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Research report

Inhibition of hyperpolarization-activated current by ZD7288 suppresses ectopic discharges of injured dorsal root ganglion neurons in a rat model of neuropathic pain

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Abstract

Peripheral nerve injury causes ectopic discharges of different firing patterns, which may play an important role in the development of neuropathic pain. The molecular mechanisms underlying the generation of ectopic discharges are still unclear. In the present study, by using in vivo teased fiber recording technique we examined the effect of ZD7288, a specific blocker of hyperpolarization-activated current (I_h), on the ectopic discharges in the dorsal root ganglion (DRG) neurons injured by spinal nerve ligation. We found that ectopic discharges of all three firing patterns (tonic, bursting and irregular) were dose- and time-dependently inhibited by local application of ZD7288. Interestingly, the extent of suppression was negatively related to frequency of firing prior to application of ZD7288. We also observed that ZD7288 could alter the firing patterns of the ectopic discharges. At 100 μM, tonic firing pattern was gradually transformed into bursting type whereas at 1 mM, it could be transformed to integer multiples firing. These results indicate that I_h might play a role in the generation of various forms of ectopic discharges in the injured DRG neurons and may thus be a possible target for neuropathic pain treatment.

Theme: Sensory system
Topic: Pain modulation: anatomy and physiology

Keywords: Neuropathic pain; Ectopic discharge; I_h; HCN channel; ZD7288; Teased fiber recording

1. Introduction

Peripheral nerve injury causes ectopic discharges originating from injured sites or dorsal root ganglion (DRG) neurons [1,13,20,26]. These ectopic discharges are widely believed to be major contributors to the development of chronic neuropathic pain following peripheral nerve injury (Refs. [20,32], but see also Refs. [10,18,38]). The mechanisms underlying ectopic discharge generation are still unclear, but changes in certain ion channels in the DRG neurons has been suggested to play a role [17]. For example, the up-regulation of $Na_v1.3$ channel in injured DRG neurons has been shown to be involved in the generation of ectopic discharges [8,16,17,37].

Interestingly, ectopic discharges from both the injured sites and the DRG neurons have strong rhythmic components. The spontaneity of different firing patterns strongly suggests that a pacemaker element underlying the generation of ectopic discharges [7]. Hyperpolarization-activated I_h current (the name of current of hyperpolarization activated, cyclic nucleotide-gated cation channels, or HCN channels, in neuron) have previously been observed in DRG neurons with patch-clamp recording and immunocytochemistry in our experiments [36] as well as in other laboratories [6,30,41]. Several characteristics of I_h, such as contributing to resting membrane potential [7,28,31] and participating in pacemaker currents [23,27,28], indicate that it may be one

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of the candidates for the ectopic discharge generation. Furthermore, ectopic discharges were found to originate mainly in medium to large-sized DRG neurons [20]. Our previous study and other investigations have demonstrated that the current amplitudes and densities of I_h in large DRG neurons are significantly larger than those in the small neurons [7,36]. Thus, the distribution of I_h in DRG neurons parallels the origin of ectopic discharges. Moreover, recent studies have reported that the amplitudes of I_h in DRG neurons were significantly increased following spinal nerve ligation [7] or chronic compression of the DRG [42], especially in the medium to large-sized neurons, implicating that I_h might contribute to the hyperexcitability of injured DRG neurons. Our recent work [35] has found that almost all ectopic discharges could be divided into three different firing patterns based on their interspike interval (ISI): tonic, bursting and irregular. In the present study, using in vivo teased fiber recording technique, we try to examine the effect of an I_h blocker, ZD7288, on the different firing patterns of ectopic discharges.

2. Materials and methods

2.1. Animals and surgery

Male Sprague–Dawley rats weighing 200–250 g were used in the present study. They were provided by the Department of Experimental Animal Sciences, Health Science Center, Peking University and were habituated for 7 days before experiments. The animals had free access to food and water during the experiments and were maintained on natural day/night cycles. All experimental protocols have been approved by the Animal Use and Care Committee of Peking University.

