Properties of an adult spinal sensorimotor circuit shaped through early postnatal experience

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Running head:
Properties of an adult spinal sensorimotor circuit

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Abstract

During development, information about the three-dimensional shape and mechanical properties of the body is laid down in the synaptic connectivity of sensorimotor systems through adaptive mechanisms. This functional adaptation occurs through alteration of connection properties. Here, we characterize the differences between strong and weak connections in the nociceptive withdrawal reflex in adult decerebrate spinal rats, representing the preserved end product of the developmental adaptation process. Stronger excitatory reflex connections from the skin onto a muscle had relatively higher gain in their input-output relations, shorter onset latencies (up to ~150 ms) and lower trial-to-trial variability in relation to response amplitude (SD ~ mean\(^{1/2}\)), than weaker pathways. Although inhibitory and excitatory nociceptive receptive fields of a muscle overlap to some degree, the results indicate that the inhibitory input is not a major determinant of the gain distribution within the excitatory receptive field and vice versa. The N-methyl-D-aspartate (NMDA) receptor antagonist, d-2-amino-5-phosphonovalerate (AP-5) (0.1–1 µg), applied topically on the spinal cord reduced the gain, whereas the response amplitude was mainly reduced by an absolute number by the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA/kainate) receptor antagonist, 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX) (1–10 µg). The results indicate that NMDA receptors have a critical role in gain regulation in the nociceptive withdrawal reflex system. It is suggested that after normal postnatal experience-dependent adaptation, the number of connections from a given skin site onto the reflex encoding interneurons is a major determinant of the difference in gain.
Introduction

Sensorimotor circuits must incorporate detailed information on the biomechanics of the body to adequately perform rapid correction of movements. How this information is acquired and stored in different neuronal circuits is a fundamental question. We have recently shown that experience-dependent adjustments that functionally adapt the nociceptive withdrawal reflexes (NWRs) take place during development (Petersson et al. 2003; Waldenstrom et al. 2003). In adult mammals, the NWR system has a modular organization (Schouenborg 2002). Each module controls a single or a few synergistic muscles and transforms sensory input from a receptive field (RF) into appropriate motor activity. The sensitivity distribution within the cutaneous excitatory RF of a module mimics its withdrawal efficiency. In rats, this imprint of the withdrawal efficiency is engraved on the reflex pathways during the first three postnatal weeks (depending on body part) through extensive adjustments whereby erroneous connections are eliminated, or reduced, and the strength of adequate connections become proportional to withdrawal efficiency (Holmberg and Schouenborg 1996b; Waldenstrom et al. 2003). This process takes approximately one week. Sensory deprivation experiments and behavioral studies, using artificial tactile feedback on spontaneous movements occurring during this time-period, indicate that the imprint of withdrawal efficiency is formed by tactile feedback on spontaneous movements (Petersson et al. 2003; Waldenstrom et al. 2003). Computer simulation has shown that an unsupervised correlation-based learning mechanism, using tactile feedback from spontaneous muscle twitches, can account for the functional adaptation of the withdrawal reflex system (Petersson et al. 2003). This novel learning mechanism was termed 'motor-directed somatosensory imprinting' (MDSI). Through MDSI, the sensorimotor transformation can adapt to neonatal alterations of both peripheral
innervation (Holmberg and Schouenborg 1996a) and movement patterns (Holmberg et al. 1997).

Although a basic knowledge of the learning mechanisms that adapt spinal circuits to the body constitution during development has been obtained, it is not known which properties constitute the difference between strong and weak connections—that is, what properties constitute the end product of the postnatal learning. For example, it is not known if inhibitory connections or the types of excitatory postsynaptic receptors differ between strong and weak connections of the reflex circuit. As a step towards clarifying this matter, a comprehensive study of the functional properties of connections with different strengths within excitatory and inhibitory RFs of the NWR system was carried out. Firstly, the excitatory RFs of single reflex modules were characterized with respect to signal gain, response latency and variability. Secondly, we analyzed the inhibitory input to these reflex modules with respect to spatial organization, latency and strength. Thirdly, the functional role of glutamate receptors (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA/kainate) and N-methyl-D-aspartate (NMDA)) in the NWR sensorimotor transformation was studied in pharmacological experiments.

