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RESEARCH ARTICLE

Three-week treadmill training changes the electrophysiological properties of spinal interneurons in the mice

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Abstract

It was shown in previous studies that endurance training enhanced excitability of rat spinal motoneurons. However, the influence of the training on the spinal interneurons remains unclear. In this study, we investigated the training effects on spinal interneurons in dorsal and ventromedial area in mice (P42–P50). The electrophysiological properties of the interneurons were recorded from spinal cord slices (T13-L6) by whole-cell patch-clamp recording. The interneurons could be classified into three types based on their response to step currents: single spike (type 1), phasic firing (type 2), and tonic firing (type 3) in both control and trained mice. Interneurons collected from control mice possessed rheobase of 11.3 ± 6.0 pA and voltage threshold (V_{th}) of -37.3 ± 4.7 mV. Treadmill training reduced the rheobase by 4.8 ± 1.5 pA and V_{th} by 3.1 ± 1.2 mV ($P < 0.05$). Furthermore, the training effects were dependent on the distribution and types of the interneurons. Treadmill training hyperpolarized V_{th} and decreased rheobase in ventromedial interneurons, while the significant change was observed only in the action potation height of the interneurons in dorsal horn. Treadmill training also hyperpolarized V_{th} and increased input resistance in type 3 interneurons, but none of these changes was shown in type 1 and 2 interneurons. Bath application of 5-HT (10−20 μM) increased the neuronal excitability in both control and trained mice. Serotonin had similar effect on membrane properties of the interneurons collected from both groups. This study suggested that treadmill training increased excitability of spinal interneurons of the mice and thus would make the spinal motor system easier to generate locomotion.

Keywords Locomotion · Treadmill training · Spinal interneuron · 5-HT modulation

Introduction

Spinal motor system has the ability to adapt to the changes of motor environment through synaptic plasticity and modulation of neuronal excitability. Exercise is one of the environment changes which is widely reported to have profound effects on the plasticity in brain and spinal cord (Gardiner et al. 2006; Ang and Gomez-Pinilla 2007; Woodrow et al. 2013). Using molecular techniques, it was systematically shown that exercise could up-regulate neurotrophic factors such as nerve growth factor (NGF), brain-derived nerve factor (BDNF) endogenously, and could change the gene expression of the transmitter receptor and ion channels (Chopek et al. 2015). Electrophysiological results indicated that exercise training improved the excitability of the motoneurons and that the effects on the motoneurons were dependent on the way of training, the intensity of the exercise and the type of the motoneurons (Gardiner et al. 2006; Power et al. 2018). With low-intensity endurance training (spontaneous wheel training and treadmill training), slow motoneurons showed changes in membrane properties. These changes including hyperpolarized resting membrane potential (RMP) and voltage threshold (V_{th}), increased rate of action potential generation, and increased amplitude of the afterhyperpolarization (AHP) (Beaumont and Gardiner 2002, 2003). The membrane properties of the motoneurons innervating the medial gastrocnemius also changed significantly after

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the compensatory overload induced in this skeletal muscle. These changes were restricted to the fast motoneurons and included a shortening of the spike rise time, an increase of the AHP amplitude and the input resistance, and a decrease of the rheobase (Krutki et al. 2015). The recruitment order of the motor units might account for these different training effects on the slow and fast type of motoneurons (Henneman et al. 1965a, b; Mendell 2005). A stronger exercise intensity might be required to recruit the fast motoneurons than the slow ones, and therefore, the electrophysiological properties of the fast motoneurons did not change significantly in the spontaneous wheel training.

In contrary to the exercise training, the excitability of spinal motoneurons was decreased when the motor activity was limited. Rat lumbar motoneurons displayed significantly elevated rheobase, lower afterhyperpolarization, depolarized spike threshold, reduced time constant, membrane capacitance, and input resistance after 2-week hindlimb suspension (Cormery et al. 2005). Similar changes in the electrophysiological properties of the spinal motoneurons were also observed in the spinal rat and paralyzed animal (Cormery et al. 2000; Chopek et al. 2013).

It is well known that central pattern generators (CPGs) are responsible for generation of motor patterns and that sensory input is crucial for the refinement of CPGs activity in response to external events (Grillner 2006). Previous studies reported that spinal interneurons located in the lamina III–V gated sensory feedback to the spinal motor system during walking and were required for the production of a fluid locomotor rhythm (Koch et al. 2017). Chopek et al. also found that passive exercise of the hindlimb could influence the gene expression in the laminal I–III in the spinal cord injury model (Chopek et al. 2018). In addition, Gozal et al. found that trace amine (TA) could generate episodic bouts of locomotor like activity in isolated neonatal rat spinal cord, providing the evidence of TAs as an intrinsic spinal cord modulatory system capable of facilitating locomotor circuits (Gozal et al. 2014). More importantly, the D1 cells settling around central canal were found to synthesis TA (Nagatsu et al. 1988), suggesting that D cells can modulate locomotor function by the unique modulator trace amine. In the study of larval zebrafish, Wyart et al. reported that the KA cells could activate locomotor circuits which were also distributed in the central canal area (Wyart et al. 2009). The frequency of the spontaneous swimming of the zebrafish was reduced when the KA cells were knocked out. In mouse spinal cord, there is also a cluster of Pitx2 neurons positioned close to the central canal (Nicholson et al. 2001). Genetic inactivation of these neurons impaired a locomotor task-dependent increase in motor neuron firing, suggesting that the Pitx 2 neuron play a neuromodulatory role in the control of locomotor activity (Zagoraiou et al. 2009). These results demonstrate that the interneurons adjacent to central canal could modulate locomotor activity. However, it remains unknown whether exercise intervention could also affect the intrinsic properties of these spinal interneurons.

Serotonin is important neurotransmitter for generation of the locomotion and plays a critical role in modulating afferent inputs for coordination in adult rats (Cazalets et al. 1992; Cowley and Schmidt 1994; Cabaj et al. 2017). Serotonergic system is highly involved in modulation of locomotor activity during treadmill training. The content of 5-HT receptor mRNA in the lumbar motoneurons of intact rats changes with different exercise regimes for 7 days and 16 weeks (Woodrow et al. 2013), and as a result of the prolonged daily treadmill training, significant decrease in mRNA content was evident for 5-HT1A receptor. In study of spinal cord injury (SCI) in which locomotor activity was limited, SCI induced up-regulation in 5-HT receptor protein and mRNA and that these changes could be reversed by exercise (Ganzer et al. 2018). The effects of 5-HT on the monosynaptic reflex (MSR) are also changed with exercise intervention (Chopek et al. 2013, 2014), but the interactions between the exercise-induced adaptation of intrinsic membrane properties of spinal interneurons and serotonergic modulations remain unclear.

For the above reasons, the purpose of this study is to investigate the effects of 3-week treadmill exercise on the electrophysiological properties of the spinal interneurons in the mice and examine serotonergic modulation on the spinal interneurons in both control and training conditions. We hypothesized that exercise training enhanced the excitability of spinal interneurons and that serotonin changed the intrinsic membrane properties of the interneurons in both control and trained mice. Preliminary data were published in abstract form (Chen et al. 2017).

Materials and methods

Experimental design

All experiments were carried out in strict accordance with the ethical requirements of the East China Normal University Public Platform for Innovation (Ethical code: 20141003). Experiments were conducted on the B6 mouse obtained from Jackson Lab. The 3 week postnatal animals were assigned to either a sedentary control group ($n = 25$) or a treadmill training group ($n = 27$) randomly. The control and trained mice were housed in standard cages without wheel. The mice assigned to the trained group received daily treadmill training at 3 week postnatal. The mice of trained group were trained 1 h/day, 6 days/week on a motor driven treadmill for 3 weeks. The speed of the treadmill training was set at 13 m/min (70% VO_2 max) and then progressively increased to a peak of 20 m/min (85% VO_2 max)

for 20–30 min during every training session (Fernando et al. 1993). The exercise animals had an adjustment period for 3 days before the 3-week training session, and the speed of the treadmill was set at low level (13 m/min). Whole-cell patch-clamp recording was performed in both control and trained groups when the animals were 6 weeks old.

