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An improved method for *in vitro* morphofunctional analysis of mouse dorsal root ganglia

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Running title: **DRG analysis in a whole-mount preparation**

Abstract

Sensory neurons in dorsal root ganglia (DRGs) are the first-order neurons along the pathway that conveys sensory information from the periphery to the central nervous system. The analysis of the morphological and physiological features of these neurons and their alterations in pathology is the necessary premise to understand pain encoding mechanisms. Here, we describe an *in vitro* procedure for combined morphofunctional analysis of mouse DRGs. Acutely excised DRGs obtained from adult mice were incubated in collagenase to dissolve the ensheathing connective capsule. The degradation of the connective tissue facilitates both the access to the neurons by classical recording glass pipettes and the penetration of primary antibodies for immunohistochemical procedures. The entire DRGs were then imaged at the confocal microscope obtaining a fine 3D representation of their cytoarchitecture without requiring tissue sectioning. Thus, our proposed whole-mount preparation represents a flexible *in vitro* approach for both functional and phenotypic analysis of DRG neurons by preserving at the same time their neuroanatomical relationships.

Keywords: nociceptors, DRG, whole-mount, patch clamp, immunohistochemistry

1. Introduction

Combining histological and functional analysis on intact samples of nervous system is a powerful approach for understanding cell function in relationship with their actual tridimensional context. Unfortunately, achieving this goal at cellular level is hampered by the intrinsic limitations of both histological and physiological techniques. Indeed, to properly investigate single cell function and phenotype, tissue slicing or dissociation is often required thus disrupting anatomical connections or requiring complicate reconstruction procedures. In recent times, a number of powerful whole-mount approaches have been developed to investigate large areas of the nervous system. In particular, CLARITY and derivate techniques, consisting in lipid extraction procedures, result in a nearly transparent light permissive brain-hydrogel hybrid which allows to visualize fluorescence-tagged neuronal networks (Chung et al., 2013) and/or identify single neurons by *ad hoc* modified immunohistochemical or in situ hybridization techniques (Yang et al., 2014). However, clearing procedures are quite demanding in terms of time and difficult to couple with functional studies at single cell level.

In the present study we describe a simple, time-saving and convenient approach to perform both functional and anatomical investigations in the intact dorsal root ganglia (DRGs) of the mouse. DRGs are small neuronal ganglia encoding sensory information at the periphery and transmitting it to the central nervous system (Scott, 1992). Neuronal and non-neuronal cell populations in DRGs form a heterogeneous set whose structure and organization is likely to have an impact on neuronal function (Wu et al., 2012; Christie et al., 2015). Nevertheless, most of the functional and pharmacological studies on DRG neurons have been performed on dissociated DRG cultures (Melli and Höke, 2009) where anatomical relationships are disrupted. Here, we show that functional and anatomical studies can be combined *in vitro* without altering DRG structure. DRGs can be thus investigated as a whole-mount preparation in which neuronal function and phenotype are examined by combining patch clamp recording and immunohistochemistry.

2. Materials and methods

2.1 Animals

Male CD1 mice (20-30 g) were housed in a controlled environment maintained on a 12/12 hour light/dark cycle with food and water ad libitum.

2.2 Ethics

All experimental procedures were approved by the Italian Ministry of Health and the Committee of Bioethics and Animal Welfare of the University of Torino. Animals were maintained according to NIH Guide for the Care and Use of Laboratory Animals.

2.3 Intact dorsal root ganglion (DRG) preparations and whole cell patch clamp recording

For preparation of intact DRGs, 2 month-old CD1 mice (n=13) were anesthetized with a lethal dose of sodium pentobarbital (30 mg/kg, intraperitoneal). DRGs dissection was performed by constantly maintaining the tissues in ice-cold artificial cerebrospinal fluid (ACSF), containing: sucrose 252 mM, KCl 2.5 mM, $NaHCO_3$ 26 mM, NaH_2PO_4 1.25 mM, D-glucose 10 mM, kynurenate 1 mM, $MgCl_2$ 3 mM, $CaCl_2$ 1.5 mM, saturated with 95% O_2-5% CO_2. The DRGs were removed after cutting the spinal column along the midline; then, they were incubated for 1 h at 37°C in constantly oxygenated ACSF containing collagenase (7 mg/mL) to degrade the outer connective layer.

