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

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Richter, Frank, Sven Rupprecht, Alfred Lehmenkühler, and Hans-Georg Schaible. Spreading depression can be elicited in brain stem of immature but not adult rats. *J Neurophysiol* 90: 2163–2170, 2003. First published June 4, 2003; 10.1152/jn.00388.2003. Spreading depression (SD), a neuronal mechanism involved in brain pathophysiology, occurs in brain areas with high neuronal density such as the cerebral cortex. By contrast, the brain stem is thought to be resistant to SD. Here we show that DC shifts resembling cortical SD can be elicited in rat brain stem by topical application of KCl but not by pricking the brain stem. However, this was only possible until postnatal day 13, and, in addition, susceptibility for SD had to be enhanced. The latter was achieved by superfusion of the brain stem for 45 min with a solution containing acetate instead of chloride ions. Transient asphyxia or hypoxia by 2 min breathing 6% O_2 in N_2 had a similar effect. Negative brain stem DC deflections were paralleled by an increase of extracellular potassium concentration ≤40 mM and were spreading, but unlike cortical SD they were not inducible by glutamate and *N*-methyl-D-aspartate (NMDA). Time course and slope of brain stem SD either resembled cortical SD or were long-lasting and sustained. The latter stopped normal breathing. Different from cortical SD, negative brain stem DC deflections were changed in their slope (mostly converted into sustained shape, peak time was significantly prolonged, decline-time and duration were prolonged), but not 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.

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 reduced, and hence Leão named this excitation wave "spreading depression" (Bureš et al. 1974; Leão 1944). CSDs are elicited by ischemic episodes in the brain where they mediate disturbances in brain function in regions adjacent to and remote from hypoxic areas (Gorji 2001). CSDs are also thought to be the neuronal mechanism underlying migraine aura in humans (Lauritzen 1994).

Under experimental conditions SD in the cerebral cortex can be reliably elicited by pin pricking the cortex (i.e., depolarization of a small brain area around the pricking site by a mechanical stimulus causing ionic leakage), by topical application of KCl to the surface (i.e., depolarization of a larger brain area by raising the $[K^+]_e$), and by topical application of excitatory amino acids (i.e., 5 mM glutamate, 20 μM kainate, or 100 μM *N*-methyl-D-aspartate (NMDA)) (Bureš et al. 1974; Lauritzen et al. 1988). The same stimuli can elicit SDs in the cerebellum and in the spinal cord but, importantly, this is only possible after "conditioning," e.g., after changing the extracellular milieu by superfusing these regions with a solution containing acetate instead of chloride ions (Lauritzen et al. 1988; Leão 1963; Nicholson et al. 1981).

By contrast, the brain stem is generally thought to be resistant to the generation of SD, and this is attributed to the low cell density (Bureš et al. 1974). In light of newer results, however, the failure of the brain stem to generate SDs should be reconsidered. First, as mentioned above, structures such as cerebellum and spinal cord show SDs only after conditioning. Second, the ability of the brain stem to generate SDs has not been investigated at a very young age before the brain stem is mature. Recordings of CSDs from postnatal developing cortex suggest that the occurrence of CSDs depends on factors such as extracellular space volume, functional ion channels, and glial coupling that change during the development of the neuronal network (Bureš and Burešova 1960; Richter et al. 1998). Because CSD is an important mechanism in pathological brain functions, we reinvestigated whether SD can be elicited in the brain stem. First, we examined whether SD can be evoked in the adult brain stem after conditioning (similar as in cerebellum and spinal cord). Second, we tested whether the generation of SDs in the brain stem is dependent on age. While pathological brain stem functions may occur at any age, sudden infant death syndrome (SIDS) suggests that brain stem malfunctions could also be particularly important at a very young age (cf. Sparks

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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 exposed by incising the neck muscles and the atlantooccipital ligament and removing the dura mater.

