Ca$^{2+}$-activated ion currents triggered by ryanodine receptor-mediated Ca$^{2+}$ release control the firing of inhibitory neurons in the prepositus hypoglossi nucleus

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Author contribution
Y.S. designed research; Y.S. and Y.Y. performed research and analyzed data; Y.S. wrote the paper; all authors approved the final version of the manuscript.

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Abstract

Spontaneous miniature outward currents (SMOCs) are known to exist in smooth muscles and peripheral neurons, and evidence for the presence of SMOCs in central neurons has been accumulating. SMOCs in central neurons are induced through Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels, which are activated through Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from the endoplasmic reticulum via ryanodine receptors (RyRs). Previously, we found that some neurons in the prepositus hypoglossi nucleus (PHN) showed spontaneous outward currents (SOCs). In the present study, we used whole-cell recordings in slice preparations of the rat brainstem to investigate the following: 1) the ionic mechanisms of SOCs, 2) the types of neurons exhibiting frequent SOCs, and 3) the effect of Ca\textsuperscript{2+}-activated conductance on neuronal firing. Pharmacological analyses revealed that SOCs were induced via the activation of small conductance-type K\textsubscript{Ca} channels (SK channels) and RyRs, indicating that SOCs correspond to SMOCs. An analysis of the voltage responses to current pulses of the fluorescence-expressing inhibitory neurons of transgenic rats revealed that inhibitory neurons frequently exhibited SOCs. The abolition of SOCs via the blockade of SK channels enhanced the frequency of the spontaneous firing of inhibitory PHN neurons. However, the abolition of SOCs via the blockade of RyRs reduced the firing frequency and
hyperpolarized the membrane potential. Similar reductions in firing frequency and hyperpolarization were also observed when Ca\(^{2+}\)-activated nonselective cation (CAN) channels were blocked. These results suggest that, in inhibitory neurons in the PHN, Ca\(^{2+}\) release via RyRs activates SK and CAN channels, and these channels regulate spontaneous firing in a complementary manner.

**Keywords**

Ca\(^{2+}\)-activated K\(^+\) channels, Ca\(^{2+}\)-activated nonselective cation channels, neural integrator, whole-cell recording, rat
Intracellular Ca$^{2+}$ affects neuronal excitability via the regulation of Ca$^{2+}$-activated ion channels responsible for potassium, chloride, and nonselective currents that suppress or promote neuronal activity (Berridge, 1998). The supply of intracellular Ca$^{2+}$ is regulated not only through the influx of external Ca$^{2+}$ via ion channels and ionotropic neurotransmitter receptors that are permeable to Ca$^{2+}$ but also through the release of Ca$^{2+}$ from internal calcium stores in structures, such as the endoplasmic reticulum (ER), via inositol 1,4,5-tris-phosphate (IP$_3$) receptors and ryanodine receptors (RyRs) (Berridge, 1998; Verkhratsky, 2005).

In peripheral neurons and smooth muscles, the release of Ca$^{2+}$ from the ER induces Ca$^{2+}$-activated K$^+$ (K$\text{Ca}$) currents, which result in spontaneous miniature or transient outward currents (SMOCs or STOCs) (Benham and Bolton, 1986; Bolton and Imamizu, 1996; Mathers and Barker, 1984; Pérez et al. 1999; Satin and Adams, 1987).

Spontaneous hyperpolarization induced by SMOCs is thought to cause the relaxation and vasodilatation of arterial smooth muscle (Nelson et al. 1995; Porter et al. 1998).

Recent evidence has accumulated regarding SMOCs in central neurons (Arima et al. 2001; Cui et al. 2004; Klement et al. 2010; Mitra and Slaughter, 2002; Shirasaki et al. 2001). SMOCs are typically induced through small conductance-type K$\text{Ca}$ channels
(SK channels) in central neurons (Arima et al. 2001; Cui et al. 2004; Klement et al. 2010), although large conductance-type $K_{Ca}$ channels (BK channels) produce SMOCs in peripheral neurons (Merriam et al. 1999), smooth muscles (Satin and Adams, 1987), and some central neurons (Mitra and Slaughter, 2002; Shirasaki et al. 2001). $K_{Ca}$ channels are activated through $Ca^{2+}$-induced $Ca^{2+}$ release from the ER via RyRs (Arima et al. 2001; Cui et al. 2004; Klement et al. 2010; Mitra and Slaughter, 2002; Shirasaki et al. 2001). Although the role of SMOCs in central neurons is poorly understood, previous research has found that a pharmacological blockade of ryanodine receptors normalizes irregular firing patterns and increases the firing frequency (Cui et al. 2004; Klement et al. 2010), suggesting that SMOCs might influence neuronal firing.

The prepositus hypoglossi nucleus (PHN) is a brainstem structure involved in maintaining horizontal gaze (Fukushima and Kaneko, 1995; Fukushima et al. 1992; McCrea and Horn, 2006; Moschovakis, 1997; Robinson, 1975, 1989). In our previous study (Saito and Yanagawa, 2010), we found that some PHN neurons exhibited spontaneous outward currents (SOCs) when the recordings were performed using a potassium-based intracellular solution. Although the SOCs appeared to be similar to SMOCs, we did not verify whether the SOCs observed in the PHN neurons corresponded to SMOCs. In the present study, we investigated SOC induction
mechanisms to confirm the correspondence between SOCs and SMOCs. Because SOC
s were not observed in all PHN neurons tested, we examined the relationship between intrinsic properties and the frequency of SOCs to clarify which types of PHN neurons exhibited frequent SOCs. Furthermore, we investigated the contribution of Ca$^{2+}$-activated ion channels triggered through Ca$^{2+}$ release via RyRs to spontaneous PHN neuron firing.
Materials and Methods

All experimental procedures were approved by the Animal Care and Experimentation Committee of Gunma University (approval number: 10-003). Every effort was made to minimize the number of animals used and their suffering.

Animals

The data were obtained from wild-type Wistar rats and vesicular GABA transporter (VGAT)-Venus transgenic rats (Uematsu et al. 2008). In VGAT-Venus rats, inhibitory neurons express Venus, a fluorescent protein brighter than enhanced GFP (Nagai et al. 2002) that makes inhibitory neurons easily identifiable with fluorescence microscopy (see Fig. 7A) (Fujiwara-Tsukamoto et al. 2010; Koyanagi et al. 2010; Shino et al. 2011; Uematsu et al. 2008). Our recent study showed that GABAergic neurons, glycinerigic neurons, and neurons with both GABA and glycine expressed Venus (Shino et al. 2011). Young wild-type and VGAT-Venus rats [aged 16–21 postnatal days (PND)] and older wild-type rats (aged 6 weeks, body weight = 160-170 g) were used in the present study. We first confirmed the expression of Venus of PHN neurons in perfusion-fixed slices by using a fluorescent microscope (Axioplan 2, Zeiss, Tokyo, Japan). The animal (PND 21) was transcardially perfused with 0.05 M phosphate
buffer (PB, pH 7.4) followed by 4% paraformaldehyde in PB under deep anesthesia with inhalation of isoflurane followed by intraperitoneal injection of sodium pentobarbital (>50 mg/kg). Frontal slices (50 μm) were coverslipped with an antifade reagent (ProLong, Invitrogen).

