Excitatory Synaptic Transmission in Neonatal Dorsal Horn: NMDA and ATP Receptors

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Postnatal development sees a strong synaptogenesis in rat superficial dorsal horn. My studies show that synapses mediated by two excitatory neurotransmitters, glutamate and ATP, are functional since the very first postnatal days. Using an electrophysiological approach, the functional properties of two receptors activated by these neurotransmitters, glutamatergic NMDA and ATP ionotropic receptors, are described.

The superficial dorsal horn is the spinal cord area involved in the elaboration of nociceptive stimuli. It is divided into lamina I and lamina II and, at complete maturation, receives the sensory input through the myelinated Aδ fibers and the small-diameter, unmyelinated C fibers. The nociceptive signal is elaborated by the local circuits of excitatory and inhibitory interneurons, which are particularly abundant in lamina II, is modulated by the descending fibers, and is finally transmitted to the higher centers by the projecting neurons, mainly located in laminae I, V, and VI.

Excitatory neurotransmitters in spinal cord dorsal horn

In the last 10 years, several electrophysiological studies have described the principal neurotransmitters and receptors that mediate synaptic transmission in superficial dorsal horn, and particularly in lamina II (Substantia gelatinosa). By electrically stimulating the primary afferent fibers contained in the dorsal root, excitatory postsynaptic responses were evoked and recorded from lamina II neurons. These studies, obtained in adult rats, showed that the principal excitatory neurotransmitter released by both Aδ and C fibers is glutamate, acting on receptors of non-N-methyl-D-aspartate (NMDA) and NMDA types (19). At membrane potentials close to the neuron resting potential, postsynaptic responses seemed to be predominantly mediated by non-NMDA receptors. Focal stimulation of the ventral region of lamina II, which preferentially activates the interneurons, evoked excitatory postsynaptic potentials, which are also mediated by glutamate and predominantly sustained by non-NMDA receptors (20).

Although these studies have indicated that glutamate is the most common fast excitatory neurotransmitter in superficial dorsal horn of adult animals, several authors have proposed, during the last 40 years, that ATP can also act as an excitatory neurotransmitter in this region. Experiments carried out on dorsal horn cultured neurons have proven that the application of ATP can excite a subpopulation of neurons (9). Despite this evidence, the role of ATP as a neurotransmitter has not been directly demonstrated and a modulatory action of ATP on glutamatergic postsynaptic responses has been suggested (12).
Postnatal development of superficial dorsal horn

The findings of the studies obtained from mature spinal cord preparations are probably not entirely applicable to the developing dorsal horn. The first postnatal weeks represent a period of intense synaptogenesis and important functional changes for rat spinal cord dorsal horn. The large-diameter, mechanosensitive Aβ fibers initially project into the superficial laminae and, over the first 3 postnatal weeks, gradually withdraw toward the deeper laminae. In the same period, C fibers form synaptic connections with lamina I and II neurons (6). The receptive fields of dorsal horn neurons in neonatal rats are larger than in the adults, and their stimulation by pinching or brushing evokes very long lasting excitatory responses in dorsal horn neurons during the first postnatal weeks (5). Experiments performed in vivo have shown that the cutaneous reflex in the newborn rat is exaggerated compared with the adult.

The synaptic mechanisms responsible for the hyperexcitability of neonatal dorsal horn neurons have not yet been investigated. The lack of fully mature inhibitory circuits is probably important; nevertheless, it is possible that particular characteristics of the excitatory synaptic transmission contribute to the genesis of these phenomena. In other central nervous system areas it has been shown that NMDA receptors undergo important changes in their functional properties during postnatal development. However, no indications are available so far about the properties of NMDA receptors in superficial dorsal horn during the first postnatal weeks.

Glutamatergic synapses mediated by NMDA receptors: “mixed” and “pure” synapses

To study the role of NMDA receptors in synaptic transmission during the early postnatal age, I have used the spinal cord slice preparation from postnatal rats and recorded from lamina II neurons and using the patch-clamp technique (Fig. 1A) (3). I have evoked excitatory postsynaptic currents (EPSCs) in lamina II neurons by focally stimulating the area surrounding the recorded cell with a glass pipette. By using the “minimal stimulation procedure,” which consists of applying the minimal stimulus intensity necessary to evoke a postsynaptic response, I have been able to activate a very low number of synaptic inputs. These synapses could be constituted either by the terminal of a primary afferent fiber or by an excitatory interneuron.