Ligation of the left L4 or L5 spinal nerve was performed as described by Kim and Chung [15]. Briefly, the rats were anesthetized with 10% chlorohydrate (0.3 ml/100 g body weight), and placed in a prone position. An incision was made left of the spine at the L4-S2 levels. The left L4 or L5 spinal nerves were then carefully isolated and tightly ligated with 6–0 silk suture 5–10 mm distal to the DRG, and cut approximately 2 mm distal to the suture.

2.2. Extracellular electrophysiological recording of ectopic discharges in vivo

Three to eight days after ligation and cut of L4 or L5 spinal nerve, rats were anesthetized with urethane (1.5 g/kg, i.p.). A tracheotomy was performed. ECG and heart-rate were monitored and body temperature was maintained at 36–37 °C using a feedback-controlled radiant heater. No paralytic agents were used. L4 or L5 dorsal root was exposed by a lower lumbar laminectomy and covered with warmed paraffin oil (36 °C) in a pool formed of skin flaps. The teased fiber recording method was used to evaluate the ectopic afferent discharges entering the spinal cord along the dorsal root. Most of the dorsal muscles supplied by the dorsal ramus of the L4 or L5 spinal nerve were removed during the laminectomy. The dorsal roots were carefully examined and any communicating branches between them and neighboring dorsal roots were cut to eliminate any afferent firing from normal receptive fields. Nevertheless, residual dorsal ramus fibers, identified by their receptive fields on the lower back, were occasionally encountered in the dorsal root. These were excluded from the present study.

Fine axon bundles (microfilaments) were teased from dorsal root using specially honed No. 5 jewelers forceps (Fine Science Tools, Swiss). Microfilaments, cut centrally but in continuity with the DRG distally, were separated from the dorsal root near its point of entry into the spinal cord, 25–30 mm central to the DRG. The cut end of the microfilament was placed on a platinum recording electrode referenced to a nearby indifferent electrode. Each microfilament was observed passively for ≥30 s. If during this period any spontaneous action potentials were noted, observation was extended and the frequency and patterns of firing were registered. Spike discrimination was performed by a window discriminator and controlled by means of an electronic delay unit. The numbers of spontaneously active nerve fibers in each microfilament, and their firing patterns were measured by observing the different spike heights in the ectopic discharges. Data was captured and analyzed with Micro1401 mk II and Spike 2 software (Cambridge Electronic Design, UK) as in our previous paper [40].

2.3. Application of ZD7288

ZD7288 (molecular weight 292.81, Tocris Cookson, UK) was dissolved and diluted in sterile 0.9% saline to reach desired concentrations (1–1000 μM) and administered topically to the DRG. The reagent was maintained at 36 °C before administration.

2.4. Statistical analysis

Data are expressed as mean ± S.E.M. The suppression of ectopic discharges by ZD7288 application was shown by the ratio. Ratio = frequency of firing after drug/frequency of firing before drug. Repeated measures analysis of variance (ANOVA) followed by the Dunnett's test and the Student's *t*-test were used for data analysis. *p*-value less than 0.05 was considered to be statistically significant.

3. Results

Topically applying ZD7288 to DRG neurons injured by spinal nerve ligation significantly suppressed ectopic discharges (Fig. 1). ZD7288 (1–1000 μM) produced a dose-dependent decrease of the frequency of ectopic discharges in DRG neurons. A significant suppression of