Preparation of slices and patch-clamp recording

Surgery: The mice were anesthetized with the diethyl ether. The animals were then decapitated and eviscerated to expose the vertebral column. Then, the torso was pinned down by the limbs onto the base of the dissecting dish which was filled with chilled dissecting ACSF and bubbled with the carbogen (95% O_2 and 5% CO_2). Ventral laminectomy was performed to expose the spinal cord. The dorsal and ventral roots of the cord were cut with the fine micro-scissors and then the meninges were stripped with the forceps. The lumbar enlargement of the cord was isolated for preparing the slices.

Slice preparation: The spinal cord lumbar enlargement was put into the space of the two parts of L-shaped agar blocks and then immobilized by the rapid glue. The agar blocks with spinal cord were glued onto the platform of the vibratome in a slice container filled with chilled dissecting ACSF and bubbled with carbogen (95% O_2 and 5% CO_2). The slices were cut at a thickness of 250 µm (about 5–7 slices per preparation) with Leica vibrating microtome (VT 1000E, Germany). The vibratory amplitude was set at 9 µm (the maximum was 10 µm), and the cutting speed to 150 µm/s. The slice chamber was surrounded with ice to keep the slice chamber cool. After cutting, the slices were transferred to the polyethylene glycol (PEG) (30% wt/vol in distilled water, 870 mOsm, Sinopharm Chemical Reagent Co, $M_n = 25322\text{-}68\text{-}3$) for 60 s. The details of the PEG exposure protocol were described previously (Carp et al. 2008; Mitra and Brownstone 2012). Each slice was then washed twice for 1 min with dissecting ACSF solution and then transferred to an incubation chamber containing dissecting ACSF in room temperature. Thirty minutes later, the slices were transferred to the recording ACSF for the next 30 min to recovery.

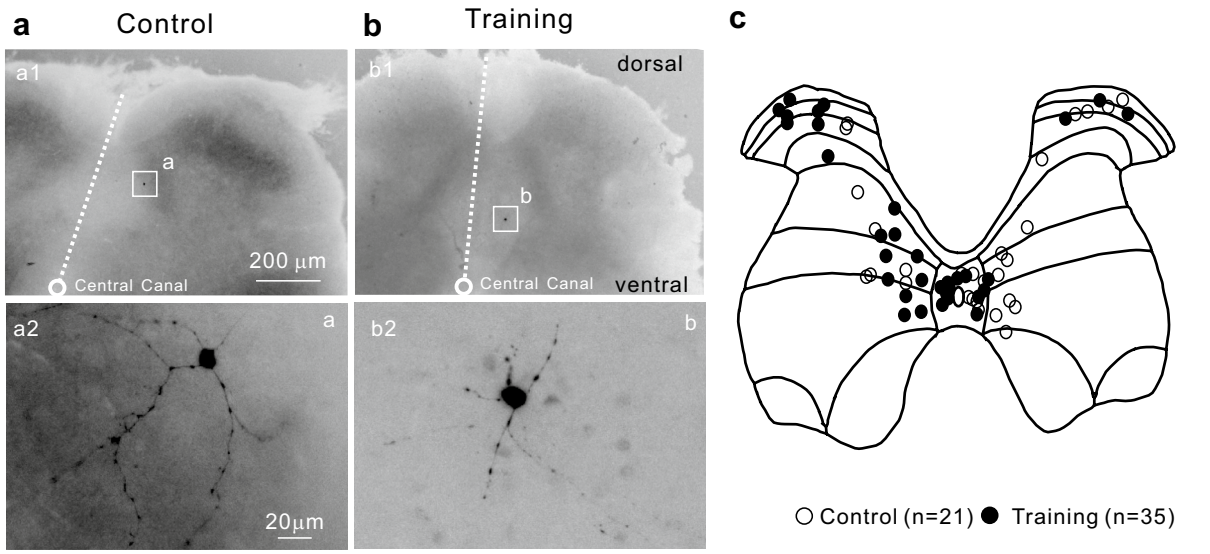
Whole-cell patch recording

The procedure for patch-clamp recording was described previously (Dai et al. 2009). Briefly, the slices were incubated for 1 h in recording ACSF for recovery and transferred to the recording chamber perfused with recording ACSF and bubbled with carbogen (95% O_2 and 5% CO_2). The perfusion was driven by gravity, and the rate was set at 2 ml/min. The Olympus BX 50 microscope with DIC was mounted upright on the top of the recording chamber. The position of the slice was adjusted at 10 × magnification; the neurons were identified at 40 × magnification. The neurons with clear edge and smooth membrane were chosen for patch-clamp recording. The recording pipettes were pulled from thick wall borosilicate glass tubing (WPI 1B150F-4) with a horizontal puller (Sutter VT1000 S), filled with intracellular solution, and had resistances of 6–8 MΩ.

The membrane properties measured and calculated in this study included rheobase (I_{th}), voltage threshold (V_{th}), resting membrane potential (E_m), input resistance (R_{in}), membrane time constant (τ_m), whole-cell capacitance (C_m), action potential (AP) height and half width, and afterhyperpolarization (AHP) depth, and half-decay time. The I_{th} was determined by the step current with 1.5-s duration and 5 pA for each step. The minimum current which evoked the action potential was taken as the I_{th}. The V_{th} was defined as the membrane potential at which the rising rate of $dV/dt \geq 10$ mV/ms. The resting membrane potentials were recorded throughout the experiments, and the reported E_m in this paper was calculated from the resting membrane potentials averaged over 500 ms prior to the step currents injected for determining the I_{th}. The action potentials (normally 3–5 spikes) evoked by the I_{th} were averaged, and the properties of the AP and AHP were calculated based on this averaged spike. The V_{th} was used as the baseline to calculate the height of the AP and the depth of the AHP. The half-decay time of the AHP was measured from the time of the AHP peak to the time of half AHP decaying from the peak. The R_{in} was calculated by the mean value of membrane potentials divided by the amplitude of the corresponding negative step current (0.5-s duration, − 10-pA step). The τ_m was determined by fitting an exponential function with form of $Y = y0 + A1*\text{Exp}(V/\tau_m)$ to the averaged voltage responses to a train of three pulses (0.5 ms, − 500 pA, 250-ms interval). C_m was calculated using formula $C_m = \tau_m/R_{in}$. The frequency–current (F–I) relationship was established through the firing frequencies versus a family of step currents, and a linear regression ($F = K*I + f$) was applied to the F–I relationship. The slope (K) and intercept (I) were used to describe the input/output relationship of the neuron. A MultiClamp 700B amplifier, Digidata 1550B A/D converter, Minidigi 1B, and pClamp (10.7) software (all from Molecular Devices) were used for data acquisition. Whole-cell patch recordings were made in current clamp mode with bridge balance. Data were low-filtered at 3 kHz and sampled at 10 kHz.