Adult male Wistar rats (300–450 g) were anesthetized with urethane (1.5 g/kg ip) to maintain areflexia to stimulation of the nictitating membrane. The animals were tracheotomized and breathed spontaneously room air. Mean arterial blood pressure was continuously monitored and body temperature was kept at 37°C. The head of the animal was fixed in a stereotaxic frame. Over the left cortex hemisphere a burr hole was made (2–3 mm diam.) and the dura over the gray matter was incised in that area. The brain stem was exposed by incising the neck muscles and the atlantooccipital ligament; the dura mater over the brain stem was removed. The study was approved by the government of Thueringen (Reg.-No. 02–04/98 and 02–10/01).

An array of two or three DC microelectrodes (<5 μm tip diam, filled with 150 mM NaCl, resistance <10 MΩ) with tips staggered vertically and laterally by 400 μm, respectively, was lowered into the brain stem $V_C/C1$ area for simultaneous DC measurements at depths of 800, 1,600, and 1,200 μm (glued with the deepest pipette in the middle, as seen in the recordings in Fig. 2B from top to bottom), providing data from three spatially different recording points in the brain stem. In a few animals this array was also moved in a vertical direction, providing data from depths of 400, 1,200, and 800 μm, respectively. In some rats another array was used consisting of one DC microelectrode glued at 400 μm tip distance vertically and 400 μm laterally to one double-barreled potassium-sensitive electrode made from theta-glass (Kuglstatter, Garching, Germany). They contained Corning K^+ exchanger 477317 (W.P.I., Berlin) within the tip and were backfilled with 100 mM KCl (calibration as described in Nicholson 1993). With this array data DC shifts and $[K^+]_e$ were measured simultaneously at two spatially different points in the brain stem. In the cortical gray matter DC potentials were also measured with a single DC electrode at a depth of 1,200 μm. All electrodes including a reference electrode (filled with 2 M KCl) on the nasal bone were connected by Ag/AgCl wires to a custom built four-channel high-impedance amplifier (Meyer, Munich, Germany). The animals were grounded through an Ag/AgCl electrode below the back skin.

Experimental protocol

Exposed areas were kept moist with regular artificial cerebrospinal fluid (ACSF; in mM: 138.4 NaCl; 3.0 KCl; 1.3 CaCl$_2$; 0.5 MgCl$_2$; 0.5 NaH$_2$PO$_4$, 2.2 urea, and 3.4 glucose, warmed to 37°C and equilibrated with 5% CO$_2$ in O$_2$). After ACSF superfusion for 1 h, we tried to elicit DC shifts in the cortex and in the native brain stem by pricking with a needle (0.5 mm tip diam.) or by applying a small KCl crystal weighing 0.1 to 0.5 mg to the surface (Bureš et al. 1974) in a distance of about 1 mm to the most superficial electrode. Prior to KCl application superfusion was stopped, the surface of the brain stem was dried with a thread of cotton wool, and then a KCl crystal was placed on the surface. After 8 to 10 min superfusion was started again for another 10 min, and then superfusion was switched for 45 min to ACSF in which 100 mM of the chloride (75%) was exchanged by 100 mM acetate. Thereafter superfusion was again stopped and pinpricks and KCl were applied as above. Superfusion was continued after the recovery of a DC shift or when a sustained DC shift resulted in a breathing arrest. In some experiments solutions containing either 12.5, 25, or 50 mM glutamate, respectively, or 100, 200, 500, or 1000 μM N-methyl-D-aspartate (L-glutamate; NMDA; Sigma-Aldrich GmbH), respectively, were applied to the brain stem and washed away after 20, 40, or 60 s.

To assess migration of negative DC deflections from one to the other side of the brain stem, in six rat pups aged 11 to 13 days after acetate conditioning a wall from vaseline paste was made between the left and the right sides of the brain stem. This wall prevented accidental migration of KCl. DC electrodes were inserted into the left side of the brain stem and attempts to elicit negative DC deflections were made by applying KCl either to the ipsilateral or to the contralateral side of the brain stem.