After incubation, each ganglion was transferred into a recording chamber through which oxygenated ACSF was constantly perfused (2 mL/min) at room temperature (RT). ACSF contained: NaCl 126 mM, KCl 2.5 mM, D-glucose 10 mM, $NaHCO_3$ 26 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 2mM, $MgCl_2$ 1.5 mM. Neurons were visualized using a fixed stage microscope (Eclipse FN-1, Nikon inc, Melville, NY) equipped with infrared gradient contrast optics and a 16x water immersion objective (Nikon). Neurons were visualized with an infrared-sensitive CCD camera (W118C, Watec Corp., Yamagata, Japan) and displayed on a video monitor. Patch-clamp whole-cell recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) connected to a Digidata interface (Digidata 1440A, Axon Instruments, Union City, CA), sampled at 10 kHz and filtered at 10 kHz. Currents were recorded and stored using pCLAMP 10.2 (Molecular Devices, Sunnyvale,CA). Patch pipettes were prepared using a horizontal puller (MP225; Sutter, Novato, CA). Pipette resistances were around 5 MΩ once filled with intracellular solution, containing: K gluconate 135 mM, KCl 5 mM, HEPES 10 mM, ATP-Na 4 mM, GTP-Na 0.4 mM, $MgCl_2$ 2 mM, pH 7.2 (with KOH). To allow post-recording visualization of recorded neurons 20 µM of Alexa Fluor 568 (Thermo Fisher Scientific, Waltham, MA) was added to the intracellular solution. Recordings were included for the

subsequent analysis only if: (i) membrane potential was more negative than -50 mV; (ii) access resistance changed less than 20% throughout the recording session.
Passive and active neuronal properties were recorded. Firing patterns were obtained by applying two different current clamp protocols: a ramp protocol, which consists in a single injection of current for 500 ms ranging from 0 nA to 1 nA; a step protocol, which consists in 12 injections of current of 20 pA in increasing steps lasting for 500 ms. Neurons were classified as small (diameter <25 µm) or medium-to-large (diameter >25 µm) (Momin et al., 2008).

2.4 Immunofluorescence

After electrophysiological recordings, entire DRGs were fixed for 30 min with 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4) and washed several times in phosphate buffered saline (PBS; 0.02 M, pH 7.4). DRGs were then pre-incubated in PBS containing 6% bovine serum albumin for 1 h, incubated overnight at 4°C in biotin-conjugated isolectin B4 (IB4; 1:250; Sigma), and finally incubated for 1 h with Extravidin-FITC (1:500; Sigma). To label calcitonin gene-related peptide (CGRP) positive neurons, DRGs were pre-incubated in PBS containing 1% Normal Goat Serum (NGS) and 0.1% Triton X-100 (TX) for 1 h, incubated overnight at 4°C in rabbit anti-CGRP (1:500; Sigma), then incubated for 1 h with anti-rabbit secondary antibody Alexa Fluor 594 or 633 (1:1000; Thermo Fisher Scientific). To label neurofilament 200 (NF200) positive neurons, DRGs were pre-incubated in PBS-1% NGS-0.1% TX for 1 h, incubated overnight at 4°C in mouse anti-NF200 (1:200; Sigma), then incubated for 1 h with anti-mouse secondary antibody Alexa Fluor 546 (1:1000; Thermo Fisher Scientific). Immunofluorescence was acquired using a Leica TCS SP5 confocal microscope with 20x lens. To obtain Z-series reconstructions, immunostained DRGs were mounted with Vectashield anti-fade medium (Vector Labs, Burlingame, CA) on slides *ad hoc* modified to preserve DRGs volume and to avoid movements. Confocal optical sections were acquired at 3.5 µm intervals along the Z-axis.

A schematic diagram summarizing the main steps of the above procedure is reported in Fig. 1.

3. Results

3.1. Active and passive membrane properties of small-size and medium-to-large-size neurons

Degrading the DRG connective capsule by collagenase pre-treatment was previously shown to permit patch clamp recordings of rat sensory neurons in intact ganglia without requiring cell dissociation procedures (Zhang et al., 1998; Hayar et al., 2008). We adapted these procedures to the mouse DRG (Fig. 1) and in the first step of our protocol we recorded active and passive membrane properties from both small and medium-to-large sensory neurons. Consistently with observations in rats (Hayar et al., 2008), small neurons exhibit lower membrane capacitance, higher input resistance and higher action potential amplitude than medium-to-large neurons (Table 1). When depolarizing steps of current (20 pA) were injected (Fig. 2 A-D), two main types of firing pattern were observed in both small and medium-to-large neurons: single spiking patterns (Fig.2 A-B), which exhibit only one spike at the beginning of the depolarization, and tonic patterns (Fig. 2 C-D), which fire repeatedly upon depolarization and for the whole pulse duration. Single spiking pattern was observed in the majority of recorded neurons (9 out of 13), which is consistent with previous observations in intact DRGs from naïve rats (Zheng et al., 2007; Hayar et al., 2008). Using a ramp protocol , which consists in a continuous injection of current from 0 to 1 nA, tonic neurons keep firing repeatedly with adapting bursts of action potentials (Fig. 2 E); some single firing pattern (5 out of 9 neurons) can also be converted into phasic pattern, with few spikes at the beginning of the ramp (Fig. 2 F) .