Statistic analysis

The data were analyzed using Axon Clampfit (10.7) and SPSS (19). Results are shown as mean ± SD. Student's t test was performed with significance defined as $P < 0.05$.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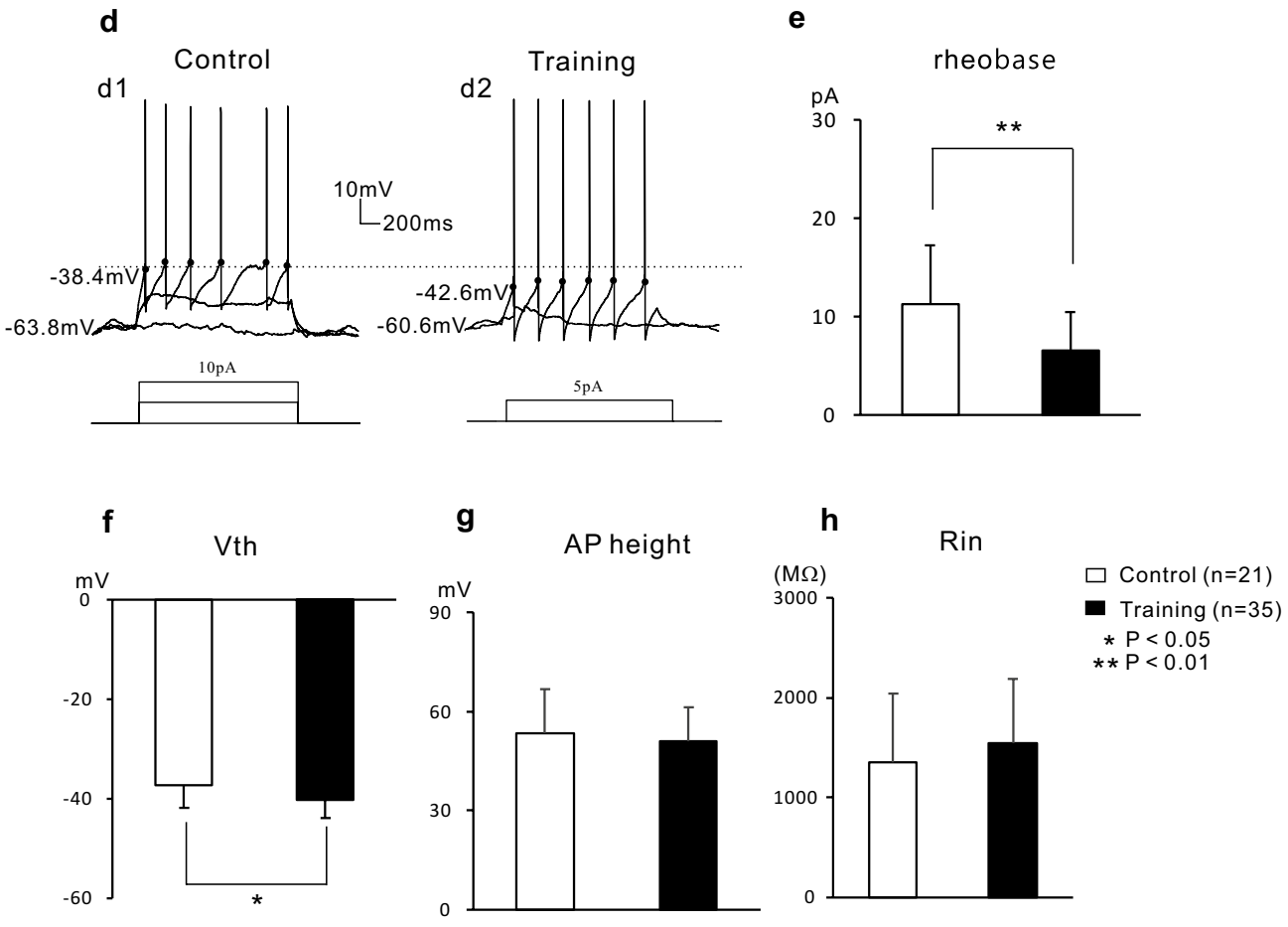
Control (n=21) Training (n=35)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Control (n=21)
Training (n=35)
* P < 0.05
** P < 0.01

◂**Fig. 1** **a**–**c** The morphology and distribution of the spinal interneurons collected in this study. The spinal interneurons labeled by Lucifer yellow in control (**a**) and trained (**b**) mice of 6 weeks. **a1** The neuron labeled in control mice marked by the white square in lamina IV. **b1** The neuron labeled in trained mice marked by the white square in lamina VI. **a2** and **b2** The detailed morphology of the neurons labeled in control and trained mice. Both of the two neurons had round soma and more than 4 stem dendrites. **c** The lamina distribution of the spinal interneurons recorded in this study. The closed circles represent the neurons collected from the trained mice and the open circles from the controls. **d**–**h** The training effects on the membrane properties of spinal interneurons. **d** Two interneurons from control (**d1**) and trained (**d2**) mice are chosen to demonstrate the training effect. These two interneurons had similar resting membrane potential and input resistance. The V_{th} of the first AP of the trained mice (− 42.6 mV) was hyperpolarized by 4.2 mV lower than that of the control (− 38.4 mV). On the other hand, the rheobase of the trained mice is smaller than the control. **e**–**h** Comparison of the rheobase, V_{th}, AP height and R_{in} of the interneurons from control (white histogram) and trained (black histogram) mice. *$P<0.05$, **$P<0.01$

Solution and chemicals

The dissecting ACSF contained (in mM) sucrose 191, K-gluconate 0.75, KH_2PO_4 1.25, choline bicarbonate 26, $MgSO_4$ 4, $CaCl_2$ 1, dextrose 20, kynurenic acid sodium salt 2, (+)-sodium L-ascorbate 1, ethyl pyruvate 5, myo-inositol 3.

The recording ACSF contained (in mM) NaCl 121, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 25, $MgCl_26H_2O$ 1.1, $CaCl_2$ 2.2, dextrose 15, (+)-sodium L-ascorbate 1, ethyl pyruvate 5, and myo-inositol 3.

The intracellular solution for current clamp recordings contained (in mM) K-gluconate 135, NaCl 10, HEPES 10, $MgCl_2$ 2, Mg-ATP 5, and GTP 0.5.

The pH of these solutions was adjusted to 7.3 ~ 7.4 by the HCl or KOH.

Drug: 5-HT 10–20 µM (H9772 Sigma).

Results

In this study, 56 interneurons were recorded from lumbar slices of 6-week mice in both control and trained animals (Fig. 1c). Of 56 interneurons, 21 were collected from control mice (open circles in Fig. 1c) and 35 were from trained mice (closed circles in Fig. 1c). These neurons were selected for data analysis based on the criteria that the $E_m \leq -50$ mV, the action potential overshoot ≥ 5 mV, and $R_{in} \geq 500$ MΩ.

The distribution of interneurons collected in present study

In this study, we selectively recorded the neurons located in the dorsal horn and ventromedial area. These neurons are candidates for integration of sensory information from super dorsal horn and contribute to modulation and generation of locomotion. Figure 1 shows two spinal interneurons in dorsal horn labeled with intracellular Lucifer yellow from control (Fig. 1a) and trained (Fig. 1b) mice of postnatal 6 weeks. The detailed morphologies show that both neurons are multipolar interneurons with 15–20-µm diameter of the soma (Fig. 1a2 for control and Fig. 1b2 for trained). Distribution of 56 interneurons collected in this study is shown in Fig. 1c.

Effects of the 3-week treadmill training on spinal interneurons

Interneurons ($n=21$) collected from control mice had a rheobase of 11.3 ± 6.0 pA and V_{th} of − 37.3 ± 4.7 mV. Training-induced increase in excitability was 4.8 ± 1.5 pA reduction of rheobase (Fig. 1d and e, $P<0.01$) and 3.1 ± 1.2 mV hyperpolarization of the voltage threshold (Fig. 1d and f, $P<0.05$). However, treadmill training did not induce significant change in the AP height and input resistance (Fig. 1g and h). In this study, we also measure other membrane properties including E_m, C_m, τ_m, AP half width, AHP depth, and AHP half-decay time. There was no significant difference in these parameters between control and trained mice. The overall effect of treadmill training on the membrane properties of the spinal interneurons is shown in Table 1 and Fig. 1d–h.