**Materials and Methods**

Adult Wistar rats (n = 31) of both sexes were used. The animals received food and water *ad libitum* and were kept in a 12-hour day-night cycle at a constant environmental temperature of 21 °C (humidity 65%). Approval for the experiments was obtained in advance from the Malmö/Lund ethical committee on animal experiments.
Surgery and preparation

The animals were anaesthetized with halothane (0.9%–2.0%), in a mixture of 65% nitrous oxide and 35% oxygen, and were ventilated artificially via a tracheal cannula. The expiratory CO$_2$ (3.0%–4.0%) was monitored continuously. An infusion of 5% glucose in Ringer acetate (pH = 7.0) at a rate of 40–80 µl/min, was administered via the right jugular vein. Mean arterial blood pressure (65–140 mmHg) was monitored continuously in the right carotid artery. Core temperature was maintained between 36.5 and 38.5 °C using a thermostatically controlled, feedback-regulated heating system. Local infiltration of 3.5 mg/ml lidocaine (Xylocaine) with 2.2 µg/ml adrenaline was used to reduce nociceptive input during surgery. A laminectomy of the tenth thoracic vertebrae was carried out and the rat was spinalized using a pair of fine scissors. A craniotomy was performed, and the brain rostral to the inferior colliculus was removed. The anesthesia was then discontinued. In experiments used for pharmacological interventions, a laminectomy of the thirteenth thoracic, first and second lumbar vertebrae was made and the underlying meninges were removed. An agar pool with artificial cerebrospinal fluid (aCSF) (Edwards et al. 1989) over the lumbar cord was then created. Experiments were terminated upon signs of deterioration, such as a precipitous drop in blood pressure or in expiratory CO$_2$ level. After termination of the experiments, the animals were given a lethal dose of halothane.

Cutaneous stimulation and electromyographic recordings

Nociceptive stimulation was performed either with CO$_2$ laser (beam diameter 3 mm, intensity 5W, pulse duration 12–28 ms (60–140 mJ)), resulting in an estimated maximal temperature of between 43 and 64 °C at a depth of 100 µm (Haimi-Cohen et al. 1983) once every 1.5–2 min or by intracutaneous cathodal electrical stimulation using fine steel needle electrodes (n = 16,
see below). No visible damage of the skin, or marked changes in response properties at the stimulation sites, was detectable at these intensities.

The needles used for skin stimulation and electromyographic (EMG) recordings were electrolytically pointed and insulated with a varnish coating except for about 40 µm and 80 µm, respectively, at the tip. A small metal plate, used as an anode, was placed subcutaneously well outside the stimulation area. For EMG recordings, a small opening was made in the skin overlying the muscle, and the electrode was inserted into the mid-region of each muscle belly. A reference electrode was inserted in an adjacent skin flap. Generally, the EMG activity in two or three hind limb muscles was recorded simultaneously in each experiment.

**Receptive field mapping**

A computerized method, termed receptive field imaging (RFI), for rapid mapping of multiple RFs and their respective sensitivity distributions was used in all experiments (Petersson et al. 2001). Automated stimulation and recording, with spike detection and counting, were performed on-line by this system. All raw data sweeps were stored to permit further off-line analyses. The RFI system allows repeated RF measurements in a time range of minutes. The RFs obtained with CO₂ laser and electrical stimulation were almost identical, as has been shown previously (Petersson et al. 2001). The same 16 stimulation sites were used in all experiments (indicated in Fig. 1). When using electrical stimulation, sites of stimulation were randomized and responses were averaged over 10 successive mappings.

**Topographical representation of receptive fields**

A topographical representation of RFs was used for illustrations. From the measured data, 36×76-matrices were computed (intermediate sites were interpolated by spatial low-pass
filtering). From these matrices, contour maps were constructed and plotted on the paw surface (using Surfer software from Golden Inc.). For details see (Petersson et al. 2001).

**Latency measurements**

An automated process was used to estimate onset latencies. The initial spike counting algorithm assigned each spike to a 2 ms time bin. Multiple raw-data files containing peri-stimulus histograms recorded at a certain stimulation intensity were summed. The accumulated peri-stimulus histogram was then smoothed by grouping the 2 ms time bins, five by five, into 10 ms time bins. The onset latency was defined as the time passed from stimulus onset until the first 10 ms bin containing at least half the number of spikes of the bin with the highest number—a method previously found to yield accurate estimates of onset latencies (Friedman and Priebe 1998). All histograms were also inspected visually and if responses were judged too small to allow reliable latency estimations these values were excluded from the automated process. A low spontaneous activity in all latency experiments enabled the use of this relatively simple algorithm, yielding estimates similar to the ones obtained by visual inspection of the spike arrival trains. Response latencies shorter than 100 ms (judged as A-fiber responses) and latencies longer than 400 ms (activity likely arising from other sources) were not included. When compensating for differences in afferent fiber length a C-fiber conduction velocity of 0.8 m/s was assumed (Gee et al. 1996).