In another nine rat pups we tested whether asphyxia or hypoxia are able to facilitate the generation of DC shifts by pinprick or KCl. In five rat pups asphyxia of 30–45 s was induced by closing the tracheal cannula before KCl was applied to the brain stem, and in four rat pups ventilation was switched from room air to a gas mixture consisting of 6% O$_2$ in N$_2$ for 2 min each at intervals of 30 min before KCl was administered.

In a further 10 rat pups aged 11 days we tested whether DC shifts can be influenced by the NMDA receptor antagonist MK-801. When a SD could be elicited in the brain stem after acetate, the rat received three ip injections of 1 mg/kg MK-801 (Sigma-Aldrich GmbH) at intervals of 30 min. Twenty minutes after the last MK-801 injection we tried to elicit DC shifts in the cortex and in the brain stem by topical administration of KCl.

Data were recorded on a PC by using custom signal acquisition programs. DC shifts were analyzed regarding occurrence, amplitude, SD peak-time (time from beginning of depolarization to maximal amplitude in the case of one peak only or to first peak in the case of several peaks), SD-decline (time from beginning of repolarization to baseline), and SD-duration (time interval between beginning of depolarization and reaching the baseline). A mechanograph (custom-built lever with a piezzo-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).

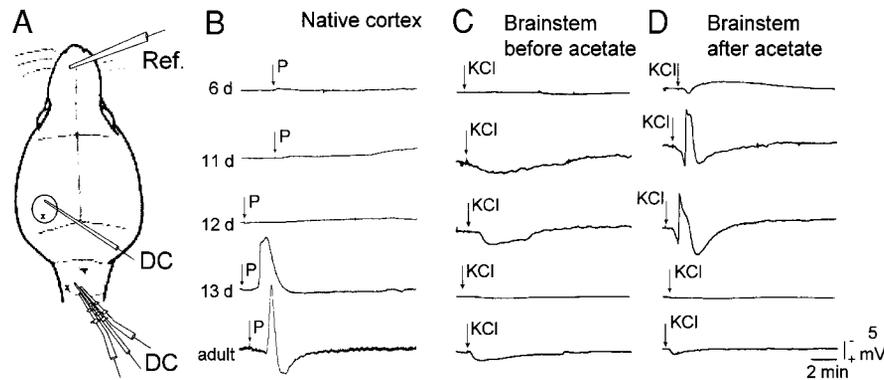In the same rats we tested whether DC shifts could be

FIG. 1. DC shifts in the cerebral cortex and in the brain stem elicited by pin prick (P) or by KCl. *A*: sites for the introduction of recording (DC) electrodes in cortex and brain stem. This schematic skull drawing resembles adults but is not to scale for immature rats. Electrodes are not drawn to scale. In the exposed cortex area (circle) the single-barrel electrode was positioned in the occipitotemporal area. In the brain stem a 3-barrel electrode was positioned near the trigeminal nucleus. The barrels were separated and staggered such that the recording sites extended 800 μm in the mediolateral direction and the tips reached depths of 400, 800, and 1,200 μm or 800, 1,600, and 1,200 μm within the brain stem, respectively. Crosses show sites where pinpricks (cortex) or KCl (brain stem) were applied. *B*: typical spreading depressions (negative DC shifts, upward deflections) in the cerebral cortex evoked by pinpricking the cortex (P) in a rat of 13 days age and in an adult rat, but not in younger rat pups. The DC potentials were recorded at depths of 1,200 μm. *C*: DC recordings from the brain stem in the same rats as in *B*. The potentials were recorded at depths of 800 μm. For stimulation KCl was administered to the brain stem surface. No negative DC shifts. *D*: DC recordings in the brain stem of the same rats and the same brain stem depths, after application of acetate to the brain stem. Negative DC shifts in rats of 6, 11, and 12 days of age.

