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Electrophysiological analysis of vulnerability to experimental ischemia in neonatal rat spinal ventral horn neurons

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ABSTRACT

To clarify the vulnerability of spinal motoneurons to excitotoxicity, we analyzed the agonal current induced by experimental ischemia in ventral lamina IX neurons of spinal cord slices from neonatal rats by using whole-cell patch-clamp. Ischemia was simulated *in vitro* by oxygen/glucose deprivation. Superfusion with ischemia-simulating medium elicited an agonal inward current, which was initially slow and then became rapid. We compared 8-, 9-, 10-, 11-, and 12-day postnatal rats and found age-dependent shortening of the latency of the rapid inward current. Furthermore, the membrane capacitance (Cm) and resting membrane potential (RMP) of the lamina IX neurons demonstrated significant negative correlations with the latency of the rapid inward current. Logistic regression analysis showed that postnatal age, Cm, and RMP were independent contributing factors to ischemic vulnerability. These results suggest that not only cell volume and ionic balance but also early postnatal maturation of the intracellular environment is vital for developing vulnerability to excitotoxicity.

Excitotoxicity is an important mechanism of neuronal death, implicated in the pathogenesis of ischemia, trauma, and neurodegenerative disorders [8,10,15]. Excitotoxic injury is mediated by glutamate, a major excitatory neurotransmitter. During pathologic insult, glutamate excessively accumulates in the extracellular space and stimulates the neurons through ionotropic receptors [9,11,14,20]; such stimulation leads to neuronal depolarization and irreversible loss of function [17].

Specific regions of the central nervous system are susceptible to developing neuronal damage after excitotoxic injury. In addition, such vulnerability critically depends on postnatal maturation [16]. Spinal cord motoneurons are also known to be particularly vulnerable during ischemia [17]; some of their molecular features are known to play important roles in cell death in degenerative motoneuron diseases [22]. However, factors contributing to the vulnerability of spinal motoneurons to ischemia and changes in susceptibility during maturation have not been well documented.

In vitro ischemia is mimicked by oxygen/glucose deprivation; this preparation has been well established in electrophysiological studies with spinal cord and brain slices [17,18,23–25]. In the present study, we investigated the influence of experimental ischemia on ventral lamina IX neurons in spinal cord slices by using the whole-cell patch-clamp method to clarify parameters contributing to the vulnerability of developing spinal motoneurons.

All experimental procedures involving the use of animals were approved by the Animal Care and Use Committee of Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan).

Slices of rat spinal cord were prepared as previously described [17]. In brief, neonatal Wistar rats (8–12 days postnatal) were anesthetized with urethane (1.2–1.5 g/kg, intraperitoneal). Dorsal laminectomy was performed, and the lumbosacral segment of the spinal cord was removed. The rats were immediately killed by exsanguination. The spinal cord was placed in pre-oxygenated ice-cold artificial cerebrospinal fluid (ACSF). After cutting all the ventral and dorsal roots near the root entry zone, the pia-arachnoid membranes were removed. The spinal cord was mounted on a metal stage of a microslicer (DTK-1500; Dosaka, Kyoto, Japan) and cut into 500-μm-thick transverse slices. Each spinal cord slice was transferred to a recording chamber and placed on the stage of an upright microscope equipped with an infrared-differential interference contrast (IR-DIC) system (E600FN; Nikon, Tokyo, Japan). The slice was fixed by an anchor and superfused at 4–6 ml/min with ACSF solution equilibrated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 36 °C by using a temperature controller (TC-324B; Warner Instruments, Hamden, CT, USA). The ACSF solution comprised (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11.5 D-glucose (pH 7.4).

Lamina regions were identified under low magnification (5× objective lens) and individual neurons were identified using a 40× objective lens with an IR-DIC microscope and monitored by a CCD camera (C2400-79H; Hamamatsu Photonics, Hamamatsu, Japan)

