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A comparison of anticonvulsant efficacy and action mechanism of Mannitol vs Phenytoin in adult rat neocortical slices

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ABSTRACT

We show that, in adult rat neocortical slices, an anticonvulsant effect comparable to that of Phenytoin can be obtained through a Mannitol-induced increase in extracellular osmolarity of only 30 mOsm/L. The anticonvulsant action of extracellular hyperosmolarity has been known for decades but has not found a feasible therapeutic application, yet. A 30 mOsm/L increase in extracellular osmolarity is already utilized in neurocritical care though not as an anticonvulsant agent: the data suggest a possible effective anticonvulsant use, too, in this setting.

We used multiple electrode arrays to characterize and compare the anticonvulsant mechanisms of Mannitol and Phenytoin. Phenytoin decreased the voltage, duration and spatial spread of rhythmic repetitive, ictal-like activity. In contrast, Mannitol did not significantly affect voltage, duration and spatial spread of rhythmic repetitive, ictal-like activity but rather it inhibited the rate of epileptiform discharges.

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1. Introduction

Epileptic discharges are generated by neurons that transiently synchronize their electrical firing. Epileptic synchrony can be facilitated by an increase in synaptic connections between neurons but can also occur through non-synaptic mechanisms (Roper et al., 1992; Dudek et al., 1990, 1998; Haglund and Hochman, 2005; Seigneur and Timifeev, 2011; Hochman, 2012).

Seizures lead to cytotoxic swelling (Kramer et al., 1987; Konermann et al., 2003; Kim et al., 2001; Hormigo et al., 2004) resulting in extracellular shrinking and heightened impedance (Vongerichten et al., 2016). The increase in physical proximity between adjacent neurons due to extracellular shrinking heightens excitatory *non-synaptic* electrical interactions between neurons and the effect of extracellular ionic changes occurring in tinier volumes. This enhancement of non-synaptic coupling between neurons is an important and established component of epileptic synchrony: indeed, treatment with hypertonic solutions to decrease neuronal swelling, has been known for decades to inhibit epileptiform activity in brain slices, in animal models of repetitive seizures (Reed and Woodbury, 1964; Baran et al., 1987; Baxter et al., 1986; Rosen and Andrews, 1990; Roper et al., 1992; Dudek et al., 1990, 1998; Hochman, 2012) and even in human patients (Haglund and Hochman, 2005).

These studies have proven that, *in principle*, hyperosmolarity does have an anticonvulsant effect but have not yet indicated how it might be utilized in clinical practice, for example, in which clinical condition and at what concentrations it would be effective and non-toxic.

Rather than treating seizures, hypertonic solutions are already routinely administered to patients with increased intracranial pressure (Qureshi et al., 1999; White et al., 2006; Cottenceau et al., 2011; Papangelou et al., 2013; Todd, 2013; Dostal et al., 2015; Schilte et al., 2015; Surani et al., 2015) so as to increase serum osmolarity up to ~30 mOsm/L.

In this paper we show that in brain slices, a Mannitol concentration increasing extracellular osmolarity by only 30 mOsm/L, does exert a sizable anticonvulsant effect. Thus, Mannitol concentrations already utilized in routine clinical care are effective to treat experimental seizures. The data presented in this study on Mannitol concentration, efficacy and mechanism of action may *pave the way* towards a practical application.

2. Materials and methods

2.1. Slice preparation

The study was performed on 43 slices obtained from 16 female rats of ages between 2 and 5 months. The rat was anesthetized with isoflurane and euthanized by decapitation. The head was immediately placed in a low calcium, high magnesium saline (NaCl

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126 mM; KCl 3.5 mM; CaCl$_2$ 0.1 mM; MgSO$_4$ 10.0 mM; NaHCO$_3$, 26 mM; NaH$_2$PO$_4$ 1.25 mM; Glucose 10 mM) at 2–3 °C, bubbled with 95% O$_2$/5% CO$_2$. The skull was opened and the brain was removed and incubated in a recovery chamber at 2–3 °C for 4–5 min. The cortex was placed over a stage with cyanoacrylate glue. The chamber was filled with ice cold saline at 2–5 °C.

Coronal slices (370 μm) of hemispheres were cut between sections corresponding to Plate 33 (Bregma 0.00) to Plate 47 (Bregma −1.72) of Paxinos and Watson atlas (2007). The lateral sectum protrusion into the ventricles was a prominent structural landmark offering easy identification of the section at which coronal slices were to be cut. Each rat's brain was cut with only 3-4 coronal slices beginning at the posterior part of the sectum. Each slice was cut only 370 μm thin to facilitate slice oxygenation. The distance between the most anterior and the most posterior slice section was about 1.5 mm along the anterior-to-posterior axis or about 6% of the anterior-to-posterior length of the hemisphere. Slices were transferred on a holding chamber at room temperature with physiological saline (NaCl 126 mM, KCl 3.5 mM, CaCl$_2$ 1.1 mM, MgSO$_4$ 1.0 mM, NaHCO$_3$ 26 mM, NaH$_2$PO$_4$ 1.25 mM, glucose 10 mM) bubbled with 95% O$_2$/5% CO$_2$. *Measured* osmolarity for this solution was 302 ± 7 mosm/L (mean $\pm$ SE, n = 5). In our experiments, the average of serum osmolarity measured in 4 rats was 298 ± 3 mOsm/L (mean $\pm$ SE, n = 4), consistent with previously reported values (Thenuwara et al., 2002). In the RESULTS section we will refer to these values as those of normal physiological extracellular osmolarity.

2.2. Extracellular field recordings in adult brain neocortical slices by electrode arrays

Recordings are performed between 2 and 12 h after the dissection. In 39 out the 43 slices utilized in this study the recordings were started within 8 hours from the end the dissection. In only four slices the recording was performed between 8 and 12 hours from the end of the dissection: the data obtained in the latter showed were homogeneous with those obtained closer to the time of the dissection.