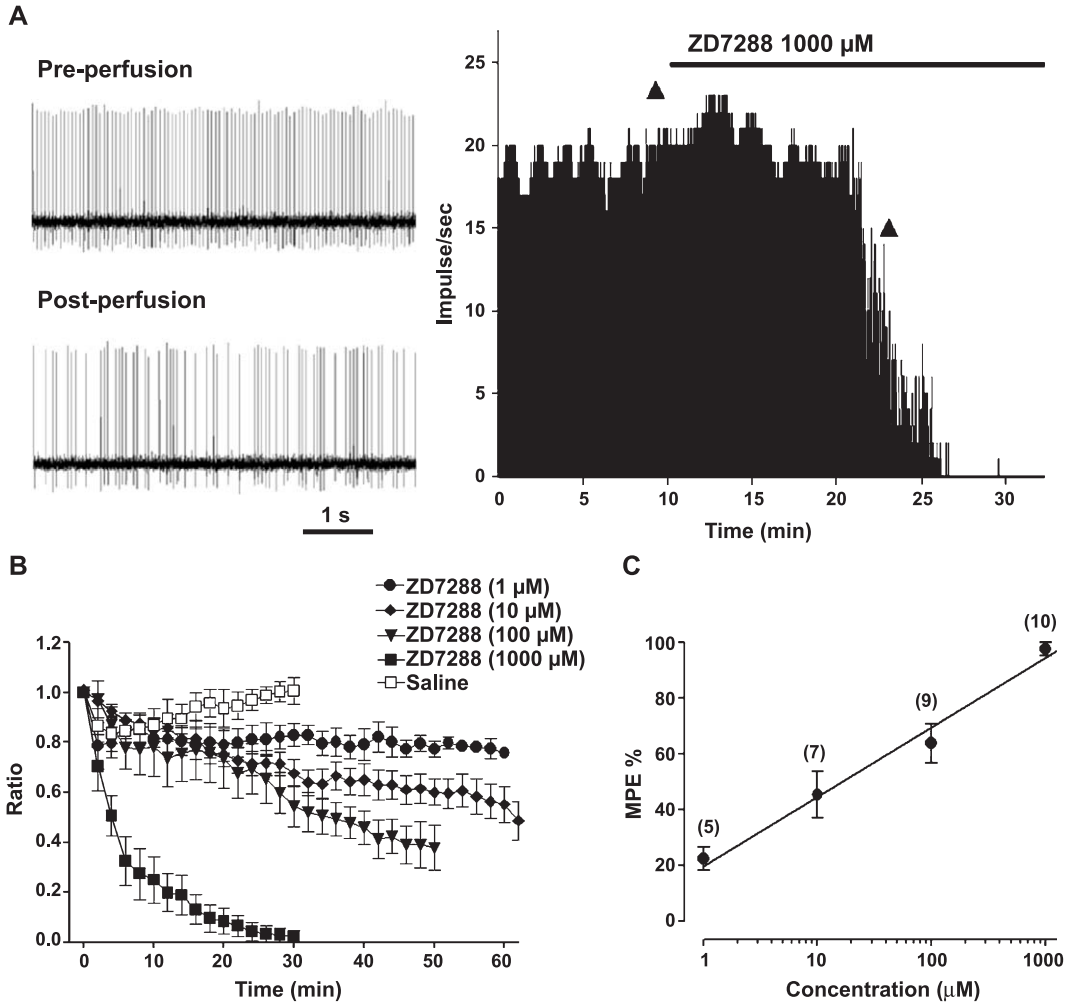

Fig. 1. Inhibition of I_h decreases the frequencies of ectopic discharges from the injured DRG neurons. (A) One example of time-histogram (*Y*-axis: impulses/s) for a single fiber recording in vivo from a fiber before and after application of 1000 μM ZD7288. The right panel illustrates an example showing gradual suppression of ectopic discharges by ZD7288. Solid triangles on the right panel indicate the sources of the left panels showing the original spike patterns before (*Pre*-) and after (*Post*-) ZD7288 application. (B) Time course of firing suppression by ZD7288 at different concentrations. When 1000 μM ZD7288 was applied, almost complete suppression of firing was observed. (C) Dose–response curves for ZD7288 in (B). *Y*-axis: percentage of maximum possible effect (MPE) (complete suppression of ectopic discharges by ZD7288 = 100%; no change from pre-ZD7288 application = 0%). The ED_{50} for firing suppression is 20 μM. The number in the parentheses beside each point represents the number of fibers examined.

