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RESEARCH ARTICLE

Changes in the expression and current of the Na^+/K^+ pump in the snail nervous system after exposure to a static magnetic field

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SUMMARY

Compelling evidence supports the use of a moderate static magnetic field (SMF) for therapeutic purposes. In order to provide insight into the mechanisms underlying SMF treatment, it is essential to examine the cellular responses elicited by therapeutically applied SMF, especially in the nervous system. The Na^+/K^+ pump, by creating and maintaining the gradient of Na^+ and K^+ ions across the plasma membrane, regulates the physiological properties of neurons. In this study, we examined the expression of the Na^+/K^+ pump in the isolated brain–subesophageal ganglion complex of the garden snail *Helix pomatia*, along with the immunoreactivity and current of the Na^+/K^+ pump in isolated snail neurons after 15 min exposure to a moderate (10 mT) SMF. Western blot and immunofluorescence analysis revealed that 10 mT SMF did not significantly change the expression of the Na^+/K^+ pump α-subunit in the snail brain and the neuronal cell body. However, our immunofluorescence data showed that SMF treatment induced a significant increase in the Na^+/K^+ pump α-subunit expression in the neuronal plasma membrane area. This change in Na^+/K^+ pump expression was reflected in pump activity as demonstrated by the pump current measurements. Whole-cell patch-clamp recordings from isolated snail neurons revealed that Na^+/K^+ pump current density was significantly increased after the 10 mT SMF treatment. The SMF-induced increase was different in the two groups of control snail neurons, as defined by the pump current level. The results obtained could represent a physiologically important response of neurons to 10 mT SMF comparable in strength to therapeutic applications.

Key words: dissociated neurons, immunofluorescence, magnet, Na^+/K^+-ATPase, whole-cell patch clamp.

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INTRODUCTION

Wide and frequent use of static magnetic field (SMF) in medicine and industry has resulted in an increased exposure of humans to this field. Consequently, the main purpose of numerous studies has been to investigate the health effects of SMF on humans and animals. Based on the obtained data from various studies, the World Health Organization and International Commission on Non-Ionizing Radiation Protection established limits for acute and chronic exposure of humans to SMF, which should not exceed 400 mT and 40 mT, respectively (WHO, 2006; International Commission on Non-Ionizing Radiation Protection, 2009). The greatest concern is related to the health effects of strong SMF (1–5 T) widely applied in medical diagnostics (Schenck, 2000; László and Gyires, 2009; Silva et al., 2006; Hartwig et al., 2009), and moderate SMF (1 mT to 1 T) because of its possible therapeutic application. It has been shown that 0.9 and 1.8 mT SMF can evoke epileptiform activity in human brain and thus may facilitate localization of primary epileptogenic focus (Fuller et al., 1995). SMF that varies between 230 and 280 mT induces similar responses to the Parkinson's disease drug candidate in the PC12 cell line, indicating that SMF can potentially be used for treatment of this neurodegenerative disease (Wang et al., 2010). Furthermore, previous studies showed that SMF of 4–85 mT, 40–56 mT and 170–200 mT strength reduces the pain of osteoarthritis of the hip and knee (Hinman et al., 2002; Harlow et al., 2004; Wolsko et al., 2004). With regard to the beneficial health effects of moderate SMF, it is important to investigate the cellular responses that it elicits, especially in the nervous system. So far, only a few studies have shown that exposure to moderate SMF can change the properties of ion channels and pumps in the membrane of cells in the nervous system. It has been shown that 125 mT SMF can change the properties of voltage-gated potassium channels in the neurons of rat trigeminal root ganglion (Shen et al., 2007). Similarly, properties of voltage-gated calcium and sodium channels in a GH3 cell line are affected by 120 and 125 mT SMF, respectively (Rosen, 1996; Rosen, 2003a). Our previous study showed that 10 mT SMF can change the activity of the Na^+/K^+-ATPase (Na^+/K^+ pump) in the nervous system of the garden snail (Nikolić et al., 2012).

The Na^+/K^+ pump is a highly conserved ubiquitously expressed enzyme in the invertebrate and vertebrate nervous systems (Blanco and Mercer, 1998; Therien and Blostein, 2000). The Na^+/K^+ pump uses energy derived from ATP hydrolysis to exchange $3Na^+$ (outwards) for $2K^+$ (inwards) ions across the plasma membrane and thus generates hyperpolarizing outward current, which is specifically blocked by the cardiac glycoside ouabain (Skou, 1957; Glitsch, 2001). The functional Na^+/K^+ pump is anchored in the plasma membrane and consists of the catalytic α- and a regulatory β-subunit

that are expressed in several isoforms (Blanco and Mercer, 1998). Isoforms of the Na^+/K^+ pump catalytic subunit characterized by different functional properties have been found in both invertebrates and vertebrates, indicating that the divergence of Na^+/K^+ pump genes occurred early in evolution (Cortas et al., 1989; Blanco and Mercer, 1998). In addition to the plasma membrane-associated pool, the Na^+/K^+ pump also exists in the latent pool in the intracellular compartments of the cell (Barlet-Bas et al., 1990; Gonin et al., 2001; Vinciguerra et al., 2003). Activity of Na^+/K^+ pump is tightly regulated. Long-term regulation involves changes in the expression of Na^+/K^+ pump subunits, while short-term regulation involves changes in the functional properties of the pump and its redistribution between intracellular compartments and the plasma membrane of the cell through phosphorylation and dephosporylation processes (Therien and Blostein, 2000). Activity of the pump varies between the neurons of vertebrates, indicating that Na^+/K^+ pump current is not uniform throughout the neuronal population (Dobretsov et al., 1999a; Anderson et al., 2010). Compared with the thoroughly investigated properties of the Na^+/K^+ pump in the neurons of vertebrates (Watts et al., 1991; Dobretsov et al., 1999a; Dobretsov et al., 1999b; Horisberger and Kharoubi-Hess, 2002; Hamada et al., 2003; Anderson et al., 2010), there is little information available with respect to the expression and activity of the pump in the invertebrate neurons (Cortas et al., 1989; Yasuhara et al., 2000; Górska-Andrzejak et al., 2009).

The neurons from the brain–ganglion complex of the garden snail *Helix pomatia*, because of their accessibility, stability and relative simplicity, are a very suitable experimental model for investigating physiological properties of neurons in general. Furthermore, as the fundamental neurophysiological processes are conserved between invertebrates and vertebrates, snail neurons can be a useful experimental model for studying the health effects of moderate SMF on cell membrane properties. Our previous findings showed that 15 min exposure to the moderate 10 mT SMF, comparable in strength to therapeutic application, increases activity of the Na^+/K^+ pump in the nervous system of the garden snail *H. pomatia* through processes of phosphorylation and dephosphorylation (Nikolić et al., 2012). Accordingly, the current study under the same conditions of SMF exposure examined the expression of the Na^+/K^+ pump in the isolated snail brain–subesophageal ganglion complex, along with the immunoreactivity and current of the Na^+/K^+ pump in isolated snail neurons. The data obtained indicate that 10 mT SMF treatment of snail neurons caused an increased immunolabeling of the Na^+/K^+ pump α-subunit in the neuronal plasma membrane area, and consequently an increase in pump current density.