The array is at the bottom of the chamber and is a 10×6 matrix (10 columns, 6 rows) of planar titanium electrodes, of 30 μm diameter, 500 μm inter-electrode distance and 30-50 kΩ impedance (Multichannel Systems, 60MEA500/30iR-Ti, Reutlingen, Germany). The outlet cable was interfaced into a Stellate Harmonie system (64 channels of E2 amplifier).

Slices were positioned in the chamber so that the cortex overlaid the location of electrodes. Excess fluid was removed and slice position was adjusted with a needle. A platinum wire anchor with a mesh kept the slice in position. The solution inflow was ~1.5 ml/min. A digital camera picture of the slice position was taken at the end of the recording. Epileptiform activity was induced by perfusion with 0.1 mM 4-Aminopyridine in a medium devoid of Magnesium following a rationale and a protocol previously utilized by this investigator (Serafini et al., 2015, 2016).

2.3. Data acquisition and initial analysis

Acquisition was at a bandwidth between 0.1 and 200 Hz using built-in EEG filters. For visual display and analysis, data were filtered to 1–15 Hz specifically to allow analysis of recordings with limited signal-to-noise ratio *preventing selection bias*. Epileptiform waveforms with established practical clinical relevance (spike-and-slow wave, sharp-and-slow waves, poly-spikes, rhythmic delta) still fall within this bandwidth. Data with highest signal-to-noise ratio were also reviewed at a bandwidth of 1–100 Hz: the spatial distribution of discharges appeared very similar to that at 1–15 Hz (not shown). Negative polarity signals were displayed

upwards and digital images defined electrodes positions on the slice.

On each recording day an initial assessment of the baseline noise was obtained with perfusion of the recording chamber with the platinum ring anchor but without a slice. We define brief electrical signals generated by the slices as *"discharges"*. Discharges are defined operationally as those waveforms that 1) do not appear in the recording of the perfused chamber without the slice; 2) in each recorded slice occur consistently and recurrently in the same channel(s) throughout the recording; 3) when occurring in several channels they do so within a time window of less than 500 msec. These signals exhibit a sustained baseline shift (with/without multiple phases) standing out from the high frequency baseline noise. They exhibit an electrographic morphology consistent with epileptic discharges described in clinical EEG practice. They also exhibit a dose-dependent effect on their frequency and are inhibited from perfusion with an anticonvulsant (see data in Serafini et al., 2015). Discharges were classified as isolated discharges (Interictal Epileptiform Discharges, or IEDs) or as rhythmic repetitive discharges (seizures) as previously described (Serafini et al., 2015, 2016), following criteria similar to those of clinical EEG practice. In each recording after the initial onset of epileptiform activity we assessed for its stability and persistence for at least 15 minutes before administering Mannitol or Phenytoin.

2.4. Quantitative analysis

The effect of treatments on convulsive activity can be quantified through several measures. The time spent in epileptiform activity, frequency of discharges and mean duration of discharges have been measured in *in vivo* models of epilepsy (Vezzani et al., 1986). The effects of a treatment on the time spent in epileptiform activity reflects its efficacy. In contrast, the effect on the mean duration of discharges or on their frequency may provide insight on whether the anticonvulsant treatment acts inhibiting the initiation of discharges or by curtailing rhythmic repetitive ictal progression. In the experiments presented in this study we have expanded these measurements, assessing them in each minute and we have measured also voltage and spatial spread of discharges.

Measured parameters are therefore: a) time spent in epileptiform activity *per minute*, calculated as the sum of the durations of all the individual discharges in each minute; b) rate of discharges *per minute*, calculated as the number of discharges in each minute; c) mean duration of discharges *per minute*, calculated as the ratio between a) and b) in each minute; d) voltage of discharges (as this appeared consistent in each subsequent epoch we have measured discharge voltages in discharges that were representative of the recording and/or of the specific pharmacological treatment); e) spread of epileptiform activity, estimated by measuring the number of electrodes exhibiting epileptiform discharges.

3. Results

3.1. Experimental approach

In experimental models of epilepsy Mannitol's anticonvulsant action is established and has been attributed to an enhancement of extracellular osmolarity. Yet, Mannitol or in general hyperosmolarity currently are not considered as potential therapeutic clinical anticonvulsants. The reasons are unclear but concerns of low efficacy and/or of toxicity are a likely explanation. We are inquiring whether hyperosmolarity might be suitable as an anticonvulsant agent: in this paper we study *whether a Mannitol dose increasing extracellular osmolarity within in a range of routinely established clinical use values may still exert any effective anticonvulsant action.*

For a rational planning of in vivo experiments, slices' recordings experiments can define i) whether the Mannitol-induced hyperosmolarity values required for an effective anticonvulsant action are in a likely toxic or non-toxic range; ii) the time-course of the effect; iii) hyperosmolarity anticonvulsant mechanism at the circuitry level through Multiple Electrodes Arrays recordings over an extended cortical surface.

Mannitol's anticonvulsant effect has been attributed to hyperosmolarity. Traynelis and Dingledine (1989) showed that in hippocampal slices seizures induced by high extracellular potassium are blocked by a variety of hyperosmolar agents such as Mannitol, Sucrose, Raffinose, L-Glucose and Dextran. These agents are equally effective in suppressing seizures. Ballyk et al. (1991) showed that in rat hippocampal slices, seizures evoked by a medium without magnesium can be blocked by enhancing extracellular osmolarity through D-Glucose. In this study Mannitol's effect on epileptiform activity *is assumed* to be due to hyperosmolarity, too.

