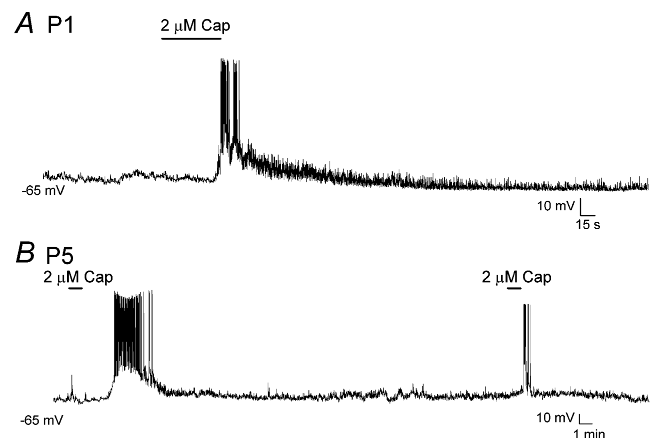

Figure 6. Capsaicin elicits action potentials in superficial dorsal horn neurons during the first postnatal week

In current clamp, cells were maintained at V_m = −60 to −65 mV with DC injection and 2 μM capsaicin was subsequently bath applied at the indicated times to P1 (*A*) or P5 (*B*) neurons.

Journal of Physiology

horn of the neonatal rat for the first time. Both the spontaneous (or background) synaptic activity and the functional maturation of synaptic inputs originating from a distinct subset of capsaicin- and menthol-sensitive nociceptive primary afferents have been investigated.

Age-dependent increase in spontaneous glutamate release and the role of presynaptic action potentials

Spontaneous excitatory postsynaptic currents (sEPSCs) mediated by glutamate were evident from P0 and demonstrated a rapid increase in frequency over the first 10 days of postnatal life, particularly between the ages of P5 and P10 (Fig. 1*A*). The frequency of TTX-resistant mEPSCs was significantly lower than the sEPSC frequency at P5–P6 and P9–P10, suggesting that presynaptic action potentials (APs) contribute to sEPSC generation in the neonatal dorsal horn *in vitro*. This has also been observed in a previous study of embryonic and early postnatal (P1–P3) lumbar motoneurons (Gao *et al.* 1998), which reported a similar contribution of APs to sEPSC frequency in the two age groups. Our results also indicate a relatively stable contribution of APs to sEPSC generation in the dorsal horn during the first 10 postnatal days, as the ratio of sEPSC frequency to mEPSC frequency was similar in all age groups examined. The role of AP invasion of the presynaptic terminal in generating dorsal horn sEPSCs seems to subsequently diminish with maturation, as application of TTX does not significantly affect EPSC frequency in the adult dorsal horn (Bao *et al.* 1998; Yang *et al.* 1998). The developmental increase in mEPSC frequency (Fig. 1*A*) may be explained by an increase in the number of synaptic release sites or age-dependent changes in the probability of transmitter release. However, we cannot conclusively exclude a role for an age-dependent recruitment of postsynaptic AMPA receptors to previously 'silent' synapses (Li & Zhuo, 1998).

Primary afferents expressing VR1 establish functional synapses in the dorsal horn from birth

Recent work has identified VR1 as the capsaicin receptor and a major transduction site for noxious thermal and chemical stimuli in a subset of small-diameter sensory neurons (Caterina *et al.* 1997; Tominaga *et al*, 1998). In the present study, the elevation in mEPSC frequency evoked by 2 μM capsaicin was mediated via VR1 receptors since it was blocked by the non-competitive antagonist Ruthenium Red (Fig. 2). The competitive antagonist capsazepine failed to block the effect of 2 μM capsaicin but did block the action of lower concentrations, which may be explained by the limited potency of the antagonist (Szallasi & Blumberg, 1999). However, it is also possible that higher concentrations of capsaicin activate capsazepine-insensitive VRs that have been documented in rat trigeminal neurons (Liu *et al.* 1998). In contrast, the effect of 2 μM capsaicin on mEPSC frequency was reportedly blocked by capsazepine in the adult dorsal horn (Yang *et al.* 1998). Whether or not this discrepancy results from a developmental difference in the expression of VR subtypes remains unclear.