**Slice preparation and whole cell patch clamp recordings**

The procedures for slice preparation and whole-cell patch clamp recordings were similar to those described previously (Shino et al. 2008, 2011). Animals were deeply anesthetized by inhalation of isoflurane (dose adequacy was judged by the absence of reflexive movements in response to toe pinches) and decapitated with scissors (young animals) or a guillotine (older animals). The brain was quickly removed, and frontal slices of the brainstem (250 μm in thickness) were cut with a Microslicer (Pro 7, Dosaka EM, Kyoto, Japan) in an oxygenated ice-cold sucrose solution containing (in mM) 234 sucrose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 10 MgSO$_4$, 0.5 CaCl$_2$, 26 NaHCO$_3$, and 11 glucose and were subsequently incubated in an oxygenated extracellular solution containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, and 25 glucose. The slices containing the rostral and caudal edge of the PHN were discarded because it was difficult to determine the boundary of the PHN there. The
other slices containing the PHN were incubated in oxygenated extracellular solution for more than 1 hour at room temperature. To minimize the number of animal necessary, the frontal slice was further separated into right and left halves at the midline prior to recording. Each half-slice was placed in a submersion-type recording chamber on an upright microscope (Leica DM LFS, Leica, Wetzlar, Germany) and was continuously superfused with the extracellular solution at a rate of 5 ml/min. The bath solution temperature was maintained at 30-32 °C using an in-line heater (SH-27A, Warner Instruments, Hamden, CT). The PHN was defined using the rat brain atlas (Paxinos and Watson, 2007). When the recordings were performed using Venus-expressing neurons, the neurons were first identified under epifluorescence optics; subsequently, whole-cell recordings were performed under observation with Nomarski optics. Neurons were sampled throughout the PHN, but we did not record the location of each neuron. Patch pipettes were prepared from borosilicate glass capillaries and filled with a K+-based internal solution containing (in mM) 120 K-methylsulfate, 20 KCl, 0.2 EGTA, 2 MgATP, 0.3 NaGTP, 10 HEPES, and 0.1 spermine pH-adjusted to 7.3 with KOH. When we recorded spontaneous firing, an intracellular solution containing (in mM) 10 KCl and 10 Na2-phosphocreatine instead of 20 KCl was used. In some experiments, we used a Cs+-based internal solution containing (in mM) 145
Cs-gluconate, 5 CsCl, 0.2 EGTA, 2 Mg-ATP, 0.3 Na-GTP, 10 HEPES, 0.1 spermine, and
5 lidocaine N-ethyl bromide (QX-314), pH 7.3. The osmolarity of the internal
solutions was 280-290 mOsm/l, and the resistance of the electrodes was 3-7 MΩ in the
bath solution. The measured liquid junction potential (K⁺-based internal solution = -5
mV, Cs⁺-based internal solution = -10 mV) was corrected. Whole-cell voltage-clamp
and current-clamp recordings were performed using an EPC-8 patch clamp amplifier
(HEKA, Darmstadt, Germany). Neurons displaying a membrane potential below -50
mV immediately after patch membrane rupture were used for further analyses. When
current recordings were performed, the holding potential was usually -75 mV. When
voltage responses to current pulses were investigated, depolarizing current pulses (400
ms in duration) were applied to the neurons at a membrane potential of -85 to -75 mV
that was maintained by the injection of constant current. Voltage and current signals
were low-pass filtered at 1-3 kHz and digitized at 5-10 kHz. Data were acquired using
a pClamp9 system (Molecular Devices, Foster City, CA). Off-line analysis was
performed with Axograph software (Molecular Devices).

Drugs
Strychnine hydrochloride, mecamylamine, atropine sulfate, apamin, nifedipine, heparin, 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid (BAPTA), and flufenamic acid (FFA) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). D-(-)-2-amino-5-phosphonopentanoic acid (APV), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX), CGP52432, (RS)-α-methyl-4-carboxyphenylglycine (MCPG), iberiotoxin, and SKF 96365 were purchased from Tocris Bioscience (Bristol, UK). ω-conotoxin GVIA, ω-agatoxin IVA, and SNX-482 were purchased from Peptide Institute (Osaka, Japan). Aminophylline dihydrate was purchased from LKT laboratories (St. Paul, MN, USA). Other drugs including picrotoxin, tetrodotoxin (TTX), ryanodine, caffeine monohydrate, and xestospongin C were purchased from Wako Pure Chemical Industries (Osaka, Japan). Picrotoxin, MCPG, and caffeine were dissolved directly in the solutions. Ryanodine, nifedipine, xestospongin C, and FFA stocks were prepared in dimethyl sulfoxide (DMSO) (2,000 times the final concentration). Other drugs were dissolved in water (1,000 times the final concentration) and stored as stock solutions at −20 °C before being diluted in the oxygenated solutions. Peptidic calcium channel blockers were applied in the presence of cytochrome c (0.1 mg/ml) to prevent non-specific peptide binding to the tubing.
Data analysis

For the analysis of SOCs, current traces were filtered up to 10 Hz to minimize noise. The presence of an SOC was defined by an outward current peak that was more than three times the standard deviation (SD) of the baseline level. SOC frequency was analyzed using 20- to 40-s segments of the current recordings and defined as the total number of events divided by the recording time. SOC amplitude was determined as the peak amplitude of SOC that was measured from the raw data (8-10 s current traces in each neuron). Comparisons of SOC frequencies were performed between the control solution and a solution containing the drug tested. In the case of the intracellular application of the drug, the comparisons were performed between at 1 min and 3 min after the patch membrane rupture. The rate of spontaneous firing before and after drug application was defined as the number of spikes over 20 s preceding and the 4-5 min following the drug application, respectively. The instantaneous firing frequency was calculated as the reciprocal of the interspike interval between successive pairs of spikes, and the ten highest instantaneous frequencies from each recording were averaged to obtain the maximal firing frequency. Firing patterns were analyzed from spike trains containing 5-10 action potentials, or 1-3 action potentials for a firing pattern.
with low threshold spike and low firing late (see below). For analysis of
afterhyperpolarization (AHP) profiles, the current pulses were adjusted to induce one
action potential during 400 ms, and 2-3 action potentials were recorded from each
neuron. The amplitude of AHP was estimated as the difference between the most
negative membrane potential of the AHP and the action potential threshold. The action
potential threshold was defined the membrane potential where the derivative of the
voltage trace reached 10 V/s (Bagnall et al. 2007). All values are shown as the mean ±
SD, and the error bars within the figures also represent the SD. The number (n)
described in the text refers to the number of neurons analyzed, unless otherwise noted.
Because the distribution of SOC frequency and SOC amplitude (Fig. 1B) did not follow
a normal distribution (p< 0.0001, the Shapiro-Wilk test), the statistical analysis of SOC
was performed using the nonparametric test (the Mann-Whitney test for unpaired data
and the Wilcoxon signed-rank test or Friedman test followed by the Wilcoxon test for
paired data). As for the spontaneous firing, the data followed normal distribution, and
thus we used Student’s paired t-test for the statistical analysis. The analyses were
performed using StatView or JMP software (Hulinks, Tokyo, Japan). The threshold for
statistical significance was set at p < 0.05 for all statistical tests.
Results

Characterization of SOCs

As shown in Figure 1A, SOCs displaying relatively slow kinetics (inset) were observed at a membrane potential of -75 mV in some PHN neurons in young wild-type rats. The analysis of 98 SOCs revealed that the rise time from 10% to 90% of the peak amplitude was 8.2 ± 2.3 ms (range, 3.9-13.7 ms) and the decay time constant was 24.4 ± 11.3 ms (range, 9.6-57.2 ms). In current clamp mode, the neurons showed spontaneous hyperpolarizations of the membrane potential (Fig. 1A2). Figure 1B1 shows a histogram illustrating the distribution of SOC frequencies in the recorded PHN neurons. SOCs were observed in 48 of the 78 PHN neurons recorded (open bars in Fig. 1B1) and were not detected in the remaining 30 neurons (filled bar in Fig. 1B1). Although the average frequency of SOCs was 1.29 ± 1.13 event/s (n = 48), more than half of the neurons exhibiting SOCs (26/48) showed a frequency below 1 event/s. The histogram in Figure 1B2 shows the distribution of the amplitudes of SOCs (331 events obtained from 48 neurons). The SOC amplitudes varied across events, and the average amplitude was 13.7 ± 6.0 pA. Figure 1C shows current traces recorded at different holding potentials. The frequency of SOCs appeared to be higher when the membrane potential was depolarized. SOCs generally disappeared at a holding potential of -105
mV (Fig. 1C; the reversal potential of potassium in this study was approximately -100 mV). In our previous study (Saito and Yanagawa 2010), SOCs were not observed when recordings were performed with intracellular solutions containing cesium. Altogether, these findings indicate that potassium channels mediate SOCs. The SOC frequency in the presence of 0.25 μM TTX was not significantly different from that in the control condition (95.8 ± 18.8% of the control, n = 7, p = 0.60) (Fig. 1D1 and 2). Moreover, the application of antagonists of AMPA-type glutamate receptors (20 μM NBQX, 104.4 ± 10.8%, n = 7, p = 0.50), NMDA-type glutamate receptors (50 μM APV, 102.2 ± 6.9%, n = 6, p = 0.91), group I/II metabotropic glutamate receptors [1 mM (s)-MCPG, 99.3 ± 11.4%, n = 8, p = 0.57], GABA_A and glycine receptors (100 μM picrotoxin & 20 μM strychnine, 99.3 ± 4.9%, n = 7, p = 0.67), GABA_B receptors (6 μM CGP52432, 97.7 ± 19.5%, n = 7, p = 0.87), nicotinic acetylcholine receptors (10 μM mecamylamine, 96.6 ± 15.1%, n = 6, p = 0.69), or muscarinic acetylcholine receptors (1 μM atropine, 100.1 ± 18.0%, n = 8, p > 0.99) (Fig. 1D2) did not significantly affect the SOC frequency. These results indicate that SOCs cannot be attributed to synaptic transmission.