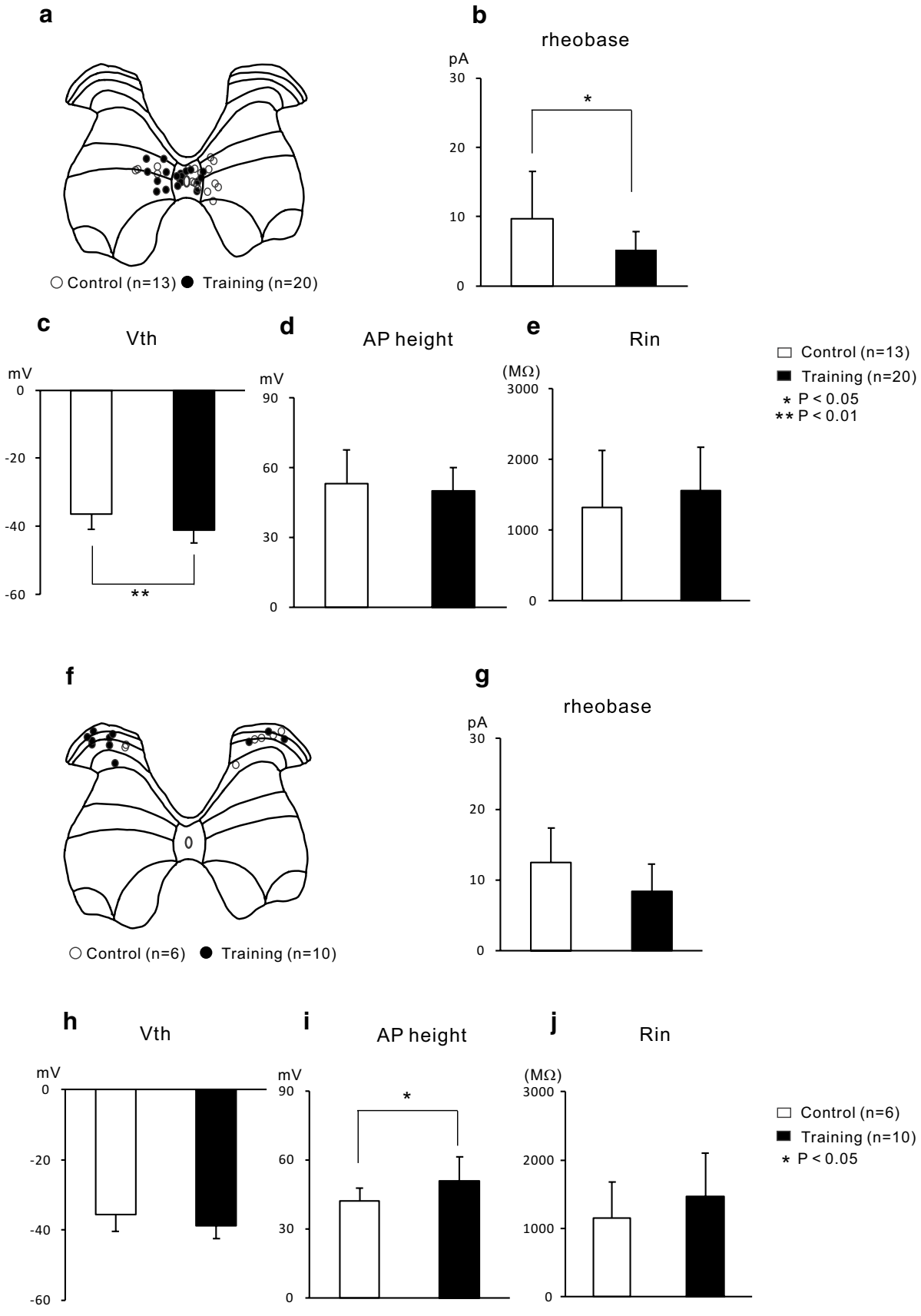
The training effect depended on the interneuron distribution

The V_{th} and rheobase of interneurons in ventromedial area of the trained mice (Fig. 2a) were shown to be 4.8 ± 1.4 mV ($P<0.01$) and 4.5 ± 2.0 pA ($P<0.05$) lower than those of control (Fig. 2b and c). On the other hand,

Table 1 Training effects on membrane properties of spinal interneurons

	Control ($n=21$)	Training ($n=35$)	Change
E_m (mV)	− 61.1 ± 5.8	− 63.2 ± 4.1	− 2.1 ± 1.6
I_{th} (pA)	11.3 ± 6.0	6.6 ± 3.9	− 4.8 ± 1.5**
R_{in} (MΩ)	1352 ± 684	1546 ± 637	194 ± 187
τ_m (ms)	39.0 ± 17.3	41.1 ± 21.3	2.1 ± 7.2
C_m (pF)	31.6 ± 12.5	28.1 ± 26.1	− 3.6 ± 7.7
V_{th} (mV)	− 37.3 ± 4.7	− 40.4 ± 3.6	− 3.1 ± 1.2*
AP height (mV)	53.3 ± 13.5	50.9 ± 10.4	− 2.4 ± 3.4
AP half width (ms)	1.3 ± 0.4	1.5 ± 0.5	0.2 ± 0.1
AHP depth (mV)	19.3 ± 4.2	18.9 ± 5.4	− 0.4 ± 1.3
AHP 1/2 decay (ms)	109.1 ± 85.9	117.4 ± 73.5	8.2 ± 30.9

*$P<0.05$, **$P<0.01$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□ Control (n=13)
■ Training (n=20)
* P < 0.05
** P < 0.01
f
○ Control (n=6) ● Training (n=10)
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□ Control (n=6)
■ Training (n=10)
* P < 0.05

◂**Fig. 2** The training effect depended on the distribution of the interneurons. **a** The distribution of the neurons recorded in ventral media area from trained (closed circles) and control (open circles) mice. **b–e** The rheobase, V_{th}, AP height, and R_{in} of the interneurons distributed in the ventromedial area from control (white) and trained (black) mice. **f** The distribution of the neurons recorded in dorsal horn from trained (closed circles) and control (open circles) mice. **g–j** The rheobase, V_{th}, AP height, and R_{in} of the interneurons distributed in the dorsal horn from control (white) and trained (black) mice. $^*P < 0.05$

however, there was no significant difference in the AP height (Fig. 2d) and input resistance (Fig. 2e) of ventromedial neurons between trained and control mice (Table 2).

16 neurons in the dorsal horn were recorded in this study (Fig. 2f), and the significant difference was found only in AP height between control and trained mice, where the AP height of the trained mice increased by 9.3 ± 4.2 mV compared with the control mice (Fig. 2i). Treadmill training induced reduction of rheobase (Fig. 2g), hyperpolarization of V_{th} (Fig. 2h), and increase of input resistance (Fig. 2j) in the dorsal horn neurons. However, none of these changes was statistically different compared with control mice (Table 3). Training-induced changes in other parameters (E_m, τ_m, C_m, AP half width and AHP depth) were not significant either. Details of these results are shown in Tables 2 and 3.

The training effect depended on the firing patterns

The spinal interneurons recorded in this study could be classified into three types: single spike (type 1), phasic firing (type 2), and tonic firing (type 3), based on their response to depolarized step currents (Fig. 3a). Bursting neurons ($n = 2$) were also observed only in lamina III and lamina V of trained mice (Fig. 3b). Burst firing is one of the important membrane properties that could contribute to the rhythmic generation during locomotion. Figure 3b shows the bursting patterns generated with the step currents. The bursting patterns occurred gradually with increasing the step currents (from top to bottom panels in Fig. 3b).

The statistical results showed that the training effect on the membrane properties of the interneurons appeared to be limited to the type 3 interneurons. Treadmill training induced a 3.5 ± 1.3 mV hyperpolarization in V_{th} of type 3 interneurons from -38.6 ± 4.7 mV (Control, $n = 10$) to -42.1 ± 3.2 mV (Training, $n = 19$, $P < 0.05$, Fig. 4a1, a2 and c). Treadmill training also increased R_{in} by 498 ± 187 MΩ from 1149 ± 573 MΩ to 1647 ± 624 MΩ ($P < 0.05$, Fig. 4a3 and e). The R_{in}, however, did not change significantly in types 1 and 2 interneurons with treadmill training (Table 4). Although training-induced reduction of rheobase and AP height were shown in type 3 neurons, these changes in membrane properties were not statistically different. The training effect on membrane properties of the three types of interneurons is summarized in Table 4.