MATERIALS AND METHODS

Isolation of snail brains and neurons

Neurons were freshly isolated from the brain–subesophageal ganglion complex of the garden snail *Helix pomatia* Linnaeus (Pulmonata: Helicidae). Animals were collected seasonally and held in the hibernating state at 7°C. Twenty days prior to the experiments, snails (shell length 25–30 mm) were kept at a temperature of 22°C, regularly wetted and fed every 2–3 days, and thus maintained in the active state. The snail brain was isolated according to the usual dissecting technique (see Nikolić et al., 2008). Enzymatic and mechanical dissociation of the ganglia complex into single neurons was performed according to a previously described procedure (Ivic et al., 1995). Here and below, unless otherwise mentioned, all chemicals were supplied by Sigma-Aldrich (Taufkirchen, Germany). Isolated snail brain was placed in a solution containing 0.5% pronase (protease from *Streptomyces griseus*, type IV) dissolved in (mmol l^{-1}): 100 NaCl, 4 KCl, 3 $MgCl_2$ and 7 $CaCl_2$, and incubated in a water bath for 2 h at 34°C. Snail brain was then rinsed several times for 5 min with extracellular solution (ECS) containing (mmol l^{-1}): 100 NaCl, 5 KCl, 5 $MgCl_2$, 7 $CaCl_2$ and 10 Hepes, pH 7.4 adjusted with NaOH. Connective tissues were cleared from the surface of the neurons in the brain–subesophageal ganglion complex and individual ganglia separated. Separated ganglia were placed in ECS supplemented with 10 mmol l^{-1} glucose, and then neurons were completely dissociated by repeated gentle suction of ganglia several times using Pasteur fire-polished pipettes of decreasing tip diameter. Dissociated neurons were kept on ice until further use in experiments. Neurons were plated (200 µl of suspension) on 15 mm circle coverslips (Manzel-Glasser, Braunschweig, Germany) coated with plant lectin concanavalin A (Con A type IV). To coat with Con A, coverslips were incubated in a solution of 1 mg of Con A per 1 ml of deionized water for 2 h and rinsed several times with ECS before use. Neurons adhered firmly to the coverslips after 30 min. Only neurons of bright appearance and pale yellow color were determined to be healthy (Ivic et al., 1995) and were further used in electrophysiological experiments. The cell body diameter of examined neurons was 20–35 µm.

Exposure of isolated snail brains or neurons to 10 mT SMF

A magnetic field was created using a permanent ferrite magnet with a cylindrical shape (diameter 4.5 cm, height 3.7 cm). This magnet provided a unidirectional field (Fig. 1A) that varied between 0.8 and 45.6 mT over a distance of 11.5 cm (measured with a GMO5 gaussmeter using a PT2837 probe, Hirst Magnetic Instruments Ltd, Falmouth, UK). The magnet was placed under a small Petri dish containing isolated snail brains or a coverslip with plated neurons with the north pole up, parallel to the vertical component of the geomagnetic field. To achieve a magnetic field strength of 10 mT, the center of the north pole surface of the magnet was positioned at a distance of 3 cm from the center of the Petri dish or coverslip (Fig. 1). At the established position, the magnetic field strength had a mean value of 10.07±0.18 mT measured across the Petri dish or surface of the coverslip (15 mm diameter), which ensured that snail brains or plated neurons were essentially exposed to 10 mT SMF (Fig. 1B). This specific SMF strength was chosen as it is comparable to the lower effective strength of the magnetic field used for therapeutic applications (Hinman et al., 2002; Wolsko et al., 2004). Isolated snail brains or neurons plated on the coverslips in ECS were mounted on the laboratory-made adapter and exposed to 10 mT SMF for a period of 15 min (corresponding to the final 15 min of adhesion of neurons to the coverslips; Fig. 1B). Our previous measurements showed that the effects of 10 mT strength SMF on the membrane properties of identified snail neurons are evident at the 15th minute of exposure period (Nikolić et al., 2008). Therefore, a specific 15 min period of exposure was chosen to further investigate the mechanisms of 10 mT SMF action on the membrane properties of snail neurons. During the exposure time, temperature changes of the ECS were not detected within the resolution of 0.1°C (measured with a DVM 401 Environment Meter, Velleman, Fort Worth, TX, USA). Unexposed control snail brains or neurons were subjected to experimental conditions identical to those of SMF-exposed brains/neurons except that the ferrite magnet was not placed under the Petri dish or coverslip. The strength of the magnetic field measured for control snail brains or neurons had a value of ~47.5 µT, which is essentially identical to the 47.798 µT background Earth magnetic field reported for 44°38′N latitude and a 20°46′E longitude by the National Geophysical Data Center. For the purpose of immunofluorescence and electrophysiological experiments, we

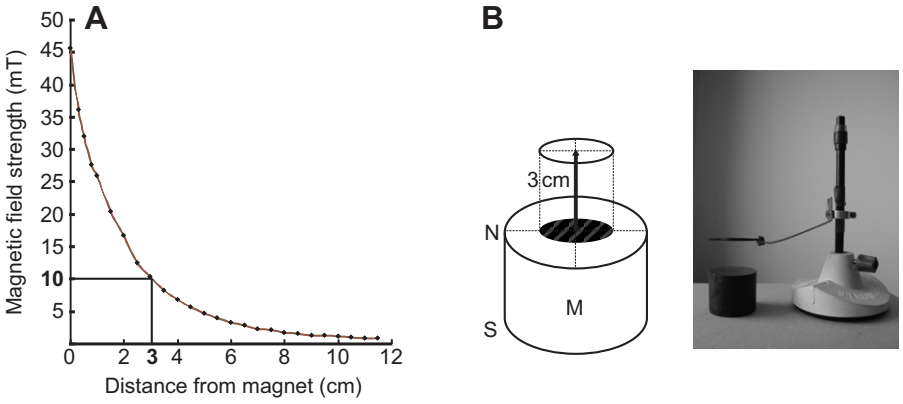

Fig. 1. Experimental system for exposure of isolated snail brains/neurons to 10 mT static magnetic field (SMF). (A) Plot of SMF strength against distance from the center of the north pole surface of the magnet. Unidirectional static magnetic field strength varied between 0.8 and 45.6 mT over a distance of 11.5 cm. As indicated, a magnetic field strength of 10 mT corresponds to 3 cm distance. (B) Schematic diagram and photograph of the experimental system used for exposure of isolated snail brains or neurons to the 10 mT SMF. The center of the small Petri dish containing snail brain or the coverslip with neurons attached was positioned at a distance of 3 cm from the center of the north pole (N) surface of the magnet (M). Measured across the surface of the Petri dish or coverslip, magnetic field strength had a mean value of 10.07±0.18 mT. S, south pole of the magnet.

always obtained coverslips with control and with SMF-exposed neurons isolated from the same animal.

Previous data showed that pronase treatment, which was exogenously applied in this study to dissociate neurons, can affect the membrane properties of isolated *Lymnea* neurons (Hermann et al., 1997). However, as control neurons and neurons exposed to 10 mT SMF were prepared from the same animals and were subjected to the same experimental procedures throughout the study, differences between them can be attributed to the effects of SMF only.