In rat midbrain dopaminergic neurons, SMOC-induced spontaneous miniature hyperpolarizations were observed in preparations obtained from neonatal animals (9-16
days after birth) but not in those obtained from adults (Seutin et al. 1998, 2000). However, SMOCs were observed in medial preoptic neurons that were prepared from older rats (20-35 days after birth) (Klement et al. 2010). Therefore, to verify whether SOCs in the PHN can be observed in older rats, we performed current recordings using PHN neurons from 6-week-old rats. In the 34 PHN neurons recorded, 25 neurons showed SOCs with a frequency of $0.74 \pm 0.63$ events/s and an amplitude of $14.6 \pm 5.6 \ pA$ ($n = 178$ events tested), and 9 did not exhibit SOCs. This result indicates that SOCs in PHN neurons are not limited to neonatal animals.

If the SOCs observed in PHN neurons correspond to SMOCs, SOCs should be induced through the same ionic mechanisms that induce SMOCs. We first investigated the contribution of $K_{Ca}$ channels to SOCs. Figure 2A shows spontaneous currents of a PHN neuron before (1) and after (2) the application of 0.1 $\mu$M apamin, a blocker of SK channels. The application of apamin abolished most of the SOCs, whereas the inward synaptic currents appeared to be unchanged. The SOC frequency in the presence of apamin ($0.12 \pm 0.13$ events/s) was significantly lower than that in the control condition ($1.76 \pm 1.02$ events/s, $n = 8$, $p = 0.012$) (Fig. 2A3). However, the application of iberiotoxin (0.1 $\mu$M), a blocker of BK channels, did not affect most SOCs (Fig. 2B1, 2). The SOC frequency in the presence of iberiotoxin ($1.65 \pm 0.98$ events/s) was not
significantly different from the control (1.68 ± 0.98 events/s, n = 8, p = 0.89) (Fig. 2B3).

The average amplitude of the SOCs in the presence of iberiotoxin (11.8 ± 2.0 pA) was also not significantly different from the average amplitude of the SOCs observed in the control (12.0 ± 2.7 pA, n = 8 cells, p = 0.48). This result confirms that SOCs are dependent on the activation of SK, but not BK, channels.

Because \( K_{Ca} \) channels are activated through increases in intracellular \( Ca^{2+} \), we investigated whether SOCs were detected when the intracellular \( Ca^{2+} \) was chelated using BAPTA. Figure 2C shows the current recording of a PHN neuron using an intracellular solution containing 10 mM BAPTA. In this neuron, SOCs were detected immediately after the start of the recording (approximately 30 s after the patch membrane rupture, Fig. 2Ca) but disappeared quickly (Fig. 2Cb). In the 12 PHN neurons tested, SOCs were not detected in 9 neurons and disappeared quickly in 3 neurons. This result supports the dependence of SOCs on intracellular \( Ca^{2+} \).

Notably, the baseline currents shifted gradually to the outward direction (Fig. 2C). This outward shift of the baseline currents was observed in all PHN neurons tested (n = 12) and was possibly caused by the block of another \( Ca^{2+} \)-activated current, as shown later.

We next investigated the contribution of RyRs to SOCs. RyR channels open
more frequently after the application of low concentrations of ryanodine, but they are blocked in the presence of high concentrations (several tens of μM) of ryanodine (Buck et al. 1992; Lai et al. 1989; Nagasaki and Fleischer, 1988). Figures 3A1 and 2 show the effects of the extracellular application of low (1) and high concentrations (2) of ryanodine on SOCs. Consistent with previous findings, the low concentration of ryanodine enhanced SOC frequency, whereas the high concentration of ryanodine abolished SOCs (Fig. 3C, open bars). When low (0.01-0.05 μM, Fig. 3B1) and high (10 μM, Fig. 3B2) concentrations of ryanodine were applied intracellularly to specify the effects of ryanodine within the cell, similar results were observed (Fig. 3C, filled bars). The baseline currents shifted gradually to the outward direction in all neurons tested (n = 9) when the recordings were performed with intracellular solutions containing 10 μM ryanodine. When a low concentration of ryanodine was applied intracellularly, the baseline currents shifted to either the outward or inward direction in some neurons (n = 5) and remained stable in other neurons (n = 2). Similarly, in 6-week-old rats, the SOC frequency in the presence of 20 μM ryanodine (0.10 ± 0.11 events/s) was significantly lower than that in the control condition (1.40 ± 0.54 events/s, n = 5, p = 0.043) (Fig. 3D).

To confirm that the SOCs are not affected through the activation of IP₃
receptors, similar to most SMOCs in central neurons, we investigated the effects of heparin and xestospongin C, which are used to block IP$_3$ receptors (Gafni et al. 1997). Although xestospongin C is permeable to the membrane, heparin is impermeable. Thus, heparin was dissolved in the intracellular solution and applied to recorded neurons intracellularly. The SOC frequency was not significantly reduced after the extracellular application of 1 μM xestospongin C (103.7 ± 7.9%, n = 7, p = 0.75) or the intracellular application of heparin (1 mg/ml) (102.3 ± 13.7%, n = 8, p = 0.80) (Fig. 3E). Previous studies have shown that SMOCs in central and peripheral neurons and smooth muscle cells are enhanced after the application of caffeine (Arima et al. 2001; Klement et al. 2010). Therefore, we investigated the effect of caffeine on SOCs. Figure 4A shows the frequency of SOCs before (1) and 2 min after the application of 3 mM caffeine (2). The SOC frequency in the presence of caffeine (2.72 ± 0.85 events/s) was significantly higher than that in the control condition (1.32 ± 0.31 events/s, n = 9, p = 0.0076) (Fig. 4A3). Although this effect of caffeine on SOC frequency may reflect the potentiation of Ca$^{2+}$ release from the ER through the activation of RyRs (McPherson et al. 1991; Miller, 1991), caffeine has several biological effects such as antagonism of adenosine receptors, inhibition of phosphodiesterases, and depression of the activity of GABA$_A$ receptors (Ribeiro and Sebastião, 2010; Yoshimura, 2005).
Because picrotoxin did not affect SOCs (Fig. 1D2), the enhancement of SOCs through caffeine is not associated with the effect on GABA_A receptors. Aminophylline, a complex of theophylline and ethylenediamine, acts not only as a nonselective phosphodiesterase enzyme (PDE) inhibitor but also as a nonselective adenosine receptor (AR) antagonist (Nakamizo et al. 2003; Soares et al. 2009). Therefore, we characterized the effect of aminophylline on SOCs to determine whether PDEs and ARs affect SOCs (Fig. 4B). Unlike the effects observed with caffeine (Fig. 4B3), SOCs did not appear to be affected by the application of 200 μM aminophylline (Fig. 4B2). The SOC frequency in the presence of 200 μM aminophylline (1.46 ± 1.1 events/s) was not different from that in the control (1.45 ± 1.1 events/s, p = 0.83) but was significantly different from that in the presence of caffeine (3.23 ± 1.5 events/s, p = 0.008) (Fig. 4B4). This result indicates that the enhancement of SOCs through caffeine reflects an increase in Ca^{2+} release due to the activation of RyRs.

In some peripheral and central neurons, Ca^{2+} influx through voltage-gated Ca^{2+} channels triggers Ca^{2+} release from the ER via RyRs and the induction of SMOCs (Cui et al. 2004; Merriam et al. 1999; Mitra and Slaughter, 2002). In this study, we observed SOCs at the membrane potential of -75 mV, at which hardly any voltage-gated Ca^{2+} channels are activated. Although this finding suggests that voltage-gated Ca^{2+}
channels hardly contribute to the induction of SOCs, we cannot rule out the possibility that voltage-gated Ca\(^{2+}\) channels on an insufficiently voltage-clamped membrane might trigger the induction of SOCs. Therefore, we investigated the effects of voltage-gated Ca\(^{2+}\) channels blockers on SOC frequency. Figure 5A shows the current traces in the control (1) and in the presence of 50 \(\mu\)M Ni\(^{2+}\) (2), a preferential blocker of T-type and R-type Ca\(^{2+}\) channels (Perez-Reyes, 2003). A comparison of the SOC frequencies before and after the application of Ni\(^{2+}\) revealed that SOC frequency significantly decreased (Fig. 5B, 73.9 ± 17.3%, \(n = 9\), \(p = 0.012\)). However, SOCs were not completely abolished through the application of Ni\(^{2+}\), and in some neurons, the SOCs were hardly affected (Fig. 5B). In addition, the SOC frequency was not significantly decreased after the application of other types of Ca\(^{2+}\) channel blockers (Fig. 5B), namely 20 \(\mu\)M nifedipine (L-type, 94.9 ± 22.0%, \(n = 9\), \(p = 0.26\)), 1 \(\mu\)M \(\omega\)-conotoxin GVIA (N-type, 85.9 ± 25.0%, \(n = 8\), \(p = 0.33\)), 20 \(\mu\)M \(\omega\)-agatoxin VIA (P/Q-type, 87.6 ± 20.3%, \(n = 9\), \(p = 0.26\)), and 200 nM SNX-482 (R-type, 108.1 ± 13.7%, \(n = 8\), \(p = 0.40\)). These results suggest that most voltage-gated Ca\(^{2+}\) channels are not involved in the induction of SOCs in PHN neurons.