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on a video monitor screen. The size of each recorded neuron was calculated from the arithmetic mean length of the diameter of the long and short axes of the soma intersecting at right angles. Whole-cell voltage-clamp or current-clamp recordings were made from large lamina IX neurons (size, 15–25 μm), which were generally observed in the ventral lateral or ventral medial areas. Whole-cell patch pipettes were constructed from borosilicate glass capillaries (1.5-mm OD; World Precision Instruments, Sarasota, FL, USA). The resistance of a typical patch pipette was 5–10 MΩ when filled with an internal solution composed of (in mM) 135 potassium gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, and 5 ATP-Mg (pH 7.2). After the whole-cell configuration was established, voltage-clamped neurons were held at −70 mV and current-clamped neurons were held at 0 pA. Signals were amplified with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA), filtered at 2 kHz, and digitized at 5 kHz. Data were stored and analyzed using a pCLAMP 9.1 data acquisition program (Molecular Devices). Membrane capacitance (Cm) and input resistance (Rin) were measured by applying hyperpolarizing voltage pulses (10 mV) from a holding potential of −70 mV with a duration of 20 ms. Cm was measured by integrating the transient capacitative currents evoked during the voltage-clamp steps. These measurements were used to estimate membrane surface area [21]. We considered the potential at which the holding current becomes zero to be the resting membrane potential (RMP). Neurons with RMPs above −50 mV were removed from the analysis [1,3].

Ischemia was simulated by superfusing the slices with ischemia-stimulating medium (ISM) consisting of ACSF solution, equilibrated with a gas mixture of 95% N_2 and 5% CO_2, in which glucose was replaced with an equimolar concentration of sucrose. Drugs and ISM were applied by perfusion via a three-way stopcock, without changing the perfusion rate or temperature. The solution in the connection tube and recording chamber having a volume of 2.5 ml was completely replaced within 35 s of the initiation of ISM perfusion. After applying the ischemia-simulating medium, we discarded the slice.

Numerical data are represented as mean ± SEM. One-way analysis of variance (ANOVA) with post hoc Tukey's honestly significant difference (HSD) test was used to compare the groups classified according to age. Pearson's correlation coefficients (r) were calculated between latency of rapid inward currents induced by ISM and age or electrophysiological membrane properties. Multiple regression analyses were performed to determine the relative contribution of different variables, and F-test was used to evaluate the significance of all independent variables. Statistical significance was defined as $P < 0.05$. When referring to electrophysiological data, n indicates the number of neurons studied.

Whole-cell voltage-clamp recordings were made from 69 neurons of which 60 exhibited RMPs below −50 mV and were included in analysis. Neuron bodies were round or multipolar, with an average soma size of 20.5 ± 0.3 μm; average Cm, 90.8 ± 4.4 pF; average RMP, −58.1 ± 0.3 mV, and average Rin, 152.4 ± 9.2 MΩ ($n = 60$). Soma size demonstrated a significant positive correlation with Cm ($r = 0.57$, $P < 0.01$).

The lamina IX neurons remained viable up to 12 h in slices perfused with pre-oxygenated ACSF solution. However, all recordings in this experiment were obtained within the first 4 h. When the membrane potential was held at −70 mV, superfusion with ISM produced an outward current of 34.1 ± 6.6 pA in 20% of the 60 neurons examined ($n = 12$); this was followed by an agonal inward current (Fig. 1A). The remaining neurons ($n = 48$) exhibited only the inward current. Meanwhile, in the current-clamp mode, ISM produced agonal depolarization followed by persistent depolarization ($n = 5$, Fig. 1B). When ISM superfusion was continued after the appearance of the agonal depolarization, synaptic activity disappeared, and it could not be returned to its previous state despite

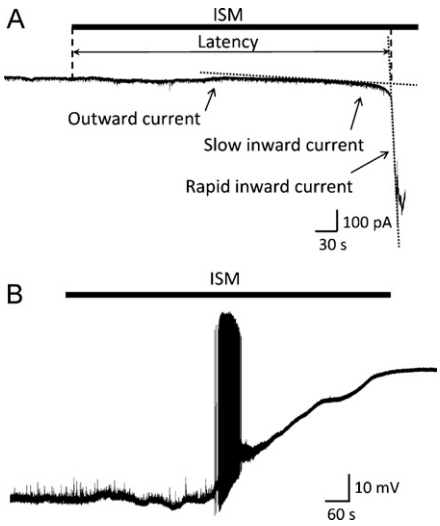

Fig. 1. Agonal current induced by ischemia-simulating medium (ISM) in a representative lamina IX neuron. (A) In the voltage-clamp mode, perfusion with ISM produced an outward current and, subsequently, an agonal inward current at −70 mV in 20% of the neurons examined ($n = 12$). The agonal inward current consisted of a slow and subsequent rapid inward current. The latency of the rapid inward current was measured from the beginning of ISM perfusion to the onset of the rapid inward current, which was estimated by extrapolating the slope of the rapid inward current to the slope of the slow inward current. The current trace shown was obtained from a neuron of an 8-day postnatal rat. (B) In the current-clamp mode, perfusion with ISM produced an agonal depolarization. The resting membrane potential was −61 mV. After the generation of rapid depolarization, the membrane continued to depolarize to 0 mV and synaptic activity disappeared. This trace was obtained from a neuron of a 9-day postnatal rat.