firing was obtained at doses of 10 μM and above, with almost complete suppression achieved at 1000 μM (Fig. 1B). The calculated ED_{50} value for ZD7288 was 20 μM (Fig. 1C). In addition, the effect of ZD7288 on ectopic discharges is time-dependent. Longer time was needed to reach maximal effects when ZD7288 was at lower doses, whereas when at higher dose, for example 1000 μM, the maximal effect was rapidly reached. The recovery of firing after ZD7288 washout was also examined. Following administration of 1000 μM ZD7288, no recovery of ectopic discharges was observed for at least 60 min in some recordings (data not shown).

In our previous study, we found that there were three different firing patterns in the injured DRG neurons after spinal nerve ligation: tonic, bursting and irregular types [35]. In the present study, the effect of ZD7288 on different firing patterns was investigated. As shown in Fig. 2, ZD7288 inhibited all three firing patterns. Interestingly, in some cases the tonic type was transformed to bursting one after 100 μM ZD7288 administration (Fig. 2B).

We also found that the effect of ZD7288 on ectopic discharges was not equivalent in neurons with different firing frequencies (Fig. 3). The ectopic discharges with low frequencies (<7 Hz) were almost completely inhibited, while those with relatively higher frequencies (>7 Hz) were inhibited only about 50% by ZD7288 (Fig. 3B).

Another interesting finding in this study was that, in three fibers with tonic firing pattern, tonic firing was transformed to integer multiples firing before complete inhibition by local application of 1000 μM ZD7288. This means that the interspike intervals (ISIs) become integer multiples of its basic ISI. As shown in Fig. 4A, before application of ZD7288, the injured afferent fiber displayed tonic firing in which ISIs maintained at the approximately same level