Electrophysiological properties of PHN neurons exhibiting frequent SOCs
SOCs were not observed in all PHN neurons, and SOC frequency varied widely among the PHN neurons exhibiting SOCs (Fig. 1B1). These findings raise the question of which types of PHN neurons frequently exhibit SOCs. Because PHN neurons are classified into heterogeneous subtypes based on different intrinsic electrophysiological properties (Bobker and Williams 1990; Idoux et al. 2006; Shino et al. 2008; Kolkman et al. 2011; for review, see Eugène et al. 2011), it may be possible that SOC frequencies are different among neuronal types with different intrinsic properties. We therefore investigated the relationship between SOC frequencies and the intrinsic properties of PHN neurons. Our classification of PHN neurons was based on their AHP profiles and firing patterns (Shino et al. 2008; 2011), as shown in Figures 6A and B. The AHP profiles are classified into three types: 1) AHP with a slow component and an afterdepolarization (ADP, arrow) [AHP(s+) with ADP]; 2) AHP with a slow component [AHP(s+)]; and 3) AHP without a slow component [AHP(s-)], whereas the firing patterns are classified into six types: 1) a repetitive firing pattern with relatively constant interspike intervals (continuous spiking, Cont); 2) a firing pattern with a delay in the generation of the first spike due to transient hyperpolarization following the onset of the depolarizing pulse (late spiking, Late); 3) a firing pattern exhibiting a cluster of two or more spikes due to a Low-Threshold calcium Spike (LTS); 4) a firing pattern
exhibiting a first interspike interval longer than the second interspike interval (First Interspike interval Long; FIL); 5) a firing pattern exhibiting few spikes during the 400 ms current injection despite sufficient membrane depolarization (Low Firing Rate; LFR); and 6) an oscillatory firing pattern (Osc). Figure 6C shows plots of SOC frequency in PHN neurons recorded from young rats separated according to AHP profiles (1) or firing patterns (2). SOCs with frequencies higher than 1 event/s were detected in all AHP types. The proportion of AHP(s-) neurons showing SOCs (5 of 6 neurons tested) was higher than those of AHP(s+) with ADP (24/46) and AHP(s+) neurons (19/26). SOCs were detected in all firing types of PHN neurons except LTS neurons (0/7). All neurons exhibiting FIL (n = 14) and oscillatory firing (n = 8) exhibited SOCs, and more than half of the neurons exhibited a SOC frequency higher than 1 event/s (FIL: 1.40 ± 0.95 events/s, oscillatory: 2.25 ± 1.38 events/s). Although most LFR neurons (5/6) exhibited SOCs, their SOC frequency was lower than 1 event/s (0.32 ± 0.16 event/s, n = 5, except for a LFR neuron that did not exhibit SOCs). More than half of the late-spiking neurons (14/22) exhibited SOCs. Of the 14 late-spiking neurons exhibiting SOCs, 8 exhibited SOCs more frequently than 1 event/s (1.40 ± 1.15 event/s, n = 14). The fraction of continuous-spiking neurons exhibiting SOCs was small (7/21). Only one continuous-spiking neuron exhibited a frequency higher than 1
event/s, and the average SOC frequency, except for 14 continuous-spiking neurons that did not exhibit SOCs, was small (0.47 ± 0.39 event/s, n = 7). These results indicate that the SOC frequency is high in late-spiking, FIL, and oscillatory firing neurons. Although some continuous-spiking neurons and most LFR neurons also exhibited SOCs, their SOCs were low in frequency. Similar relationships between SOC frequency and intrinsic properties were observed in PHN neurons recorded from 6-week-old rats (Fig. 6D). The proportions of AHP(s-) neurons showing SOCs were high (Fig. 6D1), and the SOC frequency was high in late-spiking and FIL neurons (Fig. 6D2). Notably, in the present study, we did not observe any PHN neurons exhibiting oscillatory firing in the older rats. Unlike the guinea pig PHN (more than 20%, Idoux et al. 2006), neurons exhibiting oscillatory firing were not frequently observed in the PHN of young rats (fewer than 5%, Shino et al, 2008); thus, it might be difficult to observed these neurons in adult preparations. Alternatively, because neurons exhibiting oscillatory firing are found in the rostromedial regions of the guinea pig PHN (Idoux et al. 2006), more neurons might have been recorded if we searched at the locations intensely. Taken together, the results obtained from both young rats and 6-week-old rats indicate that the preferential properties of PHN neurons exhibiting frequent SOCs are AHP(s-), late spiking, FIL, and oscillatory firing.
Our previous studies have shown that these intrinsic properties described above are the preferential properties of inhibitory neurons in the PHN (Shino et al. 2008, 2011). To verify whether inhibitory PHN neurons exhibit frequent SOCs, we analyzed the SOC frequency in neurons from VGAT-Venus transgenic rats (Fig. 7). The result of our previous study using VGAT-Venus transgenic rats demonstrated that all PHN neurons expressing Venus also expressed VGAT, although Venus was not detected in approximately 10% of PHN neurons expressing VGAT (Shino et al. 2011). We performed recordings from 45 Venus-expressing [Venus(+)] and 45 non-Venus-expressing [Venus(-)] PHN neurons (Fig. 7A). More than 85% of the Venus(+) neurons tested (39/45) exhibited SOCs, whereas 56% of the Venus(-) neurons tested (25/45) exhibited SOCs. The SOC frequency in Venus(+) neurons (1.67 ± 1.32 events/s, n = 45) was significantly higher than that in Venus(-) neurons (0.48 ± 0.71 events/s, n = 45, p < 0.0001) (Fig. 7B). When this comparison was limited to neurons exhibiting SOCs, the SOC frequency in the Venus(+) neurons (1.93 ± 1.23 events/s, n = 39) was significantly higher than that in the Venus(-) neurons (0.87 ± 0.76 events/s, n = 25, p = 0.0006). To clarify the neuronal distributions of the recorded Venus(+) and Venus(-) PHN neurons, we analyzed their AHP profiles and firing patterns (Fig. 7C, D). AHP(s-) was only observed in Venus(+) neurons, and the proportion of AHP(s+) with
ADP and AHP(s+) neurons showing SOCs were higher in the Venus(+) neurons than in the Venus(-) neurons (Fig. 7C1, D1). The SOC frequency of Venus(+) neurons exhibiting AHP(s+) with ADP (2.37 ± 1.31 events/s, n = 20) was significantly higher than that of Venus(-) neurons exhibiting the same AHP profile (1.07 ± 0.78 events/s, n = 19, p = 0.0016). Similarly, in neurons exhibiting AHP(s+), the SOC frequency of the Venus(+) neurons (1.37 ± 1.06 events/s, n = 11) was significantly higher than that of the Venus(-) neurons (0.25 ± 0.15 events/s, n = 6, p = 0.0089). The Venus(+) and Venus(-) PHN neurons did not show oscillatory firing and LFR (Fig. 7C2, D2), respectively, consistent with the results of our previous study (Shino et al. 2011). A majority of Venus(+) neurons were late-spiking neurons that exhibited frequent SOCs, whereas most Venus(-) neurons were continuous-spiking neurons that did not exhibit frequent SOCs. In late-spiking and FIL neurons, the proportion of neurons that exhibited SOCs was similar in Venus(+) and Venus(-) neurons. Taken together, these results indicate that inhibitory neurons in the PHN tend to exhibit frequent SOCs. This tendency might reflect the inhibitory neuronal populations comprising neurons that exhibit the preferential properties, such as AHP(s-), late-spiking, FIL and oscillating firing.

