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Morphophysiologic properties of delayed firing neurons in substantia gelatinosa of the rat spinal cord

Нейрони желатинозної субстанції (ЖС) спинного мозку дуже різноманітні за своїми морфологічними та фізіологічними властивостями. За типом генерації потенціалів дії (ПД), викликаних тривалою деполяризацією мембрани, нейрони можуть бути класифіковані як тонічні, адаптуючі та затриманої генерації (НЗГ). Ми досліджували властивості НЗГ у зрізах спинного мозку 3–5-тижневих щурів за допомогою методів patch-clamp реєстрації та конфокальної мікроскопії. Характерними рисами НЗГ була висока реобаза (95,7 пА ± 11,2 пА) та більш позитивний поріг генерації ПД (-37,8 мВ ± 0,7 мВ), ніж у нейронів інших типів. У режимі фіксації потенціалу всі НЗГ мали високоамплітудний вихідний калієвий струм А-типу, котрий починав активуватись уже при -70 мВ, перед натрієвим струмом. Морфологічно, більшість НЗГ мала вертикально орієнтоване дендритне дерево, а їх аксони не обмежувалися ЖС і спрямовувалися переважно в ляміну I. Дискутуються можливі фізіологічні функції НЗГ.

INTRODUCTION

Substantia gelatinosa (SG) or lamina II of the spinal cord plays an important role in the processing and relay of nociceptive and thermoreceptive information. SG cells receive monosynaptic input from thin primary afferents and relay it further to projection neurons located in other laminas (I and IV-V). Therefore, it is believed that SG is comprised of interneurons. Their structural and physiological characteristics are highly heterogeneous, however. Morphologically, there has been described a variety of neuronal types, from two or four in rat [4, 18, 21, 22] and monkey [14], to five in hamsters [7] and six in cat trigeminal lamina II [5, 6]. More straightforward might be physiological classification. Based on intrinsic firing properties, three major cell types have been shown: neurons with tonic, adapting and delayed firing [1, 2, 7, 8, 9, 11, 12, 20, 23, but see 15]. Frustratingly, there seems to be no strong correlation between individual morphological and physiological features in SG neurons [7, 11, 12, 22, but see 16]. The advantages and priorities of these two classification approaches still need to be proven.

In our earlier studies, the properties of tonic and adapting neurons were reported [1, 11, 12, 13, 16]. Here I describe distinctive electrophysiological and structural features of delayed firing neurons (DFNs).

METHODS

Patch-clamp recordings were performed on 300-mm parasagittal and coronal slices prepared from the lumbar enlargement of the spinal cord of 3–5 week-old Sprague-Dawley rats [12]. The animals were anesthetized by Na-pentobarbital (30 mg/kg ip). Slices were incubated in artificial cerebrospinal fluid (ACSF) for >30 min at 32–33°C before experiments (carried at similar temperature). Lamina II was identified as a translucent band in the dorsal horn. The neurons were visualized by infrared-differential interference contrast (IR-DIC) optics of a Zeiss FS2 microscope (Carl Zeiss, Germany). ACSF contained (in mM): NaCl, 124; KCl, 3; $CaCl_2$, 2.5;

$MgSO_4$, 1.3; NaH_2PO_4, 1.4; $NaHCO_3$, 26; glucose, 10 (pH 7.4 when bubbled with 95% O_2 - 5% CO_2). Pipettes had resistances of 5-7 MΩ when filled with an internal solution containing (in mM): K-gluconate, 125; KCl, 4; MgATP, 5; NaGTP, 0.3; EGTA, 5; HEPES, 5; neurobiotin, 0.2 % (pH adjusted to 7.25 with KOH, osmolarity adjusted to 295 mOsm). Pipette tips were filled separately, with internal solution without neurobiotin. The MultiClamp 700B amplifier was used for recordings. Signals were filtered at 3 kHz and digitized at 10 kHz using a computer interface (Digidata 1322) and pCLAMP 9.2 software (Axon Instruments, Burlingame, CA). Voltage-clamp recordings were corrected for the calculated 10 mV liquid junction potential. Series resistance was <25 MΩ and was not compensated. Membrane potential of recorded cells was adjusted to –70 mV. Firing of action potentials (APs) was evoked by 500 ms depolarizing current steps of varying intensities (range 10-650 pA). Threshold to evoke AP was determined using first derivative of a voltage response [19]. Following recordings, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 4°C for 48 h. After washing overnight in 20 % sucrose solution, unsectioned slices were incubated with neutravidin-conjugated Alexa 594 (1:300) for 1 h, washed with PBS and mounted in Prolong® anti-fading medium (Molecular Probes). Images (1 mm interval) were taken and processed with a Zeiss LSM510 confocal microscope; measurements were done by cursor. Data are presented as mean ± S.E.M. They were statistically analyzed by *t*-test, and probability level $P<0.05$ was taken as significant.