In clinical practice, treatment with Mannitol hyperosmolar solutions aims at an increase in serum osmolarity up to 320 mosm/l, though higher values are often utilized safely in neurocritical care (Diringer and Zazulia, 2004). Accordingly, we used Mannitol concentrations of 15 mM, 30 mM and 50 mM so as to increase extracellular osmolarity by values of mOsm/l similar to those utilized in clinical practice. We induced epileptiform discharges through perfusion with a medium with 0.1 mM 4-AP and with zero Mg as previously described (Serafini et al., 2015, 2016). The effect of extracellular Mannitol-induced hyperosmolarity on epileptiform activity was studied through four distinct protocols:

  i) a short pulse (5 min) of a higher Mannitol concentration (50 mM) *after* the onset of epileptiform discharges (*post*-treatment) to assess for evidence of an anti-seizure effect;
 ii) a prolonged pulse (23 min) of a lower Mannitol concentration (30 mM) *after* the onset of epileptiform discharges (*post*-treatment) to assess for an anti-seizure effect at a concentration with likely low toxicity, potentially to be applied for clinical use;
iii) continuous co-perfusion with Mannitol *after* the onset of epileptiform discharges (*post*-treatment) to assess for the maximal anti-seizure effect;
 iv) co-perfusion with Mannitol (15 mM and 30 mM) *before* the onset of epileptiform discharges (preventative *pre*-treatment).

Control is represented by epileptiform activity recorded in 10 neocortical slices from 7 adult rats perfused throughout with 0.1 mM 4-AP and with zero Mg and normal osmolarity. In addition, in experiments i) and ii) reference control to assess for the effect of Mannitol is also the average between epileptiform activity before addition of Mannitol and that measured 10 min after the return to the solution without Mannitol.

Further, we compared the anticonvulsant effect of Mannitol with that of Phenytoin to assess whether its efficacy may compare with that of an established anti-seizure drug.

Last, we characterized similarities and differences between Phenytoin and Mannitol in their anticonvulsant mechanisms.

3.2. Mannitol inhibits epileptiform discharges in a reversible fashion

In the initial experiments we administered Mannitol solutions only *after* the onset of epileptiform activity. We designate this set of experiments as *post-treatment*. In experiments i), ii) and iii) we induced epileptiform discharges in 16 neocortical slices from 6 adult rats by perfusion with a solution containing 0.1 mM 4-AP and zero Mg. Before switching to a solution with Mannitol we assessed the consistency and stability of the epileptiform activity

for at least 12 minutes. We subsequently switched transiently to a solution containing 50 mM Mannitol for 7 min (5 slices) in experiment i) or 30 mM Mannitol for 23 min (7 slices) in experiment ii) to assess for a reversible effect of Mannitol on epileptiform activity. As previously described, epileptiform activity typically manifested as isolated or quasi-periodic sharp waves occasionally followed by rhythmic repetitive discharges (Fig. 1).

For quantitative analyses we normalized the time spent in each slice in epileptiform activity per minute to the values obtained in the initial baseline. When slices were perfused with 4-AP and zero Mg but without Mannitol or Phenytoin, there was only a relatively small (∼20%) gradual decrease in time spent in epileptiform activity over 35–40 minutes of recording (Fig. 2, plots with blue lines and symbols). In contrast, hyperosmolarity resulted in a *sizable and reversible* anticonvulsant effect (Fig. 2A and B).

In a subsequent set of experiments (experiment iii) we determined Mannitol *maximal* anticonvulsant effect. In 4 slices, after inducing epileptiform discharges a persistent hyperosmolar infusion (Fig. 2C) decreased the time spent in epileptiform activity per minute by 60%.

We compared the effect of Mannitol anticonvulsant effect with that of an established anticonvulsant treatment such as Phenytoin. In 7 neocortical slices from 2 adult rats Phenytoin Sodium (25 μg/ml) decreased epileptiform activity per minute by ∼60%. The efficacy of Mannitol treatment is therefore similar to that of Phenytoin.

In order to characterize the anti-convulsant mechanism of Mannitol, we measured its effect on the rate, duration, voltage and spatial spread of epileptiform discharges.

3.3. Mannitol anti-convulsant effect is due to an inhibition of discharge rates

Perfusion with Mannitol solutions resulted in a decrease in the rate of discharges by about 50% both with 30 and 50 mM Mannitol (Fig. 3 and Table 1). In another set of experiments after inducing epileptiform discharges we administered Mannitol medium throughout the rest of the recording in order to assess for its maximal inhibitory effect on discharges (Fig. 3C and Table 1). Data show a gradual decrease in the rate of discharges up to ∼80% that develops in 20–25 min.

In slices perfused with a solution containing 0.1 mM 4-AP and zero Mg (Fig. 3, plots with blue circles and lines) and normal osmolarity there was only a slight time-dependent increase in the rate of discharges. The anti-convulsant mechanism of Mannitol is different from that of Phenytoin: slices perfused with Phenytoin Sodium 25 μg/ml exhibited no decrease in the rate of discharges. Rather there was a variable, statistically non-significant *increase,* on average of ∼70% (Table 1). As the study was focused on Mannitol effects, Phenytoin actions were not investigated further with a larger sample size. Yet, it is important to point out that this increase in discharge rate by Phenytoin is the opposite of Mannitol decrease in the discharge rates. As such, when attempting to combine several anticonvulsant treatments to obtain a synergistic therapeutic effect, Phenytoin may possibly dampen Mannitol-induced inhibition of discharge rates.

3.4. Mannitol anticonvulsant action does not affect discharge duration, voltage, or spatial spread

We have assessed whether Mannitol may affect the duration of discharges, their voltage, spatial spread or the transition from isolated discharge into rhythmic repetitive discharges.