The above results demonstrated that the types 3 interneurons appeared to be more sensitive than types 1 and 2 to the treadmill training, suggesting that they may play different functional roles during locomotion.

5-HT modulation of spinal interneurons in both control and trained mice

To investigate the training effect on spinal interneurons with 5-HT modulation, we compared the frequency–current (F/I) relationship and other membrane properties of the interneurons between control and trained mice. Two typical examples are shown in Fig. 5. Bath application of 20-μM 5-HT shifted the F/I relationships to the left with little change in F/I slopes in both control (Fig. 5a1) and trained mice (Fig. 5a2). 5-HT also hyperpolarized V_{th} and reduced AHP in both control (Fig. 5b1–3) and trained (Fig. 5c1–3) mice. Figure 5b and c shows that 5-HT induced a 4.3 mV hyperpolarization of V_{th} accompanying 2.4 mV reduction of AHP in control mice, while 5-HT also produced a 3.9-mV hyperpolarization of V_{th} with 3.2 mV reduction of AHP in trained mice. Statistical results indicated that 5-HT induced 4.2 ± 2.3 mV ($P < 0.05$) and 6.8 ± 3.3 mV ($P < 0.01$) hyperpolarization of V_{th} in control and trained mice, respectively. 5-HT also produced 3.6 ± 3.6 mV ($P < 0.05$) and 3.3 ± 3.0 mV ($P < 0.05$) reduction of AHP in control and trained mice, respectively. In addition, 5-HT-induced reduction of rheobase was observed in control mice (5.0 ± 3.5 pA, $P < 0.05$). In general, 5-HT shifted the F/I relationships to the left, depolarized the membrane potential; increased AP width, and reduced I_{th} and AHP in both control and trained mice. However, significant changes were observed only in lowering of I_{th}, V_{th}, and AHP in control and reduction of AHP and V_{th} in trained mice. The statistical results from 20 interneurons are summarized in Table 5. These data indicated that 5-HT increased neuronal excitability in both control and trained mice and that the effect of 5-HT on these interneurons appeared to be more concentrated on I_{th}, AHP and V_{th} in control mice and V_{th} and AHP in trained mice. The functional difference in 5-HT modulation of spinal interneurons between control and trained mice remains unclear.

Discussion

Using whole-cell patch-clamp technique and slice preparations, we investigated training effects on membrane properties of spinal interneurons which located in the dorsal horn and central canal areas. The most significant finding of this study is to demonstrate that treadmill training increased excitability of spinal interneurons. This exercise-induced

Table 2 Training effects on membrane properties of ventromedial interneurons

	Control ($n=13$)	Training ($n=20$)	Change
E_m (mV)	− 61.2 ± 5.9	− 63.0 ± 4.4	− 1.8 ± 2.0
I_{th} (pA)	9.6 ± 6.9	5.1 ± 2.7	− 4.5 ± 2.0*
R_{in} (MΩ)	1318 ± 812	1491 ± 625	173 ± 262
τ_m (ms)	38.9 ± 13.1	42.7 ± 24.8	3.8 ± 9.8
C_m (pF)	31.1 ± 17.6	21.7 ± 8.0	− 9.4 ± 8.4
V_{th} (mV)	− 36.3 ± 4.5	− 41.1 ± 3.8	− 4.8 ± 1.4**
AP height (mV)	53.0 ± 14.6	50.0 ± 10.1	− 2.9 ± 4.4
AP half width (ms)	1.1 ± 0.4	1.4 ± 0.5	0.3 ± 0.1
AHP depth (mV)	20.4 ± 5.3	18.0 ± 5.9	− 2.3 ± 1.9
AHP 1/2 decay (ms)	175.7 ± 121.9	166.0 ± 69.2	− 9.7 ± 74.5

*$P<0.05$, **$P<0.01$

Table 3 Training effects on membrane properties of dorsal horn interneurons

	Control ($n=6$)	Training ($n=10$)	Change
E_m (mV)	− 60.8 ± 6.0	− 64.4 ± 3.3	− 3.6 ± 2.9
I_{th} (pA)	14.2 ± 9.0	9.0 ± 3.9	− 5.2 ± 2.4
R_{in} (MΩ)	1101 ± 528	1414 ± 612	314 ± 261
τ_m (ms)	48.3 ± 22.9	37.1 ± 9.2	− 11.2 ± 11.8
C_m (pF)	33.2 ± 11.3	26.2 ± 9.3	− 7.0 ± 6.6
V_{th} (mV)	− 35.6 ± 5.0	− 38.9 ± 4.1	− 3.3 ± 2.4
AP height (mV)	42.2 ± 6.2	50.9 ± 10.4	9.3 ± 4.2*
AP half width (ms)	1.4 ± 0.3	1.7 ± 0.6	0.2 ± 0.2
AHP depth (mV)	15.5 ± 4.5	18.9 ± 5.9	3.3 ± 2.6

*$P<0.05$

alteration of membrane properties depended on the location and firing patterns of the interneurons. Furthermore, we showed that 5-HT increased excitability of spinal interneurons of both control and trained mice.

The excitability of the interneurons increased with training

The effects of exercise intervention on spinal motoneurons have been studied intensively in rodents (Cormery et al. 2000, 2005; Beaumont and Gardiner 2002, 2003; Grillner 2006). However, little was known about exercise effects on spinal interneurons, particularly the interneurons in dorsal horn and ventromedial areas. In the present study, we showed that 3-week treadmill training induced significant changes in membrane properties of spinal interneurons in the dorsal horn and ventromedial areas. These changes included the hyperpolarization of V_{th}, decrease of rheobase, and increase of input resistance. Similar observation of V_{th} hyperpolarization was reported in the rat slow motoneurons after long-term spontaneous/endurance running (Beaumont and Gardiner 2002, 2003). In addition, chronic muscle overload also decreased the rheobase of the fast motoneurons (Krutki et al. 2015). On the other hand, however, long-term spontaneous/endurance running induced an increase in amplitude of AHP and decrease in resting membrane potential in rat spinal motoneurons. These changes were not observed in the present study. The different observations between our study and Beaumont et al.'s studies could be resulted from the different animal models (adult rat vs adolescent mice) and experimental preparations (spinal cord recording in vivo vs slice recording in vitro). However, both of our studies showed that exercise training changed the excitability of spinal neurons. Therefore, we might expect that mechanisms regulating the neuronal excitability could be different due to the different functional roles in generating and adapting locomotion between the spinal motoneurons and interneurons. In the present study, the speed of the treadmill training was set at 13 m/min at the beginning and then progressively increased to a peak of 20 m/min for 20–30 min during every training session. This training plan allowed the slow and fast motor units to be activated during the exercise. However, it is not clear how the intensity of training affected the population of interneurons. A further study is required to address this issue.

Results from modeling studies suggested that up-regulation of transient sodium conductance or down-regulation of delayed-rectifier potassium conductance could cause hyperpolarization of the V_{th} in spinal neurons (Dai et al. 2002; Gardiner et al. 2006). Previous study also demonstrated that veratridine induced a hyperpolarization of the V_{th} in neonatal rat motoneurons by enhancing the sodium current (Power et al. 2012). Therefore, we might expect that modulation of ionic channels could be response for the exercise-induced changes in membrane properties. On the other hand, however, we noticed the results from recent study that no significant change was observed in expression of the sodium and potassium channels in lumbar alpha-motoneurons in the increased physical activity of rats (Woodrow et al. 2013). Therefore, in addition to channel mechanisms, we may expect that multi-factors such as synaptic plasticity, laminar distribution of interneurons, neuromuscular transmission efficacy and corticospinal effect, etc could play essential

