Postnatal development of the nociceptive withdrawal reflexes in the rat: a behavioural and electromyographic study

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1. The postnatal development of nociceptive withdrawal reflexes was studied. In awake intact rats, forelimb, hindlimb and tail reflexes were recorded on videotape. In decerebrate spinal rats, electromyography (EMG) was used to record nociceptive withdrawal reflexes in musculi extensor digitorum longus (EDL), peronei, gastrocnemius–soleus (G–S) and biceps posterior–semitendinosus (BP–ST). Thermal (short-lasting CO_2 laser pulses) and mechanical stimulation were used.

2. In adults, nociceptive withdrawal reflexes were typically well directed and reflex pathways to single hindlimb muscles had functionally adapted receptive fields. By contrast, at postnatal day (P) 1–7, the nociceptive withdrawal reflexes were often inappropriate, sometimes producing movements towards the stimulation, and EMG recordings revealed unadapted variable receptive fields. With increasing age, the nociceptive withdrawal reflexes progressively became well directed, thus producing localized withdrawal. Both withdrawal movements and spatial organization of the receptive fields were adult-like at P20–25.

3. Up to P25, reflex thresholds were more or less constant in both intact awake rats and spinal decerebrate rats, except in G–S in which no nociceptive withdrawal reflexes were evoked from P20 on. After P25, mechanical, but not thermal, thresholds increased dramatically.

4. EMG recordings revealed that during the first three postnatal weeks, the latency of the CO_2 laser-evoked nociceptive withdrawal reflexes decreased significantly in peronei and BP–ST, but not in EDL, and thereafter increased significantly in peronei, BP–ST and EDL. The magnitude of the nociceptive withdrawal reflexes in these muscles increased markedly between P7 and P20 and showed little change thereafter.

5. Possible mechanisms underlying the postnatal tuning of the nociceptive withdrawal reflexes are discussed.

Nociceptive withdrawal reflexes have served as an important model in the study of the processing of nociceptive information in the developing and adult spinal cord (Fitzgerald & Gibson, 1984; Hammond, 1989). For a long time, these reflexes were assumed to be organized as general 'flexion reflexes', denoting simultaneous activation of predominantly flexor muscles and inhibition of extensor muscles from large receptive fields (Sherrington, 1910). That some extensor muscles also have excitatory nociceptive fields on restricted skin areas was later found (Hagbarth, 1952; see also Schomburg, 1990), thus introducing an exception to the flexion reflex concept. Recently, a more differentiated organization of these reflexes has been revealed (Schouenborg & Kalliomäki, 1990; Kalliomäki, Schouenborg & Dickenson, 1992; Schouenborg, Holmberg & Weng, 1992; Schouenborg & Weng, 1994; Schouenborg, Weng & Holmberg, 1994). On the basis of differences in receptive fields, thresholds, time course and supraspinal control between the reflex responses in different muscles, it has been proposed that the withdrawal reflex system essentially has a 'modular' organization, each 'module' being concerned with the control of a single, or a few synergistic muscles (Schouenborg & Weng, 1994). The strength of cutaneous nociceptive input to each muscle was found to have a spatial pattern on the skin which directly reflects the spatial pattern of the withdrawal of the skin surface ensuing on contraction in the muscle itself (Schouenborg & Weng, 1994). For example, maximal responses in a single muscle were elicited from the skin area maximally withdrawn by the muscle. Therefore, the withdrawal movement pattern caused by a single muscle is, in a sense, 'imprinted' on the withdrawal reflex pathway to that muscle.

The developmental mechanisms underlying this 'imprint' are not known. It may be relevant, however, that immature reflex pathways have large receptive fields which appear to shrink during the last embryonic days in rats (Saito, 1979) and the first postnatal weeks in kittens (Ekholm, 1967). Also, reflex responses elicited by nociceptive stimulation at birth have been reported to be exaggerated in kittens (Ekholm, 1967) and rats (Fitzgerald & Gibson, 1984; Guy & Abbot, 1992), often involving large groups of limb and trunk muscles, i.e. quite unlike the differentiated adult reflex pattern. By contrast, the response properties and somatotopic termination pattern of thin A and C afferent fibres already appear adult-like at birth in rats (Smith, 1983; Fitzgerald, 1987*a*,*b*). At this time, motoneurones synapse with their final target muscles (see Kelly, 1983). Furthermore, at this age, heteronymous α-motoneurones do not appear to be electrotonically coupled (Walton & Navarrete, 1991). The shrinkage of receptive fields and differentiation of reflex patterns would therefore be due neither to changes in the response properties or termination pattern of afferent fibres nor to changes in the innervation of muscles. Rather, tuning of the intrinsic connections in the reflex arcs may account for the shrinkage of receptive fields and differentiation of reflex patterns. The morphological and functional changes of dorsal horn neurones during the first postnatal weeks (Bicknell & Beal, 1984; Fitzgerald, 1985) may reflect such tuning in the nociceptive reflex pathways.

However, the above-mentioned studies do not provide information on the development of the detailed spatial organization of the cutaneous nociceptive input to individual reflex 'modules'. In order to characterize further the normal postnatal development of the nociceptive withdrawal reflex system, and thus obtain a basis for future studies on the mechanisms responsible for the functional tuning of nociceptive systems, we have studied the functional organization of this reflex system at different postnatal stages. A behavioural study on nociceptive reflex responses in the forelimb, hindlimb and tail in awake intact rats and electromyographic recordings in single hindlimb muscles in decerebrate spinal rats were made. A comprehensive description of both the compound withdrawal reflex pattern and the spatial input–output relationship in single withdrawal reflex pathways was obtained at different postnatal ages. Preliminary results have been published (Holmberg & Schouenborg, 1994).

METHODS

Animals used

Eighty-four Wistar rats of both sexes, obtained from time-mated rats, were studied at different postnatal ages. Postnatal day 1 (P1) was defined as the first 24 h after birth. The rats received food and water *ad libitum* and were kept in a 12 h day–night cycle and at a constant environmental temperature of 21 °C (humidity 65%). Approval for the experiments was obtained in advance from the University of Lund Local Ethical Committee.

Studies on nocifensive behaviour in awake intact rats

Normal compound withdrawal reflex patterns were studied in awake intact rats ($n = 8$). The rats were handled by the experienced staff of the animal house until they rested quietly. They did not struggle or show any other sign of discomfort during the experiment. The baseline temperature of the hindpaw and tail skin (29–33 °C at all ages) was measured by a non-contact probe (Thermonitor C-1600 M, Linear Laboratories, Los Altos, CA, USA; infrared detection). The threshold intensity for evoking a visible reflex response on CO_2 laser stimulation (Directed Energy Inc., Irvine, CA, USA; unfocused beam: diameter, 1·1 mm; intensity, 1 W; pulse duration, 5–25 ms) was measured before documentation of reflex responses. Reflexes elicited by CO_2 laser pulses at an intensity of two times the reflex threshold were recorded on videotape. The stimulation intensities used elicited a weak and very short-lasting pricking pain when tested on the dorsal side of the experimenters' hands, and did not produce vocalization or aggressive behaviour in the rats. No visible skin damage occurred.

