Spreading Depression Can Be Elicited in Brain Stem of Immature But Not Adult Rats

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INTRODUCTION

Spreading depression (SD) in the cerebral cortex is a neuronal mechanism that is thought to be involved in pathological brain functions. Cortical SD (CSD), first described by Leão (1944), is a transient local negative DC potential shift (Leão 1947) that propagates from a focus across the cortical hemisphere. It is paralleled by a rapid and substantial increase in potassium concentration in the extracellular space (\([K^+]_e\)), water influx into cells, and shrinkage of the extracellular space volume (Gardner-Medwin 1981; Kraig and Nicholson 1978; Nicholson 1993; Somjen 2001). Such CSDs may be followed by a permanent increase in the diffusion parameters, namely extracellular volume fraction and tortuosity, and these may be caused by a reactive astrogliosis (Mazel et al. 2002). During CSD the normal electroencephalographic activity is transiently abolished by the NMDA receptor blocker MK-801. Thus we demonstrate that the immature brain stem has the capacity to generate negative DC shifts, which could be relevant as a risk factor in newborn brain stem function.

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and Hunsaker 2002). In the present study we found that SD cannot be elicited in the brain stem of the adult rat, even after conditioning. However we were able to show that SD can be evoked in the brain stem of the very young rat, after conditioning with either acetate or hypoxia.

**METHODS**

**Recording of DC shifts in the cerebral cortex and the brain stem**

Wistar rat pups of 6–14 days were anesthetized with urethane (1.5 g/kg ip, Sigma-Aldrich GmbH, Seelze, Germany), tracheotomized, and placed in a head holder. They breathed spontaneously room air. Body temperature was maintained by a feedback-controlled heating pad at 37°C. A trephination (2–3 mm diam.) was made over the occipitotemporal cortex; the dura was incised. The brain stem was monitored and body temperature was kept at 37°C simultaneously room air. Mean arterial blood pressure was continuously monitored and the animals were tracheotomized and breathed spontaneously to stimulation of the nictitans to evoked spreading negative DC shifts. After KCl only a positive DC shift was seen in the brain stem that returned slowly to baseline, and SD-duration (time interval between beginning of depolarization and repolarization). A mechanograph (custom-built lever with a piezo-electric force transducer) monitored thorax movements that were written on a y-t-chart-recorder. Thorax excursions at intervals of 15 s were counted and converted into breathing frequency per minute.

**RESULTS**

**Negative DC shifts in the cortex and brain stem of rat pups and adult rats**

In the exposed cortex (circle at left skull side) or the exposed brain stem a pinprick was applied to a stimulation site (crosses) and DC shifts were recorded with DC electrodes at adjacent and remote recording sites (Fig. 1A). Figure 1, B–D shows recordings of DC potentials in five rats of different ages. Pinprick to the cortex (P) evoked at a latency of about 1.5 min a negative DC shift (upward deflection) at the remote cortical recording electrode in a 13-day-old rat and in an adult rat, but not in rat pups that were 6, 11, and 12 days old (Fig. 1B). By contrast, in the brain stem of the same rats neither pinprick (not shown) nor the application of KCl to the brain stem surface evoked spreading negative DC shifts. After KCl only a positive DC deflection was seen in the brain stem that returned slowly to baseline (Fig. 1C). In the same rats we tested whether DC shifts could be
elicited in the brain stem after sodium acetate superfusion. After acetate, KCl evoked negative DC shifts in rats up to the age of 12 days but not in adult rats (Fig. 1D). In the five 13-day-old rats tested, we could only elicit negative DC shifts by KCl in one rat. These negative DC shifts were either comparable to those usually observed in the cortex (fast rising with time to peak < 16 s, and recovery within 2 to 4 min, see examples in the second and third traces in Fig. 1D), or they appeared as sustained negative DC deflections (slow rising, slow recovery). In 6- or 7-day-old rats, negative DC deflections rose very slowly and did not exceed amplitudes of 6 mV (see example in the first trace in Fig. 1D). Table 1 summarizes the data from all experiments and compares the numbers of attempts (KCl applications) with the numbers of elicited negative DC deflections in the different age groups. Figure 2 shows the localization of the electrode tips within the brain stem in one 12-day-old rat. The electrode tips were deep in the gray matter and the trace of the deepest electrode can be followed down to the ventral gray matter.