**Contribution of SOCs to spontaneous firing**
Most PHN neurons fire spontaneously, and this firing can be detected even in the cell-attached mode before membrane rupture. Because SOCs induce the transient hyperpolarization of membrane potentials (Fig. 1A2), we predicted that blocking SOCs would abolish hyperpolarization and thus enhance the rate of spontaneous firing. To test this possibility, we recorded the spontaneous activity of Venus(+) PHN neurons in VGAT-Venus transgenic rats because inhibitory neurons frequently exhibit SOCs in the PHN. To eliminate the remaining synaptic effects on spontaneous firing in the slices, we performed the recordings in the presence of a cocktail of antagonists for AMPA-type (20 μM NBQX) and NMDA-type (50 μM D-APV) glutamate receptors and GABA (100 μM picrotoxin) and glycine (20 μM strychnine) receptors. We first investigated the effects of abolishing SOCs via the blockade of SK channels on spontaneous firing. Figure 8A shows the spontaneous firing of a Venus(+) PHN neuron before and after the application of 0.1 μM apamin; apamin increased the firing rate. Because SK channels are not only responsible for SOCs but also for an AHP with relatively slow kinetics (Sah, 1996; Sah and Davies, 2000), the application of apamin also blocks AHPs with a slow component. The blockade of the slow AHP alone increases the firing rate of neurons exhibiting slow AHP (Saito et al. 2008). Of the 12 Venus(+) PHN neurons tested, 5 neurons showed slow AHPs [2 AHP(s+) with ADP and 3 AHP(s+)]. The application
of apamin indeed decreased the amplitude of AHP(s+) with ADP (Fig. 8B closed circles) and AHP(s+) (Fig. 8B, closed triangles) and increased the spontaneous firing rates of these neurons (Fig. 8C, closed circles and triangles). The remaining neurons (n = 7) only showed AHP with a fast component [AHP(s-)]; SK channels contribute little to this component. This result was supported by the finding that the amplitude of AHP(s-) was not decreased after the application of apamin (Fig. 8B, open circles). All the neurons exhibited SOCs (SOC frequency = 0.98 ± 0.87 events/s, range = 0.10-2.28 event/s), suggesting that PHN neurons exhibiting SOCs do not always show a slow AHP.

Of the 7 neurons that showed AHP(s-), 5 neurons showed an enhancement of firing rate after the application of apamin, and 2 neurons showed a reduction in the firing rate (Fig. 8C, open circles). In the neurons showing a reduction in the firing rate, spontaneous firing was often paused temporarily in the presence of apamin; therefore, the total number of spikes over 20 s was reduced. However, the instantaneous firing frequency during the occurrence of spontaneous firing was significantly increased after apamin application in all PHN neurons tested (n = 12) (Fig. 8D, open circles). The finding that spontaneous firing was enhanced after the application of apamin, even in neurons exhibiting AHP(s-), suggests that the abolition of SOCs via the blockade of SK channels do indeed moderate the firing of inhibitory PHN neurons.
We next investigated the effect of abolishing SOCs via the blockade of RyRs on spontaneous firing. RyRs are upstream of SK channels; thus, we predicted that the blockade of RyRs through the application of a high concentration of ryanodine (> 10 μM) would enhance the frequency of spontaneous firing, similar to the blockade of SK channels. Figure 9A1 shows the spontaneous firing of a Venus(+) PHN neuron before and 4 min after the application of 20 μM ryanodine. Contrary to our prediction, the firing rate was reduced through the blockade of RyRs. In 3 neurons, spontaneous firing disappeared after the application of 20 μM ryanodine (Fig. 9A2). All neurons tested (n = 12) showed a reduction in the firing rate in the presence of 20 μM ryanodine (control = 7.5 ± 3.4 spikes/s, ryanodine = 1.9 ± 1.9 spikes/s, p < 0.0001, Fig. 9C). This reduction in firing rate was also observed when the antagonist cocktail was not added to the control solution (control = 11.3 ± 5.7 spikes/s, ryanodine = 3.4 ± 3.4 spikes/s, n = 8, p = 0.0058). To confirm that the reduction of firing rate through ryanodine was not primarily mediated through signaling from other neuronal and non-neuronal cells, we recorded the spontaneous firing of Venus(+) PHN neurons using an intracellular solution containing 10 μM ryanodine (Fig. 9B). The firing rate decreased over time (Fig. 9B1, 2). The firing rate after 2 min (4.3 ± 3.5 spikes/s) was significantly smaller than that observed immediately after the start of recording (10.9 ± 3.6 spikes/s, n = 7, p
These results indicate that the block of RyRs reduces the firing of individual neurons.

When the baseline membrane potentials without action potentials were investigated in the presence of 0.25 μM TTX, they were hyperpolarized (5.9 ± 6.0 mV, n = 10) after the application of 20 μM ryanodine (Fig. 9D1), although the hyperpolarizing membrane potential fluctuations induced through SOCs were abolished after ryanodine application (Fig. 9D2). This finding confirms the shift of the baseline currents to the outward direction when the recordings were performed with an intracellular solution containing 10 μM ryanodine (Fig. 3B2). Further, this finding suggests that blocking RyRs hyperpolarizes the membrane potential and reduces the firing rate of inhibitory PHN neurons despite the abolition of transient SOC-induced hyperpolarization.

Because the application of a low concentration of ryanodine or caffeine increased SOC frequency (Figs. 4, 5), we predicted that these treatments would reduce the firing rate. However, when 0.1 μM ryanodine was applied, some neurons exhibited a reduction in the firing rate and others exhibited an increase in the firing rate (Fig. 9C). When 3 mM caffeine was applied, similar results to those observed with 0.1 μM ryanodine were obtained (Fig. 9C). These results suggest that the increase in SOC
frequency via the enhancement of Ca\(^{2+}\) release does not necessarily lower the firing rate of inhibitory PHN neurons.

Although blocking SK channels and RyRs abolished SOCs together, the change in the firing rate induced through the block of RyRs was opposite of that induced through the block of SK channels. When SOCs that induce hyperpolarizations are abolished through the blocking of RyRs, it is reasonable to assume that the membrane potential becomes depolarized. Alternatively, the abolishment might not alter the membrane potential because each hyperpolarization is transient. However, blocking RyRs induced hyperpolarization regardless of the abolition of SOCs. Blocking RyRs induces an inhibition of Ca\(^{2+}\) release via RyRs. The fact that the inhibition of Ca\(^{2+}\) release induced hyperpolarization suggests that the membrane potential is constantly depolarized through the increase in the intracellular Ca\(^{2+}\). If PHN neurons express other ion channels that are activated through Ca\(^{2+}\) increase and subsequently depolarize membrane potentials, then the inhibition of Ca\(^{2+}\) release could prevent the activation of the channels and the depolarization of the membrane potential. Among several Ca\(^{2+}\)-activated channels, Ca\(^{2+}\)-activated nonselective cation (CAN) channels are potential candidates because they are activated through an increase in intracellular Ca\(^{2+}\) and are capable of depolarizing the membrane potential (Fransén et al. 2006; Morisset
and Nagy, 1999; Viana Di Prisco et al. 2000). Furthermore, our previous findings indicated the presence of PHN neurons that express CAN channels (Saito and Yanagawa 2010). Therefore, we examined the effect of flufenamic acid (FFA), a CAN channel blocker (Partridge and Valenzuela, 2000), on the spontaneous firing rate of Venus-expressing PHN neurons to determine whether CAN channels contribute to spontaneous firing (Fig. 10). Figure 10A1 shows the reduction of the firing rate of a Venus(+) PHN neuron at 4 min after the application of 200 μM FFA. In 3 neurons, the spontaneous firing was abolished after the application of FFA (Fig. 10A2), as was observed after the application of 20 μM ryanodine (Fig. 9A2). The firing rate at 4 min after the application of FFA (1.62 ± 1.61 spikes/s) was significantly lower than that in the control (7.42 ± 3.88 spikes/s, n = 10, p = 0.0064, Fig. 10A3). Similar to the results observed with ryanodine, the baseline membrane potentials in the presence of TTX were hyperpolarized after the application of FFA (7.4 ± 5.9 mV, n = 10). These findings suggest that CAN channels are involved in the spontaneous firing of inhibitory PHN neurons.

Although the molecular basis of CAN channels has not been completely clarified, subtypes of the transient receptor potential (TRP) channel family, including TRPC3, TRPM4, and TRPM5, have been implicated as CAN channels (Clapham et al.
2005; Fleig and Penner, 2004; Launay et al. 2002). Because SKF 96365 (Vazquez et al. 2004) and 9-phenanthrol (Grand et al. 2008) block TRPC and TRPM4 channels, respectively, we used these agents to investigate the contribution of TRPC3 and TRPM4 channels to the spontaneous firing rate of Venus(+) PHN neurons. The application of 100 µM 9-phenanthrol decreased the firing rate of all neurons recorded (control = 5.7 ± 2.2 Hz, 9-phenanthrol = 2.0 ± 1.7 Hz, n = 10, p < 0.0001, Fig. 10B). However, the firing rate in the presence of 50 µM SKF 96365 (5.7 ± 3.0 Hz) was not significantly smaller than that in the control (6.6 ± 2.4 Hz, n = 10, p = 0.11), although two neurons showed a large decrease in firing rate (Fig. 10C). These results suggest that TRPM4 channels contribute to the spontaneous firing of inhibitory PHN neurons.