RESULTS

The recordings were obtained from 108 neurons, which were categorized based on their typical firing response (i.e., firing pattern) evoked by sustained depolarization of increasing amplitude. Electrophysiological properties did not depend on the type of slicing (i.e., coronal or parasagittal) and the results were pulled. DFNs comprised 30.6 % of all cells, they generated APs with a delay starting from the end of a depolarizing step (Fig. 1, A). The delay decreased with increasing stimulation intensity. 58.3 % were tonic and 11.1 % adapting neurons, the properties of which were reported earlier [12, 13, 16]. Distinctive features of DFNs were high rheobase (Rh, 95.7 ± 11.2 pA) and depolarized threshold (Th, -37.8 ± 0.7 mV) to evoke AP. They differed significantly from the respective parameters of tonic (Rh 27.1 ± 2.3 pA, Th –50.4 ± 0.6 mV) and adapting (Rh 33 ± 3.7 pA, Th -45 ± 1.1 mV) neurons. Resting membrane potential of DFNs was -68.6 ± 1.2 mV, similar to neurons of other types. Their input resistance of 670 ± 116 MΩ tended to be lower than in tonic (1.1 ± 0.2 GΩ) and adapting (1.3 ± 0.3 GΩ) cells [12, 13]. Current-voltage relationship of DFNs was close to linear, with a small inward rectification (Fig. 1, B). DFNs did not have slow inward H-current activated by hyperpolarization, which could be observed in some fraction of neurons with tonic firing [11]. Remarkably, in voltage-clamp recordings all DFNs expressed transient outward K^+ current of A-type (K_A) of high amplitude, which started activation before Na^+ current at about –70 mV and reached 1-2 nA at –50 mV (Fig. 1, C).

Morphology of neurobiotin-filled and reconstructed DFNs was studied using confocal microscopy. To determine morphological type of the neurons, the size and orientation of dendritic tree was used as a most important criterion [5, 6, 7]. As far as the way of slicing might distort natural 3-dimensional structure of SG neurons within the spinal cord [8], some results for parasagittal and coronal slices are presented separately. Examples of confocal images from both types of slices are shown on Fig. 2. In no DFNs reconstructed in parasagittal slices was evident a large rostro-caudal extent of their dendrites (Fig. 2, A, B), so characteristic for tonic neurons

classified mainly as central or islet types [1, 7]. Some of the dendrites of DFNs inevitably crossed the border between lamina II and III and went to deeper layers of the dorsal horn. Therefore, the neuron on fig. 2 A might be classified as of vertical type. It also had a large amount of dendritic spines, which sometimes was referred to as spiny cells [4]. The neuron in fig. 2 B had larger rostro-caudal extent of some of the dendrites, similar to neurons of central type, and therefore was judged as atypical central cell. A neuron of radial type (coronal slicing) is shown on fig. 2 C. Its prominent feature was a large number of primary dendrites going in all directions. An example of a vertical type of neuron with extremely long dendrites reaching almost the center of the spinal cord (coronal slicing) is shown on fig. 2 D. Some parameters of morphometric analysis for frequently observed cell types are summarized in table 1. Note significant differences between rostro-caudal and dorso-ventral dimensions of dendritic tree measured in two types of slices. Measurements for radial cells were obtained only in coronal slices, possibly because of a limited number of reconstructed neurons.