Confirming the data reported in adult rats, my study indicates that glutamate is also the most common excitatory neurotransmitter in neonatal dorsal horn, acting on non-NMDA and NMDA receptors. Figure 1B shows an example of EPSCs mediated, respectively, by non-NMDA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate; AMPA) and NMDA receptors evoked by the same stimulus. Although this situation is the most common, I have observed that, in postnatal lamina II, a significant percentage of glutamatergic synapses is mediated only by NMDA receptors. In some lamina II neurons, focal stimulation does not evoke an AMPA receptor-mediated response at the membrane potential of –70 mV (Fig. 2A). However, by depolarizing the membrane to +50 mV, a slow EPSC becomes apparent. This current is completely blocked by D-2-amino-5-phosphopentanoic acid (D-APV), a specific antagonist of NMDA receptors, suggesting that NMDA receptors are the only receptors mediating these synapses. Similar results were obtained in lamina II by Li and Zhuo (13). Pure NMDA synapses had been previously observed in hippocampus and in other areas of the central nervous system (4, 8). In these preparations, the expression of pure NMDA synapses (also called silent synapses) seemed to be developmentally regulated. The number of pure NMDA synapses strongly decreased during the second postnatal week, as they matured into mixed synapses, mediated by both NMDA and AMPA receptors. To study the level of expression of pure NMDA synapses in lamina II over the first 2 postnatal weeks, I have recorded from a sample of 57 neurons from rats of different ages (Fig. 2B). For every cell, I have tested the presence of pure NMDA synapses by stimulating five different points around the neuron. As illustrated (Fig. 2B, left), the number of cells expressing at least one pure NMDA synapse does not change significantly during the first 14 postnatal days. Similar results were obtained by representing, as function of age, the percentage of pure NMDA synapses over the total number of glutamatergic synapses (Fig. 2B, right). In contrast with my data, Li and Zhuo (13) have reported that pure NMDA synapses, detected in lamina II by stimulation of the dorsal root, strongly decrease after the 10th postnatal day. These authors have observed that serotonin, released by the descending fibers after the second postnatal week, is able to induce the expression of AMPA receptors at pure NMDA synapses. In a recent paper, Baba et al. (1) have

FIGURE 1. A: schematic representation of the experimental conditions. Recordings are obtained from a lamina II neuron by using a patch-clamp electrode. The area surrounding the recorded neuron is stimulated by a small glass pipette to evoke postsynaptic responses. B: examples of glutamatergic excitatory postsynaptic currents (EPSCs) recorded at a mixed synapses. Left: EPSC mediated by AMPA receptors, recorded at –70 mV. Right: EPSC evoked by the same stimulus at +50 mV and mediated by N-methyl-D-aspartate (NMDA) receptors (the AMPA EPSC was blocked by 10 μM NBQX). All recordings were obtained in the presence of 10 μM bicuculline and 5 μM strychnine to block γ-aminobutyric acid (GABA) and glycine receptors.
confirmed the presence of pure NMDA synapses in dorsal horn lamina II from neonatal rats (P2-P7), but they have not observed these synapses in mature preparations. To explain the discrepancies between my data and the studies performed by other authors, one could assume that pure NMDA synapses mature into mixed synapses mainly after the first 2 postnatal weeks (in rat spinal cord dorsal horn the complete maturation is reached only at the end of the third postnatal week); my study, focused on the first 2 weeks, could have missed the period when significant changes occur. Alternatively, the different circuit organization and the larger dendritic arborization of lamina II neurons in mature spinal cord could have made the detection of pure NMDA synapses more difficult in the adults than in neonates. However, the apparent strong decrease, or even disappearance, of pure NMDA synapses in adults would imply that these synapses have a predominant developmental role. So, similarly to other central nervous system areas (4, 8), they could strongly contribute to the activity-dependent synaptic maturation and the refinement of neural circuits occurring during postnatal development. An interesting hypothesis is that pure NMDA synapses also play a role in synaptic plasticity in adult animals under pathological conditions. Woolf et al. (18) have observed that sciatic nerve transection in adult rats causes A fibers to sprout from deeper laminae to lamina II; these fibers form immature synapses that could be, at least initially, pure NMDA synapses. This hypothesis has been tested by Baba et al. (1) by investigating the presence of pure NMDA synapses in lamina II after sciatic nerve transection. The nerve injury did not cause any significant increase in pure NMDA synapse expression, suggesting that they do not play an important role in synaptic plasticity induced by this kind of lesion.

