Infant Brain Stem Is Prone to the Generation of Spreading Depression During Severe Hypoxia

Frank Funke,1,2 Miriam Kron,2,3 Mathias Dutschmann,1,2,3 and Michael Müller1,2,3

1Deutsche Forschungsgemeinschaft Research Center for Molecular Physiology of the Brain, 2Zentrum für Physiologie und Pathophysiologie, Abteilung Neuro- und Sinnesphysiologie, and 3Bernstein Center for Computational Neuroscience, Georg-August-Universität Göttingen, Göttingen, Germany

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Funke F, Kron M, Dutschmann M, Müller M. Infant brain stem is prone to the generation of spreading depression during severe hypoxia. J Neurophysiol 101: 2395–2410, 2009. First published March 4, 2009; doi:10.1152/jn.91260.2008. Spreading depression (SD) resembles a concerted, massive neuronal/glial depolarization propagating within the gray matter. Being associated with cerebrovascular pathology, such as cerebral ischemia or hemorrhage, epileptic seizures, and migraine, it is well studied in cortex and hippocampus. We have now analyzed the susceptibility of rat brain stem to hypoxia-induced spreading depression-like depolarization (HSD), which could critically interfere with cardiorespiratory control. In rat brain stem slices, severe hypoxia (oxygen withdrawal) triggered HSD within minutes. The sudden extracellular DC potential shift of approximately −20 mV showed the typical profile known from other brain regions and was accompanied by an intrinsic optical signal (IOS). Spatiotemporal IOS analysis revealed that in infant brain stem, HSD was preferably ignited within the spinal trigeminal nucleus and then mostly spread out medially, invading the hypoglossal nucleus, the nucleus of the solitary tract (NTS), and the ventral respiratory group (VRG). The neuronal hypoxic depolarizations underlying the generation of HSD were massive, but incomplete. The propagation velocity of HSD and the associated extracellular K⁺ rise were also less marked than in other brain regions. In adult brain stem, HSD was mostly confined to the NTS and its occurrence was facilitated by hypotonic solutions, but not by glial poisoning or block of GABAergic and glycineric synapses. In conclusion, brain stem tissue reliably generates propagating HSD episodes, which may be of interest for basilar-type migraine and brain stem infarcts. The preferred occurrence of HSD in the infant brain stem and its propagation into the VRG may be of interest for basilar-type synapses. In conclusion, brain stem tissue reliably generates propagating HSD episodes, which may be of interest for basilar-type synapses.

INTRODUCTION

Spreading depression (SD) resembles a propagating wave of massive depolarization affecting neurons and glial cells. It is associated with a severe disturbance of ionic distribution and a sudden negative shift in the extracellular DC potential (Hansen 1985; Müller and Somjen 2000a; Nicholson and Kraig 1981; Somjen 2001). Since its discovery in rabbit cortex (Leão 1944), SD has been characterized in cortex (Leão 1944), hippocampus (Snow et al. 1983), cerebellum (Nicholson 1984), brain stem (Richter et al. 2003), spinal cord (Czéh and Somjen 1990), and the retina (do Carmo and Martins-Ferreira 1984). Compared with other brain regions, however, little is known about the spatiotemporal properties of SD in the vital neuronal networks of brain stem.

SD can be evoked by mechanical stimulation/injury, glutamate, high extracellular K⁺ concentration ([K⁺]), hypoxosmolarity, hyperthermia, alkalinization, tetanic electrical stimulation, and severe hypoxia/anoxia (for review, see Somjen 2001); the latter can be achieved either by oxygen withdrawal or mitochondrial poisoning by cyanide, azide, or uncouplers (Gerich et al. 2006). The SD event induced by hypoxia/anoxia shares several features with the normoxic SD, but there are also some differences. Accordingly, the hypoxic/anoxic event is commonly being referred to as anoxic depolarization, terminal depolarization, or spreading depolarization; we prefer the term hypoxia-induced spreading depression-like depolarization (HSD), introduced by the Somjen group more than a decade ago (for review, see Somjen 2001).

There is overwhelming evidence that SD is associated with pathological conditions of the human brain such as migraine (Lauritzen and Olesen 1984), traumatic brain injury (Strong et al. 2002), spontaneous intracerebral hemorrhage (Fabricius et al. 2006), subarachnoid hemorrhage and the following progressive ischemic damage (Dreier et al. 2006), ischemic stroke (Dohmen et al. 2008; Nedergaard and Hansen 1993), secondary injury within the penumbra (Hossmann 1994), and epileptic seizures (Fabricius et al. 2008; Gorji and Speckmann 2004; Van Harreveld and Stamm 1953).

The occurrence of SD causes a transient loss of membrane potential, synaptic failure, and block of axonal conduction. At the same time, it intensifies the metabolic demand of brain tissue. Accordingly, the term “depression” refers to the total loss of neuronal network function. Although in conditions such as migraine this causes a transiently blurred sight, referred to as aura, and is believed to be involved in the generation of the headache (Bolay et al. 2002; Lauritzen 1994), SD is more critical during cerebral stroke and hemorrhage, where it may worsen the outcome by aggravating secondary cell damage within the penumbra (Dohmen et al. 2008; Dreier et al. 2006; Mies et al. 1993). Assuming that SD episodes may even invade the brain stem region with its crucial role in cardiorespiratory regulation, a transient failure of these vital networks might be life-threatening.

Brain stem tissue has long been considered to be quite resistant to SD (Bureš et al. 1974). Meanwhile, however, a few reports have proved its occurrence in the brain stem. In an in vivo study on anesthetized rats, Richter and colleagues (2003) showed that extracellular application of K⁺, with acetate conditioning, induces SD in neonatal rats. Yet massive conditioning is required to force its generation in adult rat brain stem in vivo (Richter et al. 2008).
In the present study, we analyzed the conditions under which HSD can be elicited in the in vitro rat brain stem. Since previous publications suggested that SD is more prevalent in neonatal brain stem tissue, we performed a developmental study. Using acute tissue slices from infant, juvenile, and adult rats we monitored in a multiparametric approach the generation and propagation of HSD in various brain stem regions, including the hypoglossal nucleus (XII), the spinal trigeminal nucleus (Sp5), the nucleus of the solitary tract (NTS), and the ventral respiratory group (VRG). Severe hypoxia elicited HSD in brain stem slices at all developmental stages; the characteristic DC potential shifts coincided with an intrinsic optical signal (IOS, an increase in light scattering), pronounced neuronal depolarization, and a massive rise in [K+]i. The susceptibility to HSD clearly decreased with brain stem maturation. Among the putative underlying mechanisms we elucidated the functional influence of postnatal maturation of inhibitory systems, glial buffering, and the dimension of the interstitial volume (ISV). In addition we screened for complete loss of neuronal membrane potentials and less synchronized single-cell responses.