Western blotting

At the same time point, control (N=2) and SMF-exposed isolated snail brains (N=2) were immediately frozen at −80°C until use. Frozen snail brains were homogenized and lysed using ice-cold RIPA buffer containing (mmol l^{-1}): 50 Tris-HCl pH 7.4, 10 EDTA, 10 EGTA and 150 NaCl, with 0.5% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). The tissue homogenates were sonicated for 30 s and centrifuged at 14,000 r.p.m. for 25 min at 4°C. The supernatant was used to determine protein concentration (Lowry et al., 1951). Equal amounts of proteins were diluted in the same volume of Laemmli-SDS sample buffer containing 10% β-mercaptoethanol and incubated for 30 min at room temperature. Samples were loaded onto 12% SDS-polyacrylamide gel (40 µg protein per lane). Proteins were separated at 120 V (Criterion Cell, Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidine difluoride (PVDF) membrane (GE Life Sciences, Amersham, Bucks, UK) at 20 mA per gel overnight at 4°C (Criterion blotter, Bio-Rad). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween (TBST) for 90 min at room temperature. The membranes were incubated with primary mouse anti-α1 Na^+/K^+-ATPase antibody diluted in TBST (1:600, Abcam, Cambridge, UK) for 90 min at room temperature. Membranes were rinsed four times for 10 min then incubated with bovine anti-mouse (1:2000, Santa Cruz, Heidelberg, Germany) secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Membranes were rinsed four times for 10 min and the antibody was detected by chemiluminescence (exposure time 15 min). Western blot experiments were performed in duplicate and densitometric analysis of data was performed using the 'Gel analysis' function in ImageJ software (National Institutes of Health). The relative density of bands of 10 mT SMF-exposed snail brains is expressed as a percentage of the relative density of the control snail brains bands, which was set at 100%.

Previous research revealed anomalies in the electrophoretic resolution of mammalian Na^+/K^+-ATPase α-subunit isoforms (Sweadner, 1990; Cortas et al., 1991). Anomalously fast Na^+/K^+-ATPase α-subunit gel mobility has been interpreted as the consequence of excess binding of SDS to the not fully unfolded hydrophobic regions of the protein. Specifically, it has been shown that mammalian Na^+/K^+-ATPase α1-subunit migrates between 85 and 105 kDa (Cortas et al., 1991), and between 89 and 113 kDa (Sweadner, 1990), relative to its predicted molecular mass of ~112 kDa.

Immunofluorescence

Immediately after the 30 min adhesion period, at the same time point, control and SMF-exposed neurons plated on coverslips were briefly rinsed in phosphate-buffered saline (PBS) and fixed in a solution of 4% paraformaldehyde containing 7.5% sucrose for 30 min. Fixed neurons were washed in PBS three times for 5 min and placed in a blocking solution containing 10% normal goat serum (Invitrogen, Camarillo, CA, USA), 1% BSA and 0.1% Triton X-100 for 45 min. Neurons were incubated with primary mouse anti-α1 Na^+/K^+-ATPase antibody (1:50, Abcam) and rabbit anti-NeuN antibody (1:500, cat. no. ABN78, Millipore, Billerica, MA, USA) overnight at +4°C. The neurons were then rinsed three times for 10 min before incubation with fluorescently labeled secondary antibody (goat anti-mouse Alexa 555, 1:200, Invitrogen; and goat anti-rabbit Alexa 633, 1:200, Invitrogen) for 2.5 h in the dark at room temperature. Control staining was performed by omission of primary antibody. Cells were mounted on microscope slides using Mowiol medium. For quantitative measurement of Na^+/K^+ pump α-subunit expression, images were taken by a confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany) using a 63× oil-immersion objective and 543 nm HeNe laser line for excitation. A z-series of optical sections through the neuronal cell body was recorded (in 0.5 µm increments) and the section through the middle of the cell body was used for analysis of the fluorescence signal intensity (Liu et al.,

2008; Liu et al., 2011). The intensity of the fluorescence signal was measured using ImageJ software. Each image was first calibrated for the micrometer unit. To analyze the expression of the Na^+/K^+ pump in the neuronal cell body, we determined a region of interest (ROI) by tracing the signal of the α-subunit in the neuronal cell body. For each examined neuron we determined the sum of the pixel values ('Raw integrated density' in ImageJ) in the ROI. To calculate the Na^+/K^+ pump α-subunit signal density (i.e. signal intensity independent of neuron size), the sum of the pixel values was divided by the corresponding ROI area (pixel intensity μm^{-2}). Expression of the Na^+/K^+ pump α-subunit in the plasma membrane area was analyzed according to the previously described procedure (Grimes et al., 1996; Beattie et al., 2000). We marked the surface of the neuron by tracing the signal of the Na^+/K^+ pump α-subunit on the margin of the cell. The length of the line that marked the cell surface was used as the cell perimeter. Next, we outlined the region 0.5 μm interior to the first line, and determined the sum of pixel values in the ROI defined between these two lines. The sum of the pixel values in the defined ROI was divided by the perimeter of the neuronal surface to yield a value for the α-subunit signal intensity per micrometer of the neuronal surface (pixel intensity μm^{-1}). Expression of the Na^+/K^+ pump α-subunit in the cytoplasm was determined by substraction of the sum of the pixel values in the plasma membrane area from the sum of the pixel values in the neuronal cell body and then divided by the area corresponding to the cytoplasm (pixel intensity μm^{-2}). For each examined neuron, the signal density of the Na^+/K^+ pump α-subunit in the neuronal cytoplasm was calculated as a percentage of the fluorescence signal density in its cell body. The neurons used for immunofluorescence labeling were prepared from five animals and ~8–12 neurons were analyzed per group (control and SMF exposed) per animal. Analysis was performed in a 'blind' manner.

Patch-clamp recordings

All electrophysiological experiments were conducted on the freshly isolated snail neurons in ECS suplemented with 10 mmol l^{-1} glucose. Electrophysiological recordings from control and SMF-exposed neurons were obtained with the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). A coverslip with neurons attached was placed in the recording chamber on the stage of an inverted Zeiss Axiovert 10 microscope. Patch-clamp recordings in voltage-clamp mode were performed using an AM Systems 2400 amplifier and Clampex 7 software (Axon Instruments, Sunnyvale, CA, USA). Membrane currents were low-passed filtered at 2 kHz and digitized at 10 kHz using a Digidata 1200 interface (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were pulled from borosilicate glass capillaries (outer diameter 1.5 mm, inner diameter 0.86 mm; Science Products, Hofheim, Germany). Pipette resistance was 3–5 MΩ when filled with intracellular solution composed of (mmol l^{-1}): 3 NaCl, 100 KCl, 1 $MgCl_2$, 5 EGTA and 10 Hepes, pH 7.4 adjusted with KOH. Membrane capacitance (C_m), membrane resistance (R_m) and series resistance (R_s) were measured directly from the amplifier, with an upper limit for R_s of 20 MΩ. During registration in the voltage-clamp mode the membrane potential was clamped at −50 mV. The Na^+/K^+ pump current was measured as an ouabain-sensitive inward current induced by bath application of 100 μmol l^{-1} ouabain dissolved in ECS containing 10 mmol l^{-1} glucose. Prior to the application of ouabain, the physiological response of the neurons was checked by applying step (20 mV increment from −150 to +70 mV, holding at −50 mV) and ramp (from −170 to +70 mV) protocols (data not shown). In order to diminish the effect of Na^+/K^+ pump current rundown, 3 min after establishing the whole-cell patch-clamp, ouabain was applied for a period of 40 s for each examined neuron. Furthermore, all patch-clamp recordings were obtained within 20 min of complete adhesion of the neurons to the coverslips. Experimental solutions were applied to the recording chamber with a constant flow rate by a gravity-driven perfusion system. Experiments were conducted at room temperature. Whole-cell recordings were obtained from 31 neurons (18 control and 13 SMF exposed) isolated from eight animals. As the Na^+/K^+ pump inhibitor ouabain is not readily removed by washing, recordings were made from only one neuron per coverslip to be certain that residual Na^+/K^+ pump blockade was not contributing to the obtained results. Thus, the Na^+/K^+ pump current was measured from three to five neurons per animal. Responses from control and SMF-exposed neurons were always observed from the same animals throughout the experiments, and with the same stock of ouabain. The peak amplitude of Na^+/K^+ pump current was measured in Clampfit software (Axon Instruments). To obtain Na^+/K^+ pump current densities independent of neuron size, the amplitude of the Na^+/K^+ pump current was divided by C_m for each examined neuron. Membrane resistance was measured before and after inhibition of the Na^+/K^+ pump with ouabain.