**Pharmacological experiments**

The exposed lumbar spinal cord was kept in a bath of artificial cerebrospinal fluid (aCSF) (bath volume ~300µl). The response values for up to 16 sites were determined every three minutes before, during and after the period with drug effect. The baseline was defined as at least five mappings with a fixed stimulation intensity yielding values with no trend to
decrease or increase during a time-period of at least 12 minutes. After the baseline measurement the aCSF was removed by gentle suction with a syringe, then 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX) (1–10 µg) or d-2-amino-5-phosphonovalerate (AP-5) (0.1–1 µg) (Tocris) diluted in 100 µl aCSF and titrated to pH 7.4 were administered into the bath. The effect of the drug stabilized 5–20 minutes after administration, and responses remained at a lower level plateau (a marked decrease, but with reliable responses from most of the stimulation sites). At least six mappings during a time-period of at least 15 minutes were made. The drug was then gradually washed out with aCSF (see Fig. 6). The doses of the glutamate antagonists used were within the range of effective doses found previously in similar models of spinal nociceptive processing (Bach and Yaksh 1995;Zahn et al. 1998;Nishiyama et al. 1998;Jhamandas et al. 1996).

**Results**

In the first set of experiments, we examined the properties of strong and weak connections in the NWR system. Differences with regard to gain, onset latency and variability for different sites of the excitatory RFs were characterized. Skin sites of the hind paw were stimulated using a CO₂ laser (pulse duration ranging from 12 to 26 ms). RFs of NWRs of the peroneus longus muscle (PL, n = 17; pronator of the hind paw), the extensor digitorum longus muscle (EDL, n = 6; dorsiflexor of the digits and ankle) and the semitendinosus muscle (ST, n = 15; knee-flexor and adductor of hind limb) were mapped in 18 rats (Fig. 1A–C).

**Input-output relations**

In previous studies, the RFs of hind-limb muscles have been mapped using constant stimulation intensity. To obtain a more detailed description, the relative reflex gain for individual skin sites within the RFs was mapped (n = 656 mappings) by applying CO₂ laser
stimulation with different pulse duration. Thus, the entire input-output relationship was
determined for up to 16 skin sites per RF. The EMG responses from three hind limb muscles,
PL (n = 6 RFs), EDL (n = 4 RFs) and ST (n = 5 RFs), were mapped in seven animals. As can
be seen in Figure 2A, every site within each RF tended to respond with a certain fraction of
the maximum response obtained in the field focus at each stimulation intensity. As a
consequence, the intrinsic order of responsiveness of sites within the RFs was conserved
regardless of the excitability changes that occurred during the experiments (Schouenborg et
al. 1992). When gauging the responses from PL and EDL, with stimulations ranging from
near-response nociceptive threshold to near-response saturation, the response amplitudes for
sites within the RFs increased linearly with increasing stimulation intensity until a plateau was
reached (linear regression; PL r = 0.80, EDL r = 0.82). The slope of the input-output relation
is referred to below as reflex gain (Fig. 2B). Notably, no distinct difference was found
between response thresholds of strong and weak connections (threshold differences of
different sites, measured as the absolute differences in pulse duration between each site and
the median value within the RF in each experiment, were 2.0 ± 1.9 ms for PL and 2.5 ± 2.4 ms
for EDL (mean±SD)). For ST, two distinct RF areas were often found, each of which had a
relatively uniform threshold and the same input–output features as PL and EDL.

**Stimulus response latencies**

To determine if there was a specific latency distribution within the RFs we made 612
mappings (PL, n = 12 RFs; EDL, n = 4 RFs; and ST, n = 8 RFs) in 13 animals (laser pulse
duration 18–26 ms). The onset latency was measured for all sites and maps of onset latency
were constructed (see Materials and Methods for details on latency measurements). In most
experiments, individual onset latencies for sites within the RFs decreased only slightly (mean
maximum difference ~50 ms in eight experiments) with increasing stimulation intensity,
while response amplitudes at the same time differed greatly (typically >5 times) (Fig. 3C–E).
The latency differences within the RFs had a distinct spatial distribution very much akin to the
response amplitude distribution of the same muscle (Fig. 3A,B; c.f. Fig. 1A) (mean maximum
difference ~150 ms in 11 experiments) Footnote 1.