**METHODS**

**Preparation**

Brain stem slices were prepared from young Sprague–Dawley rats (postnatal day 5 [P5] to P15 or P19–P21, either sex) or adult males (4–6 wk old); in all, 78 rats were used. Deeply anesthetized rats (diethyl ether, Sigma–Aldrich) were decapitated; the brain was rapidly removed and transferred to ice-cold artificial cerebrospinal fluid (ACSF). The brain stem was isolated, glued onto an agarose block, and three to five transverse slices (400 μm thick) were cut from the area of the fourth ventricle using a vibrisslicer (752M, Campden Instruments). The slices were transferred to an Oslo-style interface recording chamber (35–36°C) and were allowed to recover for ≥90 min. Temperature settings and slice thickness are standard conditions known to ensure slice survival and to enable the repetitive generation of HSD in hippocampal preparations (Müller and Somjen 1998); therefore they were used for the brain stem experiments as well. The interface chamber was continuously aerated with carbogen (95% O2-5% CO2, 400 ml/min) and perfused with oxygenated ACSF (3–4 ml/min, 95% O2-5% CO2, pH 7.4) containing (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH2PO4, 24 NaHCO3, 1.2 CaCl2, 1.2 MgSO4, and 10 dextrose.

**Hypoxia-protocol and electrical recordings**

Severe hypoxia was induced by switching the recording chamber’s gas supply from carbogen to 95% N2-5% CO2; oxygenation of ACSF was continued. At a gas flow rate of 400 ml/min and a dead space volume of the recording chamber of about 150 ml, the pH 7.4 (Mueller and Somjen 2000a). K+-selective electrodes were calibrated before and after each experiment (0, 1, 2, 5, 10, 20, 50, and 100 mM K+). Their response slope averaged 53.1 ± 5.1 mV/ decade [K+]; their detection limit was 0.89 ± 0.58 mM K+ (n = 15). Electrode responses were recorded with an electrometer amplifier (FD 223, World Precision Instruments) and sampled at 100 Hz (Hepp and Muller 2008). Sharp microelectrodes for intracellular recordings were made from thin-walled borosilicate glass (GC 150F-10, Harvard Apparatus), filled with 2 M K-acetate + 5 mM KCl + 10 mM HEPES (pH 7.4); their resistances were about 80 MΩ. Current-clamp recordings were performed with an intracellular recording amplifier (SEC-05L, NPI Instruments, Tamm, Germany) and sampled at 2.5 kHz (Hepp et al. 2005). Bridge balance and electrode-capacitance compensation were adjusted before cell impalement and continuously controlled. Neuronal input resistance was proved every 10 s by a hyperpolarizing current (400–800 pA, 200-ms duration).

**Optical recordings**

The intrinsic optical signal (IOS) associated with SD/HSD resembles an increase in light scattering that can be followed by monitoring light reflectance at the tissue surface. The underlying molecular mechanisms are still unclear; nevertheless the IOS provides a useful tool to analyze the spatiotemporal profile of SD/HSD (Aitken et al. 1999; Andrew et al. 1999; Muller and Somjen 1999). Light reflectance at the tissue surface was monitored using a computer-controlled imaging system (Polychrome III, Till Photonics, Gräfelfing, Germany) and a sensitive CCD camera (Imago QF, PCO Imaging, Kelheim, Germany) as described earlier (Fischer et al. 2009; Hepp and Muller 2008). Interfaced slices were illuminated (white light) at an angle of about 45°. Images were taken at 2-s intervals and 15-ms exposure time using a ×5, 0.13 NA objective (Eiplan, Zeiss). Hypoxia-induced reflectance changes were visualized by off-line image subtraction, normalized to the prehypoxic baseline reflectance (Müller and Somjen 1999), and displayed in a 256 level gray-scale mode covering a range of ±10% brightness changes. The propagation velocity of HSD was calculated from the progression of the IOS wave front. The relative slice area invaded by HSD was determined at the height of the reflectance increase by counting those pixels whose brightness increase exceeded the baseline fluctuations (defined as three-fold standard deviation). To illustrate the spatiotemporal profile, the image sequence was color coded with respect to the time that has passed since HSD onset (first occurrence of an IOS). Image processing was performed with Tillvision 4.0 (Till Photonics), MetaMorph Off-line 6.1 (Molecular Devices, Sunnyvale, CA), and Matlab 7.3 (The MathWorks, Natick, MA).

**Statistics**

Up to three (neonatal) or five (adult) slices could be used from each brain stem. To ensure independence of observations, each experimental series was performed on at least three different rats. Numerical values are represented as mean ± standard deviation. The number of trials (n) indicates the slices investigated. Significance of the observed changes was tested using a two-tailed, unpaired Student’s t-test. For multiple comparisons, ANOVA followed by Fisher’s post hoc least significant difference test or the Kolmogorov–Smirnov test were used.
The level of significance was defined as $P < 0.05$; in the diagrams, significant changes are marked by asterisks ($*P < 0.05; **P < 0.01$).

**RESULTS**

Being interested in the disturbance of complex neuronal networks by reduced oxygen supply, we analyzed whether SD can be induced by severe hypoxia in acute tissue slices (400 µm) of infant (P5–P15), juvenile (P19–P21), and adult (4–6 wk) rat brain stem. The experiments were performed in an in vitro slice preparation because it allows for multiparametric recordings and, based on the intrinsic optical signal (IOS), a spatiotemporal analysis of the ignition, propagation, and tissue invasion of HSD. All experiments were performed in an interface recording chamber (35–36°C). Severe hypoxia was induced by switching the chamber’s gas supply from carbogen to 95% N2–5% CO2. Each slice was carefully rated with respect to its rostrocaudal brain stem position and the experiments were performed only on those nuclei being well contained in a given slice.