Each of the eight rats was tested on six occasions, at P1, P5, P8, P14, P21 and P60–80 (adult). At each age three sites on both the right fore- and hindpaw (Figs 1 and 2) and three pairs of sites bilaterally on the tail were stimulated. Every site on the hindpaw and tail was stimulated twice (interstimulus interval more than 10 min, thus precluding 'wind-up'; Mendell, 1966), and every site on the forepaw once. The weak and short-lasting test stimulation used is not likely to have induced significant long-lasting reflex alterations, since responses elicited by the first and second stimulation of the same skin area in each session did not differ systematically. Moreover, independent of whether one or two test stimulations were used per site and age, the maturation of reflexes of the different extremities essentially followed the same time course during development (see Results).

To evaluate the possibility that the repeated testing sessions at different ages in the same rats affected the development of nociceptive reflexes, control experiments were made in naive rats (i.e. not previously tested) at P14 ($n = 8$) and at P21 ($n = 6$). In each of these naive rats three sites on the tail and three sites on the plantar side of the right hindpaw were stimulated. Neither thermal nor mechanical reflex thresholds, nor reflex movements (analysed as described below), were found to be significantly different in the naive rats compared with rats of corresponding age that had been subjected to testing at younger ages (Mann–Whitney *U* test). Thus, it seems unlikely that these aspects of nociceptive reflex development were affected by the repeated stimulation sessions.

The videotape was replayed frame by frame and the initial evoked reflex movements were analysed by visual inspection (image magnification, ×10; time interval between each frame, 40 ms). A light-emitting diode was used to indicate stimulation onset. Movements of the limb joints were classified as flexion or extension. Ankle/wrist movements were further classified as supination (rotation round the proximo-distal axis of the paw in a direction which would cause the plantar/palmar side to face medially when starting from the standing position), or pronation (rotation round the proximo-distal axis of the paw in a direction opposite that of supination). Tail movements were classified as movements towards or away from the stimulus.

Mechanical thresholds for evoking a visible hindlimb reflex response were examined using calibrated nylon Semmes-Weinstein monofilaments, commonly referred to as 'von Frey

hairs' (North Coast Medical, Inc., San Jose, CA, USA; tested force levels were 0·4, 0·8, 1·8, 3·1, 6·3, 9·6, 14, 31, 48, 64, 74, 108 and 163 mN, calibrated using a fine balance). The reflex threshold was defined as the lowest force required to evoke a visible response in at least 50% of trials (6–7 trials per force level; interstimulus interval about 15 s) of the plantar side of distal digits 4–5.

Electromyographic recordings in decerebrate spinal rats

Preparation. Rats of all ages (none of which had been used in the behavioural study) were anaesthetized with halothane (0·5–2·0%) in a mixture of 65% nitrous oxide and 35% oxygen during surgery. A laminectomy of vertebra Th10 and a craniotomy were made and the cranial contents rostral to the inferior colliculus were removed by suction. The anaesthesia was then discontinued and the exposed spinal cord transected with a pair of fine scissors. Immediately following spinalization a small incision was made in the skin overlying the investigated muscles to facilitate insertion of the EMG needles (see below) into the muscle bellies. The exposed tissue was moisturized with saline. Local infiltration of 2·0 mg ml^{-1} lidocaine (Xylocaine) with 1·2 μg ml^{-1} adrenaline reduced nociceptive input during surgery. Experiments were terminated on signs of deterioration, i.e. precipitate drops in blood pressure/heart rate, expiratory CO_2 levels or, when artificial ventilation was not used, precipitate drops in breathing rate.

Rats examined during the first seven postnatal days were held in a fixed position using a silicon mould. The core temperature of the preparation was kept between 36·5 and 38·5 °C by heating the mould. The rats usually maintained a stable condition for more than 4 h after spinalization as judged from stable heart rate (about 300 beats min^{-1}, measured by counting electrocardiography (ECG) deflections on EMG recordings), breathing and perfusion of peripheral vessels.

Rats in the P20–25 and P60–80 groups were artificially ventilated with a mixture of 50% oxygen and 50% air via a tracheal cannula and the expiratory CO_2 was monitored continuously (3·5–4·5%). An infusion of 5% glucose in Ringer acetate solution (pH = 7·0) was given via the right jugular vein (5–10 μl min^{-1} in the P20–25 group and 30–50 μl min^{-1} in the P60–80 group). The common carotid arteries were ligated prior to decerebration to reduce bleeding. Mean arterial blood pressure was monitored continously proximal to the ligature in the right carotid artery (65–110 mmHg in the P20–25 group and 90–140 mmHg in the P60–80 group). The experiments lasted up to 6 h after spinalization in the P20–25 group and up to 12 h after spinalization in the P60–80 group.

Electromyography. Reflex responses were recorded with etched fine steel electrodes (insulated up to 50 μm from the tip; diameter at the distal end of insulation, 30–40 μm; tip diameter, less than 3 μm; length of the electrode, 6–14 mm; weight, 2–5 mg; the smallest electrodes were used in neonatal rats) inserted into the belly of the muscles. Each electrode was soldered to a delicate and flexible copper wire (diameter, 80 μm). Reference electrodes were placed in the adjacent skin. Recordings were made in up to three muscles simultaneously. To reduce the risk of response contamination from nearby muscles, care was taken to ensure that the recording electrodes were placed centrally in the muscle belly. The electrode position was checked by electrically stimulating the recorded muscles through the recording electrode (see Schouenborg *et al.* 1992). From birth onwards, the movement patterns on intramuscular stimulation were essentially adult-like. Recordings were discarded if the threshold current needed to activate nearby muscles was less than six times that for the muscle under study.

In rats examined at P1–25, visible reflex responses could always be evoked within 5 min of spinalization and there was no further obvious change in reflex excitability after 1 h. Therefore, EMG recordings always commenced 1 h after spinalization in these rats. In adult rats, the recovery of excitability after spinalization is slow (Schouenborg *et al.* 1992) and the EMG recordings commenced about 5 h after spinalization in the P60–80 rats.

Cutaneous stimulation. Thermonociceptive receptive fields were mapped using the CO_2 laser stimulation (same stimulation intensities and beam size as above). The baseline temperature of the distal hindlimb skin (range, 30–34 °C) was measured during the recording sessions. Mechanonociceptive receptive fields were mapped using calibrated forceps with flattened tips (1 mm^2 on each side) at P20–25 and P60–80. The forceps were applied to a skin flap and the pinch force was increased (1·0 N s^{-1}) up to about 1·5 N (range, 1·3–1·7 N), and thereafter kept constant for more than 1 s (for details, see Schouenborg *et al.* 1992). During the first postnatal week, the skin of the rats was easily damaged by pinch stimulation. Mechanonociceptive receptive fields were therefore not mapped during this period. For mapping of all thermo- and mechanonociceptive receptive fields, between twenty and thirty-five separate sites were stimulated (interstimulus interval about 1 min) on the plantar surface of the hindpaw (viewed under a microscope at ×10 magnification in rats examined at P1–7).

Mechanical reflex thresholds were determined in some animals by calibrated monofilaments (force levels, interstimulus interval and threshold definition as above).

Analysis. The number of clearly distinguishable motor unit spikes (i.e. spikes with an amplitude exceeding 40 μV which could be separated from background noise and ECG deflections) evoked during the first second after onset of the CO_2 laser pulse or during the first second of constant pinch force was counted using the EGAA program (RC Electronics Inc., Goleta, CA, USA). Burst discharges, which gave rise to fused deflections in which individual spikes could not be resolved, were rarely evoked by the stimulation intensities used. The possible underestimation of spike numbers due to such burst discharges must therefore have been small.