The ability of capsaicin to increase mEPSC frequency in neonatal dorsal horn neurons at concentrations that do not alter mEPSC amplitudes (Fig. 1), along with the abolition of the effect by dorsal rhizotomy (Fig. 2*C*), strongly suggests a presynaptic site of action. This is consistent with reports demonstrating that VR1 is predominantly localized to the terminals of primary afferent neurons in the rat dorsal horn (Guo *et al.* 1999, 2001). Although somatic and dendritic VR1 expression has been documented in lamina II of the adult dorsal horn (Valtschanoff *et al.* 2001), it seems unlikely to play a major role in the observed elevation of glutamate release by capsaicin. First, there is no evidence that every lamina II neuron expresses VR1 postsynaptically. The presence of TTX in the bath solution excludes the possibility that a small population of VR1-positive spinal neurons consistently contribute to the recorded mEPSC frequency via polysynaptic pathways. Thus the percentage of dorsal horn neurons that exhibit postsynaptic VR1 immunoreactivity appears insufficient to explain the present finding that capsaicin (2 μM) increased the mEPSC frequency in all neurons examined. Second, the enhancement of mEPSC frequency by capsaicin occurred despite the use of negative holding potentials (at which NMDA receptors are probably blocked by Mg^{2+}) and in the absence of extracellular Ca^{2+} (Figs 1 and 4). These conditions should prohibit Ca^{2+} entry via NMDA receptor activation and any downstream insertion of functional AMPA receptors to previously 'silent' synapses, which could potentially result in a higher mEPSC frequency via postsynaptic mechanisms (Isaac *et al.* 1995). Although we cannot completely exclude the possibility that VR1 is expressed at the presynaptic terminals of a subset of spinal interneurons and thus influences the recorded mEPSC frequency, the failure of capsaicin to modulate mIPSC frequency (data not shown) argues against widespread presynaptic expression of VR1 in dorsal horn interneurons. Thus, the most likely explanation for the increased mEPSC frequency after capsaicin involves a direct effect on the presynaptic terminals of VR1-expressing primary afferents which synapse directly onto the recorded dorsal horn neuron. Importantly, the data suggest that a subset of nociceptors expressing VR1 establish functional synapses at least as early as P0 (Fig. 1) and that chemical activation of these terminals is sufficient to evoke action potentials in neonatal dorsal horn neurons (Fig. 6).

Role for presynaptic menthol receptors in synaptic transmission within the immature dorsal horn *in vitro*

The recently cloned CMR1 receptor responds to menthol and temperatures that encompass the innocuous cool

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Journal of Physiology

(15–28 °C) and part of the noxious cold (8–15 °C) range and a significant fraction (~50 %) of menthol-sensitive sensory neurons also express VR1 and can thus be categorized as nociceptors (McKemy *et al.* 2002). The lack of a consistent effect of menthol on mEPSC amplitude, along with the observation that CMR1 RNA was absent in the spinal cord (McKemy *et al.* 2002), suggests that menthol acts presynaptically to elevate mEPSC frequency. Further experiments are necessary to clarify whether capsaicin and menthol evoke glutamate release from the same primary afferent fibres in the dorsal horn. Although CMR1 is expressed in <15 % of sensory neurons, we found that menthol increased mEPSC frequency in all cells tested by P10–P11, suggesting either that menthol activates multiple receptor subtypes or that CMR1-positive afferents exhibit considerable divergence in the dorsal horn. The present observation that capsaicin increased mEPSC frequency in all superficial dorsal horn neurons also supports the idea of extensive divergence of nociceptive inputs to the dorsal horn (Cervero *et al.* 1984).