**HSD in infant brain stem**

In brain stem slices from infant rats, severe hypoxia induced within 248.6 ± 135.4 s (time to onset) characteristic DC potential deflections within the spinal trigeminal nucleus (Sp5), confirming the occurrence of HSD. Their amplitudes averaged $-21.0 \pm 4.8$ mV and, measured at the half-amplitude level, they lasted 51.7 ± 11.7 s (Fig. 1, n = 9). On reoxygenation (started 20 s after HSD onset) the DC potential recovered within about 1 min. The HSD-associated DC potential shifts recorded within the Sp5, hypoglossal nucleus (XII), ventral respiratory group (VRG), and nucleus of the solitary tract (NTS) differed considerably in terms of shape and time to HSD onset (Fig. 1A). Since each slice did not generate a HSD, we increased the extracellular K+ level to 8 mM (20 min before hypoxia was induced), a common maneuver to enhance neuronal activity in brain stem slices (Funke et al. 2007; Smith et al. 1991). With such conditioning, HSD was evoked more reliably and the time to HSD onset became shorter and less variable (Fig. 1B). Therefore 8 mM K+ conditioning was used consistently in all further experiments; higher levels of extracellular K+ were not tested.

The incidence and the parameters of HSD depended on the brain stem region from which we recorded (Fig. 2). Well-pronounced DC potential shifts occurred in the Sp5 in 59% of slices (29/49), in the XII in 33% (10/30), and in the VRG in 36% (9/25). Most reliably, DC potential shifts could be monitored in the Sp5. They occurred within 86.8 ± 47.5 s of hypoxia, had an amplitude of $-17.5 \pm 4.6$ mV, and lasted 46.8 ± 26.5 s (n = 29, Fig. 2B). These HSD parameters did not appreciably differ among the various brain stem nuclei, except for a somewhat shorter duration in XII and VRG (summarized in Fig. 2B and Table 1).

To characterize the detailed spatiotemporal profile of brain stem HSD, we combined electrophysiological and optical recordings. Based on the IOS, detectable as an increase in light reflectance at the tissue surface, the ignition site of HSD and the subsequently invaded brain stem areas can be identified (Fig. 3A) because the optical and electrical signs of HSD are closely coupled (Müller and Somjen 1998, 1999). The IOS was analyzed within the Sp5, XII, VRG, and NTS. As known from other brain areas, the negative DC potential deflection was accompanied by an increase in tissue reflectance in the interface chamber (Fig. 3A, Supplemental Movie S1).1 The maximum reflectance increase averaged 8.4 ± 2.5% (n = 8) for the Sp5 and its intensity and time course did not differ among the various nuclei (Fig. 3B, Table 1); on reoxygenation it fully recovered.

As indicated by detailed IOS analysis in 78 infant brain stem slices, HSD was restricted to the gray matter and did not enter the fiber tracts. It was preferably ignited within the dorsal aspects of the Sp5 (69% of slices) and then mostly spread out medially and dorsally (Fig. 4). Less frequently, it was ignited in the NTS or the VRG (21 and 8% of slices, respectively). Based on IOS analysis at the height of HSD, 25 ± 14% of a given hemisphere was involved. Most frequently invaded regions were the Sp5 (90%), NTS (46%), VRG (42%), and XII (27% of slices; Fig. 4B). In 9% of slices the IOS also invaded the contralateral hemisphere (Fig. 4A). Such crossing of the midline involved a short delay until the reflectance increase arose on the contralateral side; no optical changes were detectable in the commissural fiber tracts (Fig. 4A and Supplemental Movie S1). On average, HSD propagated at a velocity of $3.1 \pm 1.5$ mm/min (n = 17) and the invaded tissue area expanded at a rate of $22.3 \pm 16.0$ mm²/min. Since the field of view of the imaging system did not allow the simultaneous observation of both complete hemispheres of a slice, detailed analyses on the bilateral occurrence of HSD were not possible.

After having identified the infant Sp5 as the preferred site of HSD ignition, we monitored the hypoxic depolarization of single Sp5 neurons during HSD (with 8 mM K+ conditioning). To verify HSD ignition, the extracellular DC potential was monitored close to the impaled neuron. The intracellular recordings revealed a pronounced but incomplete depolarization of Sp5 neurons during HSD (Fig. 5A). Their resting membrane potential was slightly depolarized, obviously due to the elevated [$K^+]_o$ of 8 mM, averaging $-53.8 \pm 12.9$ mV, and their input resistance was $12.9 \pm 5.9$ MΩ (n = 12). Once HSD was ignited, Sp5 neurons rapidly depolarized to $-35.4 \pm 12.5$ mV and their input resistance decreased to 7.5 ± 4.5 MΩ (i.e., by $40.7 \pm 20.2%$). A train of action potentials that can usually be observed in, e.g., hippocampal neurons shortly before the rapid depolarization occurred in only 2 of 12 recorded Sp5 neurons. On reoxygenation, membrane potential and input resistance recovered (Fig. 5B).

With an incomplete neuronal depolarization during HSD being evident, we used K+-selective microelectrodes to monitor extracellular K+ levels (with 8 mM K+ conditioning) within the Sp5. During hypoxia, [$K^+]_o$ already rose before HSD onset to an average level of 14.3 ± 4.5 mM and then rapidly increased to its peak level of 49.5 ± 19.4 mM as HSD was ignited. On reoxygenation, [$K^+]_o$ recovered and temporarily undershot its prehypoxic baseline, reaching a nadir of 5.3 ± 1.1 mM (n = 8, Fig. 6A, Table 1). In those cases (5 of 13 slices), in which HSD failed to be ignited during 20 min of severe hypoxia, the DC potential shifted slowly by only $-4.8 \pm 4.4$ mV and [$K^+]_o$ rose only initially to a stable level of 16.1 ± 6.3 mM. On reoxygenation, a transient undershoot of the hypoxic (8 mM) K+ baseline to 4.6 ± 1.7 mM (n = 5)

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1 The online version of this article contains supplemental data.