For each muscle in each rat, responses on stimulation of the plantar side of the foot were normalized and expressed as a percentage of the maximal response. To describe the average receptive field of a muscle in an age group, the responses in this muscle, evoked by stimulation of the hindpaw in all rats of this age group, were plotted on the corresponding stimulation sites on a standard figure of the hindpaw. For each stimulated site, a mean response value was then calculated. From these mean values, an average receptive field, divided into three areas of differing sensitivity – maximal sensitivity (70–100% of maximal response), medium sensitivity (30–70% of maximum) and low sensitivity (< 30% of maximum) – was constructed. The areas of differing sensitivity were delimited with the aid of computer-generated isoresponse lines (Kriging algorithm and contour program, software from Golden Inc., Golden, CO, USA: Grid and Topo programs; see also Schouenborg, Weng, Kalliomäki & Holmberg, 1995).

Statistical analysis

The Mann–Whitney *U* test was used for statistical evaluation. Significant differences were assumed at the level of $P < 0·05$. Values are given as means ± standard error of the mean (S.E.M.).

RESULTS

In the present study, thermal and mechanical stimulation were used to characterize the postnatal development of nociceptive withdrawal reflexes both in intact awake rats and in decerebrate spinal rats. Time-locked thermal stimulation (CO_2 laser) was used at all ages since the movements elicited by mechanical stimulation were complex in young animals.

Behavioural studies

The nociceptive reflex behaviour was examined for each of eight intact awake rats at P1, P5, P8, P14, P21 and

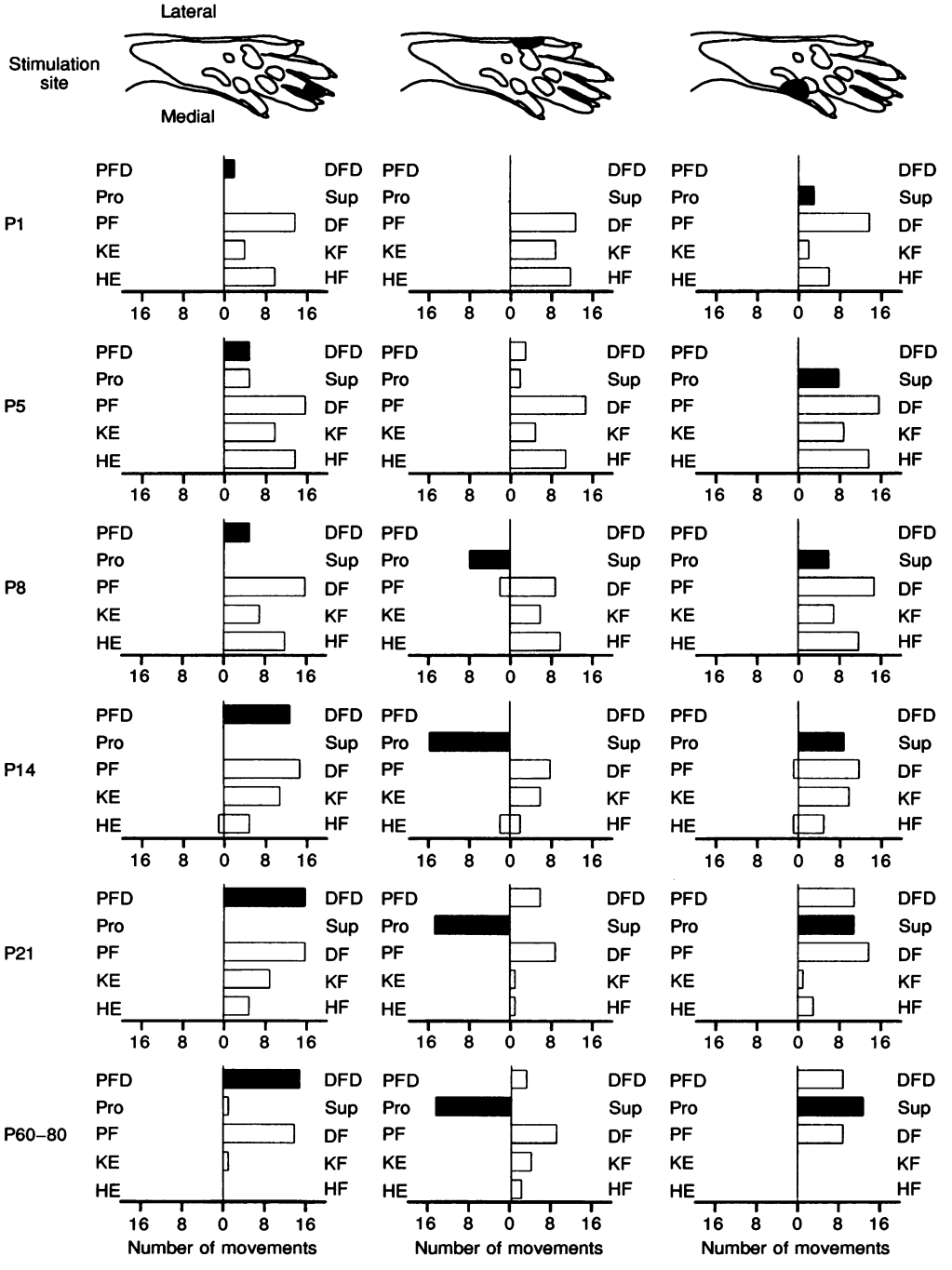

Figure 1. CO_2 laser-evoked reflex movements of the right hindlimb at different postnatal ages

Three different sites on the hindpaw (indicated above each column) were stimulated. Each site was stimulated twice at each age in each of 8 rats. Stimulation intensity was two times reflex threshold. Abbreviations: PFD, plantar flexion of digits; DFD, dorsiflexion of digits; Pro, pronation; Sup, supination; PF, plantar flexion of ankle; DF, dorsiflexion of ankle; KE, knee extension; KF, knee flexion; HE, hip extension; HF, hip flexion. Each column represents the total number of movements. Filled columns represent number of movements producing localized withdrawal from the stimulation.

P60–80 (adult). In adults, the reflexes were highly differentiated, producing localized withdrawal of the stimulated sites. Typically, pronation and supination were elicited from the lateral and medial part of the paws, respectively, and digit dorsiflexion from the distal part of the digits (filled columns in Figs 1 and 2). In adults, reflexes elicited from all sites tested on the tail were also directed away from the stimulus (filled columns in Fig. 3). In contrast, reflex responses evoked at P1 and P5 were not well differentiated and often failed to produce adequate withdrawal. After the first week of life, the frequency of localized withdrawals increased. Withdrawal reflexes in