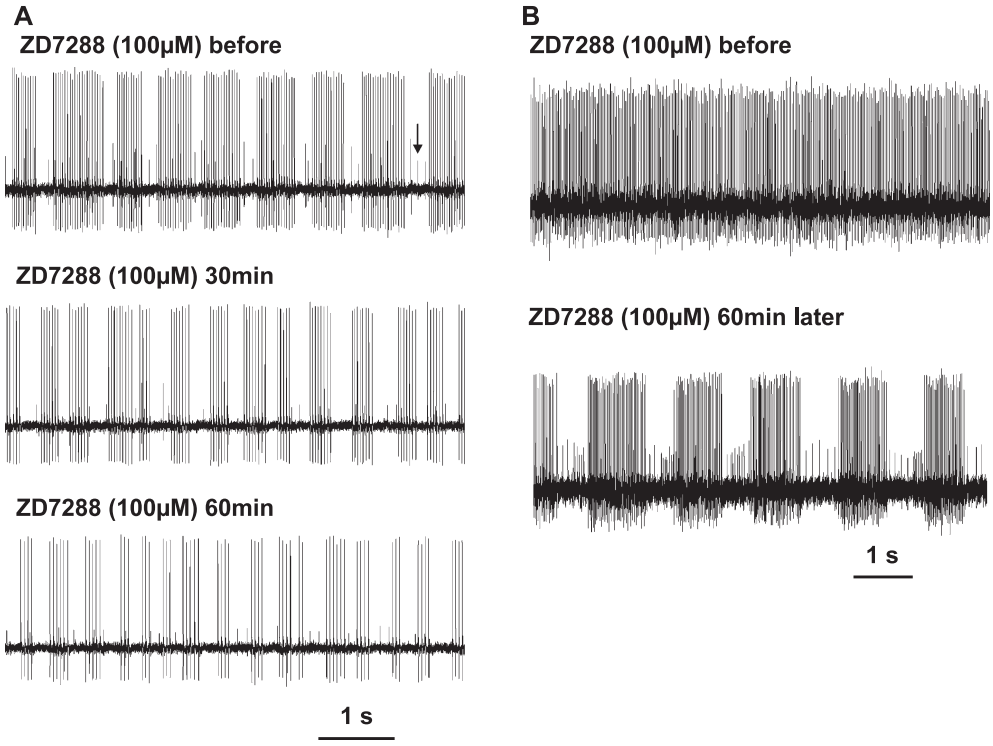

Fig. 2. Effect of ZD7288 on ectopic discharges with different firing patterns. (A) Bursting firing was partially inhibited by 100 μM ZD7288 application. Note that the arrow represents another unit with irregular firing that was completely inhibited in the same sample. (B) Tonic firing pattern was transformed to bursting by 100 μM ZD7288 application.

(around 50 ms). However, after locally applying 1000 μM ZD7288 to DRG, the ISIs changed to be integer multiples of basic interval duration until the ectopic discharges were completely inhibited (Fig. 4B). This phenomenon was not observed when ZD7288 was applied at other concentrations (1–100 μM).

4. Discussion

The major finding in this study is that inhibition of I_h current by ZD7288 suppresses the three firing patterns of ectopic discharges, and the tonic firing pattern could be transformed to bursting type by 100 μM ZD7288.

Peripheral nerve injury causes ectopic discharges in injured DRG neurons. Up to now, the molecular mechanisms underlying ectopic discharges are unclear [17]. Many previous works focused on the sodium channels. It has been repeatedly reported that the up-regulation of subtypes of sodium channels, for example $Na_v1.3$, played a key role in the generation of ectopic discharges [8,16,17,37]. This is hardly surprising since sodium channels are critical to the formation of action potentials and ectopic discharges are themselves action potentials.

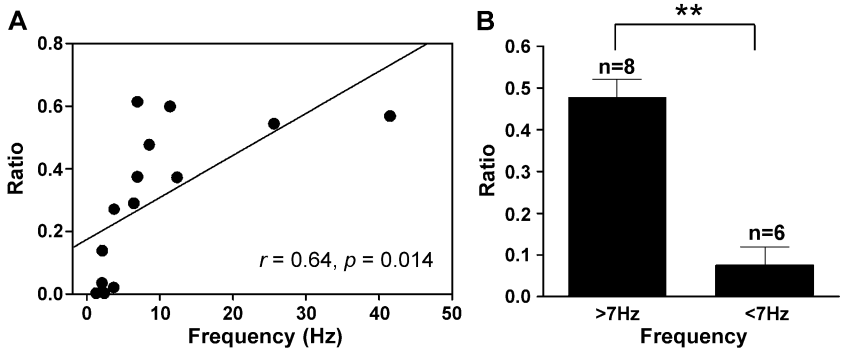

Fig. 3. Effect of ZD7288 on ectopic discharges with different firing frequency. (A) A weak negative correlation between the ratio and the firing frequency prior to 100 μM ZD7288 application. Ratio = frequency of firing after ZD7288 application/frequency before ZD7288 application. (B) Fibers sampled from (A) are arbitrarily divided into two groups (>7 and <7 Hz) according to the average frequencies before ZD7288 administration. The effect of ZD7288 was expressed as ratio. Error bars represent S.E.M. (**$p < 0.01$, unpaired t-test).