Functional classification of primary afferents mediating capsaicin effect on mEPSC frequency

The majority of dorsal root fibres destroyed by neonatal treatment with capsaicin are unmyelinated (Nagy *et al.* 1983; Jancso & Lawson, 1990; Hiura *et al.* 1999) and electron microscopy studies have confirmed that VR1-positive dorsal root afferents in the rat are predominantly unmyelinated (Valtschanoff *et al.* 2001). However, colocalization of VR1 and the A-fibre neurofilament NF200 has recently been demonstrated (Ma, 2002). Capsaicin-sensitive A-fibres have been identified in primates (Ringkamp *et al.* 2001) and constitute a significant portion of the vanilloid-sensitive afferents innervating the tooth pulp of the cat (Ikeda *et al.* 1997). Capsaicin-sensitive Aδ-fibres innervating the skin have also been reported in the adult rat (Seno & Dray, 1993), although it should be emphasized that effects of capsaicin on A-fibres were documented only at concentrations higher than 1.0 μM (Seno & Dray, 1993), while in the present study 100 nM capsaicin significantly increased the mEPSC frequency at all ages examined between P0 and P11. The possibility that capsaicin sensitivity is greater in C-fibres than A-fibres is supported by previous work demonstrating that significantly higher doses of neonatal capsaicin are required to elicit a loss of small diameter A-fibres in the dorsal root compared with the dose needed to destroy unmyelinated fibres (Nagy *et al.* 1983; Jancso & Lawson, 1990). Given that the majority of VR1 expression is located in unmyelinated fibres, it seems probable that a fraction of the synapses activated by capsaicin between P0 and P10 represent C-fibre contacts onto dorsal horn neurons. However, we cannot conclusively rule out the possibility that capsaicin elicits glutamate release solely from Aδ-fibres at P0, with a gradually increasing contribution of C-fibre synapses with later postnatal ages. In addition, it remains a possibility that a subpopulation of low-threshold A-fibres transiently expresses VR1 during early postnatal life and contributes to the elevation in mEPSC frequency by capsaicin. However, it should be noted that our preliminary results suggest the actions of capsaicin were selective for the superficial dorsal horn (Fig. 5), and previous work has demonstrated that the percentage of total DRG neurons expressing VR1 does not change during development (Guo *et al.* 2001).

Possible mechanisms of glutamate release by VR1 activation

Surprisingly, the elevation of glutamate release by capsaicin appears not to involve transmembrane influx of Ca^{2+} via the VR1 receptor, as capsaicin strongly increased mEPSC frequency in the absence of extracellular Ca^{2+} (Fig. 4). Mitochondrial and ryanodine-sensitive intracellular Ca^{2+} stores are both capable of regulating transmitter release and synaptic plasticity in various systems (Reyes & Stanton, 1996; Brodin *et al.* 1999; Scotti *et al.* 1999). However, the observed capsaicin effect persisted in the presence of caffeine, dantrolene or FCCP (Fig. 4*B*). Perfusion with BAPTA AM inhibited the electrically evoked EPSCs in the dorsal horn to a similar degree to that reported in the basolateral amygdala (11–24 %; Li *et al.* 2001; Rammes *et al.* 2001), but also failed to prevent the increased mEPSC frequency with capsaicin under zero-Ca^{2+} conditions (Fig. 4*B*). Collectively, these preliminary results suggest that the ability of capsaicin to augment glutamate release in the neonatal dorsal horn may occur via mechanisms other than intracellular Ca^{2+} release and may not depend on an increase in terminal $[Ca^{2+}]_i$. However, it should be noted that since little is known about the possible ryanodine-sensitive and mitochondrial intracellular Ca^{2+} stores in primary afferent terminals, it is difficult to verify that the above treatments sufficiently depleted these stores prior to capsaicin application. Thus additional experiments need to be performed in order to conclusively determine if capsaicin elicits glutamate release via Ca^{2+}-independent mechanisms, which have been previously implicated in the facilitation of transmitter release by trinitrobenzene sulphonic acid at the frog neuromuscular junction (Kijima & Tanabe, 1988) and by α-latrotoxin in the CA3 region of the hippocampus (Capogna *et al.* 1996).