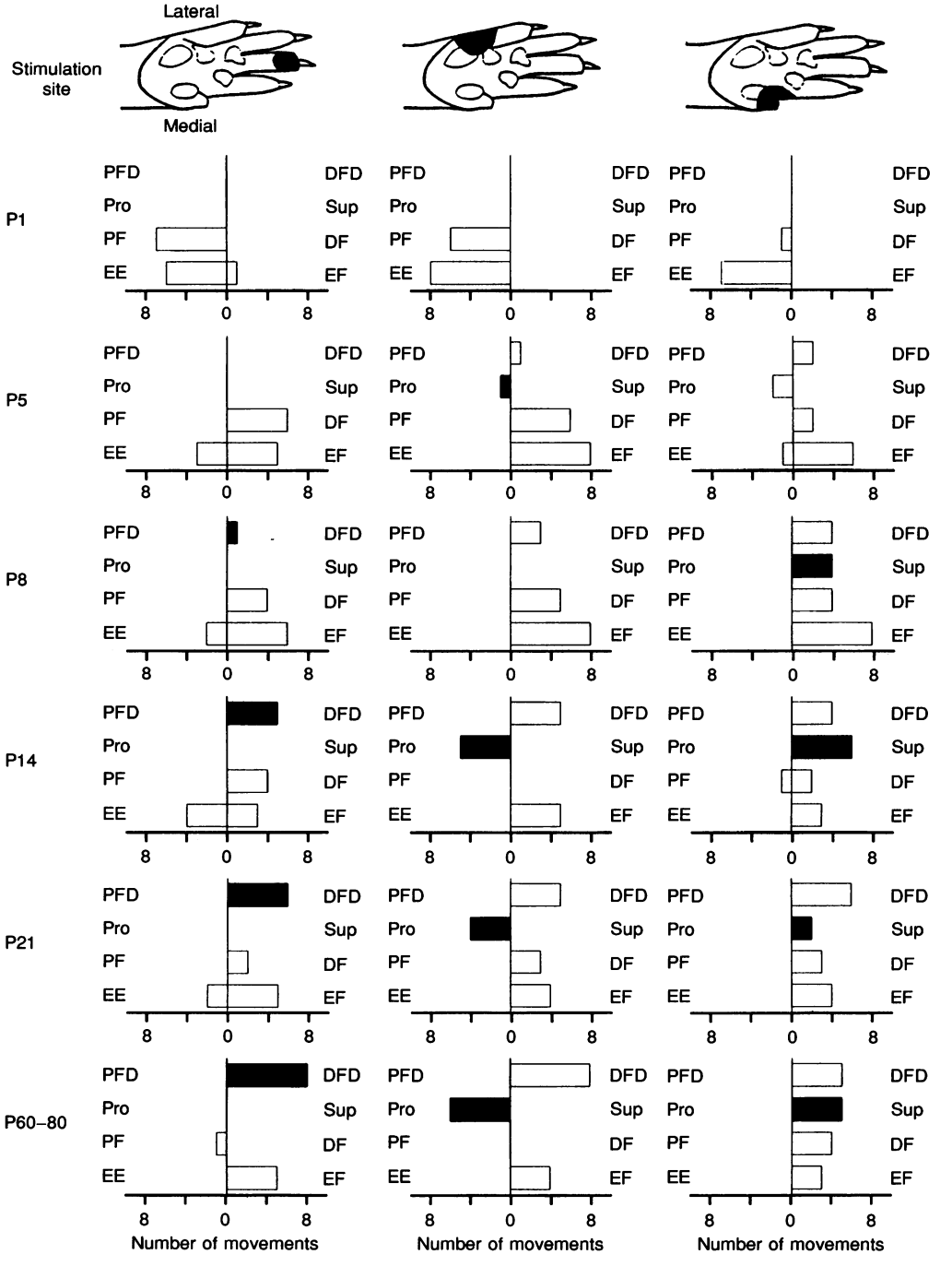

Figure 2. CO_2 laser-evoked reflex movements of the right forelimb at different postnatal ages

Three different sites on the fore paw (indicated above each column) were stimulated. Each site was stimulated once at each age in each of 8 rats. Stimulation intensity was two times reflex threshold. Abbreviations: PFD, palmar flexion of digits; DFD, dorsiflexion of digits; Pro, pronation; Sup, supination; PF, palmar flexion of wrist; DF, dorsiflexion of wrist; EE, elbow extension; EF, elbow flexion. Each column represents the total number of movements. Filled columns represent number of movements producing localized withdrawal from the stimulation.

both fore- and hindlimbs were essentially adult-like at P14, whereas reflexes elicited from the distal part of the tail were often not appropriate until P21 (Fig. 3). Note that reflexes directed towards the stimulus were often elicited from the distal and middle part of the tail at P1–14.

Reflex thresholds. The thresholds for visible reflex responses evoked by CO_2 laser and mechanical stimulation of the plantar side of digits 4–5 were assessed (Fig. 4*A*). The data indicate that mechanical and thermal thresholds do not change significantly during the first three postnatal weeks. Between P21 and P60–80, mechanical, but not thermal, thresholds increased significantly ($P < 0{\cdot}001$). There was no significant difference in baseline skin temperature between any of the age groups studied.

Electromyographic recordings

While the behavioural observations clearly show a gradual tuning of the withdrawal reflexes during the first three postnatal weeks, a detailed characterization of this process requires data on the reflex responses of single muscles. To this end, EMG recordings of reflex responses were made at P1–3, P4–7, P20–25 and P60–80 in four hindlimb muscles: musculi extensor digitorum longus (EDL), peronei, gastrocnemius–soleus (G–S) and biceps posterior–semitendinosus (BP–ST).

Reflex thresholds. CO_2 laser stimulation was used to determine thermal reflex thresholds of all four muscles, and calibrated Semmes-Weinstein monofilaments were used to determine mechanical reflex thresholds of EDL and peronei. The most sensitive area within the plantar receptive field of each muscle was stimulated. In agreement with the behavioural study (Fig. 4*A*), the EMG recordings revealed no significant difference in reflex thresholds during the first 3 weeks. EDL and peronei thresholds are shown combined in Fig. 4*B*. Furthermore, as in the behavioural study, mechanical, but not thermal, reflex

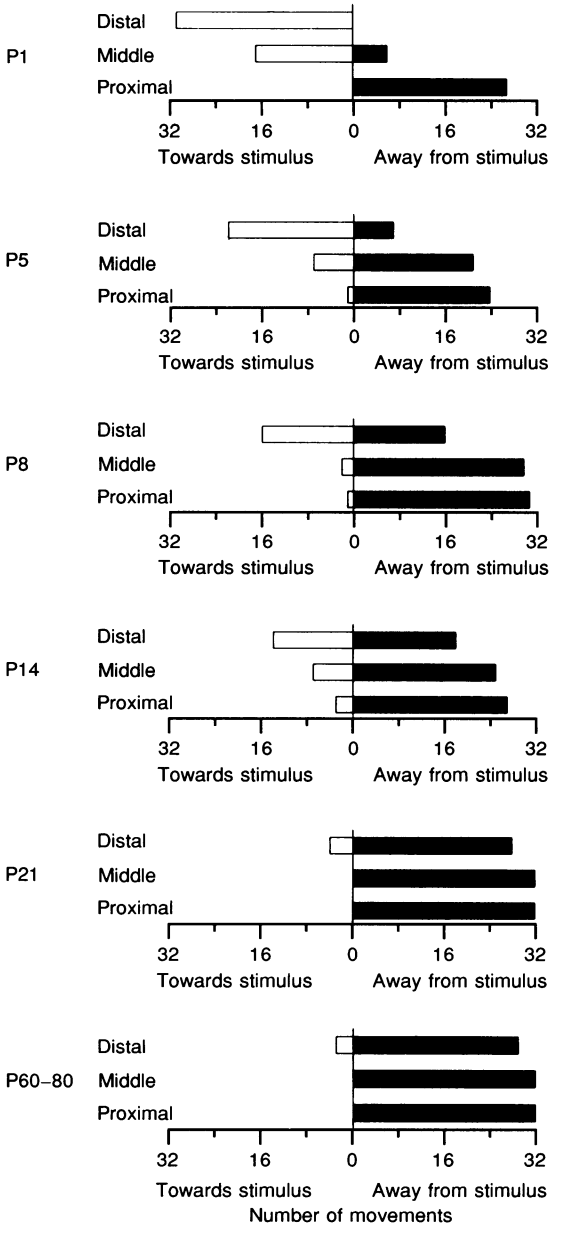

Figure 3. CO_2 laser-evoked reflex movements of the tail at different postnatal ages