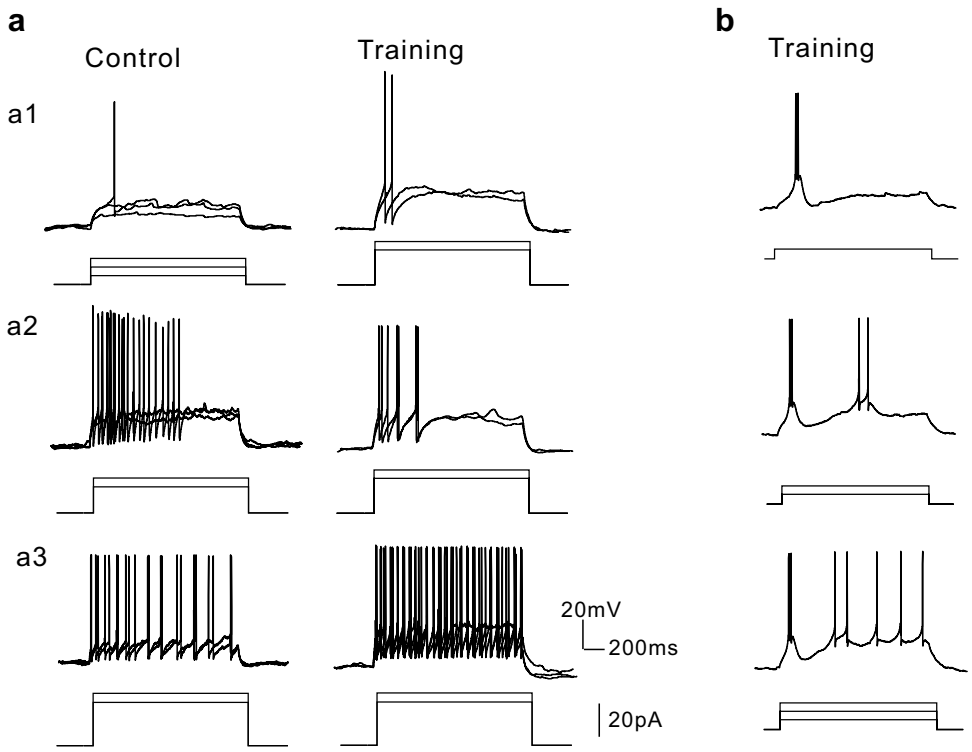

Fig. 3 Properties of the spinal interneurons collected from the trained and control mice. **a** Three types of interneurons from the 6-week control (left) and trained (right) mice. The interneurons were classified into three types based on the firing patterns in response to depolarizing step currents (1.5 s, 5 pA of step; bottom). **a1** Type 1 interneurons recorded in control (left) and trained (right) mice with injection of depolarizing current. Only one or two spikes were evoked. **a2** Type 2 interneurons fired repetitively, but it stopped firing before the termination of depolarizing step currents. **a3** Type 3 interneurons fired repetitively until the termination of depolarizing step currents. **b** Bursting interneurons obtained from trained mice. The frequency increased with depolarizing step currents

roles in modulating membrane properties of spinal interneurons in exercise (Gardiner et al. 2006; Aboodarda et al. 2015; Collins et al. 2018). Based on the previous studies on spinal motoneurons and the output of the nervous system, our study implicated that the potential training effects on spinal interneurons may have major ramifications for motor output. If the interneurons and motoneurons demonstrate higher excitability post-training, then the motor system would maintain a higher state of excitability and thus make the system easier to generate locomotion.

The training effects dependent on the distribution and types of the interneurons

Dai et al. used c-fos immunohistochemical method to localize locomotor-activated neurons in the adult cat spinal cord (Dai et al. 2005). Their results showed that treadmill-induced c-fos expressed neurons were distributed from Rexed's laminae I to X (except lamina IX), completely covering the dorsal and ventromedial areas in which we recorded interneurons in the present study. During ongoing locomotion, the incoming somatosensory information is needed for the animals to keep rhythmic stepping movements in dynamic environment. Spinal dorsal horn is the first relay station for integration of somatosensory information (Thomson et al. 1989), and there are a lot of evidences, indicating that sensory information is processed or integrated by neurons distributed in dorsal area rather than those around the central canal (Schmidt 1999). Neurons adjacent to central canal play different functional roles in modulating locomotor activity (Wyart et al. 2009; Zagoraiou et al. 2009). For this reason, we classified the interneurons into dorsal horn and ventromedial neurons, and studied their membrane properties between control and trained mice.

Interestingly, our results showed that the training effects were dependent on the laminar distribution of the interneurons. The neurons distributed in the lamina I–IV were less sensitive to treadmill training than those in the ventromedial area around the central canal. Paddock et al. (2018) found the gene expression in dorsal root ganglion neurons in rats altered significantly following 16–18 weeks of daily increased activity, suggesting that the increased activity potentially changes the sensory processing of nociceptors and proprioceptors. These changes in turn alter locomotor coordination.

Compared with small training effect on the interneurons in the dorsal horn, substantial changes (hyperpolarized V_{th} and decreased rheobase) were observed in interneurons of ventromedial area in the present study (Table 2). Interneurons in these areas have been shown to play essential roles in locomotion. The D cells, KA neurons, and Pitx2-marked cholinergic neurons which concentrated around the central