In control experiments, in slices perfused with 4-AP and zero Mg and no Mannitol there was only a slight decrease in the mean duration of discharges of ∼8% at 24 min and a further decrease
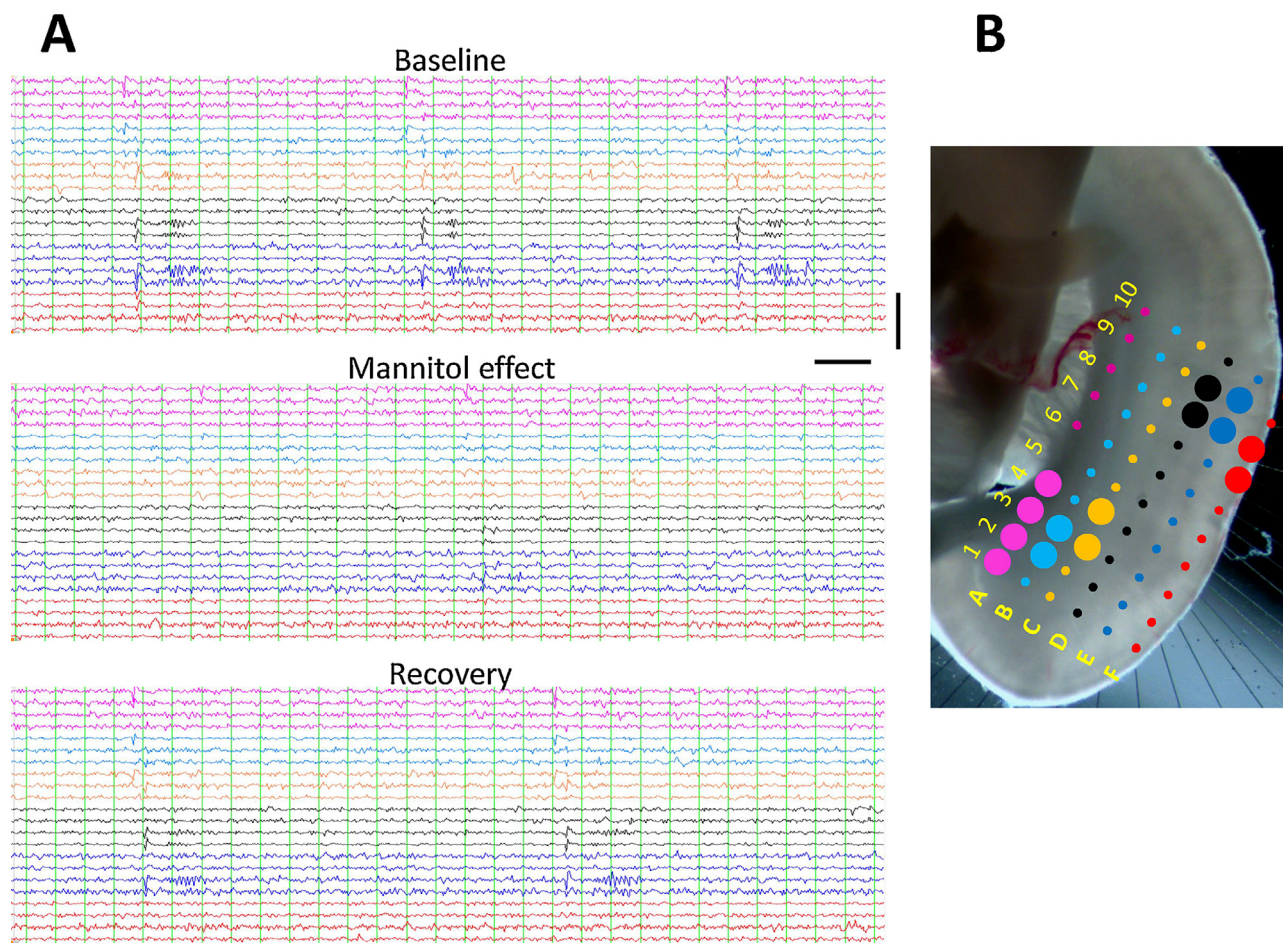
Fig. 1. Mannitol suppresses epileptiform discharges.
Panel A shows voltage traces obtained through a Multiple Electrodes Array with 60 electrodes (10 columns × 6 rows). The traces shown are from a selection of 22 electrodes that exhibit discharges. Traces of each color represent electrodes from the same row. Purple and light blue traces correspond to deep layers while red and deep blue traces correspond to superficial cortical laminae. Upper panel shows a recording of 30 seconds obtained after perfusion with a medium containing 0.1 mM 4-AP and zero Mg. Data show at least three discharges from several levels of cortical depth over rows of different colors. Discharges exhibit an initial transient which is followed by rhythmic repetitive discharges, more over the blue traces. In the middle panel, after switching to a solution containing 0.1 mM 4-AP and zero Mg containing 30 mM Mannitol there is a decrease in the occurrence of discharges. In the lower panel, after switching back to a solution containing 0.1 mM 4-AP and zero Mg but no Mannitol, discharges come back. Calibrations bars are 2 seconds and 200 microvolts.
Panel B shows a picture of the neocortical slice from which the recording shown in A was obtained. Colored dots correspond to the locations of electrodes and each row is represented by a different color matching the colors of the voltage traces. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Mannitol post-treatment decreases the rate of epileptiform discharges.