The proximal, middle and distal part of the tail were stimulated bilaterally at six different postnatal ages. Each site was stimulated twice at each age in each of 8 rats. Stimulation intensity was two times reflex threshold. Results obtained from corresponding bilateral sites are pooled. Each column represents the total number of movements. Filled columns represent number of movements producing withdrawal on stimulation of the respective sites. Proximal, proximal part of the tail; Middle, middle part of the tail; Distal, distal part of the tail.

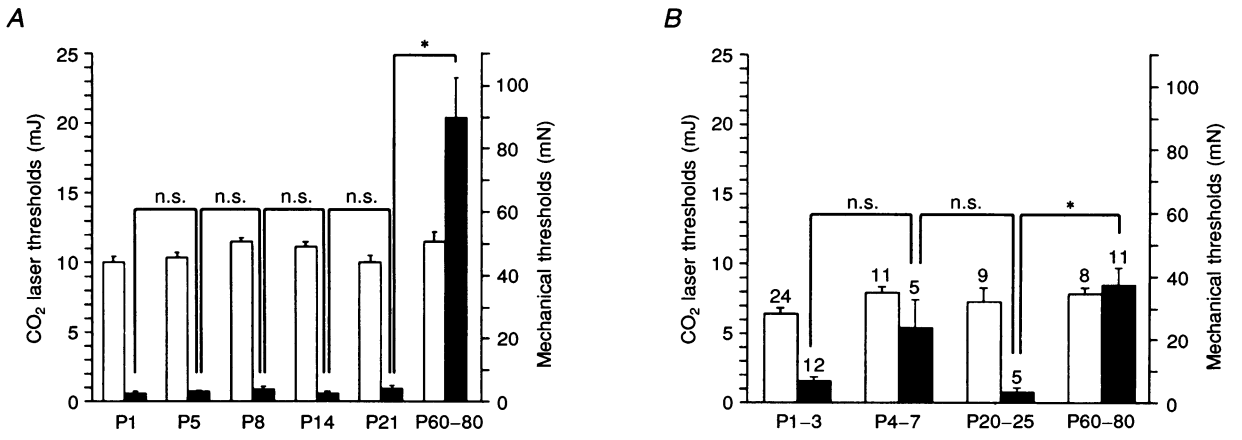

Figure 4. CO_2 laser and mechanical (Semmes-Weinstein monofilaments) thresholds for eliciting reflex responses at different postnatal ages

Each column represents mean ± standard error of the mean (S.E.M.). Statistical comparisons of mean mechanoreceptive threshold values were made between successive age groups (square brackets). n.s., not significant; * $P < 0{\cdot}001$. □, CO_2 laser stimulation; ■, mechanical stimulation. *A*, thresholds for evoking visible reflex movements of the right hindlimb in intact awake rats ($n = 8$ animals). The plantar side of digits 4–5 was stimulated. No significant difference in CO_2 laser thresholds was found between any of the age groups studied. *B*, thresholds for evoking reflex responses in peronei and EDL in spinal decerebrate rats (EMG recordings). The most sensitive site within their respective receptive fields was stimulated. No significant difference in CO_2 laser thresholds was found between any of the age groups studied. EDL and peronei threshold values did not differ and were therefore pooled. The number of rats is indicated above each column.

thresholds increased significantly ($P < 0{\cdot}001$) between P20–25 and P60–80. There was no significant difference in baseline skin temperature between any of the age groups studied.

Receptive field characteristics. At P1–3 the receptive fields of single muscles appeared unorganized, often having multiple foci. There was also considerable interanimal variability in spatial organization of the receptive fields (Fig. 5). Despite averaging results (see 'Analysis' in Methods) from all rats studied at P1–3 (EDL, $n = 19$; peronei, $n = 8$; G–S, $n = 19$ and BP–ST, $n = 18$), no clear spatial organization of the distribution of sensitivity was found within the receptive fields of any of the muscles studied at these ages (Fig. 6). In rats studied at P4–7 (EDL,

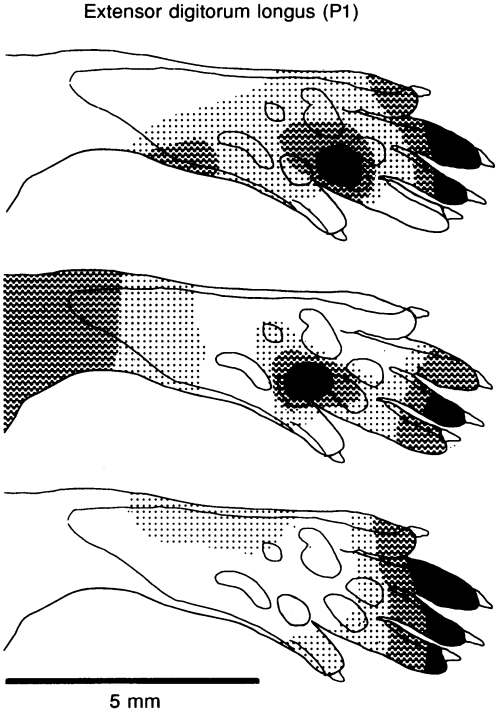

Figure 5. Samples of receptive fields of EDL obtained at P1 in three rats

Receptive fields on the hindpaw skin obtained using CO_2 laser stimulation (intensity two times reflex threshold). Low, medium and high density of dots indicates areas of the skin from which the evoked responses were 0–30%, 30–70% and 70–100% of maximal response, respectively. Note the variability of the receptive fields at this age.