ACSF reperfusion, indicating that ISM resulted in irreversible membrane dysfunction (Fig. 1B). The agonal inward current consisted of a slow followed by a rapid inward current [23]. The onset of the rapid inward current was estimated by extrapolating the slope of the rapid inward current into the slope of the slow current. The latency of the rapid inward current was measured from the onset of superfusion with the ISM to that of the rapid inward current (Fig. 1A). Such a brief superfusion with ISM is known to affect synaptic transmission for several hours in rat hippocampal CA1 neurons; therefore, only the data obtained by its primary application were included in the present study.

Twelve lamina IX neurons in each age group were exposed to ISM. To estimate the influence of an initial outward current, we compared the average age, size, Cm, RMP, Rin, and latency of the rapid inward current. No significant differences were observed in these results between neurons with (10.2 ± 0.5 days, 21.2 ± 0.6 μm, 102.1 ± 7.7 pF, −56.1 ± 1.5 mV, 134.9 ± 12.2 MΩ, 440 ± 23 s, $n = 12$) and without (10.0 ± 0.2 days, 20.3 ± 0.3 μm, 87.9 ± 5.1 pF, −58.6 ± 1.0 mV, 156.8 ± 11.1 MΩ, 454 ± 33 s, $n = 48$) ISM-induced outward currents ($P = 0.65$, 0.24, 0.20, 0.23, 0.35, 0.80, respectively). The differences in soma size and electrophysiological membrane properties between the groups are listed in Table 1. Average latencies of the rapid inward current in the lamina IX neurons were 478 ± 19 s, 448 ± 26 s, 424 ± 32 s, 391 ± 28 s, and 377 ± 18 s on postnatal days 8, 9, 10, 11, and 12, respectively. An obvious age-dependent shortening of the latency of the rapid inward currents was observed (one-way ANOVA, $P < 0.05$; post hoc Tukey's HSD test, P8 versus P12, $P < 0.05$) (Fig. 2). Because age was not found to be a significant factor in the correlation between membrane properties and latency of the rapid inward current, we analyzed these results without grouping by age. Cm of the lamina IX neurons demonstrated a significant negative correlation with the latency of the rapid inward current ($r = -0.41$, $P < 0.01$) (Fig. 3A). Furthermore, RMP of the neurons also exhibited a sig-

Table 1
Comparison of the soma size and electrophysiological membrane properties classified according to postnatal age.

Postnatal days	P 8	P 9	P 10	P 11	P 12	*P* value
n	12	12	12	12	12	
Size (μm)	20.3 ± 0.6	20.4 ± 0.7	21.5 ± 0.9	19.7 ± 0.4	20.5 ± 0.6	0.45
Cm (pF)	83.0 ± 6.8	80.2 ± 7.7	107.2 ± 15.4	87.2 ± 7.2	96.3 ± 9.4	0.30
RMP (mV)	−60.8 ± 2.1	−57.8 ± 1.7	−57.2 ± 2.1	−56.5 ± 1.5	−58.2 ± 1.9	0.56
Rin (MΩ)	146.8 ± 19.9	139.5 ± 18.6	150.8 ± 27.0	183.9 ± 17.4	141.0 ± 19.6	0.55

There was no significant difference in soma size and membrane properties by one-way ANOVA among the postnatal age groups. Cm: membrane capacitance; RMP: resting membrane potential; Rin: input resistance. Data are shown as mean ± SEM. *n* refers to the number of neurons studied.