Next we tested whether the DC shifts in the brain stem are comparable to SDs in the cerebral cortex. Cortical SDs are characterized by parallel increases of the extracellular potassium ([K+]e) and by propagation of the transient DC shift away from the stimulation site. Negative DC shifts in the brain stem (Fig. 3A, bottom trace) were paralleled by an increase of [K+]e (Fig. 3A, top trace). The [K+]e increases were in the range of 25.9 ± 9.3 mM (mean ± SD, n = 12 DC shifts in 3 rats). By contrast, slight DC deflections in a range of 0.5 ± 2.9 mV that

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**TABLE 1. Occurrence of KCl-induced negative DC shifts in the brain stem before and after acetate conditioning of rats at different postnatal ages**

<table>
<thead>
<tr>
<th>Age of rat, days</th>
<th>No. of rats tested</th>
<th>No. of rats showing negative DC shifts after acetate*</th>
<th>DC shifts†</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5/6 (sustained)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>32/68 (CSD-like)</td>
</tr>
<tr>
<td>10/11</td>
<td>14</td>
<td>14</td>
<td>0/16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19/68 (sustained)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7/16 (CSD-like)</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3/16 (sustained)</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>1</td>
<td>0/5</td>
</tr>
<tr>
<td>Adult, &gt;90</td>
<td>9</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0/29</td>
</tr>
</tbody>
</table>

* Number of rats for which negative DC shifts could be evoked by KCl.
† Positive elicitation vs all attempts. ‡ Positive elicitation are subdivided into CSD-like and sustained (sust.) negative DC deflections.
were not classified as negative DC shifts were only accompanied by increases in \([K^+]_e\), in the range of 7.7 ± 1.4 mM (mean ± SD, respectively; \(n = 7\) KCl applications in 3 rats). Furthermore, negative DC shifts were spreading. In a typical experiment KCl caused a DC shift in the brain stem that was observed first at the electrode with the tip at a depth of 800 μm (closest to the stimulation site). After a delay, the DC shifts were observed at the other 2 electrodes that had at a greater distance from the stimulation site and whose tips were located at depths of 1,600 and 1,200 μm.

Effect of negative DC shifts on breathing frequency

During prolonged negative DC shifts breathing frequency was impaired. Superfusion of the brain stem with acetate had no influence on breathing frequency. A KCl-evoked short-lasting DC shift after acetate conditioning did not substantially change breathing frequency (Fig. 5A). By contrast, respiration came to a complete stop during a KCl-evoked sustained negative DC shift (Fig. 5B). When superfusion was started to recover the animal the DC shift was reduced and respiration recovered (Fig. 5B). Figure 5C shows the relationship between SD duration and effect on breathing frequency from all experiments. The longer the negative DC shift, the higher the incidence of less frequent respiratory movements.

It is noteworthy, that in rat pups the frequency of respiratory movements was not reduced when a particular KCl application did not elicit a DC shift. Furthermore, without previous acetate conditioning, KCl neither induced negative DC shifts in the brain stem nor reduced respiration frequency (19 rats at the age of 6–13 days). Importantly, adult rats (\(n = 9\) rats) usually showed positive DC shifts after KCl but neither negative DC shifts nor reductions of respiratory frequency when KCl was applied to the brain stem after acetate.

Because DC shifts in rat pups can only be elicited after
conditioning we asked whether physiological or pathophysiological conditions rather than acetate application could trigger DC shifts. Indeed, asphyxia or hypoxia were able to trigger DC shifts. Further, the ability of the brain stem to generate negative DC shifts was lost during maturation. A major obvious consequence of the reduction of the extracellular chloride concentration was also measured at 800 μM (middle trace). A: DC shifts in the cortex and in the brain stem elicited by KCl following acetate conditioning, but before MK-801. B: KCl-evoked DC shifts and extracellular K+ rise in the brain stem after 3 mg/kg MK-801. C: Bar diagram comparing mean values ± SD of DC amplitudes in mV, peak times in s, decline times in s, and durations in s in 11-day-old rats. Hollow bars, animals before 3 mg/kg MK-801 (n = 10); black bars, animals after 3 mg/kg MK-801 (n = 10 for amplitude and peak time; n = 8 for decay time and duration). * Statistically significant differences (Wilcoxon matched-pairs test, P < 0.05).

FIG. 4. Effect of the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 on cortical and brain stem negative DC shifts in an 11-day-old rat. DC shifts in the cerebral cortex at 1200 μm (top traces) and in the brain stem at 400 and 800 μm (bottom traces) were recorded simultaneously. In the brain stem extracellular K+ concentration was also measured at 800 μM (middle trace). A: DC shifts in the cortex and in the brain stem elicited by KCl following acetate conditioning, but before MK-801. B: KCl-evoked DC shifts and extracellular K+ rise in the brain stem after 3 mg/kg MK-801. C: Bar diagram comparing mean values ± SD of DC amplitudes in mV, peak times in s, decline times in s, and durations in s in 11-day-old rats. Hollow bars, animals before 3 mg/kg MK-801 (n = 10); black bars, animals after 3 mg/kg MK-801 (n = 10 for amplitude and peak time; n = 8 for decay time and duration). * Statistically significant differences (Wilcoxon matched-pairs test, P < 0.05).