Variance
The variance of the input signal for different sites could be expected to be scaled-up
proportionally to the square of the gain in the circuit (noise amplification). If, however,
different connections vary in properties other than the relative gain, these properties could be
reflected in the relation between mean response amplitude and the variation of these
responses. We therefore studied the variability in response amplitudes over multiple mappings
using cutaneous CO₂ laser stimulation PL (n = 7 RFs), EDL (n = 3 RFs) and ST (n = 5 RFs)
in eight animals. The variance increased monotonically with the mean response amplitude of
the different sites. Approximate relationships can be formulated based on the median values
of linear regressions in the log-log plots of all experiments as $\text{Var} \sim 13.3 \times \overline{\text{response}}^{0.91}$ for
PL, $\text{Var} \sim 28.6 \times \overline{\text{response}}^{0.82}$ for EDL and $\text{Var} \sim 10.8 \times \overline{\text{response}}^{0.93}$ for ST (PL and ST are
shown in Fig. 4A,B). An almost linear relationship was evident between variance and mean
responses for different sites within the RF. The amount of variability within the RF could,
therefore, not be directly explained by the difference in relative gain for different sites.
Rather, it appears that sites with higher relative gain somehow differ in signal transduction
properties to sites with a lower gain.

When using natural stimulation some noise will be introduced at the level of the peripheral
receptors. This variance is independent of the central mechanisms and can thus be treated as a
simple addition to the total variance. Because of the modular organization of the reflex for
different muscles, the shared variance between two muscles provides a rough estimate of the
peripheral variability and can therefore conveniently be estimated by judging how much of the variance in one muscle can be explained by the variance in the other (calculating $r^2$) for each site. This part of the variance was estimated to be ~21% for laser stimulation (for comparison the corresponding value for electrical stimulation is ~3.3%). To assess the central part of the variability the peripheral part can be subtracted so that for natural stimulation the relation becomes $Var \sim 10.5 \times (\text{response})^{0.91}$ for PL and so forth.

**Organization of overlapping inhibitory input**

Previous studies have shown that the inhibitory input from the skin is organized in an analogous way to that of the excitatory input, such that the inhibition is related to the size of the movement component directed towards stimulation (Schouenborg and Weng 1994). Excitatory and inhibitory inputs have been shown to overlap to some extent (Weng and Schouenborg 1996a; Schomburg et al. 2001; Sonnenborg et al. 2000) (see also **Footnote 2**). To clarify if overlapping inhibitory inputs contribute to the differences between strong and weak connections, the inhibitory nociceptive input was analyzed using pair wise stimulation. The control stimulation, producing excitation in the reflex pathway, was caused either by stimulation of the excitatory field focus or, in alternative experiments, random stimulation within the excitatory RF. The results were similar to those found previously (Weng and Schouenborg 1996a) and additionally showed that the inhibitory RF for each muscle was independent of the site used to induce excitation (Fig. 5A,B). The onset latency of inhibition varied within the inhibitory RF in a manner similar to that of the excitatory RF, such that the shortest latencies of inhibition were found for skin sites producing maximal inhibition (data not shown). It is worth noting that this means that their respective response latency patterns cannot be explained by influences from the opposing field. These findings indicate that there are distinct inhibitory RFs in the NWR system in parallel to the excitatory RFs.
Effects of glutamate receptor antagonists

The RFI system used allowed for rapid reliable RF mapping using electrical stimulation. In this arrangement each site was stimulated multiple times in each RF mapping (compared with just one site with the laser) and mappings could be made more often without the risk of receptor sensitization/desensitization. Thus, a high sensitivity to RF changes and a good temporal resolution was reached. However, this was achieved at the cost of using a more artificial type of stimulation. In the pharmacological experiments, where a high sensitivity to RF dynamics is advantageous to reliably track the effect of the applied drugs, both natural and electrical stimulation (10 stimulations/site and mapping, starting every third minute) were used. CO₂ laser stimulation was used in seven rats PL (n = 6 RFs), EDL (n = 3 RFs) and ST (n = 3 RFs) and electrical stimulation in six rats PL (n = 4 RFs), EDL (n = 3 RFs) and ST (n = 4 RFs). Hypothetically, differences in amplitude, latency and variation within the excitatory RF could be due to differences in glutamate receptors in the reflex pathways from different skin sites. It has been suggested that the AMPA/kainate to NMDA receptor ratio (A/N) differs between strong and weak synapses (Takahashi et al. 2003). Thus, if the non-NMDA to NMDA receptor ratio differs for different connection strengths, the relative effects of selective antagonists would be expected to vary. To assess this possibility, we investigated the effects of NBQX (n = 205 mappings in nine experiments) and AP-5 (n = 202 mappings in 10 experiments) for three different muscles. The drugs were applied topically onto the spinal cord. Both antagonists caused a response decrease, but the degree to which strong and weak connections were affected differed. AP-5 caused a response decrease almost exactly proportional to the strength of the connections for all sites (Fig. 7A–C), whereas NBQX application resulted in an absolute decrease of response regardless of site (weaker sites stopped responding) and only a moderate proportional decrease (Fig. 7A–C). This was found
in experiments using laser and electrical stimulation. A similar effect of the antagonists on gain/threshold was also found for individual sites in experiments evaluating input-output relations (pulse duration 16–26 ms) (n = 2 experiments; data not shown). These results are at variance with the view that there are major differences between strong and weak connections with regard to receptor populations (see Appendix for further details).