Statistical analysis of data

Data were plotted and analyzed using SigmaPlot (Systat Software Inc., San Jose, CA, USA) and GraphPad software (GraphPad Software Inc., San Diego, CA, USA). Gaussian fitting of data, single and sum of the two Gaussian distributions, was performed in SigmaPlot and GraphPad software, and goodness of fit was tested by calculation of the coefficient of determination (R^2). Student's *t*-test and *t*-test for dependent samples were used to evaluate the differences between two groups of data that passed the normal distribution test. Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's multiple comparison procedure was used to compare three groups of data that did not pass the normal distribution test. The differences were considered significant for $P<0.05$. Data are presented as means ± s.e.m.

RESULTS

Expression of Na^+/K^+ pump α-subunit in the snail nervous system after exposure to 10 mT SMF

The Na^+/K^+ pump α-subunit in the snail brain was identified by western blotting analysis. The mouse anti-α1 Na^+/K^+-ATPase antibody specifically recognized a single ~85 kDa band in both control (N=2) and 10 mT SMF-exposed snail brains (N=2; Fig. 2A) which corresponds to the western blot data of Na^+/K^+-ATPase α1-subunit obtained from mammals (Cortas et al., 1991). Densitometric analysis of immunoblots (Fig. 2B) revealed an increase of 11% in the expression of Na^+/K^+ pump α-subunit caused by SMF, although the observed change was not significant. We next examined the effect of 10 mT SMF on the expression of the Na^+/K^+ pump in isolated snail neurons by immunofluorescence analysis. High magnification images revealed a distinct labeling pattern of the α-subunit in both control and SMF-exposed neurons. As illustrated in representative examples in Fig. 3A, more intense Na^+/K^+ pump α-subunit labeling was observed in the neuronal plasma membrane area as compared with the diffuse intracellular labeling in the neuron interior. Similar plasma membrane and intracellular labeling of the α-subunit has been demonstrated previously in the neurons of rat dorsal root ganglia (Dobretsov et al., 1999b). To examine the effect of 10 mT SMF on the expression of the Na^+/K^+ pump α-subunit we quantified the pump fluorescence signal intensity in the neuronal cell body (Fig. 3B). Our data showed that α-subunit signal density

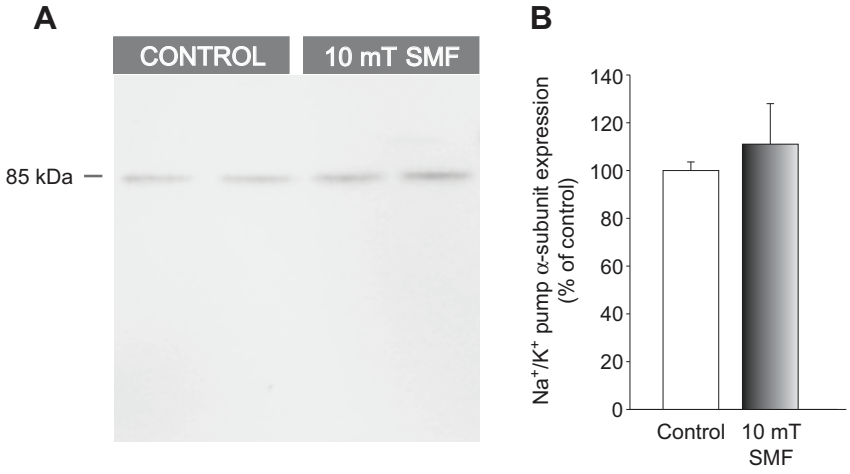

Fig. 2. Western blot analysis of Na^+/K^+ pump α-subunit expression in isolated snail brains after 15 min exposure to 10 mT SMF. (A) Representative western blot of control and 10 mT SMF-exposed snail brains probed for Na^+/K^+-ATPase α-subunit, showing a single band corresponding to ~85 kDa. (B) Expresssion of the Na^+/K^+-ATPase α-subunit was slightly increased in SMF-exposed (N=2) compared with control (N=2) snail brains, although the observed change was not statistically significant. The experiments were performed in duplicate.

of SMF-exposed neurons, although increased by 10% (1.88±0.08 pixel intensity μm^{-2}, N=55), was not significantly different from the signal density of control neurons (1.71±0.06 pixel intensity μm^{-2}, N=53). These immunofluorescence data were consistent with the western blot data obtained from snail brains. Considering that SMF in this study was applied for a relatively short period, a less prominent change in the Na^+/K^+ pump α-subunit expression in the snail brain and neuronal cell body was expected.

As our previous biochemical data (Nikolić et al., 2012) showed that in the snail nervous system 10 mT SMF increases the activity of the Na^+/K^+ pump through phosphorylation and dephosphorylation signaling pathways, we were particularly interested to examine the effect of SMF on the expression of functionally active Na^+/K^+ pump α-subunit in the plasma membrane area of snail neurons. As illustrated in Fig. 3A, Na^+/K^+ pump α-subunit was more intensely labeled in the plasma membrane area of neurons exposed to 10 mT SMF compared with control neurons. Analysis of the Na^+/K^+ pump α-subunit fluorescence signal in the plasma membrane area of 53 examined control neurons showed that the distribution of the α-subunit signal density could be best fitted (R^2=0.99) with the sum of two Gaussian distributions, indicating that expression of the α-subunit slightly differs between control neurons (Fig. 3C). However, as we were not able to clearly distinguish groups of control neurons on the basis of their α-subunit expression, the control neurons were observed as a single group. Unlike for control neurons, the intensity of the α-subunit fluorescence signal from 55 neurons exposed to the 10 mT SMF was homogeneous and was best fitted (R^2=0.96) with a single Gaussian distribution (Fig. 3C). As illustrated in Fig. 3C, superimposed Gaussian distributions of control and SMF-exposed neurons show that the peak of the homogeneous SMF distribution is shifted toward the higher values of α-subunit fluorescence signal intensity. Quantification of α-subunit fluorescence signal intensity in the plasma membrane area revealed that α-subunit expression was significantly increased in neurons exposed to SMF (2.53±0.08 pixel intensity μm^{-1}, N=55) compared with control neurons (2.23±0.08 pixel intensity μm^{-1}, N=53, P=0.01, t-test; Fig. 3C). The observed increase in the expression of the Na^+/K^+ pump α-subunit suggested that SMF could have caused a redistribution of the α-subunit from intracellular compartments toward the plasma membrane. Therefore, we next examined the Na^+/K^+ pump α-subunit fluorescence signal density in the neuronal cytoplasm. The α-subunit expression in the cytoplasm of SMF-exposed neurons (Fig. 3D) showed a trend towards a lower fraction of cell body fluorescence (90.1±1.6%), but was not significantly different from that in control neurons (94.6±1.8%). Although not significant, this change indicates that treatment with 10 mT SMF induces a trend of recruitment of Na^+/K^+ pump α-subunit near the plasma membrane.