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A: thionine (Nissl)-stained transversal slice of infant rat brain stem; anatomical landmarks and the nuclei recorded from are highlighted. The DC potential recordings obtained from different slices in 3.5 mM K\(^+\)-containing artificial cerebrospinal fluid (ACSF) confirm that HSD occurs in the spinal trigeminal nucleus (Sp5), hypoglossal nucleus (XII), nucleus of the solitary tract (NTS), and ventral respiratory group (VRG). The time to HSD onset and the shape of the DC potential shift is, however, quite heterogeneous. 4V, fourth ventricle; Amb, nucleus ambiguus; DVN, dorsal vagal nucleus; IO, inferior olive; sp5, spinal trigeminal tract.

B: comparison of the characteristic HSD parameters (DC potential amplitude, time to onset, and duration at the half-amplitude level: for definition see Sp5 trace in A) recorded from the infant Sp5 with normal (3.5 mM) and increased K\(^+\) concentration (8 mM) reveals that the time to HSD onset is decreased and less variable with 8 mM K\(^+\) conditioning. Error bars represent standard deviations; the number of trials is indicated at the bottom of the bars (**P < 0.01).


**Brain stem HSD can be repeatedly induced**

Evoking three HSD episodes, each separated by 20 min of recovery, did not reveal marked changes in the DC potential shifts or the associated changes in $[K^+]_{in}$ within the Sp5 (Fig. 6). The duration of the DC shift slightly increased to $113.7 \pm 13.6\%$ (second HSD) and $120.6 \pm 21.0\%$ (third HSD, $n = 8$), and the undershoot of the $[K^+]_{in}$ on reoxygenation was less pronounced for the second HSD (92.5 $\pm$ 10.2%) and third HSD (82.4 $\pm$ 19.3% of control, $n = 8$, Fig. 6B). The IOS tended to become slightly more intense and the relative tissue invasion increased from 22.7 $\pm$ 7.9% of a hemisphere to 33.2 $\pm$ 16.5% (second HSD) and 33.2 $\pm$ 16.3% (third HSD, $n = 15$, Fig. 6C).

**HSD in juvenile and adult brain stem**

$K^+$-induced SD in the in vivo brain stem can be elicited only up to an age of about 2 wk (Richter et al. 2003) unless massive conditioning is performed (Richter et al. 2008). We therefore elucidated whether this also applies to HSD in vitro. In brain stem slices of juvenile rats (P19–P21), severe hypoxia (with 8 mM $K^+$ conditioning) induced well-pronounced HSD episodes within the Sp5, VRG, and XII. As analyzed within the Sp5, the DC potential shift was less marked than that in infant slices, averaging only $-9.9 \pm 8.2$ mV ($n = 7$), although the time to HSD onset and the duration were comparable (Table 1).

In adult (4–6 wk) brain stem slices—despite 8 mM $K^+$ conditioning—usually only slow and moderate DC potential changes were observed during hypoxia (Fig. 7A), which averaged $-10.5 \pm 6.5$ mV in the Sp5 ($n = 13$) and recovered on reoxygenation. Similar slow DC potential changes occurred in the XII and even prolonged hypoxia ($\geq 20$ min) failed to induce characteristic HSD. An IOS in the Sp5, XII, and VRG was absent in most slices (80%), which further argues against rating these slow DC shifts as HSD. The only adult brain stem region that at least occasionally generated characteristic DC potential shifts, i.e., a sudden deflection exceeding $-10$ mV, was the NTS. In 21% of slices, characteristic DC potential deflections averaging $-19.1 \pm 4.9$ mV ($n = 7$) were observed (Fig. 7A, “NTS fast”), whereas 36% of slices showed only a slow DC potential shift, averaging $-9.7 \pm 4.8$ mV ($n = 12$; Fig. 7A, “NTS slow”). The remaining 43% of slices did not show pronounced DC potential changes during severe hypoxia. The IOS was confined to the NTS and thus affected only 1.7 $\pm$ 0.9% of the hemisphere ($n = 16$).

**Putative factors opposing HSD ignition in adult brain stem**

In the following experiments, we attempted to force HSD generation by addressing putative mechanisms underlying the reduced HSD susceptibility of adult brain stem slices. To elucidate whether glial maturation modulates HSD susceptibility, glial cells were poisoned by fluoroacetate (FAC, 5 mM, 3–5 h), a treatment causing the gradual loss of glial resting membrane potential (Largo et al. 1997). In six of nine FAC-treated slices, hypoxia induced only slow DC potential changes, even in the NTS (Fig. 7B); the time to onset did not noticeably differ from that of control slices. An IOS was observed, probably due to $K^+$ diffusion into less affected slice areas and/or the bathing medium.
detectable in only three slices and indicated an even less marked spread of HSD.

γ-Aminobutyric acid receptor type A (GABA\_A)–mediated currents are depolarizing at neonatal stages and then turn into hyperpolarizing currents as the Cl\^- equilibrium potential shifts during maturation (Rivera et al. 1999). Since modulation of Cl\^- conductances affects hippocampal HSD (Müller 2000) and synchronizes population activity in, e.g., respiration-controlling networks of the lower brain stem (Funke et al. 2008), we elucidated whether the maturational shift in Cl\^- equilibrium potential may also modulate the HSD susceptibility of brain stem. Blocking GABAergic and glycinergic synapses with bicuculline (20 μM) plus strychnine (0.5 μM, 20 min) failed, however, to facilitate the generation of HSD in the adult NTS (Fig. 7B, n = 4), nor did it noticeably affect HSD in the infant (n = 13) or juvenile Sp5 (n = 6).

The dimension of the interstitial volume (ISV) is another pivotal parameter. Hypotonic solutions decrease the ISV and synchronize population activity in, e.g., respiration-control networks of the lower brain stem (Funke et al. 2008), which did not produce HSD, [K\(^+\)]o slowly increased and stabilized to 6.4 ± 3.8 mM (i.e., by 35.4 ± 24.6%); a train of action potentials shortly before the hypoxic depolarization could not be observed (n = 12, Fig. 9B). On reoxygenation, the membrane potential recovered to −65.9 ± 9.7 mV in 10 of 12 neurons, whereas the membrane resistance tended to increase beyond control levels, to 14.4 ± 8.2 MΩ, in 9 of 12 neurons (Fig. 9B).