**DISCUSSION**

The present data show for the first time that the immature brain stem can generate negative DC shifts on stimulation. Two features of these brain stem DC shifts seem to be of particular importance. First, negative DC shifts were only elicited when excitability was enhanced by conditioning. Second, the ability of the brain stem to generate negative DC shifts was lost during maturation. A major obvious consequence of sustained negative DC shifts in the present study was the disruption of normal breathing frequency.

We observed in adult rats confirm older data by Bureš et al. (1974) who showed that in the brain stem pricks or KCl could not elicit any SD-related depolarizations although these stimuli reliably evoke SDs in the cerebral cortex. We show, however, that this is different in rat pups. After acetate conditioning KCl, but not pinprick, could indeed elicit DC deflections in the brain stem. The topical focal application of a KCl crystal to the brain stem surface induced negative DC potential shifts at electrodes located at remote sites within the gray matter. These negative DC potential shifts were accompanied by rises in [K+]o with a parallel time course. The course of many of these DC shifts resembled those in the cortex. The latency between KCl application and the beginning of the negative DC shift as well as the [K+]o increase was dependent on the distance between the application site and the particular recording site. However, the same stimulation procedure failed to evoke negative DC shifts after the age of 12–13 days.

Thus compared with other neuronal structures, the brain stem is unique in that the generation of negative DC shifts or SD occurs only transiently during an early stage of development and that the mature brain stem is resistant to this neuronal mechanism even when conditioning stimuli are used that facilitate SD generation at all other places. Why the application of acetate instead of chloride further the generation of SDs has not been fully worked out. Nicholson and Kraig (1981), who used this method to elicit SD in the rat cerebellum, supposed that the reduction of the extracellular chloride concentration impairs the metabolic and ion-pumping ability of the neurons.

The disappearance of spreading negative DC shifts in the brain stem at the age of 2 wk probably has several reasons. During maturation neuronal regions are becoming better isolated from each other by fibers and myelinisation (Lehnenkühler et al. 1993; Prokopová et al. 1997). A spreading excitation is usually only observed in regions with high cell density such as cerebral cortical layers (Bureš et al. 1974). Furthermore, transmitter/receptor systems in the brain stem such as glycineric (Paton et al. 1994; Singer et al. 1998) and GABAergic mechanisms (Xia and Haddad 1992) and AMPA (Whitney et al. 2000) and NMDA receptors (Akopian et al. 1997; Ohtake et al. 2000) are becoming fully expressed and functional in the first 2–3 wk after birth. It is likely, therefore, that the full development of the neuronal network prevents the...
capability of the brain stem for the generation of substantial negative DC shifts.

In the adult cortex, application of a KCl crystal usually causes repetitive CSDs, and these are probably initiated by local depolarization of neurons and glial cells by \( K^+ \) and excitatory amino acids (Somjen 2001; Vyskocil et al. 1972). In the brain stem, however, a small KCl crystal in most cases elicited only a single negative DC shift. Repetitive DC deflections mostly occurred following the application of larger KCl crystals.

In the cortex NMDA receptors are thought to play an important role in SD (Marrannes et al. 1988; Obrenovitch and Zilkha 1996). It is assumed that the initial depolarization by an increase in \( [K^+]_e \) triggers the release of glutamate, which then activates NMDA receptors, and that this mechanism is able to further increase \( [K^+]_e \) and induce cell swelling and migration of SD (Somjen 2001). Surprisingly the application of NMDA to the brain stem did not trigger SD and the application of NMDA receptor antagonists did not abolish SDs. The lack of effect of NMDA cannot be simply explained by the absence of NMDA receptors because it is known that NMDA receptors are becoming important in the brain stem respiratory network for the breathing rhythm between postnatal days 5 and 15 (Gozal and Torres 1997). Probably, brain stem SDs are different from cortical SDs in that they are not mediated by excitatory amino acids. Most likely, the increase in extracellular \( K^+ \) plays the major role. This implies that spatial buffering and/or neuronal ionic pump function in the immature brain stem is not

![FIG. 5. Effects of negative brain stem DC shifts on respiration frequency after acetate superfusion. A: short-lasting DC shift in a 12-day-old rat elicited by KCl application to the brain stem. Similar respiration frequency before, during, and after the DC shift. B: sustained DC shift in a 6-day-old rat elicited by KCl. During this sustained DC shift, respiration almost stopped. C: relationship between the duration of DC shifts and their effects on respiration frequency in 6- to 12-day-old rats.]