	13 min	24 min	35 min
Control (no Mannitol) (n = 10)	1.23 ± 0.31	1.41 ± 0.53	1.48 ± 0.6
	Baseline	Mannitol	Recovery
Mannitol 7 min pulse (n = 5)	1.28 ± 0.20	0.50 ± 0.10*	1.48 ± 0.06
Mannitol 23 min pulse (n = 7)	1.33 ± 0.34	0.65 ± 0.28*	2.12 ± 1.14
Mannitol prolonged perfusion (n = 4)	1.09 ± 0.18	0.37 ± 0.11*	
	Baseline	Phenytoin	
Phenytoin prolonged perfusion (n = 7)	1.01 ± 0.18	1.71 ± 0.44	

Data are means ± SE of *normalized* values of discharges per minute. In these recordings Mannitol was administered *after* the induction of epileptiform activity (post-treatment) through 0.1 mM 4-AP and zero Mg. In control recordings, there is a 12%, (statistically non-significant), time-dependent increase in the rate of discharges throughout 35 min. Perfusion with Mannitol induces a reversible inhibition in the rate of epileptiform activity. Continuous and prolonged perfusion with 30 mM Mannitol results in a 65% inhibition in the rate of discharges. Lower row shows that Phenytoin does not decrease the rate of epileptiform discharges. Mannitol-induced inhibition of epileptiform activity is therefore different from that of Phenytoin. * p < 0.05 vs corresponding Baseline and vs corresponding value of Control (Wilcoxon).

of up to 38% at 35 min (Table 2, upper rows). Conversely, in slices treated with Phenytoin Sodium 25 μg/ml, the duration of discharges shrunk down by ~83% (Table 2 lowest rows). While Phenytoin decreased sizably the duration of discharges, Mannitol resulted only in a variable and non-statistically significant effect on the duration of discharges.
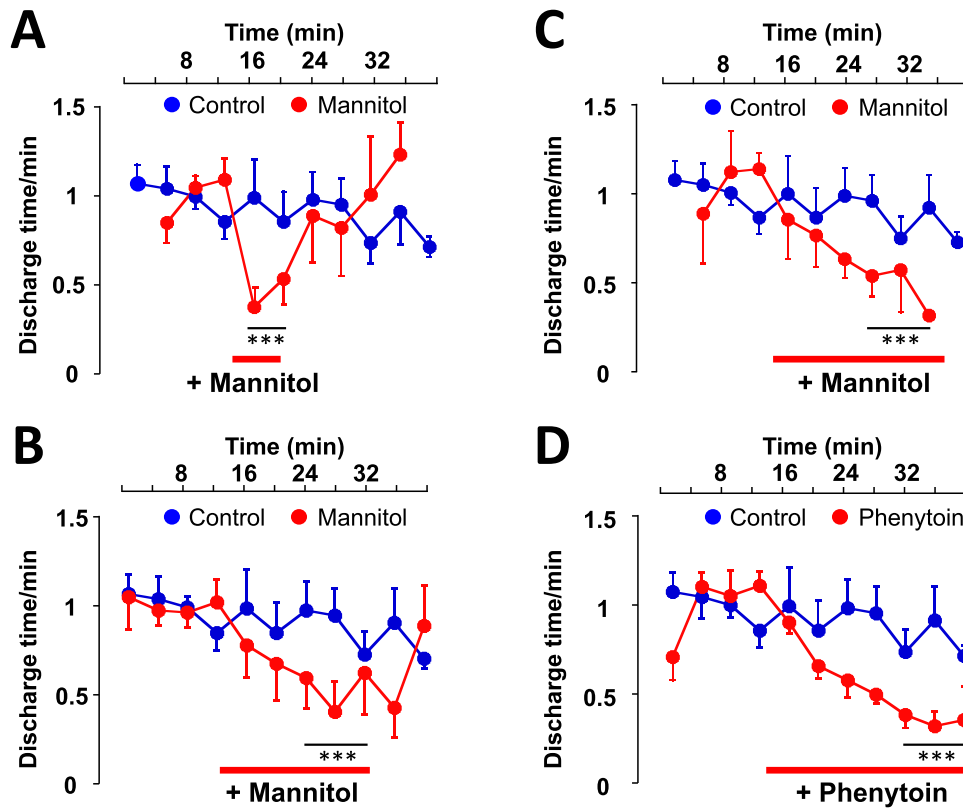
Fig. 2. Mannitol inhibition of epileptiform activity is as effective as that of Phenytoin.

Data are times of epileptiform activity per minute throughout the recordings. Each circle represent values averaged for four minutes of recordings, normalized relatively to the values observed before administration of Mannitol or of Phenytoin. Bars are SE values. Plot with blue circles represent values measured in slices perfused with 4-AP and zero Mg without Mannitol or Phenytoin. Plots with red circles represent values measured in slices perfused with 4-AP and zero Mg with the addition of Mannitol (A–C) or Phenytoin (D). In control experiments, without Mannitol or Phenytoin there is a slight run-down in epileptiform activity of 10-25% after 35–40 min of recording. Perfusion with Mannitol for 6 min with 50 mM (A) or for 23 min with 30 mM Mannitol (B) results in a transient reversible decrease in epileptiform activity. Prolonged perfusion with 30 mM Mannitol results in a ~60% decrease in time of epileptiform activity. Panel D shows that 25 mg/ml of Phenytoin results in a 60% decrease in the time of epileptiform activity. Data demonstrate that the anti-epileptic effect of 30 mM Mannitol is comparable with that of an established anticonvulsant drug such as Phenytoin.

For each treatment, statistical comparison was performed between the values at the time intervals of the maximal effect and the values of the control at the corresponding time intervals. * $p < 0.001$ ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Mannitol post-treatment does not significantly decrease the mean duration of epileptiform discharges.