**Functional properties of NMDA receptors: magnesium sensitivity and kinetics**

The analysis of voltage dependence of EPSCs recorded at pure NMDA synapses has shown that NMDA receptors are sensitive to magnesium from their very first postnatal days and that their sensitivity is very similar to that reported for NMDA receptors expressed at mixed synapses (3). I have also studied the kinetics of NMDA EPSCs at mixed and pure synapses by measuring the rise time and the decay time constants in animals at different ages (Fig. 3). Neither parameter changes significantly during the first 2 postnatal weeks at either synaptic type. I then compared the kinetic values obtained from EPSCs recorded at mixed synapses with those obtained from pure synapses. The rise time of NMDA EPSCs recorded at pure synapses is significantly slower than that at mixed synapses (P < 0.01 by 2-way ANOVA) (Fig. 3A). The decay kinetics seem to be faster in NMDA EPSCs from pure synapses. At both synapse types, the decay phase of the EPSCs recorded at +50 mV was mostly interpolated by a double exponential function (Fig. 3B). Figure 3, C and D, shows
the values of decay time constants ($\tau_1$ and $\tau_2$) as functions of age, obtained for NMDA EPSCs recorded at pure and mixed synapses. The statistical analysis has shown that there is a significant difference between mixed and pure NMDA EPSC groups for $\tau_1$ ($P < 0.01$ by 2-way ANOVA), whereas no significant difference was detected for $\tau_2$.

NMDA EPSC kinetics, and particularly decay, are strongly related to the subunit composition of NMDA receptors. During postnatal development, the kinetics of NMDA EPSCs recorded in several areas of the brain become gradually faster. This reflects a change in subunit composition of NMDA receptors, in which the subunit NR2B is substituted by NR2A. The subunit composition of NMDA receptors expressed by lamina II neurons at the postnatal age is not entirely known. Single-channel recordings on dorsal horn neurons from postnatal rats have shown that channels with different levels of conductance are present and the proposed subunit composition is NR2A, NR2B, and NR2D (15). The kinetics of NMDA EPSCs recorded at pure and mixed synapses in lamina II does not seem to change significantly during the first 2 postnatal weeks. The EPSCs decay with fast kinetics, and the time constant values obtained for both synapses are comparable to the values reported for NMDA EPSCs recorded in mature systems. This would suggest that NMDA receptors at both mixed and pure synapses in postnatal lamina II predominantly express the NR2A subunit. Also, the difference of kinetics observed between mixed and pure NMDA synapses could be related to the receptor composition. The faster decay observed at pure NMDA synapses would suggest a stronger expression of the NR2A subunit at these synapses. Further experiments with drugs specific for particular subunits (like ifenprodil) will clarify all of these aspects.

Excitatory synaptic transmission mediated by ATP

Although most excitatory synapses in postnatal lamina II are glutamatergic, I have observed, in a small portion of lamina II neurons (~5%), the presence of a fast EPSC that is not affected by glutamate receptor antagonists (6-cyano-7-nitroquinoxaline-2,3-dione and D-APV) (2). This EPSC shows a variable sensitivity to suramin (Fig. 4A), an antagonist for ATP receptors (both ionotropic and metabotropic). The application of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), an antagonist specific for ATP ionotropic receptors (named P$_{2x}$ receptors), causes a reduction of the EPSC amplitude that varies from one cell to another (Fig. 4B). The effect of PPADS and the rapid kinetics indicate that the EPSC is likely mediated by ionotropic ATP receptors.