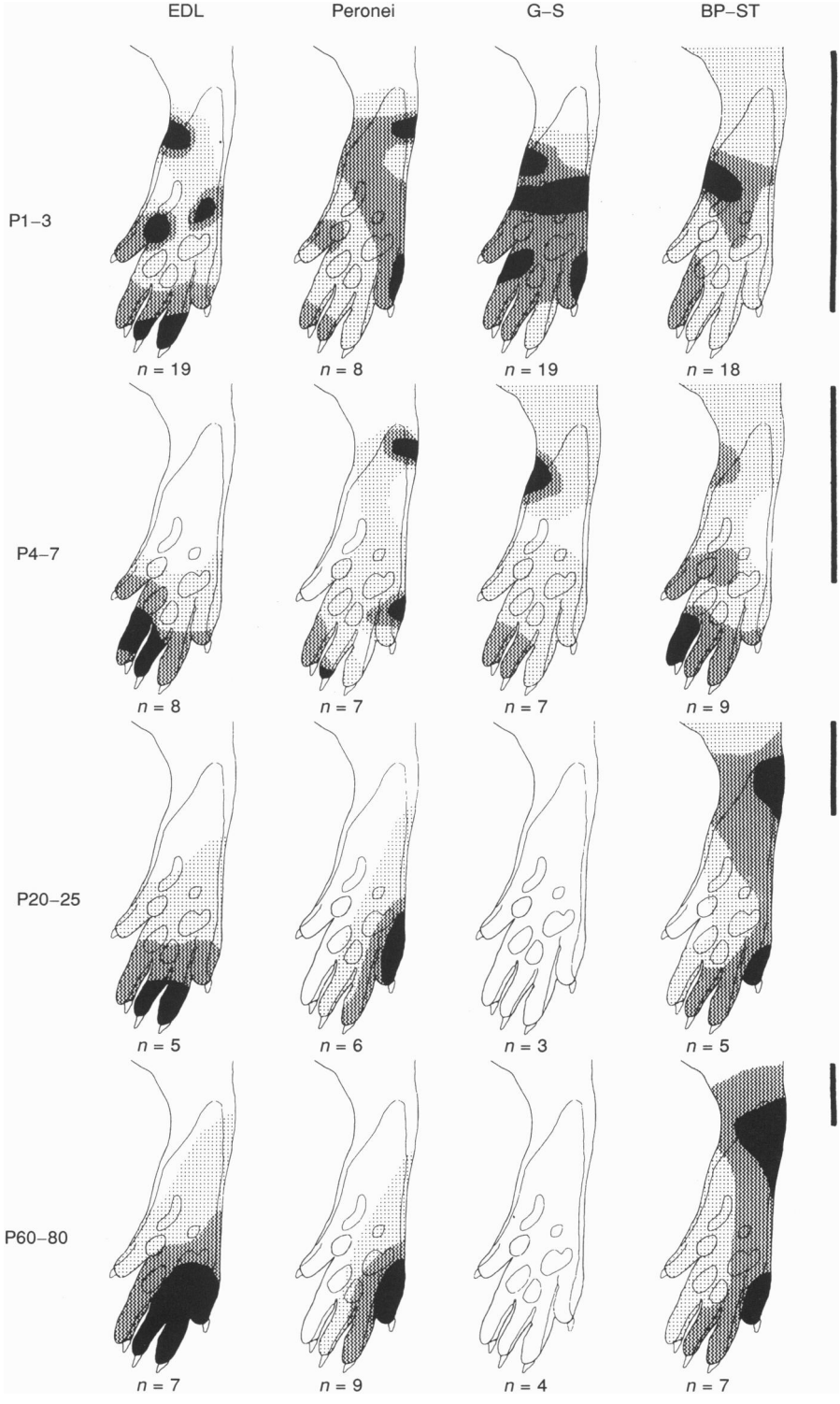

Figure 6. Averaged receptive fields of four hindlimb muscles at different postnatal ages

Averaged receptive fields (see Methods) of EDL, peronei, G–S and BP–ST obtained using CO_2 laser stimulation (intensity two times reflex threshold). *n*, number of rats. Bars indicate 10 mm as measured at P2, P5, P22 and P70. Conventions as in Fig. 5.

Table 1. Onset latency of CO_2 laser-evoked reflex responses in four hindlimb muscles at different postnatal ages

	EDL (ms)	n	P	Peronei (ms)	n	P	G–S (ms)	n	BP–ST (ms)	n	P
P1–3	170 ± 14	19		175 ± 17	8		148 ± 10	19	178 ± 11	18	
P4–7	152 ± 4	8	n.s.	177 ± 24	7	< 0·01	153 ± 22	7	159 ± 14	9	< 0·001
P20–25	136 ± 11	5	< 0·001	112 ± 8	6	< 0·001	No response	3	112 ± 9	5	< 0·001
P60–80	247 ± 11	7		189 ± 12	9		No response	4	204 ± 13	7	

For each of EDL, BP–ST and G–S, three reflex responses were sampled from each rat. For peronei, two reflex responses were sampled from each rat. The onset latency of each reflex response was counted from onset of the CO_2 laser pulse to the first evoked spike. In adult rats, reflex responses with an onset latency less than 75 ms were rare, suggesting a weak contribution from myelinated fibres at this age. The skin area from which maximal responses are evoked in the respective muscle in the adult was stimulated. Statistical comparisons of mean values in the P1–3 and P20–25 groups (square brackets) and between the P20–25 and P60–80 groups are indicated. No significant difference in mean latency was found between the P1–3 and P4–7 groups for any of the muscles studied (not indicated). Values are means ± S.E.M.; n, number of rats. n.s., not significant.

$n = 8$; peronei, $n = 7$; G–S, $n = 7$ and BP–ST, $n = 9$), the receptive fields appeared less unorganized, typically exhibiting fewer foci than in the P1–3 group.

It may be noted that one of the foci of the respective receptive field of peronei and EDL had a location corresponding to the adult focus of the respective muscles. By contrast, in intact awake rats examined during the first postnatal week, there was rarely a visible digit dorsiflexion and never any paw pronation on stimulation of the digits and lateral plantar side, respectively. It thus appears that these reflexes are too weak to produce visible movements in the intact animal at this age.

In concordance with the results from the behavioural study (see above), the distribution of sensitivity within the

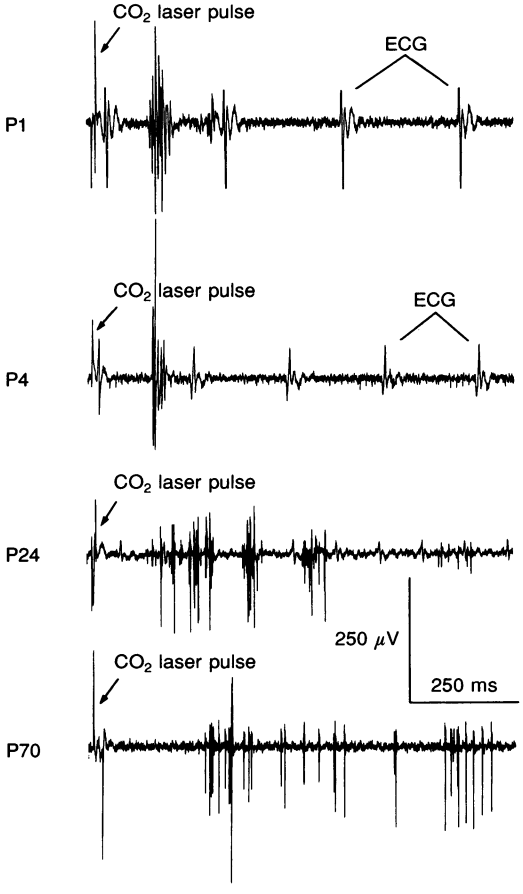

Figure 7. EMG recordings of CO_2 laser-evoked reflex responses in EDL at different postnatal days

The distal plantar part of digit three was stimulated (intensity two times reflex threshold).

thermonociceptive receptive fields of all muscles in rats studied at P20–25 (EDL, $n = 5$; peronei, $n = 6$; G–S, $n = 3$ and BP–ST, $n = 5$) was similar to that in rats studied at P60–80 (EDL, $n = 7$; peronei, $n = 9$; G–S, $n = 4$ and BP–ST, $n = 7$) (Fig. 6). This was true also for mechanonociceptive receptive fields (not illustrated). As in adult decerebrate spinal rats, no responses were evoked in G–S (more than 30 different sites on the paw were stimulated in each rat).