Na^+/K^+ pump current after exposure of snail neurons to 10 mT SMF

The increase in the expression of the Na^+/K^+ pump catalytic subunit in the plasma membrane area led us to further examine whether this change is reflected in the functional properties of the Na^+/K^+ pump in snail neurons. We performed whole-cell patch-clamp experiments on control neurons and neurons exposed to SMF in order to measure the Na^+/K^+ pump current revealed by the application of ouabain. Analysis of the data obtained (Fig. 4) revealed that after exposure to SMF, Na^+/K^+ pump current density was significantly higher (1.57 times, 0.33±0.03 pA pF^{-1}, N=13) than that of control neurons (0.21±0.01 pA pF^{-1}, N=18; P<0.01, t-test). As previous data obtained from neurons of mammals showed that the effects of Na^+/K^+ pump inhibition are diverse across the neuronal population (Dobretsov et al., 1999a; Anderson et al., 2010), we next examined the responses of control and SMF-exposed snail neurons to ouabain in more detail. Furthermore, we wanted to examine more closely the correlation of the data on the expression of the Na^+/K^+ pump α-subunit in the plasma membrane area with the Na^+/K^+ pump current in snail neurons.

The left panels of Fig. 5A,B show representative responses of control and SMF-exposed neurons to the application of ouabain that gives a measure of the Na^+/K^+ pump current obtained from neurons isolated from the same animal. It can be seen that application of ouabain induced an inward current in both control and SMF-exposed snail neurons. Thus, the Na^+/K^+ pump inhibition current from the two groups of neurons maintained a similar profile; namely, from the holding potential of −50 mV, transient inward current reached peak amplitude relatively fast, after which it slowly decayed. It can also be seen that ouabain application induced variable amplitude of Na^+/K^+ pump current in control neurons (Fig. 5A, left panel) in comparison to neurons exposed to SMF (Fig. 5B, left panel). Further analysis showed that Na^+/K^+ pump current density calculated from 18 control neurons could be best fitted (R^2=0.81) with the sum of two Gaussian distributions (Fig. 5A, right panel). The presence of two separate groups of neurons with a clear bimodal distribution of pump-related currents strongly suggested that the Na^+/K^+ pump current density differs between control neurons. However, the analysis of α-subunit expression in the plasma membrane area of control neurons (Fig. 3C) did not show clearly separated groups of

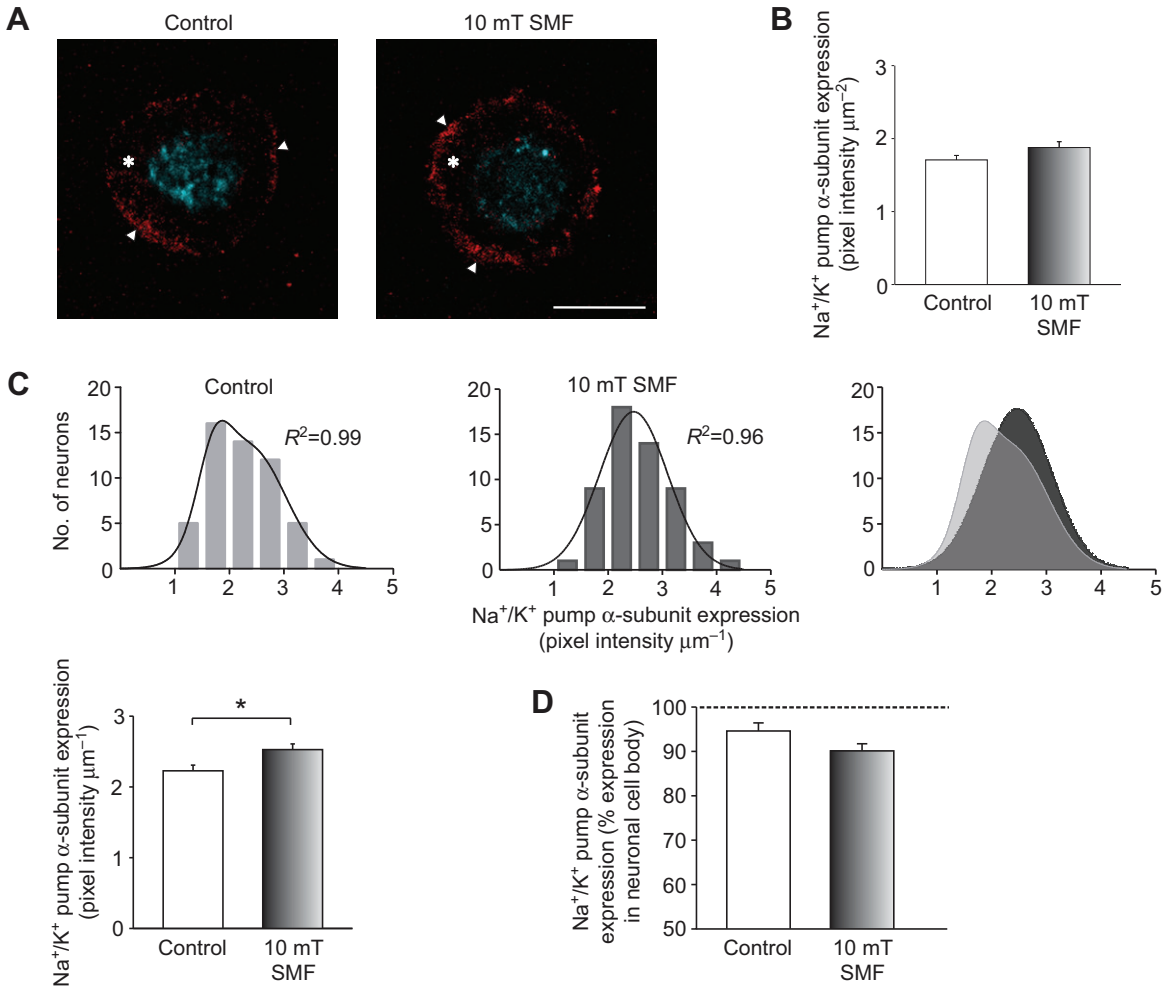

Fig. 3. Immunofluorescence analysis of Na^+/K^+ pump α-subunit expression in isolated snail neurons after 15 min exposure to 10 mT SMF. (A) Representative confocal images of a control and SMF-exposed neuron fluorescently labeled with antibody against the Na^+/K^+ pump α-subunit. Labeled Na^+/K^+ pump α-subunit (red) is found in the plasma membrane area (arrowhead) and diffusely in the neuronal interior (asterisk). Note the more intense labeling of the Na^+/K^+ pump α-subunit in the plasma membrane area of the SMF-exposed neuron. The blue labeling is neuronal marker NeuN. Control and SMF-exposed neurons were fixed at the same time points immediately after the cessation of the 15 min period of exposure to 10 mT SMF. Confocal images represent optical sections through the middle of the neuronal cell body. Scale bar, 10 μm. (B) Quantification of Na^+/K^+ pump α-subunit fluorescence signal intensity in the neuronal cell body showing that SMF did not cause a significant change in the expression of the Na^+/K^+ pump α-subunit in snail neurons (control: 1.71±0.06 pixel intensity μm^{-2}, N=53; 10 mT SMF: 1.88±0.08 pixel intensity μm^{-2}, N=55). (C) Na^+/K^+ pump α-subunit fluorescence signal intensity in the plasma membrane area of snail neurons. Quantification of signal intensity was performed within the 0.5 μm area of the neuronal surface. The Na^+/K^+ pump α-subunit expression in the plasma membrane area of control snail neurons is best fitted by the sum of two Gaussian distributions (R^2=0.99). The Na^+/K^+ pump α-subunit expression in the plasma membrane area of SMF-exposed neurons is best fitted by a single Gaussian distribution (R^2=0.96). Superimposed Gaussian distributions of control and SMF-exposed neurons (right) illustrate that the peak of homogeneous SMF distribution is shifted toward the higher values of α-subunit fluorescence intensity. The expression of the Na^+/K^+ pump α-subunit is significantly increased in the plasma membrane area of neurons exposed to 10 mT SMF (2.53±0.08 pixel intensity μm^{-1}, N=55) in comparison with control neurons (2.23±0.08 pixel intensity μm^{-1}, N=53). *P=0.01, t-test. (D) Na^+/K^+ pump α-subunit fluorescence signal intensity in the neuronal cytoplasm calculated for each neuron as a percentage of its fluorescence signal density in the neuronal cell body. The expression of the α-subunit in the cytoplasm of SMF-exposed neurons showed a trend toward the lower values (90.12±1.6%, N=55) relative to control neurons (94.57±1.8%, N=53) albeit without statistical significance.