Recordings of [K\(^+\)]o within the NTS (conditioned with 8 mM K\(^+\) hypotonic solution) showed an initial increase to 13.2 ± 1.9 mM early during hypoxia followed by a rapid rise to 50.8 ± 11.4 mM when HSD was ignited. On reoxygenation, [K\(^+\)]o transiently undershot its hypoxic baseline (8 mM), reaching a nadir of 5.6 ± 0.7 mV (n = 5, Table 1). In those slices, which did not produce HSD, [K\(^+\)]o slowly increased and stabilized at 21.4 ± 6.7 mM; on reoxygenation it transiently undershot its hypoxic baseline, reaching 3.9 ± 1.6 mV (n = 15).

Inducing HSD repeatedly did not appreciably affect the DC potential shift or the changes in [K\(^+\)]o. The duration of the DC shift slightly increased to 111.1 ± 4.5% (second HSD) and 125.2 ± 18.8% (third HSD), the onset of HSD was somewhat postponed for the third HSD episode (127.3 ± 26.0%), and [K\(^+\)]o reached a higher peak level during the second HSD (116.3 ± 8.5% of control, n = 5, Fig. 10A). The tissue invasion

### Table 1. Summary of the HSD parameters recorded in various brain stem nuclei at different levels of maturation

<table>
<thead>
<tr>
<th>Conditioning stimulus</th>
<th>Infant</th>
<th>Juvenile</th>
<th>Adult</th>
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<tr>
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<tr>
<td>Sp5</td>
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<td>Sp5</td>
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<tr>
<td>Conditioning stimulus</td>
<td>8 mM K(^+)</td>
<td>8 mM K(^+)</td>
<td>8 mM K(^+)</td>
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<tr>
<td>Amplitude, mV</td>
<td>−17.5 ± 4.6</td>
<td>−18.7 ± 3.8</td>
<td>−16.5 ± 9.0</td>
</tr>
<tr>
<td>Time to onset, s</td>
<td>86.8 ± 47.5</td>
<td>84.2 ± 45.7</td>
<td>92.4 ± 15.9</td>
</tr>
<tr>
<td>Duration, s</td>
<td>46.8 ± 26.5 (n = 29)</td>
<td>27.6 ± 18.3 (n = 10)</td>
<td>24.2 ± 20.8 (n = 9)</td>
</tr>
<tr>
<td>Incidence</td>
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<td></td>
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<tr>
<td>DC</td>
<td>59.2%</td>
<td>33.3%</td>
<td>36.0%</td>
</tr>
<tr>
<td>IOS</td>
<td>89.7%</td>
<td>26.9%</td>
<td>42.3%</td>
</tr>
<tr>
<td>IOS intensity [K(^+)]o</td>
<td>8.4 ± 2.5% (n = 8)</td>
<td>6.2 ± 1.4% (n = 7)</td>
<td>7.3 ± 1.7% (n = 9)</td>
</tr>
<tr>
<td>Threshold, mM</td>
<td>14.3 ± 4.5</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>Peak, mM</td>
<td>49.5 ± 19.4</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>Undershoot, mM</td>
<td>5.3 ± 1.1 (n = 8)</td>
<td>n.d.</td>
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Values are means ± standard deviation; n = number of slices, n.d., not determined. Summarized are the parameters of the DC potential shifts (amplitude, time to onset, duration), the incidence of HSD (fraction of slices generating HSD, detected as either DC shift or IOS), the intensity of the IOS (increase in tissue reflectance), and the characteristic parameters of the extracellular K\(^+\) changes (threshold level at HSD onset, peak level at the height of HSD, transient undershoot of the baseline level upon reoxygenation).

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tended to increase slightly with every HSD episode from 2.3 ± 1.7% (first HSD) to 3.2 ± 1.6% (second HSD) and finally to 5.4 ± 3.1% (third HSD), but the IOS intensity was not affected (n = 5; Fig. 10B).

**DISCUSSION**

The present study focuses on the susceptibility of rat brain stem to the generation of SD during severe hypoxia. It is the first study characterizing HSD in in vitro brain stem and performing a detailed multiparametric analysis of the events associated with this depolarizing wave in the vital neuronal networks crucially involved in respiratory, cardiac, and circulatory control.

**Hypoxia elicits HSD in brain stem slices, even without prior conditioning**

We triggered SD by severe hypoxia because it can be reliably induced within seconds and, in contrast to SD-stimu-
lating chemical compounds, it can be reversed just as quickly. Furthermore, no mechanical injury or chemical poisoning of the tissue occurs, an important issue when repetitive SD episodes and drug effects are studied. Importantly, however, since the entire slice is being exposed to the SD-generating stimulus, those areas with the highest HSD susceptibility can be identified and the HSD propagation analyzed.

Brain stem is considered a structure of low SD susceptibility (Bureš et al. 1974; Somjen et al. 1992). The assumed reasons include less compact neuronal packing, wider ISV, and higher content of glia (Bureš et al. 1974), all of which oppose the generation of SD and HSD. Earlier studies analyzing the effects of hypoxia or other metabolic compromise on brain stem networks (Ballanyi et al. 1992; Cowan and Martin 1992; Jiang et al. 1992; Xia et al. 1992) were performed in superfusion chambers and, in part, at lower temperatures and/or increased glucose levels (30 mM), all of which are less favorable for HSD generation (Gerich et al. 2006; Schurr et al. 1987; Takaoka et al. 1996). In dorsal vagal neurons, for example, we obtained a massive neuronal depolarization resembling the hypoxic depolarization associated with HSD only when the duration of chemical anoxia (cyanide) was extended to several minutes (see discussion in Müller et al. 2002) or when glycolysis was blocked (Müller and Ballanyi 2003).