**Discussion**

As mentioned in the introduction, the sensitivity distributions within the receptive fields of withdrawal reflex modules emerge during development as a consequence of learning and constitute 'imprints' of the withdrawal movement patterns of the output muscles. The present study provides a comprehensive description of this imprint and thus information about the end product of the learning process. In particular, differences between connections of different strengths with respect to gain, latency, variance, inhibitory inputs and effects of glutamate antagonists were analyzed. It should be kept in mind that this study used EMG recordings. Thus, overall properties are studied, not the details of individual connections.

*The reflex network studied*

For nociceptive C-fiber evoked reflexes, which dominate the CO$_2$ laser responses studied (Bromm et al. 1983; Schouenborg et al. 1992; Weng and Schouenborg 1996a), a network consisting of two interneurons, of which the second-order interneuron, termed reflex-encoder, projects to the motoneurons, is assumed (Schouenborg 2002). The first-order interneurons, thought to be located in substantia gelatinosa, receive a direct input from cutaneous nociceptive C-fibers and often exhibit rather small receptive fields (Cervero et al. 1979), whereas the reflex-encoders exhibit the same receptive fields as single muscles in the withdrawal reflex (Schouenborg et al. 1995). Differences in properties for strong and weak
connections found in the present study are assumed to reflect properties of connections prior to the reflex-encoders since the reflex-encoders, but not substantia gelatinosa neurons, exhibit receptive fields similar to those of the muscles.

**Role of inhibitory input in shaping the receptive fields**

In the present study, a detailed analysis of the inhibitory and excitatory receptive fields showed that, although overlapping to some extent, the organization of the excitatory and inhibitory receptive fields was clearly different and there was no trace of overlap in the more sensitive parts of the respective fields (see also Footnote 2). Thus, the inhibitory inputs only marginally affect the spatial distribution of reflex gain within the more central parts of the excitatory receptive field and vice versa. Moreover, the onset latency of the inhibitory input increased towards the excitatory field focus and arrived too late to underlie the differences in onset latency of the excitatory input. Nevertheless, the inhibitory inputs curtail responses from the peripheral part of the excitatory receptive fields and also limit expansion of the excitatory receptive fields in high excitability situations (Petersson et al. 2001; Weng and Schouenborg 1996b).

**Difference in response properties of excitatory connections with different strengths**

Given that inhibitory inputs cannot exclusively explain the gain maps it follows that differences in the excitatory connections underlie differences in gain. A recent study on the somatotopic organization of the dorsal horn indicates that strong connections simply produce stronger synaptic currents than weak connections (Levinsson et al. 2002). The monosynaptic tactile field potentials in the dorsal horn exhibit a convergence pattern within restricted regions that is similar to that of the withdrawal reflex pathways. For any location within a given region of the dorsal horn, the convergence pattern and weight of input is very similar to
a reflex module. Since the spread of synaptic currents is quite limited in the CNS (Nowak and Bullier 1996; Mitzdorf 1985), this would also suggest that the difference between connections of different strength is conserved throughout the region, at least on a macro-level. In the present study, the relative reflex gain of different connections was found to be independent of stimulation strength and excitability in each animal tested (Schouenborg et al. 1992). Hence, the order of gain between different skin sites, and thus the spatial distribution of sensitivity, is a very stable feature of this system. The stability of the imprints, *per se*, may suggest that structural differences underlie relative gain of different connections in this system. For example, the number of primary afferent fibers mediating the input from the skin, the number of substantia gelatinosa neurons contacting reflex-encoders, or the size of synaptic connections, may differ. Structural changes do accompany changes in connection strength in the withdrawal reflex system of *Aplysia* (Kandel 2001). In that system, the number of synaptic contacts decrease/increase in parallel with behavioral changes in long-term habituation/sensitization protocols.