When current recordings were performed with a Cs⁺-based intracellular solution, neither ryanodine-sensitive transient inward currents nor SOCs were observed (data not shown). This fact may suggest that the Ca²⁺-activated conductances including CAN channels, which underlie the inward currents, are consecutively activated not only through Ca²⁺-release via RyRs but also through Ca²⁺ increase via other pathways. In addition, the fact that the activation of CAN channels induces sustained depolarization (Fransén et al. 2006; Morisset & Nagy, 1999; Viana Di Prisco et al. 2000) might make it impossible to discern the individual inward currents mediated
Finally, we investigated the effects of the increase in Ca\(^{2+}\) release on the firing rate when SOCs were abolished. Figure 11A shows the spontaneous firing of a Venus(+) PHN neuron in which SOCs were abolished using apamin before (left trace) and after the application of caffeine (right trace). Of the 8 Venus(+) PHN neurons tested, 5 neurons showed an increase in firing rate after the application of caffeine as shown in Figure 11A (130 ± 48% of the control; range, 102 - 206%). The remaining neurons (n = 3) showed a loss of action potential after the application of caffeine (Fig. 11B). However, the action potentials were restored when the baseline membrane potential was hyperpolarized through current injection (Fig. 11B, “Hyperpolarized”), suggesting that the loss of action potentials was attributable to the inactivation of Na\(^{+}\) channels. These results suggest that the depolarization of the baseline membrane potential and the firing rate are further promoted through the activation of CAN channels when SOCs are abolished.
Discussion

Spontaneous outward currents in PHN neurons

The present physiological and pharmacological analyses revealed that SOCs were 1) independent of synaptic transmission, 2) dependent on the activation of SK, but not BK, channels, and 3) dependent on the activation of RyRs but not IP$_3$ receptors. The intracellular application of BAPTA abolished the induction of SOCs, indicating that their induction requires increases in the intracellular Ca$^{2+}$. Together, these findings strongly suggest that SOCs correspond to the SMOCs that are observed in other central neurons (Arima et al. 2001; Cui et al. 2004; Klement et al. 2010). Although we did not investigate the relationship between the induction of SOCs and increases in intracellular Ca$^{2+}$ directly, previous studies have demonstrated that the induction of SMOCs is accompanied by an increase in intracellular Ca$^{2+}$ (e.g., Imaizumi et al. 1999; Pérez et al. 1999; ZhuGe et al. 1999).

As it occurs in muscle cells, the Ca$^{2+}$-induced Ca$^{2+}$-release mediated via RyRs in neurons is activated through cytoplasmic Ca$^{2+}$ (Shmigol et al. 1995; Verkhratsky and Shmigol, 1996). Because the Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels can activate RyRs (Berridge, 1998), SMOCs might be triggered through the activation of voltage-gated Ca$^{2+}$ channels. Indeed, the Ca$^{2+}$ influx through L or N-type Ca$^{2+}$...
channels triggers SMOCs in peripheral neurons (Merrian et al. 1999; Mitra and Slaughter, 2002) and T-type Ca\(^{2+}\) channels trigger large-amplitude SMOCs in dopamine neurons (Cui et al. 2004). In PHN neurons, blocking several types of Ca\(^{2+}\) channels hardly affected the SOC frequency of PHN neurons; however, the SOC frequency was increased when the membrane potential was depolarized (Fig. 1C). This finding suggests that in PHN neurons, Ca\(^{2+}\) channels facilitate the induction of SOCs rather than trigger their induction, as suggested in Meynert neurons (Arima et al. 2001) and medial preoptic neurons (Klement et al. 2010). Because blocking Ca\(^{2+}\)-permeable neurotransmitter receptors, such as NMDA-type glutamate and nicotinic acetylcholine receptors, did not also abolish SOCs, it is likely that most SOCs in PHN neurons are not triggered through an extracellular Ca\(^{2+}\) influx. The repetitive induction of SOCs might be triggered through the spontaneous release of Ca\(^{2+}\) from the ER via RyRs in a spark-like manner (Cheng and Lederer, 2008; Imaizumi et al. 1999).

Control of spontaneous firing in inhibitory PHN neurons

SOCs induce the transient hyperpolarization of membrane potentials, which can inhibit the generation of action potentials in PHN neurons. Therefore, the abolition of SOCs should enhance the repetitive firing. The abolition of SOCs through the blockade of
SK channels indeed enhanced the rate of spontaneous firing (Fig. 6); however, the abolition of SOCs through the blockade of RyRs reduced the firing rate (Fig. 7). This decrease in the firing rate reflected the hyperpolarization of the baseline membrane potential; thus, we speculate that the cessation of Ca\(^{2+}\) release via RyRs might remove the sustained depolarization induced through Ca\(^{2+}\)-activated conductances. Similar to ryanodine application, the application of FFA reduced the firing rate and hyperpolarized the baseline membrane potential. Although FFA has been used to block the CAN channels, which are primarily members of the TRP channel family (Clapham et al. 2005; Fleig and Penner, 2004; Launay et al. 2002), FFA also affects Ca\(^{2+}\)-activated Cl\(^{-}\) channels (Frings et al. 2000), other TRP channels that do not constitute CAN channels (Peña and Ordaz, 2008), various ion channels (Gardam et al. 2008; Jones and Palmer, 2011; Yau et al. 2010), gap junctions (Harks et al. 2001) that are involved in the spontaneous firing of the medial vestibular nucleus neurons neighboring the PHN (Beraneck et al. 2009), and protein kinases (Chi et al. 2011). However, the application of 9-phenanthrol, which blocks TRPM4-constituted CAN channels, decreased the firing rate. This finding supports the idea that inhibitory PHN neurons express CAN channels that are associated with the activation of RyRs, although we do not exclude the possibility that other FFA-sensitive conductances are also present in these neurons and
contribute to their firing.

The activation of SK channels induces SOCs and hyperpolarizing fluctuations of the membrane potential, leading to the suppression of spontaneous firing. However, the activation of CAN channels depolarizes the baseline membrane potential, leading to an enhancement of spontaneous firing. The fact that blocking RyRs hyperpolarizes membrane potentials and reduces the firing rate may be attributable to the different properties of SK and CAN channels. The activation of CAN channels generates sustained depolarization (Fransén et al. 2006; Morisset and Nagy, 1999; Viana Di Prisco et al. 2000), whereas the activation of SK channels induces transient hyperpolarization (see, Fig. 1A2). Therefore, blocking the sustained depolarization might have a stronger effect on neuronal firing than blocking the transient hyperpolarization when both SK and CAN channels are blocked. However, the finding that the application of a low concentration of ryanodine or caffeine enhanced or reduced the firing rate suggests that there may be different distributions or expressions of SK and CAN channels within each neuron.

The finding that blockade of the CAN channels lowers spontaneous firing indicates that the activation of CAN channels through Ca$^{2+}$ release via RyRs is necessary to induce spontaneous firing in inhibitory neurons in the PHN. However,
when Ca\(^{2+}\) release was enhanced through caffeine in the absence of SOCs after SK channels were blocked, the firing rate was significantly increased. In some neurons, the membrane potentials were constantly depolarized, and the generation of spikes was abolished due to inactivation of Na\(^{+}\) channels. This finding suggests that the activation of CAN channels in the absence of SOCs forces enormous depolarization and firing. Therefore, SOCs induced via SK channels may be crucial for the induction of adequate spontaneous firing in inhibitory PHN neurons. In summary, SK and CAN channels, both of which are triggered through Ca\(^{2+}\) release via RyRs, may show complementary and opposing effects on the induction of adequate firing in inhibitory PHN neurons. Therefore, we suggest that the temporal patterns of action potentials in inhibitory neurons are regulated through RyRs, and SK and CAN channels, although other ion conductances and synaptic transmissions also affect neuronal firing (Häusser et al. 2004; Hille, 2001; Llinás, 1988).

The PHN functions as an oculomotor neural integrator that transforms eye or head velocity signals into eye position signals (Fukushima et al., 1992; Fukushima and Kaneko, 1995; Moschovakis, 1997; Robinson, 1975, 1989). Previous studies have suggested the involvement of excitatory networks in the function of neural integrators (Aksay et al. 2001, 2003; Saito and Yanagawa, 2010). Because most inhibitory
neurons in the PHN fire spontaneously, it is possible that they provide tonic inhibition to the surrounding neurons. Tonic inhibition is necessary to prevent the integration of background noise and the unnecessary spread of neural excitability (Robinson, 1989). However, excitatory networks might be difficult to activate if this inhibition is too strong. Therefore, the strength of the inhibition must be suitable for both the prevention of the noise and the activation of the excitatory networks. The modest spontaneous firing of inhibitory PHN neurons, controlled through SK and CAN channels via RyRs, might be suitable for keeping the strength of the inhibition adequate for the effective functioning of integrator circuits. The fact that SOCs occur preferentially in inhibitory PHN neurons might reflect the significant role for SOCs in controlling the strength of tonic inhibition together with CAN channels.