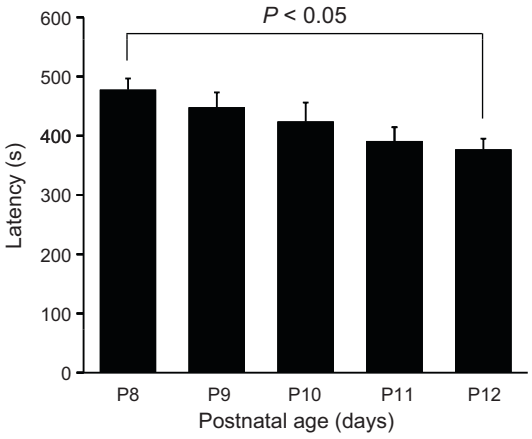

Fig. 2. Comparison of the latency of the rapid inward current induced by experimental ischemia between the different age groups. An obvious age-dependent shortening of the latency of the rapid inward current was observed (one-way analysis of variance $P < 0.05$; post hoc Tukey's honestly significant difference test, P8 versus P12, $P < 0.05$).

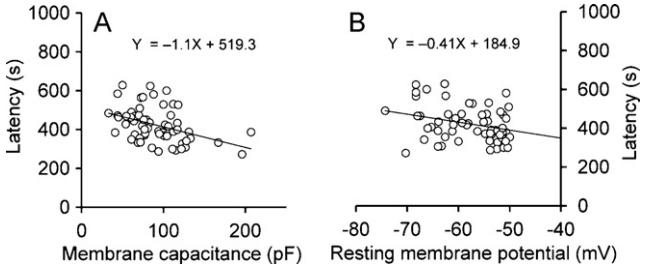

Fig. 3. Correlation between electrophysiological membrane properties and the latency of the rapid inward current as generated by ISM perfusion in lamina IX neurons (8–12 days postnatal, $n = 60$). Membrane properties were measured prior to ISM perfusion. (A) There was a negative correlation between membrane capacitance and latency ($r = -0.41$, $P < 0.01$). (B) There was also a negative correlation between resting membrane potential and latency ($r = -0.30$, $P < 0.05$).

nificant negative correlation with the latency of the rapid inward current ($r = -0.30$, $P < 0.05$) (Fig. 3B). On the other hand, Rin did not correlate with the latency ($r = -0.11$, $P > 0.05$). Logistic regression analysis showed that postnatal age, Cm, and RMP were independent contributing factors to vulnerability to ISM exposure (Table 2). The adjusted determination coefficient was 0.42, and the *F*-test showed the fitted line to be significant ($P < 0.00001$).

In this study, we reported three novel findings. First, we have demonstrated age-dependent shortening of the latency of the rapid inward current in the spinal lamina IX neurons. Surprisingly, this vulnerability develops quickly over a short neonatal period. Second, we found that RMP and Cm correlate with the latency of the rapid inward current. Third, we confirmed that age, Cm, and RMP are independent factors contributing to vulnerability.

It is generally accepted that ISM superfusion produces an outward current followed by an agonal inward current, which comprises a slow and subsequently rapid inward current. Once ISM is applied, intracellular ATP concentration decreases and intracellular Ca^{2+} concentration increases, which elicits an outward current in a portion of neurons because of the activation of both ATP-sensitive K^+ channels and Ca^{2+}-dependent K^+ channels [25]. Glutamate accumulates in the interstitial space and partially mediates the slow inward current via receptor activation [23]. In addition, the increase in intracellular Ca^{2+} and extracellular K^+ concentrations due to the inhibition of Na^+/K^+-ATPase activity contributes to the production of slow current [23]. Meanwhile, the rapid inward current is accompanied by a sudden increase in the intracellular Ca^{2+} concentration [23]. In addition to the action of glutamate receptors, the increase in the intracellular Ca^{2+} concentration is associated with Ca^{2+} influx via voltage-dependent Ca^{2+} channels, reversed operation of Na^+/Ca^{2+} transporters, and Ca^{2+} release from intracellular storage sites [7]. Furthermore, the rapid inward current is likely to be due to a nonselective increase in neuronal permeability of all participating ions; this will occur only in pathological conditions such as ischemia [23].

In this study, we found that the latency of ISM-induced rapid inward current drastically shortened between postnatal days 8 and 12. A parallel age dependency has also been reported in the spinal lamina V dorsal horn neurons of rats [6]. These findings suggest that maturation-dependent spinal neuronal vulnerability to ischemia is determined in the early postnatal period. Because no significant differences were found in soma size or passive electrophysiological membrane properties among the age groups, we conclude that age-dependent shortening of the latency is not because of the effects of increase in cell size or alteration in membrane properties. Interestingly, the number of ionotropic glutamate receptors is known to initially increase during the first 7 postnatal days prior to a decline to adult levels [4], which raises the possibility that glutamate, which plays a major role in excitotoxic cell death, is not

Table 2
Relationships between latencies of rapid inward current and contributing factors evaluated by multiple regression analysis.