neurons, as observed at the electrophysiological level. The first group of control neurons (arbitrarily marked as C1) had a significantly smaller (P<0.05, ANOVA on ranks) Na^+/K^+ pump current density (0.16±0.01 pA pF^{-1}, N=10) in comparison with the second group (arbitrarily marked as C2, 0.28±0.01 pA pF^{-1}, N=8) as shown in Fig. 5C. Both, the lower and higher Na^+/K^+ pump current responses in control conditions, corresponding to the C1 and C2 groups of neurons, were always observed from neurons isolated from the same animals throughout experiments. These data indicate that Na^+/K^+ pump current density is not uniform throughout the snail nervous system, which is in agreement with data obtained from neurons of mammals (Dobretsov et al., 1999a; Anderson et al., 2010). In comparison to the control conditions, Na^+/K^+ pump current density calculated from 13 neurons exposed to SMF (Fig. 5B, right panel) showed a homogeneous distribution and was best fitted with a single Gaussian distribution (R^2=0.96). Thus, the existence of homogeneous group of neurons, revealed by immunofluorescence and electrophysiological data, suggests that SMF effects on Na^+/K^+ pump expression at the plasma membrane and pump current density are exerted through common pathways.

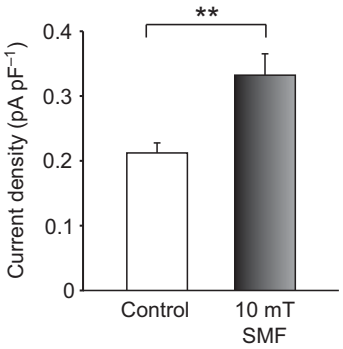

Fig. 4. Na^+/K^+ pump current density in isolated snail neurons after 15 min exposure to 10 mT SMF. Whole-cell voltage-clamp recordings from control and 10 mT SMF-exposed neurons revealed that Na^+/K^+ pump current density is significantly increased after treatment with SMF (0.33 ± 0.03 pA pF^{-1}, N=13) in comparison with control conditions (0.21 ± 0.01 pA pF^{-1}, N=18). **P<0.01, t-test.

The existence of two clearly electrophysiologically separate groups of control neurons defined by the level of response to ouabain enabled us to characterize the effect of 10 mT SMF on snail neurons in more detail. The Na^+/K^+ pump current density of SMF-exposed neurons had a higher mean value of 0.33 ± 0.03 pA pF^{-1}, compared with C1 and C2 groups of control neurons. Although increased, Na^+/K^+ pump current density of SMF-exposed neurons was significantly different only from the current density of C1 control neurons (P<0.05, ANOVA on ranks), demonstrating that SMF exerts a differential effect on C1 and C2 control neurons (Fig. 5C). There were no significant differences between Na^+/K^+ pump current densities measured from neurons isolated from different animals.

We next assessed several other electrophysiological properties of C1 and C2 control and SMF-exposed groups of neurons. It can be seen from Table 1 that the groups of neurons did not significantly differ in size (C_m value). Data presented in Table 1 also show that R_m values of C2 and SMF-exposed neurons although higher are not significantly different from the value of R_m of C1 neurons, or from each other.

Membrane resistance of C1, C2 and SMF-exposed snail neurons after inhibition of Na^+/K^+ pump

To examine whether the change in Na^+/K^+ pump activity affects the properties of the neuronal membrane, we next measured the membrane resistance of snail neurons after inhibition of the pump with ouabain. After application of ouabain, membrane resistance in C1, C2 and SMF-exposed neurons increased from 352.6±47.1, 511.6±117.6 and 532.7±145.9 MΩ to 448.0±67.1, 697.9±159.3 and 733.6±68.6 MΩ, respectively (Fig. 6). The increase of membrane resistance in C1 neurons, characterized by a lower Na^+/K^+ pump current density, was not significant (N=10, Fig. 6). However, following the application of ouabain, the increase in the membrane resistance of C2 control and SMF-exposed neurons, with higher Na^+/K^+ pump current density, was more pronounced and significant (C2: N=8, P=0.016, paired t-test; SMF: N=13, P=0.003, paired test; Fig. 6). These data indicate that the contribution of the pump to membrane resistance is more pronounced in neurons characterized by a higher Na^+/K^+ pump current density.

DISCUSSION

Active transport of Na^+ and K^+ ions across the plasma membrane, an important task performed by the Na^+/K^+ pump, is essential for the maintenance of the membrane resting potential and the regulation of electrical activity of neurons (Therien and Blostein, 2000). Hence, the change in the current generated by the Na^+/K^+ pump affects the physiological properties of neurons. This study is in line with our previous investigations on the effect of 10 mT SMF on the activity of the Na^+/K^+ pump (Nikolić et al., 2012) and demonstrates further that Na^+/K^+ pump expression in the plasma membrane area and pump current density are changed after exposure of snail neurons to 10 mT SMF.

Short-term regulation of Na^+/K^+ pump activity by phosphorylation and dephosphorylation is achieved by altering the functional properties (e.g. the turnover rate) of the pumps already present at the plasma membrane, as well as by modulating Na^+/K^+ pump expression at the cell surface (Therien and Blostein, 2000). The latter is accomplished by recruiting an intracellular protein pool of the pump to the plasma membrane, and by internalization of the plasma membrane-associated pool (Barlet-Bas et al., 1990; Chibalin et al., 1999; Gonin et al., 2001; Budu et al., 2002; Efendiev et al., 2003; Vinciguerra et al., 2003; Efendiev et al., 2007). Our previous findings (Nikolić et al., 2012) and results obtained in the present study indicate that the SMF-induced increase in Na^+/K^+ pump activity can be mediated through short-term mechanisms of pump activity regulation. Our results suggest that SMF acted to redistribute the Na^+/K^+ pump from intracellular compartments toward the plasma membrane, as the expression of the Na^+/K^+ pump α-subunit was significantly increased in the plasma membrane area while it showed a trend towards a decrease in the cytoplasm. Based on our previous findings (Nikolić et al., 2012), such an effect of SMF could be mediated by phosphorylation and dephosphorylation signaling pathways that regulate expression of the Na^+/K^+ pump at the plasma membrane. In fact, our data indicate that a 13% increase in the expression of the Na^+/K^+ pump α-subunit at the plasma membrane could not be solely responsible for the 57% increase of the Na^+/K^+ pump current density observed after 10 mT SMF exposure. This discrepancy could thus be attributed to the aforementioned phosphorylation and dephosphorylation processes (Nikolić et al., 2012) that directly affected activity of the pumps already present in the plasma membrane. Previous data showed that 15 min exposure to moderate SMF followed by 30 min and 1 day recovery caused prominent changes in the expression of genes involved in cellular metabolism in the human embryonic cell line and cultured hippocampal rat neurons, respectively (Hirai et al., 2002; Wang et al., 2009). In our experiments the overall expression of the Na^+/K^+ pump α-subunit was not significantly changed after 15 min of exposure to 10 mT SMF. This results could be anticipated, as the Na^+/K^+ pump α-subunit expression in our study was analyzed immediately after the cessation of the 15 min exposure to 10 mT SMF.