The first evidence that normoxic SD can be induced in anesthetized infant rats by topical application of KCl onto the exposed brain stem—given that prior conditioning with sodium acetate diminishes Cl⁻-mediated inhibition—was provided by Richter and colleagues (2003). Such conditioning, which is also required to induce SD in cerebellum (Nicholson 1984) and to facilitate its propagation in the retina (Martins-Ferreira et al. 1974), shifts the balance of excitation and inhibition in favor of excitation, thereby decreasing the ignition threshold of SD. In the present study, we conditioned brain stem slices by increasing [K⁺]₀ around 20 min before hypoxia was induced. Such elevation of [K⁺]₀ has been shown to potentiate the persistent sodium current (Somjen and Müller 2000), which could further increase neuronal excitability. In computer simulations, eleva-
the more heterogeneous cellular structure of brain stem a sufficiently high degree of synchronization of the neuronal depolarizations is obtained. Sustained negative DC shifts, as have been reported for KCl-induced SDs with acetate conditioning (Richter et al. 2003), were not observed; all slices fully recovered on reoxygenation. During HSD, [K\(^+\)]\(_o\) rose to about 50 mM, which exceeds the 26 mM increase in [K\(^+\)]\(_o\) reported for in vivo brain stem in anesthetized rats (Richter et al. 2003), but is less than the K\(^+\) peak we quantified for hippocampal HSD in the very same recording chamber (77–78 mM; Hepp and Müller 2008; Müller and Somjen 2000b). One possible reason for the less intense K\(^+\) levels at the height of HSD might be the incomplete hypoxic depolarization of the single neurons proven by the intracellular recordings. Despite showing a massive depolarization, the single neurons still maintained an average membrane potential of \(-35\) mV (infant Sp5) and \(-36\) mV (adult NTS) at the height of HSD; even corrected for the extracellular DC potential shift, their membrane potentials still measured \(-22.8\) ± 14.1 mV (n = 12) and \(-24.9\) ± 10.9 mV (n = 12), respectively. Also the decreases in input resistance were less marked in Sp5 and NTS neurons than the severe (83–89\%) reduction observed in hippocampal CA1 neurons (Hepp et al. 2005; Müller and Somjen 2000a).

Consistent recordings of the IOS enabled a detailed spatiotemporal analysis of brain stem HSD. If just microelectrodes were used, several HSD episodes would have remained undetected, especially in adult brain stem, where HSD was confined to the NTS. IOS analysis identified the infant Sp5 as the preferred site for HSD ignition and provided evidence for the invasion of the NTS, VRG, XII, and occasionally even the contralateral hemisphere. The consistent occurrence of HSD in the Sp5 corresponds to the in vivo experiments by Richter and colleagues (2003), who recorded SD in the transition region of the trigeminal subnucleus caudalis/upper cervical cord (Ve/C1). Furthermore, they observed changes in heart rate and arterial blood pressure as well as disturbed respiration (Richter et al. 2008), which confirm that SD in vivo also affected the cardiorespiratory control centers of the rat brain stem. A more detailed spatiotemporal analysis could not be performed with their triple electrode array, but a propagation of SD into the contralateral hemisphere was excluded (Richter et al. 2003). Nevertheless, it should be kept in mind that the detailed spread of HSD episodes may vary in vivo due to the more complex tissue geometry, vascular effects during hypoxia and HSD, and a more efficient oxygen supply via nearby capillaries.

The peak intensity of the IOS, 6–8\% reflectance increase for the various infant nuclei, and the propagation velocity of HSD (3.1 mm/min) were less notable than those in hippocampal slices (12–13\% reflectance increase, 7–11 mm/min propagation velocity; Gerich et al. 2006; Hepp and Müller 2008), even though the amplitudes of the DC potential shifts are similar in brain stem and hippocampus. Since the IOS is most pronounced in the dendritic layers of, e.g., the hippocampal formation (Aitken et al. 1999; Fayuk et al. 2002; Müller and Somjen 1999), the more heterogeneous structure and less organized neuronal arrangement of brain stem may be responsible for the less intense IOS. The very mechanisms generating the IOS are not entirely understood (Aitken et al. 1999; Andrew et al. 1999; Fayuk et al. 2002; Gerich et al. 2006; Müller and Somjen 1999), but the absence of a reflectance increase during the slow DC potential changes in adult brain stem.

As we have clearly proved, HSD in infant brain stem slices can be elicited at normal (3.5 mM) K\(^+\) levels. In contrast, in anesthetized rats hypoxia alone (6% O\(_2\) in N\(_2\)) was insufficient to induce brain stem SDs. Only combined with topical application of KCl to the brain stem surface did it elicit SD (Richter et al. 2008), suggesting a lower ignition threshold of HSD in acute brain stem slices than that under in vivo conditions. A possible reason might be the anesthesia during in vivo recordings. Other reasons for the increased HSD susceptibility in the in vitro brain stem may arise from the absence of blood flow. Oxygen supply is diffusion limited and also the clearance of K\(^+\) from the extracellular space can be considered to be less efficient. Nevertheless, a major advantage of the slice preparation is that it does allow for multiparametric recordings and a detailed noninvasive optical analysis of the spatiotemporal HSD profile.

The DC potential shifts recorded from infant brain stem slices showed the typical profile known from other brain regions. Their amplitudes of up to \(-25\) mV verify that despite the decrease in input resistance amounts to about 40\%.

Table 1. The hypoxic depolarization of infant trigeminal neurons during HSD is pronounced but incomplete. A: extracellular DC potential shift \((V_o)\) and sharp electrode (current-clamp) recording of the hypoxic depolarization of a trigeminal neuron. Negative deflections in the current-clamp trace were evoked by \(-400\)-pA test pulses applied every 10 s to probe for changes in input resistance. Note that the recorded neuron appreciably depolarizes as soon as the DC potential shift occurs, but at the height of HSD still maintains a membrane potential of \(-31\) mV. B: membrane potential \((V_m)\) and input resistance \((R_m)\) changes during HSD. Plotted are the averaged parameters \((n = 12)\) determined during hypoxic control conditions \((a)\), at the height of HSD \((b)\), and on posthypoxic recovery \((c)\), as defined in the current-clamp trace in A. The decrease in input resistance amounts to about 40\%.