![FIG. 6. Effects of negative brain stem DC shifts on respiration frequency elicited by KCl after hypoxia or asphyxia (shaded bars). A: repetitive short-lasting DC shifts after KCl in an 11-day-old rat following 60 s hypoxia. Breathing frequency was reduced to about 50%. B: long-lasting DC shift after KCl in a 10-day-old rat, following 30 s asphyxia (shaded bar). The breathing frequency was reduced almost to 0. C: relationship between the duration of the DC shifts and their effects on respiration frequency in 9- to 11-day-old rats.](http://jn.physiology.org/content/90/6/2168/F5)
sufficient to reduce high extracellular K+ concentrations quickly below a level that ignites SDs. In rat cortex the spatial buffering function by coupling of glial cells becomes mature only in the second postnatal week (Binnmöller and Müller 1992; Fischer and Kettenmann 1985; Nadarajah and Parnavelas 1999). A similar time course was found in rat spinal cord (Pastor et al. 1998) and we assume, therefore, that maturation of glial coupling in the brain stem takes place in the same time course.

A further consequence may be that SDs are not spreading as far as cortical SDs. When DC shifts were recorded in one side of the brain stem and KCl was applied to the contralateral side we never observed a negative DC shift on the ipsilateral recording side. Unfortunately there was not enough space to insert the electrode array in the rostrocaudal direction, and therefore it was not possible to test how far SDs would actually spread on the side of the recording electrode, e.g., in the rostrocaudal direction.

We have observed, however, that SDs in the brain stem were more sustained after MK-801. This finding suggests that NMDA receptors are involved in the termination of SDs. The precise mechanism underlying the prolongation of SDs under MK-801 is unclear at the moment.

A potentially important consequence of negative DC shifts or SD in the brain stem is the disturbance of respiratory movements. The breathing rhythm is generated by a neuronal network in the rostral and the caudal ventrolateral medulla that is established at birth (Arata et al. 1998; Ballanyi et al. 1999; Onimaru et al. 1995). Because we were able to show the spreading of negative DC shifts from a stimulation focus to different sites in the brain stem it is likely that the DC shifts invade also the region containing the respiratory network. In fact, we have observed that respiratory movements were markedly disturbed during sustained DC shifts. This indicates temporal alterations in the rhythm generation. It is unlikely that respiration failure was directly due to KCl application and that negative DC shifts were an epiphenomenon. After KCl respiratory movements were not impaired 1) when the particular KCl application did not elicit a DC shift, 2) when no conditioning was used, and 3) when rats were adult. The data suggest, therefore, that it is in fact the negative DC shift that causes disruption of regular respiration rhythm.

However, negative DC shifts were only generated by the coincidence of conditioning and excitatory stimuli. Hypoxia and asphyxia alone, at least those of short duration, do not elicit negative DC shifts in the brain stem that could be related to SD. This is similar to the adult rat cortex where ventilation with a CO2–O2 gas mixture did not elicit, but even stopped propagation of a CSD wave (Gardner-Medwin 1981). In the cortex asphyxia, if lasting long enough, led only to a terminal negative depolarization but did not evoke a reversible CSD at this site (Staschen et al. 1987). Indeed, in vitro slice preparations the neonatal brain stem had a greater capability to resist even long-lasting anoxia and to maintain the respiratory rhythm than the adult brain stem, suggesting that the immature brain stem is more able than the adult brain stem to survive and to maintain its metabolic needs via anaerobic mechanisms (Ballanyi et al. 1992). However, this relative protection may be overcome when additional stimuli coincide with periods of hypoxia and asphyxia. In the adult rat, hypoxia can facilitate SDs in the cortex when combined with depolarizing stimuli, most likely by induction of vulnerability by metabolic processes (Bureš and Buršová 1960; Somjen 2001), and this should be also possible in the immature brain stem as long as the capability for the generation of negative DC shifts exists.

In summary, we have observed that the brain stem is not resistant to SD throughout life. Negative DC shifts can be generated by topical application of KCl in the brain stem of rat pups under certain conditions (replacing extracellular chloride ions, transient hypercapnia, or hypoxia) but not in rats older than 2 wk. Thus SD may not be an important mechanism of pathological brain stem functions in the adult rat. However, the ability of the immature brain stem to generate negative DC shifts could be a risk factor for respiratory disturbances at a very young age. Negative DC shifts migrate in the immature brain stem similar to SD. Their ignition and migration depends on extracellular K+, but different from the cortex NMDA receptor blockade, does not abolish them. Potassium ions obviously play a pivotal role in this transient depolarization.

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DISCLOSURES

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REFERENCES