Characteristics of PHN neurons exhibiting SOCs

Idoux et al. (2006) classified PHN neurons into neurons exhibiting monophasic AHP (type A), biphasic AHP (type B), or oscillatory firing (type D) and observed that type B and D neurons showed bistable plateau-like responses through a NMDA-dependent mechanism. This property can be useful for the robustness of neural activities in the integrator network; therefore, these neurons might be involved in the functions of the
network. Furthermore, it has been suggested that type B neurons receiving glutamatergic inputs from the paramedian pontine reticular formations may play a role in generating eye position signals through an acetylcholine-dependent mechanism (Navarro-López et al. 2004, 2005). Recently, Kolkman et al. (2011) investigated the electrophysiological properties of PHN neurons using three transgenic mouse lines (YFP-16, GlyT-2, and GIN). In the YFP-16 line, most of the YFP-expressing glutamatergic and glycinergic neurons showed a biphasic AHP, whereas most fluorescence-expressing neurons in the GlyT-2 line (glycinergic neurons) and the GIN line (a subset of GABAergic neurons) showed a monophasic AHP. This finding suggests that type A PHN neurons include a subset of GABAergic neurons and a large portion of glycinergic neurons, whereas type B PHN neurons include glutamatergic neurons and a portion of glycinergic neurons. Although it is difficult to determine an complete association between the classifications of Idoux et al (2006) and our somewhat different classifications, it seems that neurons exhibiting AHP(s-) and AHP(s+) correspond to type A and neurons exhibiting AHP(s+) with ADP correspond to type B (Rössert and Straka, 2011). Neurons exhibiting oscillatory firing correspond to type D, and neurons exhibiting other firing patterns may represent subcategories of type A and B neurons.
The relationship between intrinsic properties and SOC frequency revealed that SOC

cells were observed in neurons exhibiting every AHP profile, suggesting that all
types of neurons (type A, B, and D) exhibit SOCs. However, PHN neurons exhibiting
specific properties, such as AHP(s-), late spiking, FIL, and oscillatory firing showed
frequent SOCs. Late-spiking neurons exhibit all three AHP profiles and FIL neurons
exhibit either AHP(s+) with ADP or AHP(s+) (Shino et al. 2008, and the present study),
and thus it is likely that a subset of type A, including AHP(s-) and late spiking neurons,
type B, including late spiking and FIL neurons, and most type D exhibit frequent SOCs.
If type B and D PHN neurons, which are involved in oculomotor integration
(Navarro-López et al. 2004, 2005; Idoux et al. 2006), indeed show frequent SOCs, then
SOCs might be involved in not only tonic inhibition but also oculomotor integration.
To clarify the involvement of SOCs in the integration, it needs to be determined whether
the activation of the excitatory networks in the PHN affects SOCs.

Conclusions

PHN neurons show SMOCs associated with those reported in other central neurons.
The inhibitory PHN neurons exhibit frequent SMOCs. The firing behavior of
inhibitory PHN neurons is regulated through SMOCs that are induced via SK channels
and sustained inward currents that are induced via CAN channels, both of which are triggered through Ca$$^{2+}$$ release from the RyRs. These findings can provide additional information concerning the effects of intracellular Ca$$^{2+}$$ on neuronal excitability via the regulation of Ca$$^{2+}$$-activated ion channels in central neurons.
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References


Cui G, Okamoto T, Morikawa H. Spontaneous opening of T-type Ca2+ channels contributes to the irregular firing of dopamine neurons in neonatal rats. *J Neurosci* 24:


Idoux E, Serafin M, Fort, P, Vidal PP, Beraneck M, Vibert N, Muhlethaler M, Moore L
E. Oscillatory and intrinsic membrane properties of guinea pig nucleus prepositus

Imaizumi Y, Ohi Y, Yamamura H, Ohya S, Muraki K, Watanabe M. Ca2+ spark as a

Jones SM, Palmer MJ. Pharmacological analysis of the activation and receptor
properties of the tonic GABA(C)R current in retinal bipolar cell terminals. *PLoS One* 6:
e24892, 2011.

Klement G, Druzin M, Haage D, Malinina E, Arhem P, Johansson S. Spontaneous
ryanodine-receptor-dependent Ca2+-activated K+ currents and hyperpolarizations in rat

Kolkman KE, Moghadam SH, du Lac S. Intrinsic physiology of identified neurons in

Koyanagi Y, Yamamoto K, Oi Y, Koshikawa N, Kobayashi M. Presynaptic interneuron
subtype- and age-dependent modulation of GABAergic synaptic transmission by

Lai FA, Misra M, Xu L, Smith HA, Meissner G. The ryanodine receptor-Ca2+ release
channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a


Miller RJ. The control of neuronal Ca\(^{2+}\) homeostasis. *Prog Neurobiol* 37: 255–285,


Navarro-López Jde D, Alvarado JC, Márquez-Ruiz J, Escudero M, Delgado-García JM, Yajeya J. A cholinergic synaptically triggered event participates in the generation of


Verkhratsky A. Physiology and pathophysiology of the calcium store in the endoplasmic...


Figures legends

Figure 1. Spontaneous outward currents (SOCs) in PHN neurons.

A1: spontaneous currents in a PHN neuron. The holding potential of the neuron was -75 mV. Inset: a faster sweep of the current that is indicated by the arrow. A2: spontaneous fluctuations of membrane potentials of the same neuron. B: histograms showing the distributions of the frequency (1) and amplitude (2) of recorded SOCs. The filled bar indicates neurons that did not exhibit SOCs. C: current traces at different holding potentials. The holding potentials are shown on the left. D1: current traces in the control (upper) and in the presence of 0.25 μM TTX (lower). D2: effects of TTX (0.25 μM, n = 7), NBQX (20 μM, n = 7), D-APV (50 μM, n = 6), MCPG (1 mM, n = 8), picrotoxin (100 μM) & strychnine (20 μM, n = 7), CGP52432 (6 μM, n = 7), mecamylamine (10 μM, n = 6), and atropine (1 μM, n = 8) on the frequency of SOCs. The plots indicate the data obtained from individual neurons, and the bar represents the average value. The ordinate indicates the ratio (percentage) of the frequency in the presence of each drug to that in the control.

Figure 2. Contribution of Ca$^{2+}$-activated K$^+$ channels to SOCs

A: current traces in the control (1) and in the presence of 0.1 μM apamin (2), and the
comparison between the SOC frequencies in the control solution and the solution containing apamin (3, Apa). Lines connect individual plots corresponding to results obtained from the same neuron (n = 7). Asterisks indicate a significant difference between groups (*, p < 0.05). B: current traces in the control (1) and in the presence of 0.1 μM iberiotoxin (2) and the comparison between the SOC frequencies in the control solution and the solution containing iberiotoxin (3, Ibe). Lines connect individual plots corresponding to results obtained from the same neuron (n = 8). C: current trace of a PHN neuron. The recording was performed with an intracellular solution containing 10 mM BAPTA. Lower traces indicate the faster-sweep records of segments of a and b in A. The averaged baseline currents of a and b are -69 pA and -51 pA, respectively. Although SOCs (arrows) were seen immediately after the start of the recording, they disappeared quickly.

Figure 3. Contribution of ryanodine receptors to SOCs

A: current traces in the control (upper) and in the presence of 0.1 μM ryanodine (1, lower) and 20 μM ryanodine (2, lower). B: spontaneous currents of a PHN neuron that were recorded with an intracellular solution containing 0.1 μM (1) or 10 μM (2) ryanodine. The recording started approximately 30 s after the patch membrane rupture.
The faster-sweep records indicated by a and b are shown at right. The averaged baseline currents of a and b are -112 pA and -99 pA (B1), and -54 pA and -45 pA (B2), respectively.  

**C**: effects of the extracellular (white bars) and intracellular (black bars) application of the different concentrations of ryanodine on the SOC frequency. The plots indicate data obtained from individual neurons, and the bar represents the average value.  0, L, and H indicate zero (DMSO only), low (0.01-0.05 μM), and high (10 μM) concentrations of ryanodine. Asterisks indicate a significant difference between the control and the presence of ryanodine or at 1 min and 3 min after rupture [*: p < 0.05, **: p < 0.01, p = 0.017 (bath 0.05 μM), p = 0.0076 (bath 0.1 μM), p = 0.084 (bath 0.5 μM), p = 0.18 (bath 1 μM), p = 0.0077 (bath 10 μM), p = 0.0022 (bath 20 μM), p = 0.0012 (bath 50 μM), p = 0.14 (intra 0), p = 0.017 (intra L), p = 0.0076 (intra H)].  