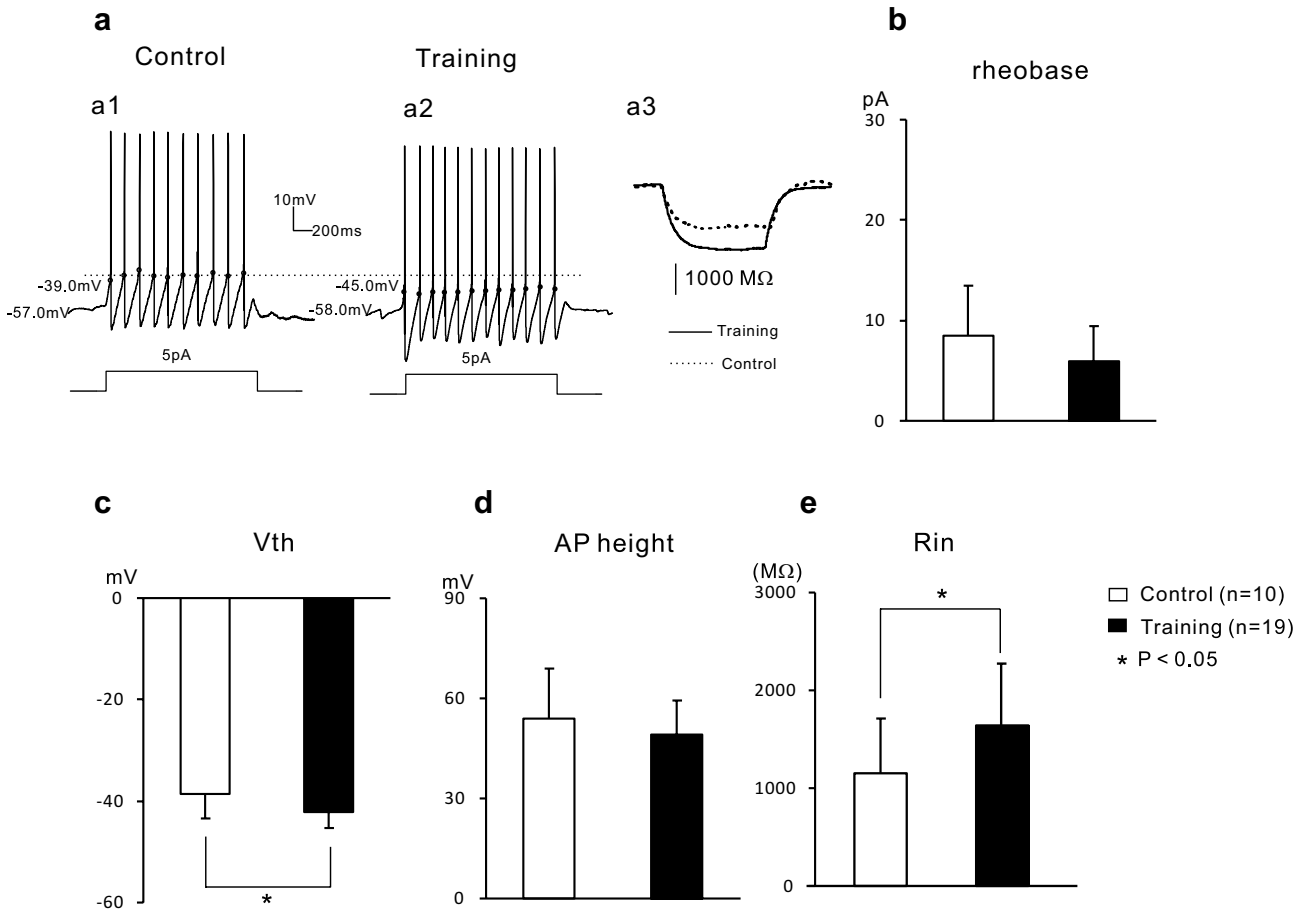

Fig. 4 The training effect on type 3 interneurons. **a** The V_{th} and R_{in} recorded in control and trained mice. **a1** A family of action potentials (top) was evoked by 1.5-s step current of 5 pA (bottom) in a type 3 interneuron from control mice, and the voltage threshold (black dots) of the first spike was − 39.0 mV; **a2** The voltage threshold (black dots) of the first spike recorded from the trained mice is − 45.0 mV. **a3** The voltage deflection evoked by the hyperpolarizing current (− 10 pA, 0.5 s) from the type 3 interneurons recorded in control (dashed line) and trained (solid line) mice. **b–e** The rheobase, V_{th}, AP height, and R_{in} of the type 3 interneurons from control (white) and (black) trained mice. $^*P < 0.05$

canal were deemed as important clusters of interneurons in modulating the locomotion. Genetic inactivation of the KA neurons in lamprey and Pitx2-marked cholinergic neurons in rat could impair the locomotor activity (Wyart et al. 2009; Zagoraiou et al. 2009), whereas the trace amine synthesized by the D cells was shown to be capable of promoting recruitment of locomotor circuits without the descending monoamines (Gozal et al. 2014). These results emphasized the importance of ventromedial interneurons for locomotor activities. They also suggested that interneurons characterized in the present study in dorsal horn and ventromedial areas around central canal could be heterogeneous population, which play different functional roles in modulating locomotion.

Our data also showed that the training effects on the interneurons were different with firing patterns. The significant changes in the membrane properties (V_{th} and R_{in}) were observed only in the type 3 interneurons, implicating that the type 3 interneurons might be more sensitive than types 1 and 2 to the treadmill training and more easily adapt to the endurance locomotor activities. Due to limit of type 1 interneurons collected in the present study, we could not verify this conclusion in the present study. As to the distribution of interneurons with cell types, our data showed that

Table 4 Training effects on membrane properties of different type neurons

	Type 1			Type 2			Type 3		
	Control (2)	Training (3)	Change	Control (10)	Training (13)	Change	Control (10)	Training (19)	Change
E_m (mV)	− 58.5 ± 12.3	− 65.1 ± 9.0	− 6.7 ± 7.6	− 62.4 ± 6.4	− 63.5 ± 9.8	− 1.1 ± 3.5	− 60.2 ± 5.4	− 62.2 ± 3.9	− 2.1 ± 1.9
I_{th} (pA)	13 ± 5.7	23.3 ± 14.4	10.3 ± 8.7	13.5 ± 6.3	10.4 ± 5.2	− 3.1 ± 2.4	8.4 ± 5.1	6.0 ± 3.5	− 2.5 ± 1.4
R_{in} (MΩ)	689 ± 53	691 ± 125	2 ± 65	1474 ± 832	1518 ± 684	45 ± 329	1149 ± 573	1647 ± 624	498 ± 187*
τ_m (ms)	48.3 ± 30.9	55.8 ± 21.6	7.5 ± 23.5	29.6 ± 11.8	34.6 ± 14.2	5.0 ± 5.6	37.0 ± 6.2	41.2 ± 21.1	4.2 ± 5.9
C_m (pF)	25.6 ± 8.5	65.7 ± 74	40.1 ± 52.5	23.9 ± 13.8	20.3 ± 6.3	− 3.7 ± 4.9	32.4 ± 13.6	21.8 ± 9.3	− 10.5 ± 5.7
V_{th} (mV)	− 30.1 ± 1.0	− 33.4 ± 3.6	− 3.3 ± 2.6	− 39.4 ± 7.0	− 37.8 ± 4.9	1.5 ± 2.6	− 38.6 ± 4.7	− 42.1 ± 3.2	− 3.5 ± 1.3*
AP height (mV)	43.7 ± 7.4	55.0 ± 15.2	11.3 ± 11.0	51.3 ± 13.3	46.4 ± 15.4	− 5.0 ± 6.0	53.9 ± 15.1	49.1 ± 10.2	− 4.8 ± 4.1
AP half width (ms)	1.8 ± 0.1	1.9 ± 0.7	0.1 ± 0.4	1.5 ± 0.5	1.8 ± 0.5	0.3 ± 0.2	1.5 ± 1.1	1.6 ± 0.5	0.03 ± 0.3
AHP depth (mV)	22.8 ± 9.6	16.5 ± 8.1	− 6.3 ± 6.9	19.8 ± 4.6	17.3 ± 5.6	− 2.5 ± 2.1	15.2 ± 9.2	16.4 ± 7.9	1.1 ± 2.7
AHP 1/2 decay (ms)	25.9 ± 6.3	31.6 ± 11.1	5.7 ± 7.7	159.3 ± 22.1	104.2 ± 88.1	− 55.2 ± 26.8	154.9 ± 76.4	155.1 ± 53	0.3 ± 33.8

*$P < 0.05$

interneurons with different firing patterns were randomly distributed in the spinal cord in both control and trained mice, similar to the reports from previous studies (Thomson et al. 1989; Szucs et al. 2003).

5-HT modulation on control and training mice

It has been shown that 5-HT plays an essential role in initiating locomotion in isolated neonatal spinal cord in rodents (Cazalets et al. 1992; Cabaj et al. 2017). Serotonergic system is highly involved in modulation of locomotor activity during treadmill training. The content of 5-HT receptor mRNA in the lumbar motoneurons of intact rats changes with different exercise regimes for 7 days and 16 weeks (Woodrow et al. 2013), and as a result of the prolonged daily treadmill training, significant decrease in mRNA content was evident for 5-HT1A receptor. In a study of spinal cord injury (SCI) in which locomotor activity was limited, SCI induced up-regulation in 5-HT receptor protein and mRNA, and these changes could be reversed by exercise (Ganzer et al. 2018).

In this study, we analyzed 5-HT modulation of spinal interneurons in dorsal and ventromedial areas around central canal. Our data demonstrated that 5-HT induced hyperpolarization of V_{th} and reduction of AHP amplitude in both control and trained mice. 5-HT-induced reduction of rheobase was observed only in control. Looking at detail of the data, we found that interneurons from trained mice appeared to be more sensitive to serotonergic modulation in terms of V_{th} hyperpolarization (trained, 6.8 ± 3.3 mV, $P < 0.05$; control, 4.2 ± 2.3 mV, Table 5). Compared with exercise-induced changes in membrane properties, 5-HT modulation of interneurons was not dependent on laminar distribution of the interneurons, suggesting that the serotonergic system should be evenly participated in modulating the interneurons in dorsal and ventromedial areas in both control and trained mice.

The exercise-induced hyperpolarization of V_{th} was accompanied by reduction of AHP, similar to the results from 5-HT experiments, where 5-HT induced the hyperpolarization of V_{th} with reduction of AHP in both control and trained mice (Fig. 5b and c; Table 5). These results suggested that the exercise-induced changes in membrane properties of spinal interneurons could be mediated through spinal modulatory systems such as serotonergic pathway. The exercise-induced changes in neuromodulator systems have been reported in previous studies. For example, the expression of the BDNF increased significantly in spinal cord of trained rats (Gomez-Pinilla et al. 2001, 2002; Joseph et al. 2012), and these neurotrophies could largely improve the neuronal plasticity in spinal cord. Based on these results, we could expect that the activity-dependent activation of serotonergic pathway and neuromodulation of ionic channels could be potential mechanisms underlying the increased excitability of spinal interneurons in 3-week treadmill trained mice (Dai et al. 2002; Gardiner et al. 2006; Dai et al. 2018). A further experiment is required to test this hypothesis.