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. 1*D*). 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. 1*D*), 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. 1*D*). 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. 3*A*, *bottom trace*) were paralleled by an increase of [K^+]$_e$ (Fig. 3*A*, *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

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

Age of rat, days	No. of rats tested	No. of rats showing negative DC shifts after acetate*	DC shifts†	
			Before acetate	After acetate‡
6	2	2	0/2	5/6 (sustained)
				32/68 (CSD-like)
10/11	14	14	0/16	19/68 (sustained)
				7/16 (CSD-like)
12	4	4	0/4	3/16 (sustained)
				4/15 (CSD-like)
13	5	1	0/5	1/15 (sustained)
Adult, >90	9	0	0/6	0/29

* Number of rats for which negative DC shifts could be evoked by KCl. † Positive elicitations vs all attempts. ‡ Positive elicitation are subdivided into cortical spreading detection-like and sustained negative DC deflections. In the third column the numbers of rats are given in which negative DC shifts could be evoked by KCl. Numbers in the fourth and fifth column give positive elicitations versus all attempts. In the fifth column positive elicitations are subdivided into CSD-like and sustained (sust.) negative DC deflections.
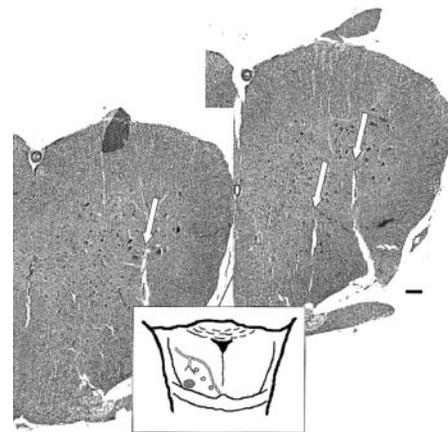

FIG. 2. Photomicrographs of two transverse sections through the brain stem in a 12-day-old rat, view from rostral, surface at top. *Inset*: (not to scale) shows the alignment of the 3 electrodes parallel to a vessel forming an angle to the left. In this particular animal the most rostral pipette was at a depth of 800 μm, the middle one was at a depth of 1,600 μm, and the most caudal one was at a depth of 1,200 μm (small open circles). The gray shaded area in the inset gives the site where KCl was applied to the surface of the brain stem. The left section shows the slit (white arrow) made by the most rostral pipette, the right section was made more caudally, showing two slits (white arrows) made by the most rostral and the middle pipettes. Scale bar = 100 μm.
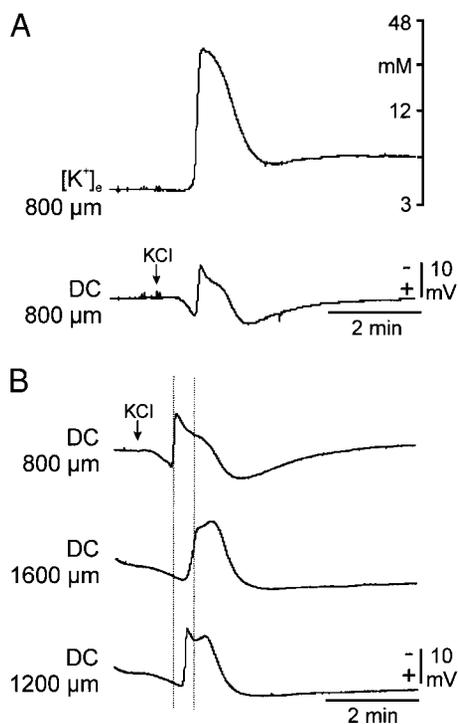
FIG. 3. Characteristics of negative DC shifts in the brain stem. *A*: recordings in an 11 day-old rat. *Bottom trace*: typical negative DC shift at a depth of 800 μm after KCl application to the brain stem surface. *Top trace*: parallel increase of the extracellular potassium concentration $[K^+]_e$. *B*: propagation of negative DC shifts in the brain stem of a 12-day-old rat. Simultaneous recording of DC shifts with a 3-barreled electrode at 3 different sites after KCl application to the brain stem surface. Traces show the migration as recorded in the brain stem with the deepest electrode in the middle. The negative DC shift appeared 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.