The important neuronal characteristic related to directionality of information flow in the dorsal horn is the axon trajectory. In 28 out of 33 of reconstructed DFNs an axon-like process was evident (indicated by arrows on Fig. 2, A, B, D). In 19 out of 28 cells the presumed axons went to lamina I, in seven cells

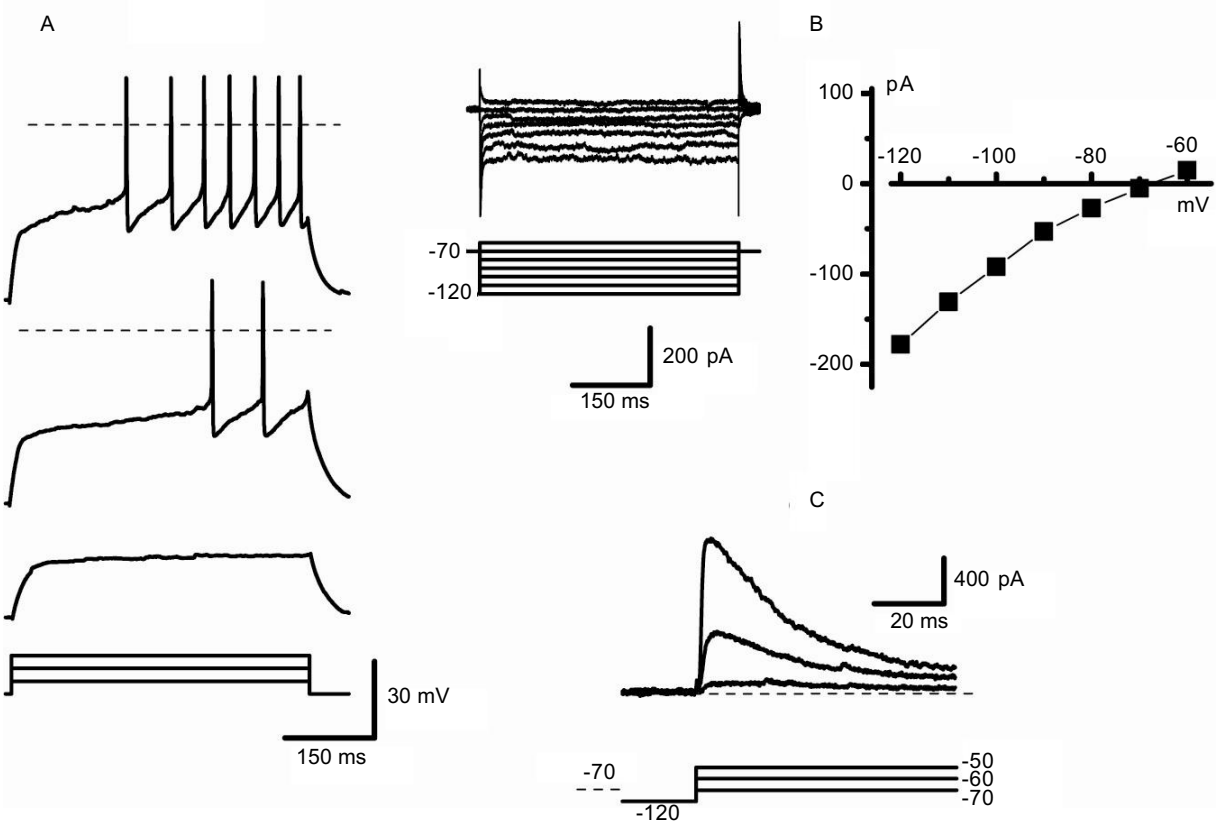

Fig. 1. Main electrophysiological properties of delayed firing neurons (DFNs). A – voltage response of DFN to depolarizing current of increasing intensity (protocol is shown below traces). Dashed line in upper two traces indicates level of 0 mV. B – input current-voltage (I-V) relationship of a DFN around resting membrane potential (~-70 mV). *Right* – I-V plot, *left* – membrane currents used for I-V plot (voltage protocol is shown below traces). C - transient outward K^+ currents evoked at voltages subthreshold for generation of inward Na^+ current (voltage protocol is shown below). Dashed line indicates level of 0 current

it stayed within lamina II and in two cases it went to lamina III.