3.2. Collagenase pre-treatment allows morphofunctional analysis of the whole DRGs

The second step of the method consisted in the morphological analysis of DRG neurons. After fixation, the entire DRGs were processed for immunohistochemistry (see methods) to indentify the main populations of sensory neurons. To investigate whether collagenase pre-treatment affects the quality of the staining, we processed in parallel collagenase-treated and untreaded lumbar DRGs. DRG nociceptors were labeled with classical peptidergic (CGRP) and non peptidergic (IB4) markers (Salio et al., 2014). As shown in Fig. 3, CGRP+ and IB4+ neurons can be easily tracked along the z-stacks in collagenase-treated DRGs (Fig. 3 A and C). Conversely, the quality of staining in absence of collagenase is strongly reduced and fluorescent signal along the Z-axis fades away and becomes less specific (Fig. 3 B and D). Thus, pre-treating DRGs with collagenase makes the tissue more accessible to antibodies and neuronal markers allowing the identification of the neuronal phenotype in the entire ganglion.

By adding a third marker for large mechanical sensitive neurons (NF200), we were able to perform in the same DRG a complete phenotypic characterization of the main populations of sensory neurons (Fig. 4). The possibility to easily identify cell populations in the entire DRG after electrophysiological analysis sets the ideal ground for combined morphological and functional investigations. Indeed, by adding a fluorescent tracer to the recording pipette the phenotype of recorded neurons can be directly addressed. In the example shown in Fig. 4 F, a single DRG neuron was recorded after collagenase incubation and was filled during the recording session with the neuronal tracer Alexa Fluor 568. Following double labeling against the nociceptor markers IB4 and CGRP, the recorded neuron was phenotypically identified as a IB4-positive non-peptidergic nociceptor. The phenotype of the recorded neuron was also consistent with its firing pattern evoked by ramp protocol. Indeed, the neuron showed an adapting pattern which has been previously described in IB4+ neurons (Choi et al., 2007).

4. Discussion

In the present study we propose a simple and time-saving method for investigating DRG neuronal function and phenotype in a whole-mount preparation.

Our method is based on a previously described approach aimed to record neurons from entire DRGs (Zhang et al., 1998; Hayar et al., 2008). In these studies, collagenase was used to remove the DRG fibrous connective capsule from acutely excised DRGs, thus allowing a easier access to DRG neurons through the recording pipette. In our study we took advantage from this model to combine electrophysiological investigations with immunohistochemistry. Our data clearly show that collagenase pre-treatment not only allows electrophysiological recordings but also improves antibodies and markers penetration into intact DRGs without requiring further slicing procedures. Time saving is one of the major advantages of using such an approach. In less than 48 hours DRG neurons can be recorded, filled with intracellular tracers, fixed, stained and analyzed by conventional confocal microscopy to investigate relationships of DRG neuronal populations. Instead, other whole-mount hydrogel-based techniques typically require longer processing time (Chung et al., 2013) and tissues should be fixed in the earlier phases of the process thus making uneasy to couple morphological and phenotypic investigations with functional studies at the single cell level.

More importantly our method allows to investigate DRG neurons *in vitro* without altering anatomical relationships between cells. Most of the functional and pharmacological studies on sensory neurons have been performed on dissociated cell cultures (Malin et al., 2007; Melli and Höke, 2009; Owen and Egerton, 2012). Investigating sensory neurons *in vivo* is technically challenging and poorly versatile, whereas neurons in culture represent a simple and expedient model for high-throughput pharmacological screening and fine analysis of neuronal physiology. Yet, changes occur in neurons during culture. Cultured neurons may

acquire new properties and exhibit increased excitability similar to that observed in nerve injury models (Ma and Lamotte, 2005; Zheng et al., 2007). As observed by Hayar and coll. (2008) and confirmed in our study, physiological properties of sensory neurons in the entire DRG are in good agreement with measurements made *in vivo* by sharp electrodes recordings (Villière and MacLachlan, 1996; Fang et al., 2005; Boada and Woodbury, 2007). This is particularly evident when firing activity is investigated. Indeed, delivering prolonged current injections in increasing steps induced phasic or single responses in most of the neurons which is in line with previous evidence from intact DRGs rather than from dissociated cultures (Zheng et al., 2007).