In the present study, the mean of the maximal differences in onset latency of CO₂ laser-evoked reflex responses differed within the receptive fields by ~150 ms. Moreover, the pattern of these onset latencies was very similar to the gain pattern. However, if the stimulation intensity of two different skin sites with different relative gain was matched to produce the same response amplitude, the difference in onset latency was relatively unchanged. Hence, onset latency is not simply a function of response amplitude or stimulation strength. Considering the often low discharge frequency of substantia gelatinosa neurons (Cervero et al. 1979), it is conceivable that the relationship between gain and onset latency reflects the number of substantia gelatinosa neurons contacting a given reflex-encoder neuron, as the reflex-encoder may need much longer time for summation of inputs to reach activation threshold. The finding that the relative variance is smaller for sites with stronger gain is also
consistent with the possibility that the number of substantia gelatinosa to reflex-encoder connections determines gain and onset latency (when the reflex-encoder samples and integrates the activity of many substantia gelatinosa cells representing the peripheral stimulus the average sum should be less variable (Shadlen and Newsome 1998)). An alternative possibility would be that dynamic response properties in the substantia gelatinosa neurons mediating nociceptive C-fiber input to reflex-encoders determines the gain and latency of the reflex response. However, this would require that substantia gelatinosa neurons are, to a large extent, dedicated to single reflex modules.

**Role of AMPA and NMDA receptors**

It has been shown that glutamatergic synapses change their receptor constituents after application of long-term potentiation protocols (Shi et al. 1999). This has spurred great interest in the importance of AMPA receptor cycling. It has been implicated that AMPA receptors are inserted in the action of potentiating a synapse and conversely removed in long-term depression (Heynen et al. 2000; Galan et al. 2003; Ji et al. 2003) that the ratio of AMPA to NMDA receptors scales with the size of the synapse (Takumi et al. 1999). However, other results indicate that AMPA and NMDA receptors are, in the long-term, tightly co-regulated in active synapses, even after synaptic scaling, such that the current ratio is unchanged regardless of synaptic strength (Watt et al. 2000; Umemiya et al. 1999). Yet, it is not known whether these mechanisms are also of importance for the retention of long-term memories (Bailey et al. 2000; Malenka and Nicoll 1999). Moreover, little is known about the role of glutamatergic receptors in shaping the spinal circuits, although NMDA channels seem to have a critical role (Beggs et al. 2002). The present model system is interesting in this context since it offers an opportunity to study the role of glutamatergic receptors in an adult reflex network shaped through learning mechanisms during postnatal development. Indeed,
the drug data and simulation of these data are consistent with a co-regulation of AMPA and NMDA receptors in connections of different strength. It should be kept in mind, though, that this conjecture is based on a system level analysis, and information that is more detailed would require experiments at a cellular level.

Importantly, the present results show that the antagonist to NMDA receptors specifically affects the reflex gain whereas the antagonist to AMPA receptors mainly affects the threshold. Hence, it is conceivable that NMDA receptors have a critical role in gain regulation in these pathways. NMDA channels are known to play a role in nociceptive transmission, in particular for central sensitization after injury (Woolf and Salter 2000). Intrathecally applied NMDA antagonists block frequency dependent potentiation in some of the nociceptive dorsal horn neurons (Dickenson et al. 1997) and in ascending spinal nociceptive pathways to cortex cerebri (Kalliomaki et al. 2003). Moreover, mice with a deficient NMDA receptor complex, due to a deficient L1 adhesion molecule, show a marked hypoalgesia (Thelin et al. 2003). Thus, the present results add to the evidence that NMDA receptors have an important role in setting the gain in nociceptive transmission.