The distribution of the Na^+/K^+ pump α-subunit in the plasma membrane area as well as the pump current density in neurons exposed to 10 mT SMF was described with the single Gaussian fit. In comparison, the sum of two Gaussian fits was needed to describe the distribution of the Na^+/K^+ pump α-subunit in the plasma membrane area and pump current density in control neurons, indicating that the population of control neurons is composed of two groups. Thus, the SMF-induced increase in the expression of the Na^+/K^+ pump α-subunit in the plasma membrane area and the increase in the pump-related currents caused the population of neurons to become homogeneous. Compared with the immunofluorescence data, the two groups of control neurons were clearly distinguishable at the electrophysiological level. The ability to clearly distinguish groups of control neurons based on their

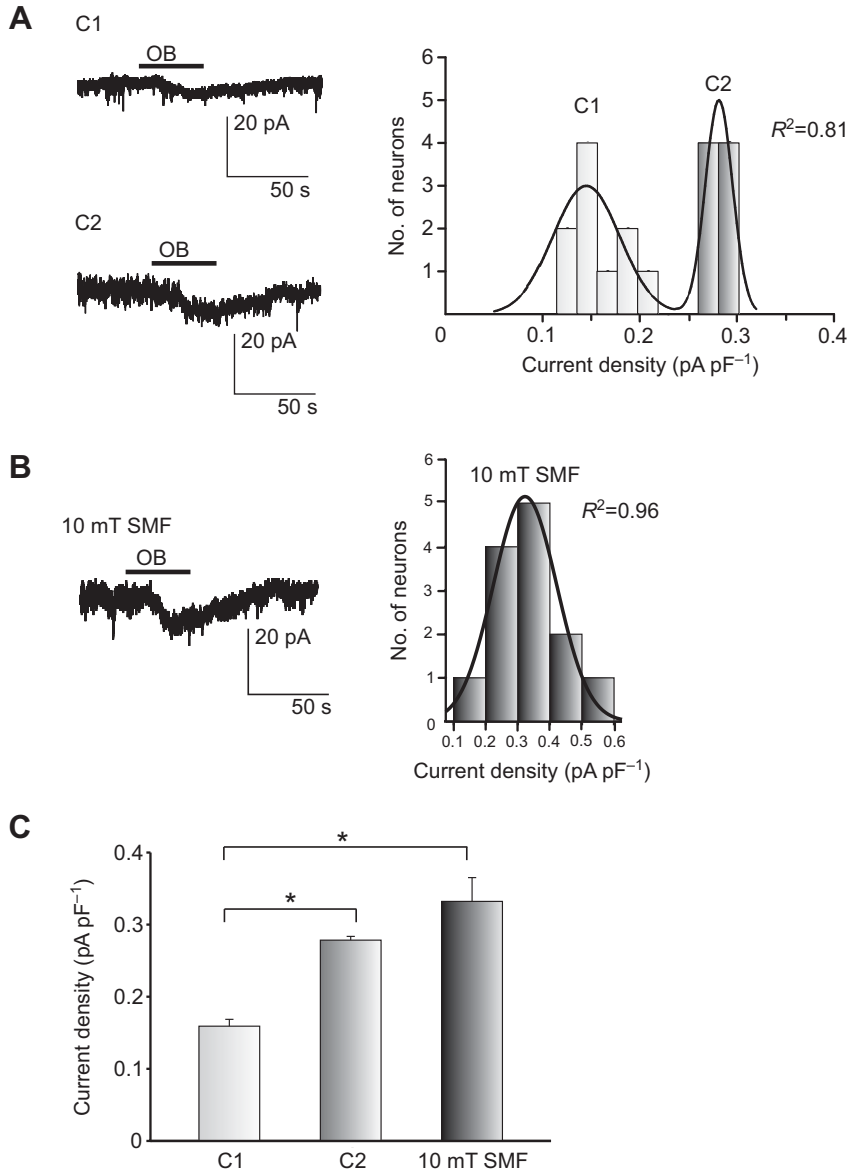

Fig. 5. Differential effects of 10 mT SMF on the Na^+/K^+ pump current density in isolated snail neurons. (A) Left panel: representative traces of whole-cell voltage-clamp recordings from control neurons of approximately the same size (membrane capacitance C_m≈45 pF) showing variable ouabain-sensitive inward current responses to the application of 100 μmol l^{-1} ouabain (black bar, OB). Holding potential, −50 mV. Right panel: Na^+/K^+ pump current density of control neurons is best fitted by the sum of the two Gaussian distributions (R^2=0.81). Note that control neurons are clearly separated according to the Na^+/K^+ pump inhibition current into a group showing small current density (arbitrarily marked C1, 0.16±0.01 pA pF^{-1}, N=10) and neurons showing a large current density peak (arbitrarily marked C2, 0.28±0.01 pA pF^{-1}, N=8). (B) Left panel: representative trace of whole-cell voltage-clamp recordings of ouabain-sensitive current response elicited by the application of 100 μmol l^{-1} ouabain (black bar, OB) in a neuron exposed to 10 mT SMF (C_m=25 pF). Holding potential, −50 mV. Right panel, Na^+/K^+ pump current density of SMF-exposed neurons is best fitted by single Gaussian distribution (R^2=0.96). (C) Calculated Na^+/K^+ pump current density is significantly different between C1 control neurons (N=10) and SMF-exposed neurons (N=13), and between C1 neurons and C2 neurons (N=8) (*P<0.05, ANOVA on ranks).

Na^+/K^+ pump current density could be attributed to the phosphorylation and dephosphorylation processes that regulate the functional properties of the pumps already present at the plasma membrane. Furthermore, it can be argued that control neurons have different compositions of functional Na^+/K^+ pump α-subunit isoforms. The invertebrate and vertebrate α-subunit isoforms have similar properties; namely, the α-subunit isoforms of both differ in their sensitivity toward the Na^+/K^+ pump inhibitors and concentrations of Na^+ and K^+ ions (Cortas et al., 1989; Blanco and Mercer, 1998). Although there is no detailed knowledge on the isoforms of Na^+/K^+ pump α-subunit in the snail nervous system, based on existing data (Cortas et al., 1989; Blanco and Mercer, 1998), it would be reasonable to assume that snail neurons express different Na^+/K^+ pump α-subunit isoforms that have certain similarities with α-subunit isoforms in mammalian neurons. It has been shown that neurons of mammals express α1 and α3 isoforms of the Na^+/K^+ pump catalytic subunit (Hieber et al., 1991; Watts et al., 1991; Pietrini et al., 1992; Brines and Robbins, 1993). Furthermore, previous research revealed grouping of different types and even subtypes of mammalian neurons based on their Na^+/K^+ pump current density (Dobretsov et al., 1999a; Anderson et al., 2010). Our data are in agreement with those findings as we identified two groups of snail neurons (C1 and C2) differentiated by the level of response to ouabain. However, the correlation of the responses from the arbitrarily assigned C1 and C2 neuronal groups with the specific neuronal type needs further electrophysiological study. Furthermore, our results reveal that C1 and C2 neuronal groups responded differently to the applied 10 mT SMF, as the induced increase in the Na^+/K^+ current density was significant only in the C1 population of neurons. Nevertheless, the overall effect of SMF was a significant increase in the Na^+/K^+ pump current density in the total population of examined neurons (C1 with C2). We could not observe any differences in the membrane resistance and neuronal size between C1 and C2 groups of neurons. Furthermore, the obtained data did not allow us to identify C1 and C2 groups as neurons of a particular bioelectric activity and function in the snail nervous system. In this study, we examined the expression of Na^+/K^+ pump α1 isoform, which has been shown to be a dominant isoform in the rat central nervous system (Watts et al., 1991). However, previous data also showed that mammalian neurons expressing α1