Besides altered intrinsic neuronal properties, sensory neurons in culture also miss their anatomical context. Little work has been done so far to understand the impact of the anatomical organization of DRGs on sensory neurons physiology, however a number of evidence has been brought to suggest that neuronal function in DRGs is influenced by neuron-to-neuron interactions (Devor and Wall, 1990) and by neuron-glia communications (Hanani, 2012; Huang et al., 2013; Christie et al., 2015), both of which are lost in dissociated cell cultures settings. In our model, not only the anatomical relationships between cell populations are preserved, but it is also possible to investigate cell phenotypes by conventional immunofluorescence techniques. The treatment with collagenase, together with the small size of the mouse DRGs, allows antibodies and cellular markers to easily penetrate into the tissue. Thus, the specific phenotype of recorded neurons can be recognized and their relationship with other cell populations addressed. As an increasing number of transgenic mice lines expressing fluorescent tag in specific sensory neuron populations have been developed in recent years (Le Pichon and Chesler,, 2014), our approach can be also applied to analyze function and anatomical context of identified sensory neurons.

In conclusion, we show that sensory neurons can be efficiently analyzed *in vitro* without renouncing to some of the advantages of *in vivo* and anatomical studies, and namely, context and phenotype information. Sensory neurons properties can be thus advantageously investigated keeping together single cell resolution and relationships with neighboring neurons.

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Legends

Figure 1. Graphic representation of the experimental procedure. Spinal column was cut along the midline and DRGs, located lateral to the spinal cord (A arrows), were removed; DRGs were incubated in constantly oxygenated ACSF containing collagenase (7 mg/mL) to remove the outer connective layer (B). After incubation, DRG neurons were placed in the recording chamber (C) and electrophysiologically recorded (D). DRGs were then fixed in 4% PAF, stained and mounted with anti-fade mounting medium in a modified slide to preserve their volume (E). Images were acquired with a confocal microscope (F).

Figure 2. Firing patterns of DRG neurons elicited in current clamp. (A-D) representative firing patterns obtained with a step protocol consisting in 12 injections of current of 20 pA in increasing steps, each lasting 500 ms; at the bottom, the protocol used and, in bold lines, currents corresponding to the recordings showed at the top. Examples of recordings of small diameter neurons (<25 µm; A and C) and of medium-to-

large diameter neurons (>25 µm; B and D). Examples of single spiking patterns (A and B), regular tonic (C) and irregular tonic patterns (D). pA values indicates the current injected. (E and F) Representative firing patterns obtained by ramp protocol, consisting in a continuous injection of current from 0 to 1 nA; on the bottom, the protocol used. Neurons with tonic firing to the step protocol, fire repeatedly also to the ramp protocol with adapting responses characterized by either a reduction of action potential amplitude (E, above) or frequency (E, below). Neurons with single firing pattern to the step protocol may exhibit a phasic pattern to the ramp (F).

Figure 3. Morphofunctional analysis of collagenase-treated and untreaded DRGs. A-D: Representative z-stacks of two DRGs acquired with a confocal microscope and sampled every 7µm. DRG incubated in ACSF containing collagenase (7 mg/mL) and then stained for CGRP and IB4 (A and C). DRG incubated in ACSF without collagenase and then stained for CGRP and IB4 (B and D). c.: collagenase; n.c.: no collagenase. Scale bars=100 µm.

Figure 4. Phenotypic characterization of the main populations of sensory neurons in the intact DRGs. (A-E) z-series reconstructions obtained by superimposing all the optical sections of DRGs in the stack: IB4+ non-peptidergic nociceptors (A), CGRP+ peptidergic nociceptors (B) and NF200+ mechanical sensitive neurons (C). Double staining of IB4+/CGRP+ neurons (D) and NF200+/CGRP+ neurons (E). (F) single stack of a DRG with the recorded cell filled with the neuronal tracer Alexa Fluor 568 (red circle) and a double staining for IB4+/CGRP+ cells. The recorded neuron is IB4+ exhibits an adapting response to the ramp stimulation (*inset*; vertical bar 30 mV, horizontal bar 50 ms). Scale bars=100 µm.