Thus, morphophysiologic properties of DFNs were described here. Their electrophysiological landmarks were high rheobase and depolarized threshold to evoke APs. Along with typical long delay before APs, those features can be likely explained by powerful K_A current, which activates at voltages more negative for APs. This outward K_A current effectively counterbalances the external stimulation current and inward Na^+ current, thus resulting in increased rheobase and threshold. The K_A current inactivates rapidly (Fig. 1, C), which would lead to appearance of APs at the end of a sustained depolarising stimulus, when the current decreases to a minimal value. However, despite obvious simplicity of this proposed mechanism of K_A current in shaping electrogenesis of DFNs, it still needs to be proven by pharmacological blockade experiments [3] or by simulation studies.

In accordance to popular morphological

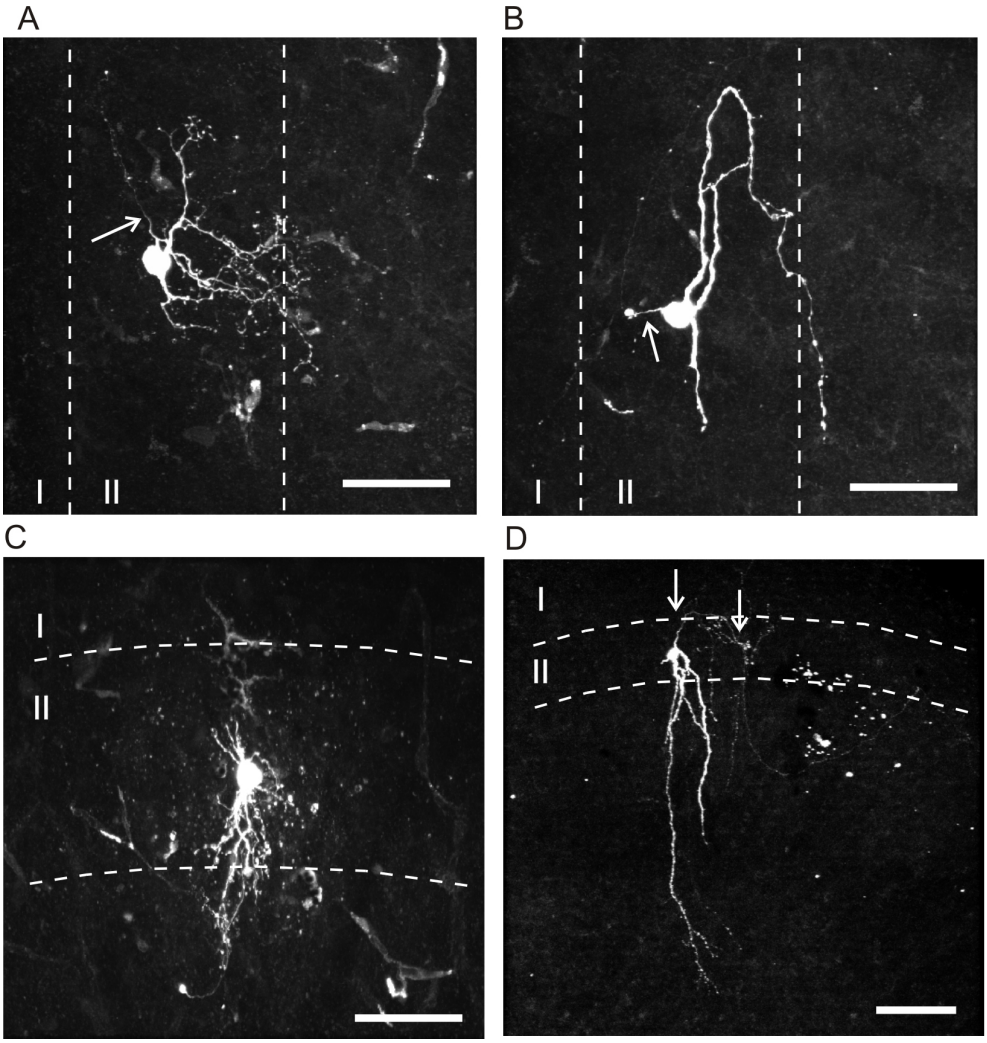

Fig. 2. Morphologic appearance of delayed firing neurons (DFNs) in parasagittal (A-B) and coronal (C-D) slices. Confocal images of cells are presented as summed Z-projections. A – neuron of vertical type, which could be also classified as spiny cell (see text). B – atypical central cell. C – neuron of radial type. D - neuron of vertical type with extremely long dendrites. Dashed lines indicate borders between laminas (numbered). Arrows point to axon-like processes. Calibration bars – 50 mm (A-C), 100 mm (D)

classification scheme [7], rat and mice SG neurons can be subdivided on four types: islet, central, radial, vertical [8, 10, 22]. Structurally, the large part of DFNs (47 %) was of vertical type. This is likely even an underestimation of cells with vertically oriented dendritic tree, because other cell types (radial, 29.5 %; atypical central, 23.5 %) also had some of their dendrites penetrating to lamina III. Another cause of underestimation is distortion of true dorso-ventral and rostro-caudal extent by parasagittal and coronal slicing, respectively (see Table 1). This likely resulted from squeezing of a slice during its processing (e.g., by coverglass). Such distortion would affect primarily the smallest extent of the spinal cord, i.e. its dorso-ventral dimension. Thus, it seems likely that the dendrites of majority of DFNs have vertical orientation. In this respect, DFNs differ substantially from tonic firing neurons, the most numerous functional group of SG cells [1, 11, 12]. The latter belongs mainly to central and islet types with their predominant rostro-caudal orientation of the dendrites, however about 32 % of tonic neurons were of vertical type [11]. The presumed axons of DFNs typically went to lamina I (~85 %), where projection (i.e., spinothalamic) neurons are located. This also contrasts with tonic neurons, the axons of which stay within SG.