![Figure 5](http://jn.physiology.org/ by 10.220.247 on September 29, 2016)
FIG. 6. HSD in infant brain stem is accompanied by a massive rise in [K⁺]ₒ and can be repeatedly evoked. A: recording of the extracellular DC potential (Vₒ) and K⁺ concentration ([K⁺]ₒ) within the Sp5. The slice underwent 3 HSD episodes, separated by a nearly 20-min recovery. Obvious changes in the HSD parameters or the characteristic extracellular K⁺ levels (threshold at HSD onset, peak, and undershoot on reoxygenation) were not observed. B: statistical comparison confirmed the consistency of the single HSD episodes. Only a minor increase in the duration of the DC potential shifts was observed for the 2nd and 3rd HSDs and the transient undershoot of the K⁺ baseline level was somewhat less marked. Plotted are the averages of 8 slices. C: inducing HSD repeatedly did not appreciably affect the IOS either. The IOS peak intensity recorded in the Sp5 at the height of HSD tended to rise slightly (n = 6) and the invaded tissue area moderately increased for the 2nd and 3rd HSD episodes (n = 15).
stem suggests that a fast DC shift—i.e., a high degree of neuronal synchronization—is required to generate the increase in light scattering. Furthermore, the initial decrease in light scattering (reduced tissue reflectance), which is evident in hippocampal slices shortly before HSD onset and obviously reflects cell swelling early during hypoxia (Aitken et al. 1999; Fayuk et al. 2002; Müller and Somjen 1999), could not be observed in brain stem slices. This may indicate a lesser degree of cell swelling during HSD in brain stem. It should be noted here that the IOS in vivo is even more complex due to a prominent contribution of hemoglobin-associated light absorption. This renders the in vivo IOS sensitive to changes in blood flow as well as the degree of hemoglobin oxygenation.

During brain stem maturation HSD susceptibility is fading

In contrast to slowly maturing higher brain circuits, the brain stem—being devoted to respiratory/circulatory control—has to be functional at birth. This may explain why HSD can be reliably induced in neonatal brain stem. Surprisingly, with maturation the brain stem then converts into a relative SD-resistant network. The HSD susceptibility of neocortical and hippocampal networks is also age dependent, albeit less pronounced. Picrotoxin, 4-aminopyridine, and low Mg²⁺ solutions elicit SD most efficiently in juvenile (P13–P16) rat neocortical slices (Wong and Yamada 2001), and 4-aminopyridine most reliably induces hippocampal SD at an age of P11–P20 (Psarropoulou and Avoli 1993). In both studies, the
probability of SD induction declined with age, although SD still can be easily induced in older animals. The same applies to \( \text{K}^+ / \text{H}^{+} \)-induced seizures followed by SD in rat hippocampus, whose incidence peaks at P18–P22 (Kreisman and Smith 1993). Hippocampal slices of premature rabbits (P8–P12) were even reported to generate spontaneous SD episodes (Haglund and Schwartzkroin 1984).

Our finding that HSD is more difficult to elicit in adult brain stem slices extends previous studies (Bureš et al. 1974; Richter et al. 2003) in which SD could not be induced in adult brain stem by mechanical stimulation (pin-pricking) or topical KCl application. Only massive conditioning (acetate substitution of 70% of Cl\(^{-}\), 10 mM K\(^{+}\), and 10 mM tetraethylammonium) rendered adult brain stem susceptible to SD and, only then, did topical application of KCl crystals or deep KCl injections trigger propagating SD episodes (Richter et al. 2008).

In our experiments on adult slices, hypoxia (with 8 mM K\(^{+}\) conditioning) induced just slow DC potential shifts; only the NTS occasionally generated the characteristic DC potential profile. This suggests that only within the NTS could a sufficient degree and synchronization of the neuronal depolarization be achieved to drive the generation of HSD. In accordance with our earlier published flow chart model of HSD (Müller and Somjen 2000b), it is the positive feedback provided by the contributing interacting variables (neuronal depolarization, extracellular K\(^{+}\) accumulation, glutamate receptor activation, cell swelling, activation of voltage-gated Na\(^{+}\) and Ca\(^{2+}\) inward currents, etc.), which drives the self-regenerative generation of...
HSD and its propagation. Accordingly, in adult brain stem, except for the NTS, a sufficient degree of “positive feedback” was not achieved; HSD failed to be ignited or to propagate into the other nuclei. On the single-cell level, this lack of synchronization and feedback obviously resulted in either incomplete and slow or only minor hypoxic depolarizations. These in turn were able to give rise to only the slow and moderate DC potential changes, that were observed in part in the adult NTS and always in the adult Sp5 and XII. An obvious consequence of such reduced synchronization and feedback is the dissociation of the hypoxic neuronal depolarization and the rapid DC potential shift that was occasionally observed in the adult NTS. In view of potential neuronal injury the failure of HSD ignition/propagation and massive neuronal depolarization is clearly advantageous. Neuronal injury may arise from the secondary Ca\(^{2+}\)/H\(^{+}\) influx mediated by N-methyl-D-aspartate (NMDA) receptors and voltage-gated Ca\(^{2+}\) channels (Lipton 1999; Siesjö 1986). Accordingly, in the absence of a massive depolarization, the neurons are also spared from the massive Ca\(^{2+}\) load (Müller and Ballanyi 2003).

Since the amount of glial cells and their K\(^{+}\) buffering capacity modulate the susceptibility to SD (Bureš et al. 1974; Kager et al. 2002), we attempted to force the generation of HSD by glial poisoning. In contrast to the hastened HSD onset achieved in hippocampal slices (Largo et al. 1997; Müller and Somjen 1999), however, FAC poisoning failed to facilitate HSD onset in adult brain stem slices. The already severely restricted propagation of HSD seemed even more limited, suggesting that FAC may have functionally impaired brain stem neurons as well.

The maturational switch from depolarizing to hyperpolarizing GABA\(_A\)-mediated currents arises from a shift in the Cl\(^{-}\) equilibrium potential based on K\(^{+}\)/Cl\(^{-}\) cotransporter KCC2 expression (Rivera et al. 1999). In mouse VRG this occurs during the first postnatal week (Ritter and Zhang 2000). Yet block of inhibitory synapses by strychnine plus bicuculline treatment failed to force the generation of HSD in adult brain stem, nor did it affect HSD in infant slices. Accordingly, maturational changes of inhibitory synapses seem unlikely as a primary cause for the low HSD susceptibility of adult brain stem.