One intriguing possibility is that the depolarization of the terminal membrane via VR1 activation leads directly to glutamate release from primary afferents. Recent experiments have pointed to a Ca^{2+}-independent but voltage-dependent exocytosis of vesicles from the somata of rat DRG neurons via an undetermined mechanism (Zhang & Zhou, 2002). A possible link between changes in membrane potential and vesicular exocytosis are the N-type voltage-dependent Ca^{2+}-channels (VDCC) which interact directly with the SNARE proteins involved in

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Journal of Physiology

neurotransmitter release (Sheng *et al.* 1994, 1996; Bajjalieh & Scheller, 1995). A recent study documented a voltage-dependent, Ca^{2+}-independent enhancement of transmitter release in cultured rat superior cervical ganglion neurons, which was blocked when the interaction of the N-type VDCC and SNARE proteins was disrupted (Mochida *et al.* 1998).

Differential effects of capsaicin on spontaneous *vs.* evoked glutamate release and its implications for *in vivo* activation of spinal VR1 receptors

Consistent with earlier reports in the adult spinal cord (Yang *et al.* 1999), we found that capsaicin inhibited the EPSCs evoked by electrical stimulation of the dorsal root (Fig. 5), despite eliciting an increase in the spontaneous, asynchronous release of glutamate and resulting mEPSC frequency. It is not clear if the reduction in the evoked EPSC is due to a direct block of primary afferent conduction in the dorsal root by capsaicin as has been described previously for C-fibres in rat sciatic nerves (Wall & Fitzgerald, 1981), or due to a depolarization of the primary afferent terminals which could reduce evoked, synchronous neurotransmitter release (Rudomin & Schmidt, 1999). Presynaptic depolarization via $GABA_A$ receptors was recently shown to have opposite effects on spontaneous *versus* evoked transmitter release at glycinergic terminals in the rat spinal cord (Jang *et al.* 2002).

The present results cast light on the possible functions of central VR1 receptors in the intact neonatal spinal cord. Activation of presynaptic VR1 receptors on nociceptive primary afferents by endogenous VR1 ligands could result in increased levels of background synaptic activity in dorsal horn neurons, as well as a reduction in the efficacy of peripheral inputs to the dorsal horn resulting from noxious stimuli. However, recent studies found that intrathecal application of capsazepine significantly inhibited both Aδ- and C-fibre-evoked responses in adult dorsal horn neurons, which would suggest that VR1 activation facilitates evoked synaptic transmission at nociceptive synapses *in vivo* (Kelly & Chapman, 2002). The reasons for this discrepancy remain unknown. However, it should be noted that the *in vitro* experiments used capsaicin at 1–2 μM to examine its effects on evoked EPSCs, while both the identity and concentration of the endogenous ligand is unknown. One possibility is that VR1 agonists exert a biphasic effect on synaptic transmission such that low levels of VR1 activation potentiate evoked EPSCs by increasing intraterminal Ca^{2+}, while higher concentrations produce maximal VR1 activation and presynaptic inhibition via primary afferent depolarization.

The identification of the endogenous VR1 ligand(s) has been the focus of many recent investigations. Anandamide and lipoxygenase derivatives are potential candidates, but both exhibit a much lower potency than capsaicin at VR1 (Zygmunt *et al.* 1999; Hwang *et al.* 2000). In contrast, the dopamine derivative *N*-arachidonoyl-dopamine (NADA) shows a similar potency to that of capsaicin at rat and human VR1 receptors expressed in human embryonic kidney cells (HEK) and is a potent agonist at native vanilloid receptors in rat DRG neurons (Huang *et al.* 2002). Importantly, Huang *et al.* report that synthesis of NADA occurs in the CNS *in vivo*, although the relative level of expression in the spinal cord is presently unclear.