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 $\pm$ 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 800 μm depth and after 13 s at the electrode with the tip at 1,600 μm depth that had a mediolateral distance of 400 μm from the electrode at 800 μm. Finally, the DC shift arrived at the third electrode with the tip at a depth of 1,200 μm (Figs. 2 and 3*B*). Since electrodes had their tips at differing depths and thus probably in brain stem areas of differing cellular density, the traveling velocity between pairs of electrodes varied to some extent but was usually in the range of 2.4–2.8 mm/min, similar to CSD. The distribution of blood vessels at the surface of the brain stem limited the positioning of the electrode array. It was not always possible to align the electrode perpendicular to the KCl application site (see also Fig. 2, *inset*).

Notably, propagation of DC shifts toward the recording electrodes was only observed when the KCl crystal was placed to the side of the recording but not to the contralateral side. This was particularly tested in six rats. The ipsilateral application of KCl after acetate conditioning evoked a negative DC shift in 15 of 16 trials. The contralateral application of KCl after acetate conditioning was tested 11 times but never evoked a negative DC shift at the ipsilateral recording electrodes.

However, after contralateral KCl, the DC potential in the remote brain stem shifted transiently in a positive direction by 1–2 mV and returned slowly to baseline.

Substantial differences between brain stem and cortical SD were observed. As mentioned, pinprick can easily elicit CSD but not brain stem SD. A further difference was noted in the effects of glutamate, NMDA, and the NMDA antagonist MK-801. Topical application of glutamate (12.5–50 mM) to the brain stem in rat pups did not cause DC shifts ($n = 13$ applications, 3 rats of 11–13 days of age). The application of NMDA (100 μM to 1 mM) to the brain stem evoked only four short and two sustained DC shifts in 33 applications (tested in 7 rats of 7–13 days of age), when solutions of 500 μM or 1 mM were applied.

The NMDA receptor antagonist MK-801 reduced SD in the cortex but did not abolish negative DC shifts in the brain stem evoked by KCl (tested in 10 rats aged 11 days). An example is shown in Fig. 4. DC recordings were performed in parallel in the cortex and in the brain stem before (Fig. 4*A*) and after (Fig. 4*B*) ip application of MK-801. In addition $[K^+]_e$ was measured in the brain stem. The brain stem was superfused with acetate before KCl stimulation. In total, 24 attempts were made to elicit SD in the brain stem by KCl prior to MK-801, and in 19 cases a negative DC shift was evoked. Ten of these shifts could be classified as SD (8 with CSD-like shape, 2 "sustained"), the other nine were only small and short-lasting negative shifts without a migration to the neighboring electrode. After application of 3 mg/kg MK-801, one further attempt was made in each rat to elicit SD in the brain stem and in the cortex. After MK-801, no SD-related, but only slowly rising DC shifts in the cortex were seen, but in all animals a negative DC shift was evoked in the brain stem. Three negative DC shifts had a CSD-like shape, and seven were "sustained." In two rats SDs did not recover and produced a fatal breathing arrest. Figure *4C* shows slope parameters and amplitudes of SDs before and after application of MK-801.

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. 5*A*). By contrast, respiration came to a complete stop during a KCl-evoked sustained negative DC shift (Fig. 5*B*). When superfusion was started to recover the animal the DC shift was reduced and respiration recovered (Fig. 5*B*). Figure 5*C* 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 respiration frequency when KCl was applied to the brain stem after acetate.