	13 min	24 min	35 min
Control (no Mannitol) (n = 10)	1.00 ± 0.21	0.92 ± 0.24	0.62 ± 0.11
	Baseline	Mannitol	Recovery
Mannitol 7 min pulse (n = 5)	1.01 ± 0.59	0.36 ± 0.24	0.27 ± 0.04
Mannitol 23 min pulse (n = 7)	1.00 ± 0.37	0.68 ± 0.29	0.46 ± 0.16
Mannitol prolonged perfusion (n = 4)	1.00 ± 0.22	0.40 ± 0.40	
	Baseline	Phenytoin	
Phenytoin prolonged perfusion (n = 7)	1.00 ± 0.42	$0.17 \pm 0.04^*$	

In the recordings from which the data were obtained, Mannitol was administered *after* the induction of epileptiform activity (post-treatment) through 0.1 mM 4-AP and zero Mg. Data are means ± SE of normalized values of the mean duration of discharges measured in each minute, calculated averaging values for four consecutive minutes. In order to allow comparison of recordings with different baseline duration of discharges, values were normalized to the duration of discharges observed at the beginning of each recording. In the experimental groups shown in the table, these ranged between 2.9 and 6.7 seconds. Data shown in this Table differ from those shown in Fig. 4 where Mannitol was administered before the onset of epileptiform activity (pre-treatment).

In control slices, there is a 38%, statistically non-significant, time-dependent decrease in the duration of discharges throughout 35 min of recording. Perfusion with Mannitol induces a variable, statistically non-significant effect in the duration of epileptiform discharges. Lowest row shows that Phenytoin decreases by 83% the duration of epileptiform discharges. Mannitol-induced inhibition of epileptiform activity is therefore different from that of Phenytoin, as the latter is due mostly to a decrease in the duration of discharges. * $p < 0.05$ vs corresponding Baseline value (Wilcoxon).

In control experiments, with slices perfused with 4-AP, zero Mg and no Mannitol, the voltage of epileptiform discharges remained constant throughout the recording (Table 3, upper rows). Perfusion with Mannitol medium resulted in a non-statistically significant decrease in the amplitude of discharges of 30-50%. In contrast, co-perfusion with Phenytoin did not influence the voltage of isolated discharges but selectively reduced the amplitude of rhythmic repetitive activity (seizures) by ~50% (Table 3 lower rows).
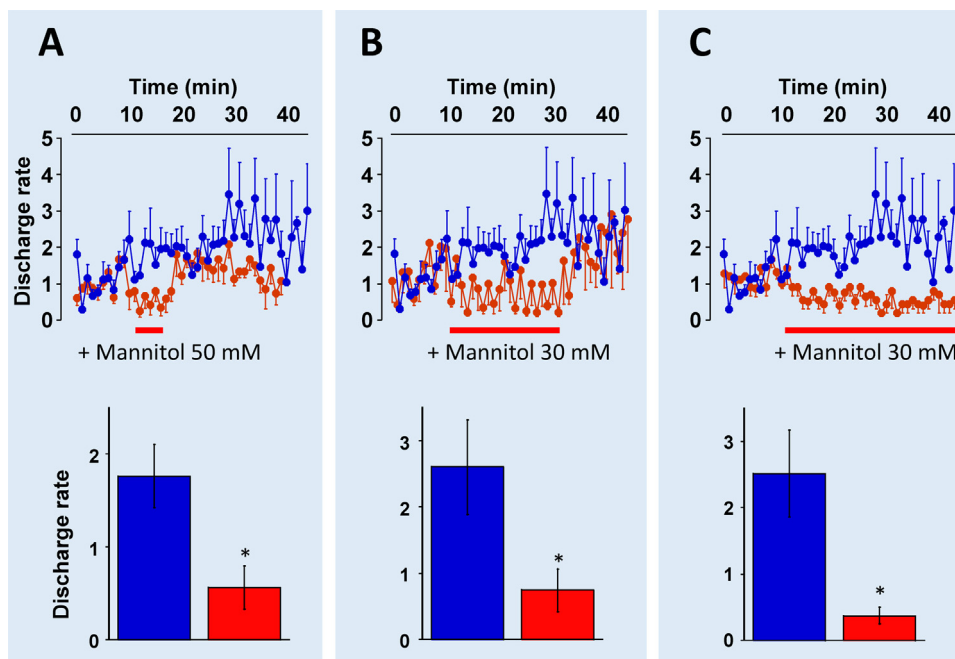
Fig. 3. Post-treatment with Mannitol decreases the rate of epileptiform activity.

Panel A shows the inhibitory effect of 30 mM Mannitol on epileptiform activity induced by perfusion with 0.1 mM 4-AP and zero Mg. Each point reports the rate of discharges per minute. Bars are SE values. The plot over the upper panel shows the time course of inhibition. Plot with blue circles and lines refer to the discharge rates without Mannitol while the red circles and lines refer to the recordings with the addition of 50 mM Mannitol. Note, that in the control plot, there is a *gradual* time-dependent increase in the discharge rate, more after 25 min of recording. Data show that within about 5 min after the start of Mannitol there is a drop by about 50% in the rate of discharges. Mannitol is perfused for 7 min: after returning to a solution without Mannitol within 10 min there is a resumption of the previous discharge rate. In the plot of the time course of the discharge rate with 0.1 mM 4-AP and zero Mg but without Mannitol, the discharge rate remains approximately constant, though with some increase after 30 min. Lower panel shows statistics of the inhibitory effect induced by 30 mM Mannitol. Data are mean ± SE. The degree of Inhibition is of ~69%.

Panel B shows the reversible inhibition of epileptiform activity induced by a prolonged perfusion of Mannitol. The plot over the upper panel shows the time course of inhibition. Within 5 min after Mannitol administration there is a decrease in the rate of discharges. Mannitol is administered for 23 min. After switch to perfusion with a medium with 4-AP and zero Mg but without Mannitol, the rate of discharges increases. Histogram in the lower panel shows statistics of the inhibitory effect induced by 30 mM Mannitol.

Panel C shows the maximal inhibitory effect of Mannitol. After the onset of epileptiform discharges, Mannitol was perfused throughout the rest of the recording (>30 min) so as to estimate the maximal inhibitory effect on the rate of discharges. In comparison with the curve of discharge induced by a medium without Mannitol (blue circles and lines), in the presence of Mannitol, the rate of discharges is inhibited by ~85%. Maximal effect is obtained in 20–25 minutes. * p < 0.05 ANOVA vs corresponding value in time of control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Mannitol post-treatment does not affect voltage of epileptiform activity.