Temporal characteristics and magnitude of the reflex responses. To characterize the time course and magnitude of the reflex responses at different ages, averaged post-stimulus time histograms (PSTHs) of responses in the investigated muscles were made (the number of rats used is indicated in Fig. 8 and Table 1). The skin area corresponding to the adult receptive field focus of the respective muscle was stimulated with the CO_2 laser (parameters as above). In the case of G–S, in which no responses were evoked in adult spinal rats ($n = 4$), the heel was stimulated since this

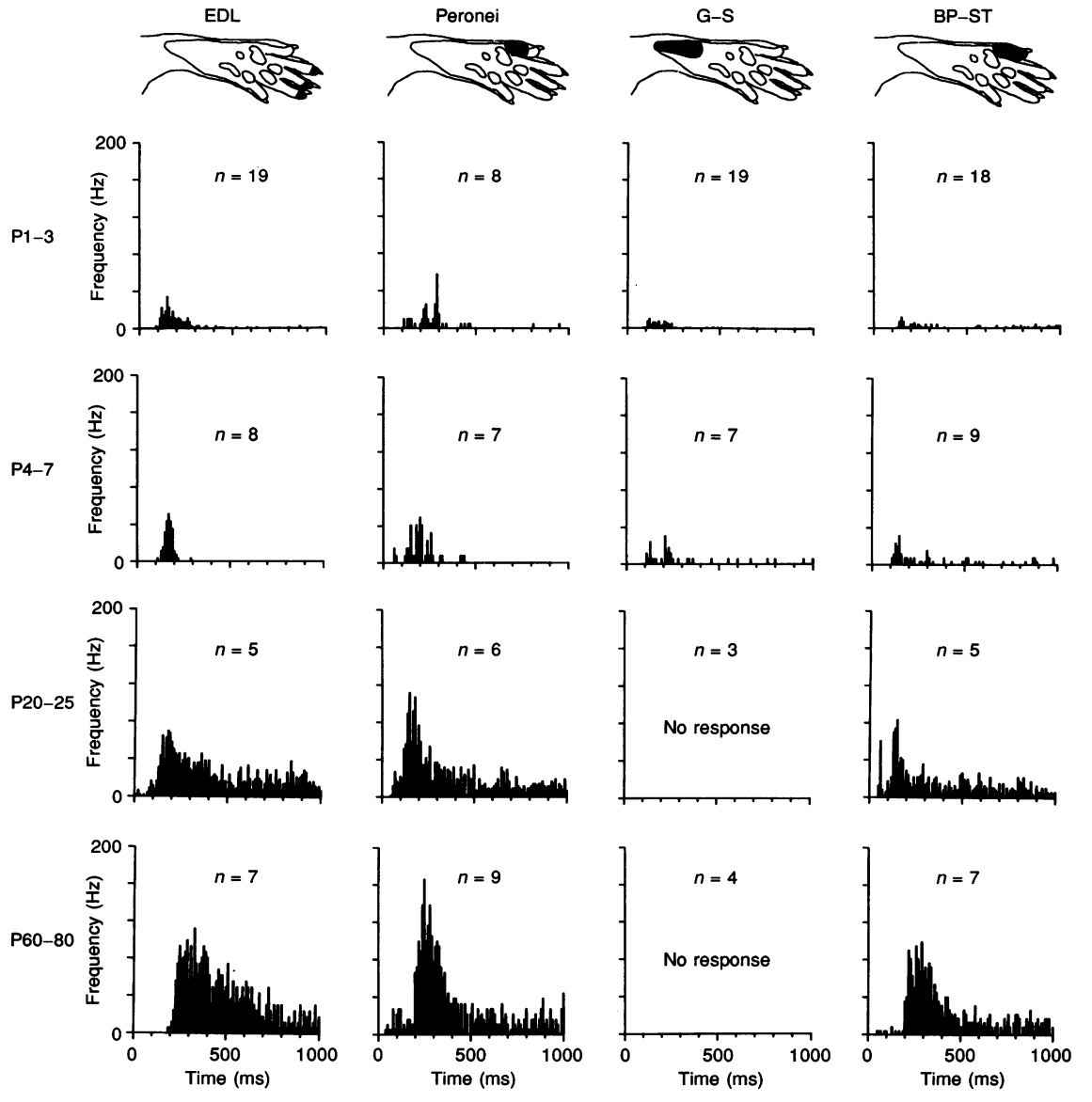

Figure 8. Averaged post-stimulus histograms of CO_2 laser-evoked reflex responses in four hindlimb muscles at different postnatal ages

The skin area from which maximal responses are evoked in the respective muscle in the adult (indicated above each column) was stimulated (intensity two times reflex threshold). For each of EDL, G–S and BP–ST, three reflex responses were sampled from each rat. For peronei, two reflex responses were sampled from each rat. Note that no responses were evoked in G–S in rats examined at P20–25 and P60–80.

area provides maximal input in intact adult rats (Schouenborg & Kalliomäki, 1990; Schouenborg & Weng, 1994; Schouenborg *et al.* 1995). Typical reflex responses evoked by CO_2 laser stimulation and averaged PSTHs are shown in Figs 7 and 8, respectively. During the first postnatal week, no significant change in mean onset latency or mean magnitude of the reflex responses (number of motor unit spikes during the first second after stimulation onset) was found for any of the muscles studied. During the following 2 weeks, the mean reflex onset latency decreased in peronei ($P < 0{\cdot}01$) and BP–ST ($P < 0{\cdot}001$), but did not change significantly in EDL (see Table 1). Response magnitudes increased eightfold in EDL and peronei and elevenfold in BP–ST ($P < 0{\cdot}001$ in all three muscle groups). By contrast, no reflex response was evoked in G–S in the P20–25 and P60–80 age groups (3 rats tested at P20–25 and 4 rats tested at P60–80). Between P20–25 and P60–80, the onset latency increased significantly ($P < 0{\cdot}001$) in EDL, peronei and BP–ST. During this period, reflex magnitude showed little change, although a relatively small increase (40% increase, $P < 0{\cdot}01$) was found for BP–ST.

DISCUSSION

The present study provides a detailed characterization of the postnatal development of a well-defined spinal nociceptive system in the rat. The major finding from both the behavioural and EMG studies was that the spatial input–output transformation performed by this reflex system undergoes profound changes during the first two to three postnatal weeks. The spatial tuning of reflexes in all extremities followed essentially the same time course, although additional refinement of tail reflexes could be demonstrated during the third week. In addition, a pronounced and selective increase in mechanoreceptive reflex thresholds occurred after the establishment of an adult-like spatial organization, suggesting delayed modality tuning.

During development, many central sensory pathways initially form crude representations of their peripheral sensory apparatus. This process appears to be chemically guided, the ingrowth of axons to their targets being dependent on trophic substances and chemical markers (Fields & Nelson, 1992). A refinement of connections then takes place, resulting in precisely organized topographical maps. This refinement appears to be activity dependent in many sensory systems (Fields & Nelson, 1992; Simon, Prusky, O'Leary & Constantine Patton, 1992; Schlaggar, Fox & O'Leary, 1993), possibly operating according to a Hebbian-like learning mechanism (see Singer, 1990). There is, however, little information on how higher-order connections in sensorimotor systems develop. The present study may provide a useful basis for investigating this fundamental problem.