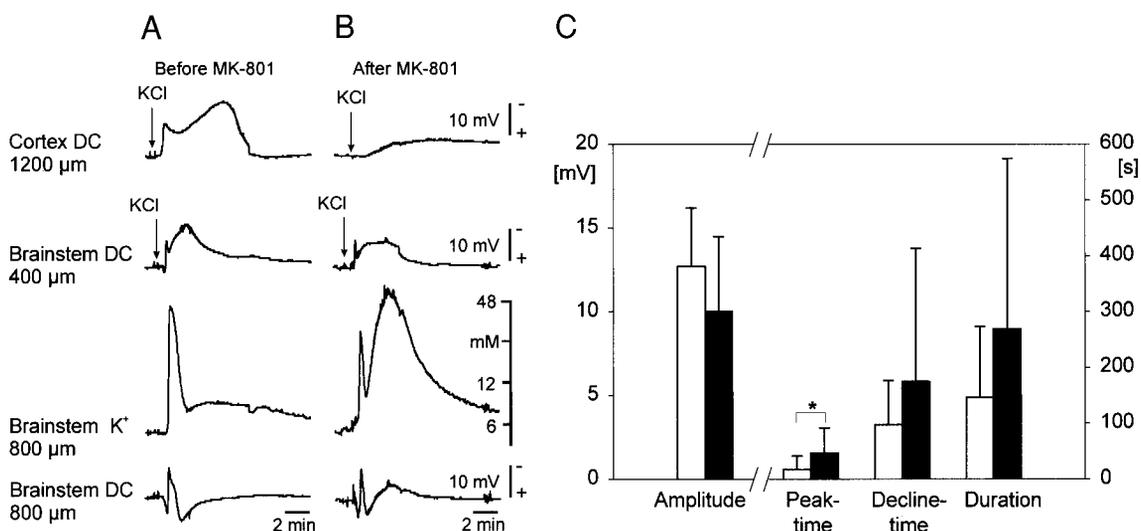Because DC shifts in rat pups can only be elicited after

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 $\pm$ 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, $P < 0.05$)

conditioning we asked whether physiological or pathophysiological conditions rather than acetate application could trigger DC shifts. Indeed, asphyxia or hypoxia were able to trigger KCl-induced DC shifts. Neither asphyxia (closing the tracheal respiration cannula for 30–45 s; $n = 5$ rats) nor hypoxia (breathing a gas mixture of 6% O_2 in N_2 for 2 min; $n = 4$ rats) caused DC shifts by themselves (Fig. 6*A* and *B*). However, the subsequent application of KCl to the brain stem evoked effects. In most cases first a positive DC shift was observed but then negative DC shifts were elicited. These were either repetitive (Fig. 6*A*) or long-lasting (Fig. 6*B*). During these DC shifts breathing frequency was reduced. Figure 6*C* shows that most of these DC shifts had a long duration and that the reduction of breathing frequency was dependent on the duration of the DC shifts.

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.

Our experiments 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 $[K^+]_e$ 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 $[K^+]_e$ 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 furthers 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 (Lehmenkühler 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 glycinergic (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
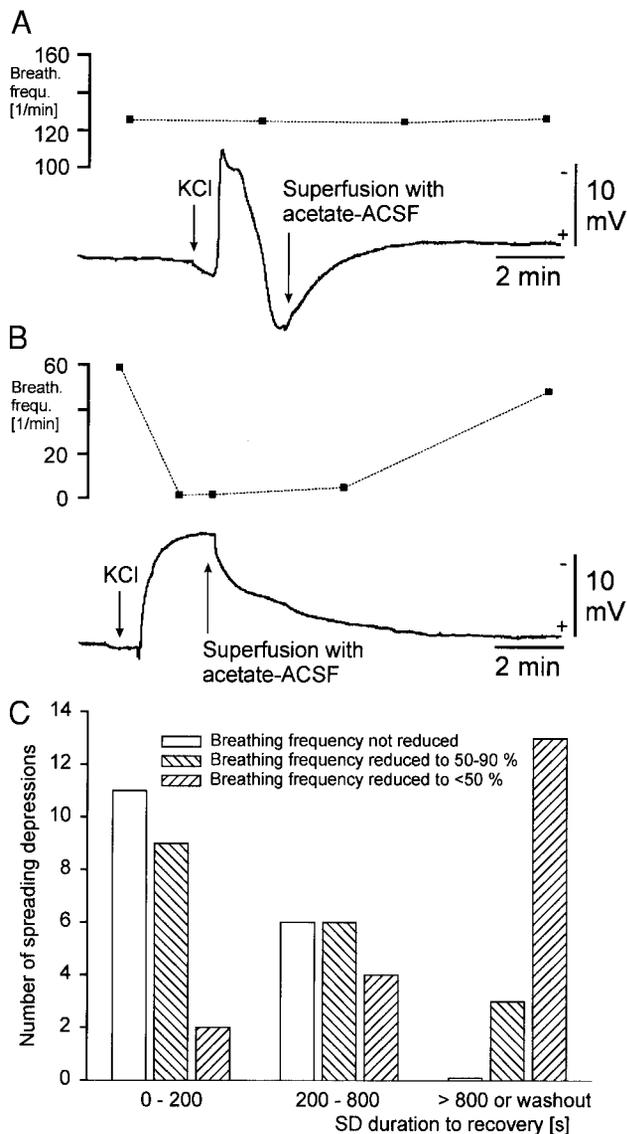
FIG. 5. Effects of negative brain stem DC shifts on respiration frequency after acetate superfusion. *A*: short-lasting DC shift in a 12-day-old rat elicited by KCl application to the brain stem. Similar respiration frequency before, during, and after the DC shift. *B*: sustained DC shift in a 6-day-old 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.