Maturation of C-fibre synapses and nociceptive transmission in the dorsal horn

The present results suggest that the failure of C-fibre electrical stimulation to evoke APs in superficial dorsal horn cells before P10 (Fitzgerald, 1988; Jennings & Fitzgerald, 1998) may not necessarily indicate an absence of C-fibre synapses during this period. The presence of synaptic contacts between VR1-expressing sensory neurons and dorsal horn neurons at P0, along with the likelihood that spinal VR1 activation occurs at least partially in unmyelinated neonatal afferents, suggest the possibility that C-fibres begin forming synapses in the region before birth. These nascent synapses are functional in that they are capable of releasing glutamate in response to chemical stimuli and subsequently activating postsynaptic AMPA/kainate receptors on spinal neurons. There are many possible explanations for why these C-fibre inputs appear to remain subthreshold *in vivo* until the second postnatal week. First, the mEPSC frequency after capsaicin application increases dramatically between the ages of P5 and P10, suggesting that the majority of functional C-fibre connections are formed during this time period. During the first few postnatal days, the number of C-fibre synapses or their release probabilities may be too low to sufficiently depolarize the postsynaptic neuron to threshold under most circumstances. Also, it should be noted that the release of neurotransmitter documented in the present study occurs asynchronously and independently of transmembrane Ca^{2+} influx. Thus, one possibility is that by the first day of postnatal life, immature C-fibre synapses are already in place and can release glutamate spontaneously but lack the ability to synchronously release large numbers of presynaptic vesicles in response to a rapid intracellular Ca^{2+} transient. The synchronization of transmitter release during early postnatal development has been previously described at synapses in the medial nucleus of the trapezoid body (MNTB) and seems to involve the maturation of Ca^{2+} extrusion and buffering mechanisms within the presynaptic terminal (Chuhma *et al.* 2001; Chuhma & Ohmori, 2002). Identification of the factors that govern the strengthening of C-fibre inputs to the superficial dorsal horn will be essential to gaining a better understanding of how the developing nervous system processes painful stimuli.

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REFERENCES

Baccei M, Bardoni R & Fitzgerald M (2002). Capsaicin elicits glutamate release in the superficial dorsal horn of the neonatal rat spinal cord. International Association for the Study of Pain, 10th World Congress on Pain Abstracts, p. 70.

Bajjalieh SM & Scheller RH (1995). The biochemistry of neurotransmitter secretion. *J Biol Chem* **270**, 1971–1974.

Bao J, Li JJ & Perl ER (1998). Differences in Ca^{2+} channels governing generation of miniature and evoked excitatory synaptic currents in spinal laminae I and II. *J Neurosci* **18**, 8740–8750.

Bennett DL, Averill S, Clary DO, Priestley JV & McMahon SB (1996). Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. *Eur J Neurosci* **8**, 2204–2208.

Brodin L, Bakeeva L & Shupliakov O (1999). Presynaptic mitochondria and the temporal pattern of neurotransmitter release. *Philos Trans R Soc Lond B Biol Sci* **354**, 365–372.

Capogna M, Gahwiler BH & Thompson SM (1996). Calcium-independent actions of alpha-latrotoxin on spontaneous and evoked synaptic transmission in the hippocampus. *J Neurophysiol* **76**, 3149–3158.

Carroll SL, Silos-Santiago I, Frese SE, Ruit KG, Milbrandt J & Snider WD (1992). Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation *in utero*. *Neuron* **9**, 779–788.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD & Julius D (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824.

Cervero F, Shouenborg J, Sjolund BH & Waddell PJ (1984). Cutaneous inputs to dorsal horn neurones in adult rats treated at birth with capsaicin. *Brain Res* **301**, 47–57.

Chuhma N, Koyano K & Ohmori H (2001). Synchronisation of neurotransmitter release during postnatal development in a calyceal presynaptic terminal of rat. *J Physiol* **530**, 93–104.

Chuhma N & Ohmori H (2002). Role of Ca(2+) in the synchronization of transmitter release at calyceal synapses in the auditory system of rat. *J Neurophysiol* **87**, 222–228.

Fitzgerald M (1988). The development of activity evoked by fine diameter cutaneous fibres in the spinal cord of the newborn rat. *Neurosci Lett* **86**, 161–166.

Fitzgerald M & Gibson S (1984). The postnatal physiological and neurochemical development of peripheral sensory C fibres. *Neuroscience* **13**, 933–944.

Fitzgerald M, King AE, Thompson SW & Woolf CJ (1987). The postnatal development of the ventral root reflex in the rat; a comparative *in vivo* and *in vitro* study. *Neurosci Lett* **78**, 41–45.