Several P$_{2x}$ receptor subunits have been located both on dorsal root ganglion (DRG) and dorsal horn neurons. In particular, three ATP receptor subunits have been detected in the superficial laminae of spinal cord dorsal horn: P$_{2x2}$, P$_{2x4}$, and
P2x6. (17). Although P2x2 is sensitive to both suramin and PPADS, P2x4 and P2x6 are relatively insensitive. The variable block of the fast EPSC obtained in the presence of the two antagonists is consistent with the expression of a heterogeneous population of ATP receptors in lamina II neurons.

My data indicate, for the first time, that ATP is released in lamina II and is able to evoke fast EPSCs. However, the use of focal stimulation to evoke the ATP-mediated EPSCs did not permit us to determine the synaptic source of ATP. An interesting study performed by Jo and Schlichter (11) has shown that ATP is coreleased with γ-aminobutyric acid from cultured dorsal horn neurons obtained from superficial laminae. This finding suggests that the same synapse could have both an inhibitory and an excitatory role that can be modulated without rearranging the anatomic organizations of the synaptic circuits.

Recent studies have investigated the role of ATP acting on P2x receptors in spinal cord dorsal horn. Gu and MacDermott (7) have shown, in a DRG-dorsal horn coculture system, that ATP has a presynaptic site of action, since the activation of P2x receptors on DRG presynaptic terminals causes an increased frequency of spontaneous glutamate release and can elicit action potentials. Stanfa et al. (16) report that the intrathecal administration of suramin and PPADS in adult rats, either normal or after nerve ligation, does not alter the responses evoked by electrical stimulation of C fibers. On the other hand, the administration of the two antagonists after the induction of carrageenan inflammation causes a significant inhibition of the C fiber-evoked response. These data suggest that ATP plays an important role in modulating synaptic transmission only under particular conditions, such as during inflammation, but only a marginal role in normal or neuropathic animals. The low percentage of neurons presenting ATP-mediated EPSCs observed in my study would confirm these results, although the insensitivity to suramin and PPADS of some P2x receptors expressed in spinal cord dorsal horn leaves the issue still open.

### Future perspectives

My work suggests that glutamate and ATP are two excitatory neurotransmitters released on lamina II neurons during the first postnatal weeks. Many aspects of the synaptic transmission mediated by these two neurotransmitters need to be clarified in the near future.

An important issue will be the understanding of the physiological role of pure NMDA synapses. The classic view is that, due to the block exerted by magnesium at negative potentials, NMDA receptors require the activation of AMPA receptors to become functional. For this reason, pure NMDA synapses are usually called silent, because they cannot be activated at the neuron resting potential. Li and Zhuo (13) have shown that serotonin can induce the expression of AMPA receptors at these synapses; nevertheless, it could be interesting to test whether these pure synapses are really silent before AMPA receptor induction. Responses mediated by NMDA receptors can summate in a nonlinear way if the synapses are stimulated at a sufficiently high frequency. By using different stimulation protocols, it will be possible to determine whether NMDA receptors are functional even in the absence of AMPA receptors and can have a significant impact on the neuron excitability. Another issue is related to the involvement of synapses mediated by NMDA receptors in general, and pure NMDA synapses in particular, in the phenomenon of central sensitization during postnatal development. In the adult, NMDA receptors have been shown to contribute to several forms of synaptic plasticity and sensitization (14). Fewer data have been reported about sensitization mechanisms in spinal cord dorsal horn during postnatal development. Repetitive stimulation of Aβ fibers in neonatal rats produces an increase in background firing of dorsal horn neurons, a phenomenon that decreases after the first postnatal week (10). A contribution of NMDA receptors to the genesis of this phenomenon is very likely, but the role of receptors at pure NMDA synapses has still to be clarified.

My data have shown that, besides glutamate, ATP acts as an excitatory neurotransmitter in lamina II in neonatal rats. It is still not completely clear, however, under which conditions ATP is released and is more effective in modulating neuronal activity. Two characteristics make ATP a particularly interesting neurotransmitter. After release, ATP is rapidly degraded to adenosine, which can also act as a neurotransmitter and modulate the transmitter release at presynaptic terminals. Also, ATP ionotrophic receptors are highly permeable to calcium ions and, unlike NMDA receptors, are also functional at negative potentials. Therefore, ATP receptors may represent a new route for synaptically gated calcium entry into dorsal horn neurons at the cell resting potential and could be involved in the phenomena of synaptic plasticity and central sensitization.

### References