	Regression coefficient	95% CI for regression coefficient		
		Lower	Upper	*P* value
Age (days)	−0.345	−0.574	−0.115	0.004
Cm (pF)	−0.015	−0.024	−0.006	0.038
RMP (mV)	−0.054	−0.104	−0.003	0.002

The adjusted determination coefficient was 0.42 and the *F*-test showed the fitted curve to significant ($P < 0.00001$). Postnatal age, Cm and RMP were independent contributing factors of vulnerability to ISM exposure.

involved in the development of vulnerability to ischemia during maturation.

Spinal motoneurons have large somas and long axonal processes; these characteristics greatly differ from those of many other types of neurons. Since these large neurons have high-energy demands for maintaining cytoskeletal components, they may be more susceptible to ischemic insult. Here, we demonstrated a significant correlation between Cm and the latency of ISM-induced rapid inward currents. Cm, which represents the surface areas of the recorded neurons, correlated with soma size, suggested the possibility that latency might also correlate neuronal volume; indeed, vulnerability to experimental ischemia with cell volume has been reported in the spinal cord ventral horn neurons [17,19] and the brain [13]. However, no significant correlation has been observed between the Cm and latency of rapid inward currents in the spinal dorsal horn neurons [17] while in the brain, large CA3 pyramidal neurons display high tolerance to ischemia [12]. These inconsistent findings implicate the involvement of other factors contributing to neuronal vulnerability.

In addition, we found a weak but significant correlation between RMP and current latency. In general, deprivation of oxygen and glucose impairs mitochondrial ATP production, thus compromising ionic gradients across the neuronal cell membrane [9]. In response to ISM superfusion, the membrane is gradually depolarized, leading to the activation of voltage-dependent calcium channels. Ca^{2+} accumulates in the intracellular space, subsequently inducing neuronal death [5,9]. Our finding suggests that lamina IX neurons with a higher hyperpolarized RMP, distant from the threshold of voltage-dependent Ca^{2+} channels, are more resistant to ischemic insults. However, the fact that all recordings were obtained at −70 mV raises doubts about RMP-dependent shortening of latency. One explanation is that incompletely voltage-clamped membrane distant from the patch electrode contributes to RMP-dependent vulnerability.

Furthermore, with logistic regression analysis, the contribution of postnatal age to susceptibility to experimental ischemia was found to be independent of the electrophysiological membrane properties. The determination coefficient (the squared value of the multiple correlation coefficient) has a value of 0.42, indicating that almost half of the variation in neuronal vulnerability for ischemia is explained by these factors. It has been reported that the number of mitochondria increases 4-fold between postnatal days 1 and 21, and is paralleled by an increase in respiratory enzyme content per mitochondrion [2]. This raises the possibility that postnatal development-dependent changes in the intracellular environment, such as metabolism or mitochondrial function, are involved in neuronal vulnerability.

Our study had certain limitations. First, we did not evaluate the age-dependent effect in adult rats. In order to identify large lamina IX neurons under IR-DIC microscope, it was necessary to exclude older rats, whose spinal neurons are obscured by highly developed fibrous tissue. Second, there was a possibility that the presence of the ISM-induced outward current affected the estimation of the slope of the slow inward current and thus the latency of the raid inward current. However, because the slope of the rapid inward current was quite precipitous, we considered that an error in determining the slope of the slow inward current would not greatly affect the estimation of the onset of the rapid inward current. Third, because the spinal lamina IX neurons studied included propriospinal interneurons, our observations may not exclusively apply to spinal motoneurons. Further studies with adult rat spinal motoneurons are required.

In conclusion, we determined that age, cell volume, and RMP may independently contribute to the susceptibility of spinal ventral lamina IX neurons to ischemia. These results emphasize that each contributing factor must be taken into consideration while evaluating spinal motoneuronal injury during development. We further propose that early postnatal maturation of the intracellular environment is an important factor affecting the development of vulnerability to excitotoxicity.

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