Friede RL & Samorajski T (1968). Myelin formation in the sciatic nerve of the rat. A quantitative electron microscopic, histochemical and radioautographic study. *J Neuropathol Exp Neurol* **27**, 546–570.

Gao BX, Cheng G & Ziskind-Conhaim L (1998). Development of spontaneous synaptic transmission in the rat spinal cord. *J Neurophysiol* **79**, 2277–2287.

Guo A, Simone DA, Stone LS, Fairbanks CA, Wang J & Elde R (2001). Developmental shift of vanilloid receptor 1 (VR1) terminals into deeper regions of the superficial dorsal horn: correlation with a shift from TrkA to Ret expression by dorsal root ganglion neurons. *Eur J Neurosci* **14**, 293–304.

Guo A, Vulchanova L, Wang J, Li X & Elde R (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur J Neurosci* **11**, 946–958.

Hall AK, Ai X, Hickman GE, MacPhedran SE, Nduaguba CO & Robertson CP (1997). The generation of neuronal heterogeneity in a rat sensory ganglion. *J Neurosci* **17**, 2775–2784.

Hiura A, Nakagawa H, Koshigae Y, Yoshizako A, Kubo Y & Ishizuka H (1999). Age-related changes in the response to thermal noxious heat and reduction of C-fibers by neonatal treatment with capsaicin. *Somatosens Mot Res* **16**, 115–121.

Hori Y & Watanabe S (1987). Morphine-sensitive late components of the flexion reflex in the neonatal rat. *Neurosci Lett* **78**, 91–96.

Huang SM, Bisogno T, Trevisani M, Al Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros TJ, Krey JF, Chu CJ, Miller JD, Davies SN, Geppetti P, Walker JM & Di MV (2002). An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. *Proc Natl Acad Sci U S A* **99**, 8400–8405.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D & Oh U (2000). Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc Natl Acad Sci U S A* **97**, 6155–6160.

Ikeda H, Tokita Y & Suda H (1997). Capsaicin-sensitive A delta fibers in cat tooth pulp. *J Dent Res* **76**, 1341–1349.

Isaac JT, Nicoll RA & Malenka RC (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15**, 427–434.

Jackman A & Fitzgerald M (2000). Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. *J Comp Neurol* **418**, 281–298.

Jancso G & Lawson SN (1990). Transganglionic degeneration of capsaicin-sensitive C-fiber primary afferent terminals. *Neuroscience* **39**, 501–511.

Jang IS, Jeong HJ, Katsurabayashi S & Akaike N (2002). Functional roles of presynaptic GABA(A) receptors on glycinergic nerve terminals in the rat spinal cord. *J Physiol* **541**, 423–434.

Jennings E & Fitzgerald M (1998). Postnatal changes in responses of rat dorsal horn cells to afferent stimulation: a fibre-induced sensitization. *J Physiol* **509**, 859–868.

Kelly S & Chapman V (2002). Spinal administration of capsazepine inhibits noxious evoked responses of dorsal horn neurons in non-inflamed and carrageenan inflamed rats. *Brain Res* **935**, 103–108.

Kijima H & Tanabe N (1988). Calcium-independent increase of transmitter release at frog end-plate by trinitrobenzene sulphonic acid. *J Physiol* **403,** 135–149.

Li H, Chen A, Xing G, Wei ML & Rogawski MA (2001). Kainate receptor-mediated heterosynaptic facilitation in the amygdala. *Nat Neurosci* **4**, 612–620.

Li P & Zhuo M (1998). Silent glutamatergic synapses and nociception in mammalian spinal cord. *Nature* **393**, 695–698.

Liu L, Szallasi A & Simon SA (1998). A non-pungent resiniferatoxin analogue, phorbol 12-phenylacetate 13 acetate 20-homovanillate, reveals vanilloid receptor subtypes on rat trigeminal ganglion neurons. *Neuroscience* **84**, 569–581.

Ma QP (2002). Expression of capsaicin receptor (VR1) by myelinated primary afferent neurons in rats. *Neurosci Lett* **319**, 87–90.

McKemy DD, Neuhausser WM & Julius D (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52–58.