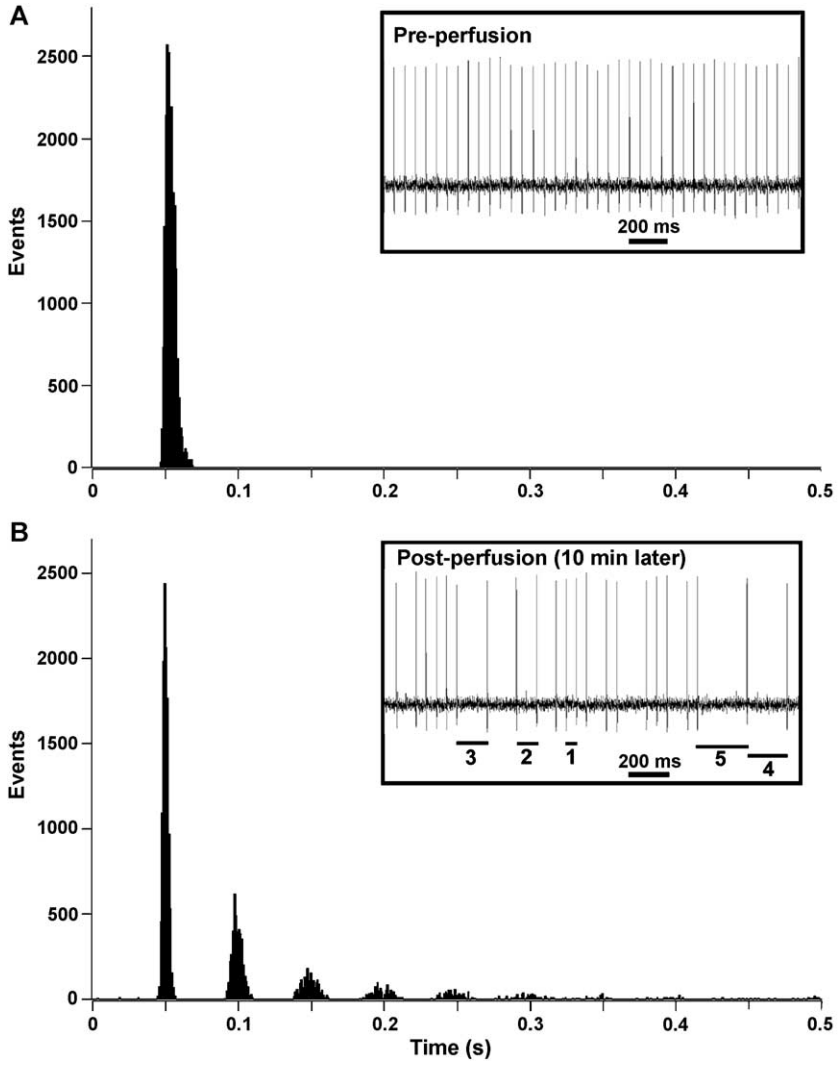

Fig. 4. ZD7288-induced integer multiple firing. (A) The interspike interval (ISI) histogram before 1000 μM ZD7288 application. Bin width is 1 ms. The distribution peak is about 50 ms before ZD7288 application. The inset shows the original recording of the tonic firing. (B) ISI histogram after 1000 μM ZD7288 application. The first distribution peak corresponds to the basic ISI (about 50 ms) before ZD7288 application and other peaks to the multiple integer multiples of basic ISI. The original recording of firing is shown in the inset. The underlined numbers in the inset represent the integer multiples of basic ISI.

One interesting question is however whether up-regulation of sodium channels alone is sufficient to induce ectopic discharges. It has been reported that I_h, also known as the "pacemaker current", contributes to the pacemaker activity in the heart [12,22,31,33,34] and the spontaneous activities of neurons in the central nervous system, for example, in cochlear pyramidal neurons and hippocampal stratum oriens-alveus interneurones [23,27]. In the present study, we examined whether I_h was also involved in the generation of ectopic discharges in the DRG neurons after peripheral nerve injury. When ZD7288, a specific blocker of the I_h current, was applied locally to the DRG in nerve-injured rats, we found that ectopic discharges were dose- and time-dependently inhibited. Evidence supporting the contribution of I_h to ectopic discharge also comes from other investigations. For example, two recent studies demonstrated that the amplitude of the I_h in the DRG neurons was significantly up-regulated after spinal nerve ligation or chronic compression of the DRG [7,42]. Interestingly, the amplitude of up-regulation in large to medium-sized DRG neurons was larger than that in small neurons [7]. These results may partially explain why ectopic discharges mainly originate from large to medium-sized myelinated neurons [20].