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
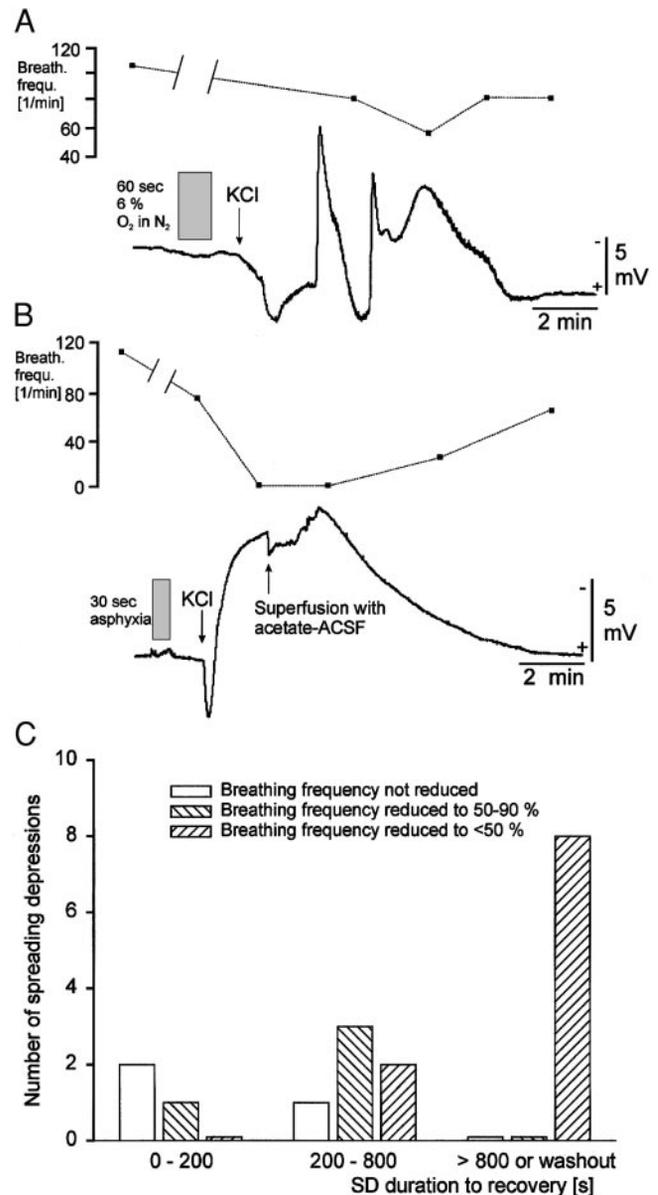

FIG. 6. Effects of negative brain stem DC shifts on respiration frequency elicited by KCl after hypoxia or asphyxia (shaded bars). *A*: repetitive short-lasting DC shifts after KCl in an 11-day-old rat following 60 s hypoxia. Breathing frequency was reduced to about 50%. *B*: long-lasting DC shift after 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.

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 (Binmöller 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 CO_2-O_2 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 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 Burešova 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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