Physiological role of DFNs in processing sensory information is not very clear. Recordings from pairs of neurons have recently shown that at least some DFNs in SG make excitatory synapses with lamina I neurons, including identified projection neurons [10]. Paired recordings specifically between SG neurons revealed that DFNs could form either excitatory or inhibitory connections [17]. Present morphological data support physiological studies mentioned above. The presumed axons of DFNs went to lamina I and II suggesting that sensory information flows from SG predominantly to lamina I. The dendrites of DFNs had vertical orientation reaching even deep laminas of the dorsal horn (Fig. 2, D). This indicates that DFNs might receive sensory input from superficial and deep layers of the dorsal horn. Functionally, DFNs had high rheobase and were difficult to trigger APs. It seems likely then that DFNs might serve as coincidence detectors in sensory processing and summation of many synaptic events is needed to excite them. Thus, considering both morphological and physiological data it might be proposed that, within circuitry in the dorsal horn, at least some DFNs in SG are last-order interneurons integrating synaptic input from many laminas and transmitting their output to projection cells.

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MORPHOPHYSIOLOGIC PROPERTIES OF DELAYED FIRING NEURONS IN SUBSTANTIA GELATINOSA OF THE RAT SPINAL CORD

Substantia gelatinosa (SG) neurons of the spinal cord are highly heterogeneous in their morphologic and physiologic properties. Based on characteristic firing response evoked by sustained depolarization, neurons can be categorized on three main types: tonic, adapting and delayed firing (DFNs). Here, properties of DFNs in spinal cord slices from 3-5 weeks-old rats were studied with the use of patch-clamp recording and confocal microscopy. Distinctive features of DFNs were increased rheobase (95.7 ± 11.2 pA) and depolarized threshold to evoke action potential (-37.8 ± 0.7 mV) than in neurons of other types. In voltage-clamp mode all DFNs expressed high amplitude outward A-type potassium current (K_A), which started activation at ~ -70 mV, before Na^+ current. Structurally, the majority of DFNs had vertically oriented dendritic tree, while their axons were not restricted to SG and projected predominantly to lamina I. Possible physiological functions of DFNs are discussed.

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