Table 1 Active and passive membrane properties of small-size and medium-to-large-size neurons

Parameters	C_m (pF)	R_m (Mohm)	Area (um^2)	V_m (mV)	Rheobase (pA/pF)	Threshold (mV)	AP height from thr (mV)	Latency (ms)	n
Small size neurons (<25 um)	29.90 ± 2.90	381.80 ± 76.24	355.39 ± 22.38	-74.80 ± 2.58	7.16 ± 2.02	-36.91 ± 2.18	104.00 ± 5.99	51.04 ± 32.78	10
Medium to Large size neurons (>25 um)	46.33 ± 16.84	198.33 ± 10.93	706.86 ± 117.81	-68.87 ± 4.33	4.84 ± 2.53	-40.66 ± 4.18	98.10 ± 3.63	16.53 ± 5.41	3

C_m: Membrane Capacitance; R_m: Membrane Resistance; V_m: Membrane Voltage; AP height from thr: Action Potential height from threshold.

Figure

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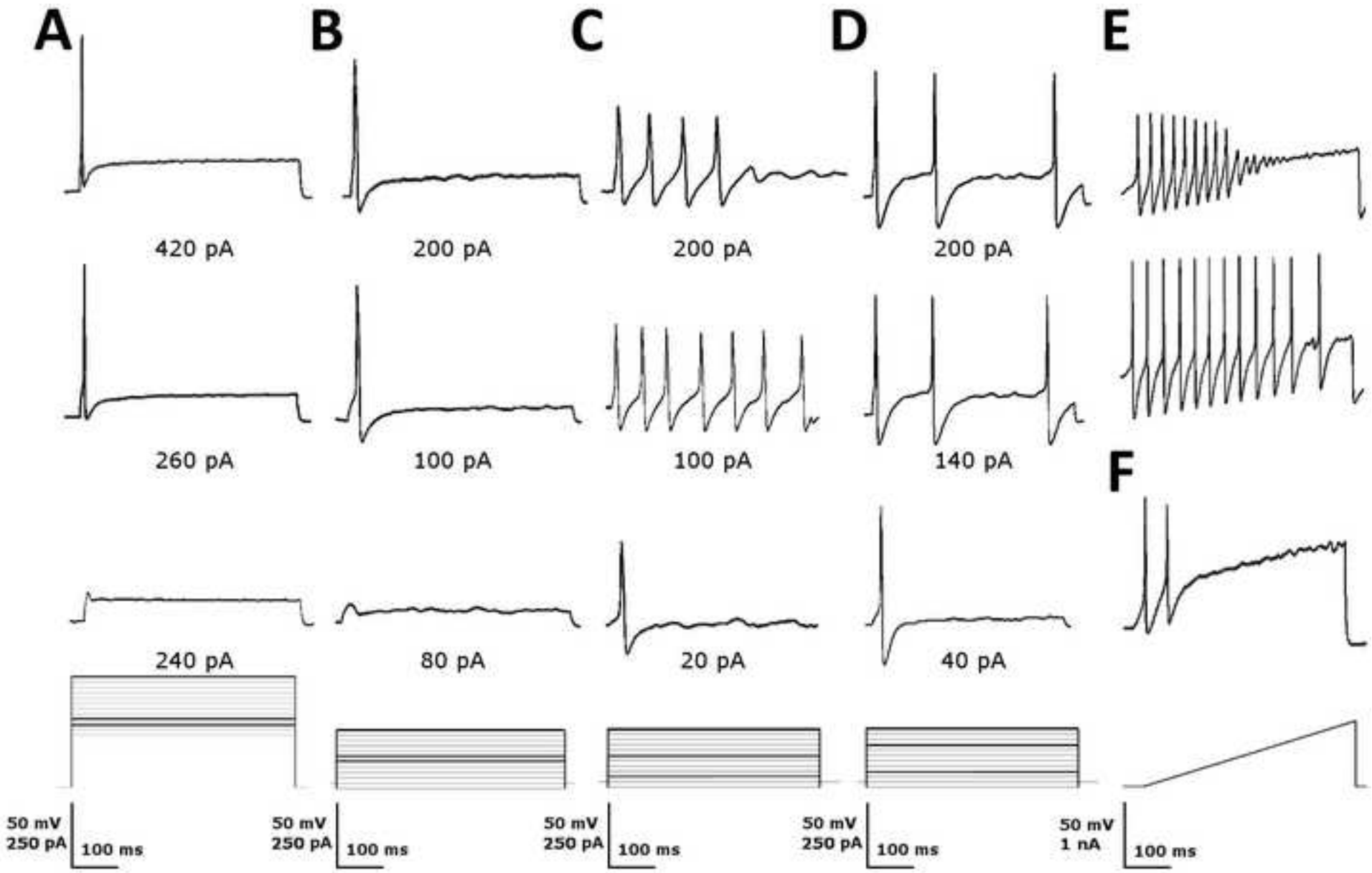

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