Mochida S, Yokoyama CT, Kim DK, Itoh K & Catterall WA (1998). Evidence for a voltage-dependent enhancement of neurotransmitter release mediated via the synaptic protein interaction site of N-type Ca^{2+} channels. *Proc Natl Acad Sci U S A* **95**, 14523–14528.

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Nagy JI, Iversen LL, Goedert M, Chapman D & Hunt SP (1983). Dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat. *J Neurosci* **3**, 399–406.

Rammes G, Eder M, Dodt HU, Kochs E & Zieglgansberger W (2001). Long-term depression in the basolateral amygdala of the mouse involves the activation of interneurons. *Neuroscience* **107**, 85–97.

Reyes M & Stanton PK (1996). Induction of hippocampal long-term depression requires release of Ca^{2+} from separate presynaptic and postsynaptic intracellular stores. *J Neurosci* **16**, 5951–5960.

Ringkamp M, Peng YB, Wu G, Hartke TV, Campbell JN, & Meyer RA (2001). Capsaicin responses in heat-sensitive and heat-insensitive A-fiber nociceptors. *JNeurosci* **21**, 4460–4468.

Rudomin P & Schmidt RF (1999). Presynaptic inhibition in the vertebrate spinal cord revisited. *Exp Brain Res* **129**, 1–37.

Scotti AL, Chatton JY & Reuter H (1999). Roles of Na^{+}–Ca^{2+} exchange and of mitochondria in the regulation of presynaptic Ca^{2+} and spontaneous glutamate release. *Philos Trans R Soc Lond B Biol Sci* **354**, 357–364.

Seno N & Dray A (1993). Capsaicin-induced activation of fine afferent fibres from rat skin *in vitro*. *Neuroscience* **55**, 563–569.

Sheng ZH, Rettig J, Cook T & Catterall WA (1996). Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. *Nature 379*, 451–454.

Sheng ZH, Rettig J, Takahashi M & Catterall WA (1994). Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**, 1303–1313.

Sima A (1974). Studies on fibre size in developing sciatic nerve and spinal roots in normal, undernourished, and rehabilitated rats. *Acta Physiol Scand* suppl. 406, 1–55.

Soyguder Z, Schmidt HH & Morris R (1994). Postnatal development of nitric oxide synthase type 1 expression in the lumbar spinal cord of the rat: a comparison with the induction of c-*fos* in response to peripheral application of mustard oil. *Neurosci Lett* **180**, 188–192.

Szallasi A & Blumberg PM (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol Rev* **51**, 159–212.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI & Julius D (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21**, 531–543.

Trudeau LE, Doyle RT, Emery DG & Haydon PG (1996). Calcium-independent activation of the secretory apparatus by ruthenium red in hippocampal neurons: a new tool to assess modulation of presynaptic function. *J Neurosci* **16**, 46–54.

Valtschanoff JG, Rustioni A, Guo A & Hwang SJ (2001). Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. *J Comp Neurol* **436**, 225–235.

Wall PD & Fitzgerald M (1981). Effects of capsaicin applied locally to adult peripheral nerve. I. Physiology of peripheral nerve and spinal cord. *Pain* **11**, 363–377.

Williams S, Evan G & Hunt SP (1990). Spinal c-fos induction by sensory stimulation in neonatal rats. *Neurosci Lett* **109**, 309–314.

Yang K, Kumamoto E, Furue H, Li YQ & Yoshimura M (1999). Action of capsaicin on dorsal root-evoked synaptic transmission to substantia gelatinosa neurons in adult rat spinal cord slices. *Brain Res* **830**, 268–273.

Yang K, Kumamoto E, Furue H & Yoshimura M (1998). Capsaicin facilitates excitatory but not inhibitory synaptic transmission in substantia gelatinosa of the rat spinal cord. *Neurosci Lett* **255**, 135–138.

Yoshimura M & Nishi S (1993). Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. *Neuroscience* **53**, 519–526.

Zhang C & Zhou Z (2002). Ca(2+)-independent but voltage-dependent secretion in mammalian dorsal root ganglion neurons. *Nat Neurosci* **5**, 425–430.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di M, V, Julius D & Hogestatt ED (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**, 452–457.

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