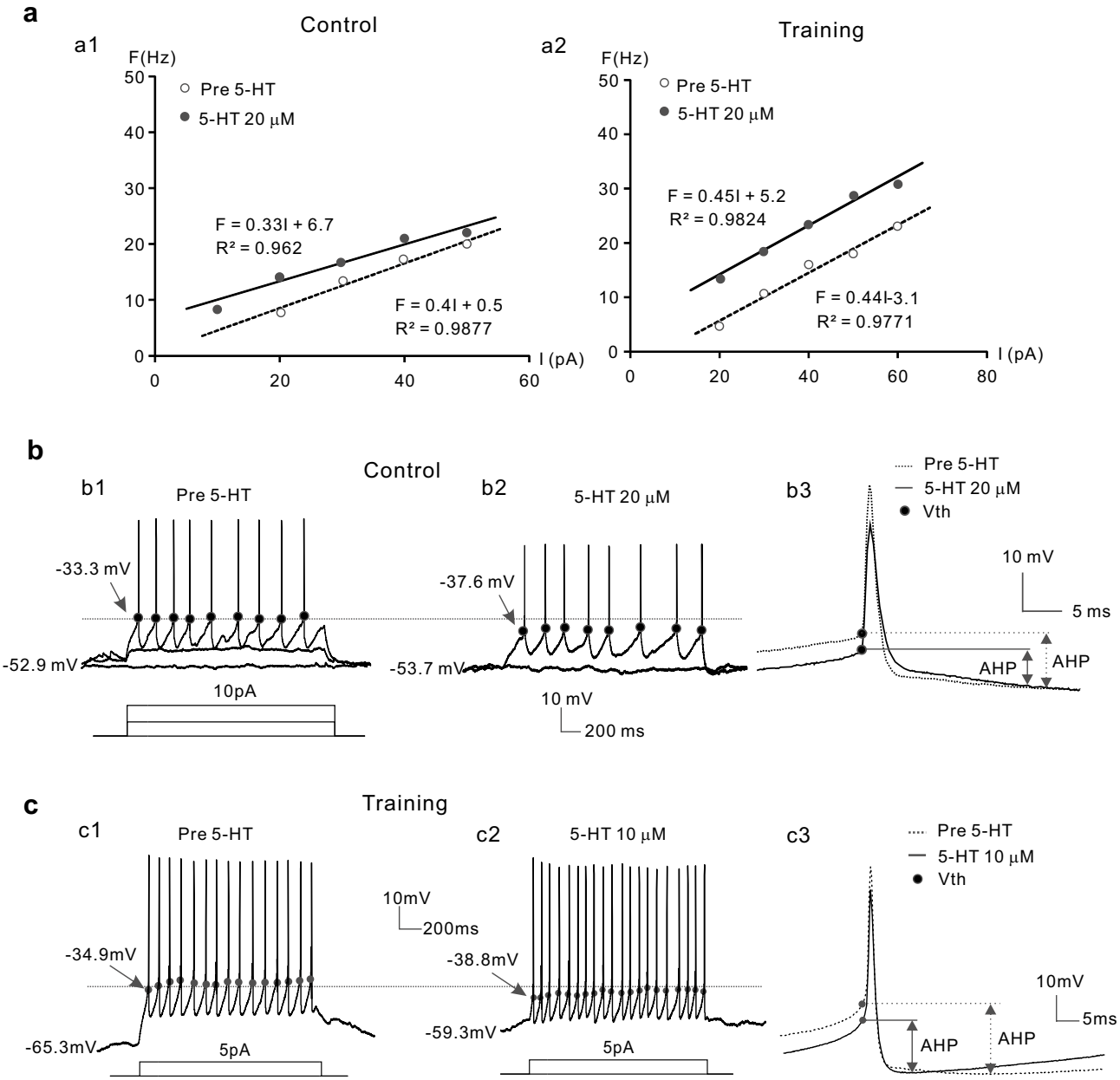

Fig. 5 5-HT modulation of membrane properties of spinal interneurons recorded from control and trained mice. a: F–I relationship of the spinal interneurons from control and trained mice were plotted for steady-state firing with bath application of 5-HT (20 μM for control and trained). The F–I curves of control (**a1**) and trained (**a2**) shifted to left with little change in slopes in both groups (open circle for pre-5HT and closed circle for 5-HT). **b** A typical example recorded from control mice. **b1** The voltage threshold (black dots) for the first action potential evoked by 1.5-s step current of 10 pA (bottom) was − 33.3 mV and the I_{th} was 10 pA in this neuron. **b2** Bath application of 20-μM 5-HT resulted in a hyperpolarization of voltage threshold of all the spikes with 5 pA lowering of step current for spike elicitation. The voltage threshold was hyperpolarized by 4.3 mV in the first spike and the resting membrane potential was hyperpolarized by 0.8 mV. **b3** The averaged spike of **b1** and **b2** were overlapped on each other. It shows that 5-HT lowered the V_{th} by 4.3 mV and reduced the AHP by 2.4 mV. **c** The similar results collected from the trained mice. **c1** A family of action potentials (top) was evoked by 1.5-s step current of 5 pA (bottom) in a spinal interneuron from trained mice. The voltage threshold (black dots) of the first spike was − 34.9 mV. **c2** bath application of 10-μM 5-HT resulted in a hyperpolarization of voltage threshold of all the spikes. More spikes were elicited with the same step current. The voltage threshold was hyperpolarized by 3.9 mV in the first spike and the resting membrane potential was depolarized by 6 mV. **c3** The averaged spike from **a1** and **a2** were overlapped on each other. It shows that 5-HT hyperpolarized the V_{th} by 3.9 mV and reduced the AHP by 3.2 mV

Table 5 5-HT modulation of membrane properties of spinal interneurons

	Control ($n=9$)			Training ($n=11$)		
	Pre 5-HT	5-HT	Change	Pre 5-HT	5-HT	Change
E_m (mV)	− 54.5 ± 3.7	− 51.9 ± 8.3	2.6 ± 7.6	− 63.2 ± 4.7	− 62.9 ± 5.2	0.3 ± 6.0
I_{th} (pA)	9.0 ± 6.5	4.0 ± 4.2	− 5.0 ± 3.5*	5.9 ± 3.0	4.5 ± 3.5	− 1.4 ± 4.5
R_{in} (MΩ)	1138 ± 656	1295 ± 784	157 ± 469	1598 ± 407	1686 ± 437	88 ± 360
V_{th} (mV)	− 36.7 ± 6.1	− 40.9 ± 6.3	− 4.2 ± 2.3*	− 38.6 ± 6.2	− 45.4 ± 6.8	− 6.8 ± 3.3**
AP height (mV)	48.6 ± 16.2	52.5 ± 16.4	3.9 ± 11.7	49.9 ± 6.4	44.5 ± 8.3	− 5.4 ± 9.3
AP width (ms)	1.5 ± 1.3	1.6 ± 1.2	0.2 ± 0.2	1.5 ± 0.5	1.6 ± 0.6	0.2 ± 0.2
AHP depth (mV)	20.1 ± 4.7	16.5 ± 4.6	− 3.6 ± 3.6*	17.7 ± 4.7	14.4 ± 6.7	− 3.3 ± 3.0*

*$P<0.05$, **$P<0.01$

Conclusion

Treadmill training changed the membrane properties of spinal interneurons. The training effects depended on the firing patterns and laminar distributions of the interneurons. 5-HT increased the excitability of the spinal interneurons in both control and trained mice. Treadmill training could enhance motor system and allow the system easier to generate locomotion.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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