		13 min	14 min	35 min
Control (no Mannitol) (n = 10)	IED	1.00 ± 0.24	1.08 ± 0.27	1.11 ± 0.24
	Seizure	0.95 ± 0.10	0.86 ± 0.10	0.86 ± 0.11
		Baseline	Mannitol	Recovery
Mannitol 7 min pulse (n = 5)	IED	1.00 ± 0.26	0.70 ± 0.17	1.00 ± 0.28
	Seizure	0.82 ± 0.25	0.50 ± 0.20	0.52 ± 0.26
Mannitol 23 min pulse (n = 7)	IED	1.00 ± 0.10	0.84 ± 0.12	0.82 ± 0.13
	Seizure	1.06 ± 0.17	0.72 ± 0.14	0.69 ± 0.10
Mannitol prolonged perfusion (n = 4)	IED	1.00 ± 0.31	0.58 ± 0.17	
	Seizure	1.63 ± 0.36	1.18 ± 0.25	
		Baseline	Phenytoin	
Phenytoin prolonged perfusion (n = 7)	IED	1.00 ± 0.22	0.92 ± 0.25	
	Seizure	0.66 ± 0.1	0.33 ± 0.10*	

In these recordings Mannitol was administered *after* the induction of epileptiform activity (post-treatment) through 0.1 mM 4-AP and zero Mg. In order to allow comparison of recordings with different baseline amplitude of discharges, values were normalized to the voltage of the interictal epileptiform discharges observed at the beginning of each recording. In the different experimental groups shown in the table the mean baseline values ranged between 45 and 131 μV. Data are means ± SE of *normalized* values. Data shown in this Table differ from those shown in Fig. 4 where Mannitol was administered before the onset of epileptiform activity (pre-treatment). Control slices showed no time-dependent change in the voltage of discharges throughout 35 min of recording. Perfusion with Mannitol results in a statistically non-significant decrease in the voltage of discharges ranging between 18 and 42% of epileptiform discharges. Lower row shows that Phenytoin does not affect the voltage of interictal discharges but decreases by 50% the amplitude of rhythmic repetitive activity. Thus, Mannitol and Phenytoin differ in their effect on the voltage of epileptiform activity. * p < 0.05 vs corresponding Baseline value (Wilcoxon).

Table 4
Mannitol post-treatment does not significantly affect the spread of epileptiform activity.

		13 min	24 min	35 min
Control (no Mannitol) (n = 10)	IED	1.00 ± 0.19	0.99 ± 0.17	0.87 ± 0.13
	Seizure	0.60 ± 0.15	0.57 ± 0.13	0.35 ± 0.09
		Baseline	Mannitol	Recovery
Mannitol 7 min pulse (n = 5)	IED	1.00 ± 0.25	0.94 ± 0.3	1.02 ± 0.30
	Seizure	0.68 ± 0.36	0.60 ± 0.29	0.52 ± 0.22
Mannitol 23 min pulse (n = 7)	IED	1.00 ± 0.24	0.69 ± 0.21	0.90 ± 0.21
	Seizure	0.63 ± 0.20	0.33 ± 0.16	0.32 ± 0.11
Mannitol prolonged infusion (n = 4)	IED	1.00 ± 0.24	0.76 ± 0.24	
	Seizure	0.75 ± 031	0.58 ± 0.26	
		Baseline	Phenytoin	
Phenytoin prolonged infusion (n = 7)	IED	1.00 ± 0.23	0.77 ± 0.18	
	Seizure	0.80 ± 0.21	0.39 ± 0.09*	

Spread is estimated by measuring the number of electrodes exhibiting discharges in each slices. In order to allow comparison of recordings with different baseline spatial spread of discharges, values were normalized to the values observed at the beginning of each recording. In the different experimental groups shown in the table, the mean baseline values ranged between 8 and 19 electrodes. Data are means ± SE of *normalized* values. In these recordings, Mannitol was administered *after* the induction of epileptiform activity (post-treatment) through 0.1 mM 4-AP and zero Mg. In control slices, there is no time-dependent change in the spatial spread of discharges, throughout 35 min of recording. Perfusion with Mannitol results in a statistically non-significant variable effect in the spread of discharges. Lower row, shows that Phenytoin does not affect the spread of interictal discharges but decreases by ∼50% the spread of rhythmic repetitive activity. Thus, Mannitol and Phenytoin differ in their effect on the spread of ictal-like activity. * $p < 0.05$ vs corresponding Baseline value (Wilcoxon).

In control experiments, with slices perfused with 4-AP, zero Mg and no Mannitol, the spatial spread of discharges exhibited a slight run-down during the course of the recording by 13-20% (Table 4). Perfusion with Mannitol medium resulted in a non-statistically significant decrease by 20% in the number of electrodes with isolated discharges, and in a non-statistically significant decrease by 14% in the number of electrodes exhibiting rhythmic repetitive ictal-like discharges. In contrast, co-perfusion with Phenytoin resulted in a ∼50% statistically significant decrease in the spatial spread of rhythmic repetitive discharges but in no significant inhibition of interictal discharges.

In sum, the data show that Mannitol post-treatment may exert only a relatively small and variable effect on the duration, voltage and spatial spread of discharges. Rather, Mannitol anti-convulsant effect can be accounted for by a sizable inhibition in the rate of initiation of epileptiform discharges. In this regard, the anticonvulsant mechanism of Mannitol differs from that of Phenytoin. The latter inhibits epileptiform activity specifically by inhibiting rhythmic repetitive ictal-like discharges, shrinking their duration and voltage without affecting isolated discharges.