Table 1. Properties of the neuronal cell membranes characterized by different Na^+/K^+ pump current densities

Group	No. of cells	C_m (pF)	R_m (MΩ)
C1	10	33.07±4	352.6±47.1
C2	8	25.21±2.92	511.6±117.6
SMF	13	29.25±5.63	532.7±145.9

'Group' was based on the Na^+/K^+ pump current density.
C_m, membrane capacitance; R_m, membrane resistance. Values are means ± s.e.m.

isoform also express α3 Na^+/K^+ pump isoform in their plasma membrane (Dobretsov et al., 1999b; Matsumoto et al., 2011). Therefore, we suggest that clearly separated responses of C1 and C2 snail neurons to ouabain are associated with coexpressed Na^+/K^+ pump α-subunit isoforms combined in different ratios in the plasma membrane of these neurons. Thus, it is possible to speculate that the different susceptibility of C1 and C2 neuronal groups to 10 mT SMF may be related to a particular Na^+/K^+ pump isoform. This assumption can be supported by further investigation of the expression of different Na^+/K^+ pump α-subunit isoforms in snail neurons.

The change in the activity of the Na^+/K^+ pump can alter the activity of the Na^+ gradient-dependent exchangers, voltage-dependent ion channels and ATP-dependent K^+ channels (Glitsch, 2001). In this study we found that the change in the Na^+/K^+ pump activity affects the properties of the neuronal membrane. After inhibition of the Na^+/K^+ pump with ouabain, membrane resistance was increased in each group of examined neurons, but significantly in C2 and SMF-exposed neurons. It is plausible that the change in the activity of the Na^+/K^+ pump solely contributes to the change in the membrane resistance of snail neurons, whereby this contribution is more pronounced in C2 and SMF-exposed neurons both with higher pump current density. However, we assume that the observed change in the membrane resistance can be interpreted as the contribution of both the change in the Na^+/K^+ pump activity and the change in the conductances affected by the Na^+/K^+ pump. Furthermore, it has been shown that blocking of the Na^+/K^+ pump by ouabain reduces outward currents in the neurons of the rat nodose ganglion (Matsumoto et al., 2011) and mouse hippocampal interneurons (Richards et al., 2007). It is important to mention that a previous study indicated that the effects of moderate SMF on the membrane resistance of identified snail neurons are mediated by glial cells, as their removal by treatment of snail brain with 1% pronase abolished the observed effect (Balaban et al., 1990). However, in our study a change in membrane resistance could still be observed in SMF-exposed neurons after treatment of snail brain with 0.5% pronase. A possible explanation for the discrepancy in the observed SMF effects on the membrane resistance of snail neurons could be the difference in the preparation of the examined neurons. In contrast with the dissociated and individual neurons examined in our study, the study by Balaban and colleagues investigated identified neurons within the snail ganglia that had their synaptic connections preserved (Balaban et al., 1990). Therefore, it may be possible that other neurons through synaptic inputs, and glial cells through interaction with neurons, modulate the response of an examined neuron to the SMF. In our study, however, such interplay between neurons was excluded. Nevertheless, a contribution of glial cells to the effects of SMF on the membrane resistance of the snail neurons observed in our study remains to be determined.

Our study does not provide evidence on the primary cellular site of 10 mT SMF action. However, according to the data in the literature, the SMF-induced change in the properties of membrane-embedded ion channels and pumps can be the consequence of a magnetic field influence on the plasma membrane fluidity due to the diamagnetic properties of the phospholipid bilayer (Braganza et al., 1984; Rosen, 2003b; Petrov and Martinac, 2007). Thus, it has been shown that the properties of the phospholipid bilayer affect the activity of the Na^+/K^+ pump (Johannsson et al., 1981). Furthermore, in addition to its fundamental ion-pumping function, recent research has revealed an additional role of the Na^+/K^+ pump in signal transduction in various signaling pathways (Reinhard et al., 2013). It is plausible that the change in the activity of the Na^+/K^+ pump induced by SMF influences phosphorylation and dephosphorylation signaling pathways. Based on our previous (Nikolić et al., 2012) and present research, we suggest that the SMF-induced increase in the activity of the Na^+/K^+ pump is mediated by phosphorylation and dephosphorylation signaling pathways that regulate expression and functional properties of the pump in the plasma membrane. Furthermore, as revealed by the change in membrane resistance, the SMF-induced increase in the Na^+/K^+ pump current density most probably affected the Na^+ gradient-dependent ionic conductances. Thus, all these changes induced by SMF would certainly influence the firing pattern of snail neurons.

The effects of moderate SMF examined in various studies are considered to be therapeutically beneficial although the appropriate strength of SMF and duration of exposure still need to be precisely determined. In this study we found that Na^+/K^+ pump expression in the plasma membrane area and pump current density are changed by 10 mT SMF. Our findings are significant as they reveal the cellular responses elicited by a SMF comparable in strength to the

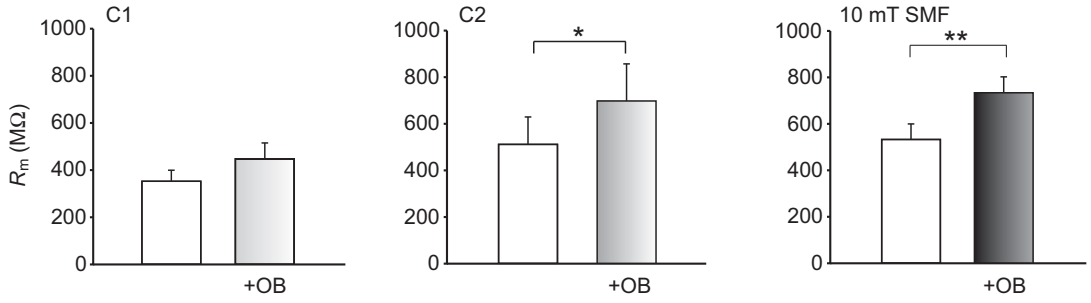

Fig. 6. Membrane resistance of C1 and C2 control neurons and neurons exposed to 10 mT SMF after application of ouabain. Inhibition of the Na^+/K^+ pump with 100 μmol l^{-1} ouabain (OB) increased membrane resistance (R_m) in all examined groups of neurons, but significantly in C2 and SMF-exposed groups (C1 N=10; C2, N=8, *P=0.016 paired t-test; SMF, N=13, **P=0.003 paired t-test).

magnetic field used for therapeutic purposes. The importance of our results is highlighted by reports of decreased Na^+/K^+ pump activity observed in numerous nervous system disorders (Lees, 1991). By observing the SMF-induced increase in Na^+/K^+ pump activity, we may begin to understand the beneficial effects of a therapeutically applied magnetic field on the nervous system, especially as Na^+/K^+ pumps show an essential similarity among neurons of diverse animal species.

AUTHOR CONTRIBUTIONS

Lj.M.N., M.N., D.T. and B.J. conceived and designed the experiments. Lj.M.N. and D.B. performed the experiments and analyzed the data. Lj.M.N., D.B. and P.R.A. interpreted the results, and wrote and revised the article.

COMPETING INTERESTS

No competing interests declared.

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