The susceptibility of HSD is also determined by the dimension of the ISV (Bureš et al. 1974; Chebabo et al. 1995). Decreasing the ISV by moderate hypotonic treatment facilitated the generation of HSD, at least within the NTS of adult brain stem. In adult rat hippocampal slices, omitting 20 mM NaCl from the ACSF reduced ISV by 24% (Fayuk et al. 2002). Since we omitted only 15 mM NaCl from the ACSF, the ISV restriction was probably even less, but still it facilitated the occurrence of HSD in the adult NTS. It should be mentioned though that ISV restriction is quite unspecific, affecting all extracellular ion levels, transmitter levels, and extracellular current flow. Nevertheless, the increased possibility of generating sudden DC potential deflections in the NTS suggests that

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**FIG. 10.** In the NTS of adult brain stem HSD episodes can be repeatedly evoked. **A:** inducing HSD repeatedly (hypotonic 8 mM K\(^{+}\) conditioning) did not reveal marked changes in the electrical HSD parameters or the characteristic K\(^{+}\) levels. Only a minor increase in the duration of the DC potential shift was observed for the 2nd and 3rd HSDs and the onset of the 3rd HSD was slightly postponed. During the 2nd HSD the peak level of [K\(^{+}\)], was somewhat higher (n = 5). **B:** repeated induction of HSD did not appreciably affect the IOS either. The IOS intensity did not change significantly (n = 5) and the tissue invasion tended to increase slightly. Nevertheless, HSD remained confined to the NTS.
hypotonic treatment improved the synchronization of the hypoxic depolarizations of the single neurons. Accordingly, a relatively wide ISV may be one of the putative parameters contributing to the lower HSD susceptibility of brain stem in general. Yet further widening of the ISV during maturation is unlikely because in rat cortex the ISV decreases during maturation, approaching its final extent around P21 (Lehmenkühler et al. 1993). No such detailed analyses have ever been performed for the brain stem, but it seems reasonable to assume that a decrease in ISV also occurs during brain stem maturation. Accordingly, the differing HSD susceptibility of infant and adult brain stem slices seems unlikely to be the consequence of maturational ISV changes.

The NR2 subunit composition of brain stem NMDA receptors also undergoes postnatal changes. The NR2D subunit expression is most intense about 1 wk after birth and then dramatically decreases with maturation (Kron et al. 2008; Monyer et al. 1994). Interestingly, incorporation of this subunit results in a weak Mg\(^{2+}\) block and long decay time constants of the NMDA receptor-mediated currents (Monyer et al. 1994). With the central role of NMDA receptor-mediated excitation in the generation of SD and HSD (Marrannes et al. 1988; Müller and Somjen 2000b), such changes in receptor subunit composition may contribute to the attenuation of HSD susceptibility during brain stem maturation.

Na\(^+/K\(^+\)\) ATPase activity also changes during maturation. Due to its reduced activity in premature networks (Haglund et al. 1985), repetitive stimulation leads to higher [K\(^+\)\]o levels in premature (P16–P30) than in mature (>P40) rat neocortex (Hablitz and Heinemann 1989). Also the different SD susceptibility of the hippocampal CA1 and CA3 subfields is assumed to arise from different levels of Na\(^+/K\(^+\)\) ATPase activity (Haglund and Schwartzkroin 1990).

Yet another putative factor may be changes in connexin expression. Both interneuronal and glial gap junctions seem to contribute to the propagation of SD (Largo et al. 1997; Shapiro 2001; Somjen 2001). In the brain stem, connexin expression changes (decrease in connexin26 and increase in connexin32) are already evident during the second and third postnatal weeks and become more pronounced in adulthood. Since connexin26 is expressed in neurons and astrocytes, whereas connexin32 is present in neurons only (Solomon et al. 2001a,b), it seems that with brain stem maturation glial gap junctions are especially down-regulated.

**Functional implications of HSD in brain stem**

Due to the crucial role of brain stem in cardiorespiratory control, a transient loss of function associated with the occurrence of SD may be of devastating systemic impact. Indeed, brain stem SD in adult rats was paralleled by a decrease in heart rate and an increase in cerebral blood flow and arterial blood pressure (Richter et al. 2008). Respiratory was disturbed during long DC shifts in neonatal brain stem and came to a stop during sustained DC shifts (Richter et al. 2003). This is in accordance with our observations that especially in infant and juvenile rats, HSD episodes often invaded the VRG. A detailed analysis on the impact of brain stem HSD on cardiorespiratory control, however, would require in vivo approaches.

Brain stem SD has already been assumed earlier to be involved in basilar-type migraine or to play a speculative role in sudden infant death syndrome (Cutrer and Baloh 1992; Richter et al. 2003, 2008). There are several indications for an indirect and direct involvement of the trigeminal nucleus in migraine. For example, hippocampal and cortical SDs lead to afferent activation of the caudal trigeminal nucleus, which then contributes to nociception and vasodilation of the middle meningeal artery by stimulating rostral brain areas and the superior salivatory nucleus, respectively (Bolay et al. 2002; Kunkler and Kraig 2003). A direct causal link may exist for basilar-type migraine, a rare subtype of migraine mainly occurring in young adults (Bickerstaff 1961). It is paralleled by symptoms arising from brain stem or both cortical hemispheres, i.e., dysarthria, vertigo, tinnitus, loss of hearing, double vision, cerebellar-type ataxia, bilateral paresthesias, perioral numbness, and decreased levels or even loss of consciousness (Bickerstaff 1961; Kirchmann et al. 2006). The decreased level of consciousness, especially, has been proposed to arise from a SD event (Cutrer and Baloh 1992).

Speculating about an involvement of brain stem SDs in sudden infant death syndrome (SIDS) would even render an SD event life-threatening. SIDS is characterized by a spontaneous arrest of breathing, assumed to be the fatal consequence of genetic predisposition combined with hyperthermia, prone sleep position, and bed sharing (for review, see Moon et al. 2007). Just recently a link of SIDS and 5-hydroxytryptamine (serotonin [5-HT]) pathology has been assumed, potentially including abnormal 5-HT neuron firing, 5-HT synthesis, release, and clearance, as well as reduced 5-HT\(_{1A}\) receptor binding (Audero et al. 2008; Paterson et al. 2006). Interestingly, in parietal cortical slices of adult rats 5-HT\(_{1A}\) receptor stimulation attenuates the duration of SD episodes (Krüger et al. 1999), suggesting that intact serotonergic signaling decreases the susceptibility of neural tissue to SD. In addition, hyperthermia—being assumed to be a risk factor in SIDS—increases the susceptibility to SD and may even be sufficient to elicit spontaneous SD episodes (Wu and Fisher 2000). In view of these findings the proven occurrence of SD and HSD, especially in the infant brain stem, could be a causal link to SIDS and deserves further analyses.

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