**D1**: current traces in the control (upper) and in the presence of 20 μM ryanodine (lower) in PHN neurons in a slice obtained from a 6-week-old old rat.  **D2**: comparison between the frequencies of SOCs in the control solution and a solution containing ryanodine (n = 5).  

**E1**: current traces in the control (upper) and in the presence of 1 μM xestospongin C (lower).  **E2**: effects of bath application of xestospongin C (n = 7) and the intracellular application of heparin (1 mg/ml, n = 8).
Figure 4. Effect of caffeine on SOCs

A: current trace of a PHN neuron before (1) and after (2) application of 3 mM caffeine. Inward currents are truncated. A3: comparison between the SOC frequencies in the control and in the presence of caffeine (n = 9). B: current traces of a PHN neuron in the control (1) and in the presence of 200 μM aminophylline (2) or 3 mM caffeine (3). B4: comparison of SOC frequencies in the control and in the presence of aminophylline or caffeine (n = 9). Asterisks indicate a significant difference between groups (**, p < 0.01).

Figure 5. Partial contribution of voltage-gated Ca\textsuperscript{2+} channels to SOCs

A: current traces in the control condition (1) and in the presence of 50 μM Ni\textsuperscript{2+} (2). B: effects of Ni\textsuperscript{2+} (100 μM, n = 9), nifedipine (20 μM, n = 9), ω-conotoxin GVIA (1 μM, n = 8), ω-agatoxin IVA (200 μM, n = 9), and SNX-482 (200 μM, n = 8) on the frequency of SOCs. The plots indicate data obtained from individual neurons, and the bar represents the average value. The gray broken line corresponds to 100%. Asterisks indicate a significant difference between the control and the presence of the Ca\textsuperscript{2+} channel blocker (*, p < 0.05).
Figure 6. Relationship between SOC frequency and membrane properties

A, B: the three afterhyperpolarization (AHP) profiles of PHN neurons: AHP with a slow component and an afterdepolarization (ADP) [AHP(s+) with ADP], AHP with a slow component [AHP(s+)], and AHP without a slow component [AHP(s-)]. On the graphs, AHP(s+) with ADP, AHP(s+), and AHP(s-) are described to ADP, S+, and S-.

B: the six firing patterns of PHN neurons: continuous spiking pattern (Cont), late spiking pattern (Late), low-threshold calcium spike pattern (LTS), longer first interspike interval pattern (FIL), low firing rate pattern (LFR), and oscillatory firing pattern (Osc).

C, D: comparisons among the SOC frequencies observed in PHN neurons exhibiting the different AHP profiles (1) and firing patterns (2) in young (C) and adult rats (D). The plots indicate data obtained from individual neurons, and the bar represents the average value. The numbers given in the parentheses indicate (the number of neurons exhibiting SOCs) / (the total number of each neuron type).

Figure 7. Comparison of SOC frequency between Venus-expressing and non-expressing PHN neurons.

A1: fluorescent photomicrograph of the PHN in a perfusion-fixed tissue section obtained from a VGAT-Venus transgenic rat. The dashed line shows the approximate location of the PHN.
boundary of the PHN. Scale bar = 100 μm. Abbreviations: 4V; fourth ventricle, D: dorsal, L: lateral. A2: high-magnification photomicrograph of the PHN in the area outlined by the rectangle in A1. Scale bar = 20 μm. B: comparison between the SOC frequencies in Venus-expressing (n = 45) and non-Venus-expressing PHN neurons (n = 45). Asterisks indicate a significant difference between groups (**, p < 0.01). C, D: comparisons among the SOC frequencies observed in Venus-expressing (C) and Venus-non-expressing PHN neurons (D) exhibiting the different AHP profiles (1) and firing patterns (2). The plots indicate data obtained from individual neurons, and the bar represents the average value. The numbers given in the parentheses indicate (the number of neurons exhibiting SOCs) / (the total number of each neuron type).

Figure 8. Effects of apamin on spontaneous firing in Venus-expressing PHN neurons. A: spontaneous firing of a Venus-expressing PHN neuron in the presence of the antagonist cocktail (upper) and the antagonist cocktail plus 100 nM apamin (bottom). B1, 2: expanded traces during spontaneous firing of a neuron exhibiting AHP(s-) (1) and that exhibiting AHP(s+) (2). Two traces recorded in the presence of the cocktail (light line) and the cocktail plus apamin (dark lines) are superimposed. Each action
potential is truncated. **B3**: effects of apamin on the amplitude of AHP. Open circles, closed circles, and closed triangles indicate AHP(s-), AHP(s+) with ADP, and AHP(s+), respectively. **C, D**: comparisons between the firing frequencies (C) and the instantaneous firing frequencies (D) in the cocktail and in the cocktail plus apamin. The symbols are same as B3. The average firing frequency was 6.5 ± 3.8 spikes/s in the cocktail and 10.3 ± 7.0 spikes/s in the cocktail plus apamin (n = 12 cells, p = 0.0171). The average instantaneous firing frequency was 7.6 ± 4.6 spikes/s in the cocktail and 15.1 ± 8.9 spikes/s in the cocktail plus apamin (n = 12 cells, p = 0.0007).

**Figure 9. Effects of ryanodine on the spontaneous firing of Venus-expressing PHN neurons.**

**A1**: spontaneous firing of a Venus-expressing PHN neuron in the presence of the antagonist cocktail (upper) and in the antagonist cocktail plus 20 μM ryanodine (bottom). **A2**, abolition of the spontaneous firing of a Venus-expressing PHN neuron after application of 20 μM ryanodine. **B1**: spontaneous firing of a Venus-expressing PHN neuron recorded with an intracellular solution containing 10 μM ryanodine. The recording started approximately 30 s after the patch membrane rupture. **B2**: faster-sweep records indicated by a and b (5 s records). **B3**: comparison between the
frequency of firing over 10 s immediately after (0-10 s) and 2 minutes after the start of recording (120-130 s). C: the effect of high (20 μM), low (0.1 μM), and zero (DMSO only) concentrations of ryanodine and 3 mM caffeine on the frequency of spontaneous firing. The firing frequency was significantly reduced by the application of 20 μM ryanodine (p < 0.0001, n = 12). Some neurons showed an enhancement of firing frequency and others showed a reduction in firing frequency after the application of 0.1 μM ryanodine (p = 0.68, n = 9) or 3 mM caffeine (p = 0.72, n = 8). The firing frequency was not significantly altered in the presence of DMSO only (p = 0.83, n = 4).

D1: a voltage trace of a Venus-expressing PHN neuron recorded in the presence of the antagonist cocktail plus 0.25 μM TTX. D2: faster sweeps of the membrane potential of before (left) and after application of 20 μM ryanodine (right).

Figure 10. Effects of FFA and TRP channel blockers on the spontaneous firing of Venus-expressing PHN neurons.

A1: spontaneous firing of a Venus-expressing PHN neuron in the presence of the antagonist cocktail (upper) and the antagonist cocktail plus 200 μM FFA (lower). A2: abolition of the spontaneous firing of a Venus-expressing PHN neuron after application of FFA. A3: comparison between the firing frequencies in the antagonist cocktail and
in the cocktail plus FFA (n = 10). **B1, C1**: spontaneous firing of a Venus-expressing PHN neuron in the presence of the antagonist cocktail (upper, **B1, C1**) and the antagonist cocktail plus 100 μM 9-phenanthrol (lower, **B1**) or 50 μM SKF 96365 (lower, **C1**). **B2, C2**: comparison between the firing frequencies in the antagonist cocktail and in the cocktail plus 9-phenanthrol (n = 10, **B2**) or SKF 96365 (n = 10, **C2**). Asterisks indicate significant differences between groups (**, p < 0.01**).

**Figure 11. Effects of apamin and caffeine on the spontaneous firing of Venus-expressing PHN neurons.**

**A**: spontaneous firing of a Venus-expressing PHN neuron in the presence of 0.1 μM apamin (1) and apamin plus 3 mM caffeine (2). **B**: spontaneous firing of another Venus-expressing PHN neuron in the presence of 0.1 μM apamin (1) and apamin plus 3 mM caffeine (2). Note the abolition of repetitive firing in the presence of apamin plus caffeine, which is attributable to the inactivation of Na⁺ channels. The gray dashed lines in A and B indicate -50 mV. **3**: Recovery of repetitive firing after the application of hyperpolarizing currents.
Fig. 2  Saito & Yanagawa
Fig. 3  Saito & Yanagawa
Fig. 4  Saito & Yanagawa
Fig. 5  Saito & Yanagawa
Fig. 7  Saito & Yanagawa
Fig. 8 Saito & Yanagawa
Fig. 9  Saito & Yanagawa
Fig. 10  Saito & Yanagawa