Conclusions

The present study shows that connections of different relative gain differ with respect to temporal aspects and response variance. These differences appear to be due primarily to differences in the excitatory connections, although inhibitory connections do contribute to shape the contours of the receptive fields. It has also demonstrated that NMDA receptors have an important role in setting the gain in the nociceptive system studied. The present study may also suggest that differences in the gain, onset latency and response variation reflect differences in number of excitatory connections. Further studies at the cellular level are needed to clarify the exact nature of these differences.
Footnote 1
In several neuronal systems, the distribution of inter-spike intervals (ISIs) of the first few spikes in each spike train approximately follow an exponential probability density so that the time to the next spike depends on the mean firing rate, but is otherwise random (a Poisson process)(Shadlen and Newsome 1998). However, in the NWR system a Poisson process description seems not to be applicable as the onset latency is, in many cases, independent of stimulation intensity (in contrast to the response amplitude), but has clear dependence on site of stimulation. To verify this, ISIs of the first spikes in individual spike trains were assessed in three experiments in order to control for the possible role of differing initial firing rates on the measured onset latencies. It was confirmed that the differences in mean ISIs were typically smaller than 10 ms—that is, considerably less than most measured latency differences between different sites. This finding supports the notion that latency differences are not due to different mean firing frequencies, but rather, are one of the specific features of the connections from each site. Notably, the onset latency, in itself, carries the information about which site within the RF has been stimulated and could therefore potentially be used by other systems to quickly read out the cutaneous nociceptive input (c.f. Garwicz et al. 2002).

Footnote 2
Previous studies have shown that the excitatory withdrawal reflexes are adapted to the withdrawal movement pattern in a standing position with the plantar side of the paw in contact with the ground (Petersson et al. 2003;Levinsson et al. 1999;Schouenborg 2002). Moreover, NWRs also have inhibitory input from the skin that are weighted in relation to the loading movement pattern (instead of unloading movement pattern for the excitatory input) of
the muscle (Weng and Schouenborg 1996a). Both the excitatory and inhibitory reflex pathways would therefore be mainly organized as negative feedback loops when the foot is on the ground. It may be worth noting, however, that in some positions when the foot is lifted from the ground, the excitatory and inhibitory reflex circuits to antigravity muscles would work as positive feedback loops, as has been suggested by Schomburg (Schomburg et al. 2001). However, the functional consequence of this is not known.

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**Appendix: Modeling the effect of glutamate antagonists for different receptor populations**

*Model assumptions and parameter values*

A simulation of how excitatory pathways with different connection strengths and different AMPA/NMDA receptor populations were affected by NBQX and AP-5 was made to facilitate interpretation of the present pharmacological data. In the simulations, the same basic cellular processes were assumed to be present for all the excitatory cells in the pathway. Thus the modulation of the responses in the model can be viewed as taking place at a single cell or being distributed over all the cells in the pathway. It was assumed that the excitatory synaptic transmission was primarily mediated by AMPA and NMDA receptors (You et al. 2003) and that the total charge mediated by the NMDA receptors upon opening was three times as large
as that mediated by the AMPA receptors (Umemiya et al. 1999). Synapses covering a range of one to 100 receptors of each type were simulated (with no distinction being made between whether they were distributed over a number of synapses or all located in a single synapse). Two scenarios with different receptor distributions were simulated: 1) A/N (AMPA to NMDA ratio) <1 in weak synapses and >1 in strong synapses (N_{AMPA} \in U[1,100], N_{NMDA} = 30); and 2) A/N = 1 in all synapses (N_{AMPA} \in U[1,100], N_{NMDA} \in U[1,100]). The opening of at least 10 AMPA receptors was assumed to be needed for NMDA receptor activation (this threshold was chosen arbitrarily). The amount of depolarization resulting from activation of the receptors was assumed to directly scale with number of action potentials emitted by the neurons (Reyes 2001) (this assumption was based on the fact that most experiments were done in a highly excitable situation, >3h after spinalization (Schouenborg et al. 1992)).

This can be formally stated as: \text{Response} = (N_{AMPA} \times 1 + N_{NMDA} \times 3) \times \Theta(N_{AMPA} - 10);

(where \Theta(<0) = 0 and \Theta(t \geq 0) = 1).

The N_{AMPA} and N_{NMDA} values were chosen to be within a biologically feasible range (Nusser et al. 1998), but altering them did not change the basic outcome of the simulation. Simulations were implemented in MATLAB (Mathwork Inc.).

**Simulated effect of glutamate antagonists**

In the simulations, we assume that the glutamate concentration is similar in different glutamatergic synapses (Auger and Marty 2000). Moreover, we assume that there is no systematic difference in drug concentration, at steady state (Fig. 6), in synapses of different strengths. The latter assumption was based on the findings that AP-5 caused a response decrease almost exactly proportional to the strength of the connections for all sites (Fig. 7A,B), whereas NBQX application resulted in an absolute decrease of response regardless of site (weaker sites stopped responding) and only a moderate proportional decrease (Fig. 7A,B).
Under these conditions, the effect of NBQX (competitive AMPA/kainate receptor antagonists) or AP-5 (competitive NMDA receptor antagonists) could be predicted for different hypothetical distributions of AMPA/kainate and NMDA receptors in different reflex connections. In Fig. A:1A,B it can be seen that the effect of AP-5 is not proportional for connections of different strengths in the first case (Fig. A:1A) whereas in the second case it is (Fig. A:1B). Furthermore, the effect of NBQX is similar to the experimentally obtained data in the second case. The simulation would thus suggest a co-regulation of AMPA/kainate and NMDA receptors in connections of different strengths.