On the topographical organization of the withdrawal reflexes in the adult

In previous studies, the adult organization of the nociceptive withdrawal reflexes in single hindlimb muscles was investigated in anaesthetized intact rats and decerebrate spinal rats (Schouenborg & Kalliomäki, 1990; Schouenborg *et al.* 1992). A 'modular' organization was revealed (see introduction section), each module essentially acting on a single muscle. Due to receptive field overlap, many modules would be expected to contribute to the compound reflex movements evoked by cutaneous stimulation. Strong responses would be elicited in the muscles that produce a significant withdrawal of the stimulated area. The present observations *in adult awake intact rats* confirm these predictions. For example, pronation of the hindpaw (mainly due to contraction in peronei) was evoked from the lateral side of the hindpaw, whereas supination (mainly due to contraction in the tibialis anterior muscle) was evoked from the medial distal part of the hindpaw.

On the spatial tuning of the receptive field organization during development

It has been suggested that a relative strengthening of inhibitory connections over excitatory ones underlies the emergence of the adult reflex response patterns (Fitzgerald, 1991; Guy & Abbot, 1992). This suggestion was based on the reported decrease of excitatory receptive field size and increase of inhibitory receptive field size of flexion reflexes in kittens (Ekholm, 1967), and on the decrease of receptive field size of rat dorsal horn neurones during the first postnatal weeks (Fitzgerald, 1985). The finding that inhibition of rat multireceptive dorsal horn neurones is not consistently evoked by electrical stimulation of the dorsolateral funiculi until the end of the third postnatal week (Fitzgerald & Koltzenburg, 1986) added further support to this notion.

In the present study, a small, but clear, decrease in receptive field size was noted for some of the muscles. In addition, pronounced changes in the distribution of reflex gain for cutaneous input from *within* the receptive fields occurred during the period P1–25. At P1–7, the distribution of sensitivity within the receptive fields was unadapted, exhibiting variable and multiple foci. In fact, misdirected reflex movements, bringing the stimulated area of the skin towards the stimulus, were often evoked. By contrast, from the third postnatal week onwards, localized, well-differentiated reflex responses producing adequate withdrawal, were typically evoked. Thus, inappropriate connections in the reflex arcs become depressed, or eliminated, during P1–25. Similarly, during early postnatal life, the number of inappropriate synapses in monosynaptic Ia reflexes have been reported to be significantly reduced in the rat (Seebach & Ziskind-Conhaim, 1994). In addition, the magnitude of reflex

responses evoked by CO_2 laser stimulation of the skin areas corresponding to the adult receptive field foci increased considerably in all but the extensor muscles. This increase may reflect increasing strength in the adequate excitatory reflex connections, although maturation of motoneurones is also likely to contribute (Fulton & Walton, 1986).

The very fact that the receptive field of single muscles attains a spatial organization that directly reflects the pattern of withdrawal ensuing on contraction of the muscles themselves may provide an important clue to the understanding of the developmental tuning of the withdrawal reflex pathways (Schouenborg & Weng, 1994). Since the responses in most mechanoreceptors reflect changes in pressure on the skin surface, the feedback from such receptors resulting from muscle contraction would exhibit a spatial pattern related to the withdrawal movement. Hence, the cutaneous sensory feedback ensuing on muscle contraction may be instrumental in shaping the reflex connections during postnatal development (Schouenborg & Weng, 1994).

The spinal circuitry of the withdrawal reflexes has not yet been elucidated in detail. However, a group of putative interneurones, that can encode the spatial input–output relationship of the nociceptive withdrawal reflexes of single muscles, was recently identified in laminae 4–6 of the dorsal horn (Schouenborg *et al.* 1995). The 'imprint' of the withdrawal movements caused by a single muscle thus appears to be engraved on the receptive fields of such neurones and would therefore reflect plasticity at the interneuronal level.

On the afferent input to withdrawal reflexes during development

The long latency of the CO_2 laser-evoked withdrawal reflexes after the third postnatal week suggests an important contribution from nociceptive C fibres (Devor, Carmon & Frostig, 1982). However, latency measurements cannot distinguish between the contributions from different afferent fibres in the neonate since myelination of the sciatic nerve only begins around birth in the rat (Sima, 1974; Ziskind-Conhaim, 1988). Myelination is rapid during the first three postnatal weeks (Sima, 1974), presumably explaining why the reflex latency decreases despite a substantial increase in body size. The increase in latency observed after P20–25 may well reflect that the rate of myelination of axons within the sciatic nerve no longer compensates for the rapid growth of the rat.

It was recently reported that large, presumably mechanoreceptive, afferents have transient projections to the superficial dorsal horn that retract to their adult termination laminae after the first three postnatal weeks (Fitzgerald, Butcher & Shortland, 1994). This could result in diminished mechanoreceptive input to nociceptive reflex circuits and may thus be related to the selective increase of mechanoreceptive thresholds seen after P20–25 in the present study. A prominent increase in mechanical reflex threshold has previously been reported to occur after the third postnatal week in the rat (Fitzgerald, Shaw & Macintosh, 1988).

On the supraspinal control of withdrawal reflexes

During the first 3 weeks after birth, visible reflex responses in peronei, EDL, G–S and BP–ST were evoked shortly after transection of the spinal cord. This is consistent with previous studies reporting little acute effects of spinal transection before P15 in the rat (Weber & Stelzner, 1977). The marginal effects of spinalization in the early postnatal period have been thought to reflect a limited functional contribution of descending systems to spinal circuits at this age (Stelzner, 1971; Commissiong, 1983).

Following spinalization in adult rats, reflex excitability in peronei, EDL and BP–ST slowly increases after a short period (10–20 min) of abolished transmission ('spinal shock') (Schouenborg *et al.* 1992). As shown in the present study, reflexes in G–S are exceptional in that they remain abolished for up to 12 h after spinalization in adults and up to 6 h after spinalization in the P20–25 group. The marked difference in G–S reflex transmission after spinal transection during the first compared with the fourth postnatal week may suggest that over the first postnatal weeks, G–S reflexes are brought under a crucial descending control, the integrity of which is a requisite for nociceptive reflex transmission to these muscles.

On the generality of the findings

The present study reveals that the task-specific adult organization of the nociceptive withdrawal reflex system is the result of profound postnatal changes. In this process, inappropriate reflex connections become depressed, or eliminated, and appropriate connections appear to be strengthened. Previous behavioural and electrophysiological studies suggest that other types of nociceptive reactions, such as shaking, paw-licking and orienting movements also become adult-like during the first three postnatal weeks (Ekholm, 1967; Stelzner, 1971; Guy & Abbot, 1992). Thus, mechanisms similar to those underlying the tuning of the nociceptive withdrawal reflex system may also be involved in the development of other central nociceptive systems.

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Acknowledgements

This work was supported by Swedish Medical Research Council Projects Nos. 10569 and 1013, the Medical Faculty of the University of Lund, Crafoordska Stiftelsen of Lund, The Royal Physiographic Society in Lund, Elsa and Thorsten Segerfalks Stiftelse, Greta and Johan Kocks Stiftelser and Magn. Bergvalls Stiftelse.

Received 22 June 1995; accepted 20 December 1995.