In our previous study, using teased fiber recording method, we recorded three different firing patterns, including tonic, bursting and irregular types, from dorsal roots connected with injured DRG [35]. We also found that the proportion of these three patterns and average frequencies of firing changed dramatically at different postoperative time points, suggesting that the excitability of the injured DRG neurons changes over time after spinal nerve ligation [35]. Previous work in vitro demonstrated that the irregular firing pattern could be inhibited by ZD7288, but the other two types have not been examined [7]. In this study, we extended these observations by examining the effect of

ZD7288 on other firing patterns. We observed that all three firing patterns could be inhibited by ZD7288. Interestingly, in some cases the tonic firing pattern was transformed to bursting by 100 μM ZD7288 application. This result implicates that the excitability of DRG neurons with bursting firing is lower than those with tonic firing as seen when the excitability of neurons was suppressed by ZD7288. Similar phenomenon has previously been observed in other structures of the central nervous system [4]. For example, previous studies on subthalamic and cerebellar Purkinje neurons revealed that I_h contributed to firing pattern transformation and when I_h was inhibited in some subthalamic neurons, the tonic pattern was transformed to bursting. At the same time, the membrane potential was hyperpolarized by application of ZD7288 [4]. The resting membrane potential in DRG neurons were also hyperpolarized about 5–10 mV by ZD7288 [7,29,30,36,41]. Thus, it is possible that the same mechanism underlying the firing pattern transformation in the DRG and subthalamic neurons.

The second interesting finding in the present study is that there is a weak negative relationship between the frequency of ectopic discharges and the ratio of suppression. The underlying mechanism of this phenomenon is unclear, but there are some possible explanations. First of all, the amplitude of I_h might be relatively high in the DRG neurons with low frequency and play a relatively more important role, so that the suppression rate is higher in these neurons. Secondly, the excitability of neurons with low frequencies may be lower than that of neurons with high frequencies and they would thus be easier suppressed by ZD7288.

The third major finding in this study is that, rather than ISI being directly lengthened by local application of 1000 μM ZD7288, the tonic firing was transformed gradually to integer multiples firing (ISI becomes integer multiples of the basic interspike interval) before they were completely suppressed. Similar phenomenon has also been reported when locally applying TTX to injured DRG neurons [39]. The mechanism of integer multiples firing generation is not well understood, although a hypothesis has been proposed in that subthreshold membrane oscillation (STMO) may account for them [39]. Interestingly, I_h in a variety of neurons in the central nervous system has been reported to contribute to STMO [3,9,12,25]. In addition, STMO of the injured DRG neurons has been shown to be necessary for the generation of bursting firing pattern of ectopic discharges after spinal nerve ligation [1,2,19], thus the role of I_h in STMO warrant further studies. I_h has been hypothesized to contribute to the subthreshold depolarization phase of the ectopic discharges [7]. Although we could not exclude this possibility, it is more likely that the membrane potential depolarized by tonically activated I_h contributes to the generation of ectopic firing. If I_h participates in ectopic discharges by contributing to the subthreshold depolarization phase, it will be expected that the ISI should directly be lengthened by ZD7288 application, rather than firing pattern transformation or integer multiple firing (Figs. 2B and 4).

Although ZD7288 was widely believed to inhibit I_h specifically [5], one recent study demonstrated that ZD7288 could also inhibit T-type calcium channels in mouse spermatogenic cells at relative high doses [11]. Moreover, it has been shown that frequency of ectopic discharges could be diminished when T-type calcium channels in DRG neurons were inhibited [21]. Therefore, we could not rule out the possibility that the effect of ZD7288 at the highest dose as employed in the present study is, at least partially, derived from its inhibition on T-type calcium channels. It needs to be noted however that T-type calcium currents were profoundly decreased after spinal nerve ligation [14,24].

In summary, we found that inhibition of I_h by ZD7288 suppresses the frequency of ectopic discharges in the injured DRG neurons. These results suggest that I_h, or HCNs, contribute to the generation of ectopic discharges following spinal nerve ligation, and provide a possible target for neuropathic pain treatment.

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