Figure A:1
Simulated effects of glutamate antagonists for different proportions of AMPA/kainate (A) and NMDA (N) receptors in strong and weak connections. A) A/N <1 in weak connections and >1 in strong connections; and B) A/N = 1 in all connections. Dashed line = NBQX 70% block, continuous line = AP-5 70% block and dotted line 1:1 (no effect of drug).
Figure legends

Figure 1
Maps of the excitatory receptive fields of three muscles. A–C) Typical receptive fields of the peroneus longus (PL), the extensor digitorum longus (EDL) and the semitendinosus muscle (ST) (median responses from 10 experiments using 20–26 ms laser pulses). The stimulation sites used in all mapping experiments are marked with cross hairs. Although not present in the illustration, the receptive field of ST often exhibited an additional focus on digit 5. Responses are presented both with the mean number of action potentials (next to the cross hair of each site) and as percentages of the respective maximal response (shaded areas correspond to 0–30%, 31%–70%, 71%–100% of the maximal response).

Figure 2
Connection gains for different sites. A) A single mapping experiment showing the input-output relations for different sites of the PL receptive field. Linear regression lines shown were used to estimate the individual gains (r = 0.94±0.06 mean±SD; range 0.79–0.99); location of the sites are shown in B with corresponding symbols). B) Normalized gain for different sites of the PL receptive field (n = 282 mappings in six animals, mean r = 0.80) plotted as an iso-gain contour map.

Figure 3
Onset latencies of the PL muscle. A) Median time of onset latency above the shortest onset latency within the excitatory receptive field in each experiment (ms). B) The data in
A) are compensated for the delay in conduction time depending on spatial location (0.8 m/s conduction velocity assumed) (mean SD of latencies for the same sites in different experiments = 48 ms). C–D) Effects of varying stimulation intensity shown for a single experiment. C) Response amplitudes increase with increasing stimulation intensity for the six sites shown; while in D) latency measurements in the same mappings show no clear relationship between latencies and stimulation intensity. E) location of the six sites within the PL receptive field in this experiment. Symbols in E correspond to those in C–D.

Figure 4

Trial-to-trial variability of response amplitudes of different stimulation sites for A) PL (n = 7 rats) and B) ST (n = 5 rats) (n>20 mappings). A close to linear relationship between response amplitude and variability in responses was found for the different sites of the RF, as indicated by the inclination of ~1 for the graphs plotted in the log-log diagram for the two muscles (PL, r = 0.91±0.07 (mean±SD), range 0.77–0.96; ST, r = 0.88±0.07 (mean±SD), range 0.76–0.93), (ln (Var): natural logarithm of response variance and ln (R): natural logarithm of the response amplitude).

Figure 5

Inhibitory receptive fields of PL. Inhibitory receptive fields were similar regardless of the site used to provide the excitatory input. A) excitatory drive from distal part of digit 5; and B) excitatory drive from the lateral side of the paw. Filled circles indicate sites of excitatory co-stimulation.

Figure 6
Receptive field imaging during topical drug application. RF characteristics under drug influences were sampled during the plateau (t = 18–30). Data are from a single experiment, each value is the mean of 10 responses. The electrical stimulation used was started every three minutes. Downward arrow indicates the time of topical application of NBQX (10 µg). Horizontal bar indicates wash out period.

Figure 7
Effects of glutamate antagonists. A–D) Experimental data from seven animals showing differential effects of glutamate antagonists. A–B) Effect of NMDA receptor antagonists (AP-5) on responses elicited by CO₂ laser (n = 5) and electrical stimulation (n = 12), respectively. C–D) Effect of AMPA/kainate receptor antagonists (NBQX) on responses elicited by CO₂ laser (n = 6) and electrical stimulation (n = 13), respectively. Response values under drug treatment were plotted against pre-drug administration values (dotted line corresponds to 1:1, corresponding to no effect of the drug).
Figure 7

A) CO₂-laser
B) electrical stimulation

AP5

C) NBQX

D) # APs before drug

# APs during drug

# APs before drug
Reference List