3.5. Mannitol exerts a dose-dependent preventative effect on epileptiform activity

In a different set of experiments in 10 neocortical slices from 3 adult rats Mannitol was perfused throughout the recording rather than after the onset of epileptiform activity. Here we compared the rate of discharges in two groups: 1) slices perfused with 0.1 mM 4-AP and zero Mg and 15 mM Mannitol; 2) slices perfused with 0.1 mM 4-AP and zero Mg and 30 mM Mannitol. In three slices perfused with 15 mM Mannitol epileptiform discharges occurred at a rate of 1.3 ± 0.2 per min (μ ± SE). In contrast, in seven slices perfused with 30 mM Mannitol, epileptiform discharges were evoked only in four slices (Fig. 4). In the previous sections we showed that Mannitol *post-treatment* (Mannitol infusion *after* discharges have started) can inhibit the rate of discharges by ∼50% though all the slices still exhibit a residual epileptiform activity. In contrast, with Mannitol *pre-treatment* (perfusion with hyperosmolar solutions *before* discharges have started), about half of the slices showed complete suppression of epileptiform activity. Thus Mannitol as a

preventative agent is more effective in inhibiting epileptiform discharges, though its effect is less uniform.

4. Discussion

4.1. Main findings

An increase in Mannitol extracellular concentration similar to that utilized in neurocritical care inhibits epileptiform activity in brain slices by about 60% . However, when Mannitol is perfused *before* the onset of discharges, suppression of epileptiform activity is complete in nearly half of the slices. This Mannitol anticonvulsant effect is due to a decrease the rate of discharges without significantly affecting their duration, voltage or spatial spread. It is therefore a mechanism different from that of a sodium-channel blocker such as Phenytoin.

4.2. Neuroimaging and physiological evidence that seizures induce cytotoxic edema and extracellular shrinking

Brain MRI demonstrates that seizures lead to cytotoxic swelling (Kramer et al., 1987; Henry et al., 1994; Konermann et al., 2003; Kim et al., 2001; Hormigo et al., 2004). The occurrence of neuronal cytotoxic edema with seizures is corroborated by evidence of enhanced impedance of the extracellular space, consistent with a shrinking of the extracellular space of the brain (Vongerichten et al., 2016). Shrinking of the extracellular space may increase the physical proximity between the membranes of adjacent cells, facilitating non-synaptic interactions, increasing the electrical coupling of neurons to one another.

4.3. Extracellular shrinking facilitates epileptic hyper-synchrony

The spread of epileptiform activity may involve large cortical areas: for example, at least 9 cm² of cortex must be involved for an epileptiform discharge to be detectable by scalp electrodes (Ebersole, 2014). In addition, the typical duration of an interictal epileptiform discharge ranges between 20 and 200 msec (Koubessi and So, 2014). The mechanisms underlying epileptic synchronization must account for the spreading over a large cortical surface within a short time window. In models of experimental epilepsy the
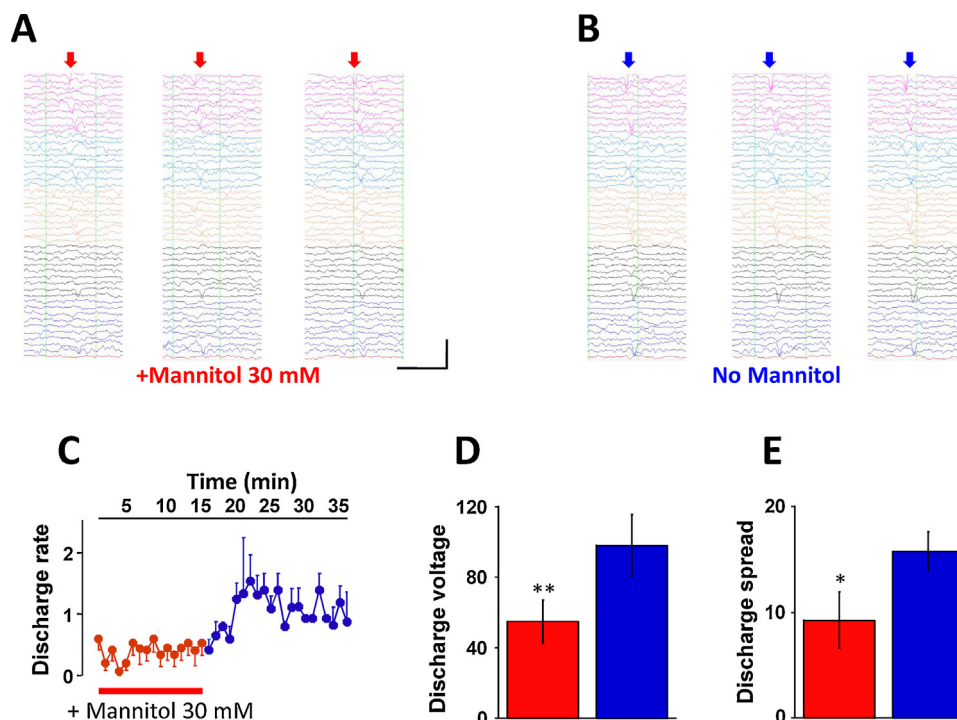
Fig. 4. Pre-treatment with Mannitol constrains discharges into a lower rate, smaller voltage and narrower spatial spread.

Data show the effect of Mannitol on epileptiform activity when Mannitol is administered before the onset of discharges (pre-treatment). We perfused 7 slices with 0.1 mM 4-AP, zero Mg and 30 mM Mannitol. In 3 slices Mannitol was administered throughout the recording and no epileptiform discharge were recorded. In 4 slices, discharges were observed within 10 min from the start of the recording. In these, after recording discharges for 15 min we switched to a medium with 0.1 mM 4-AP, zero Mg but without Mannitol. In less than 5 min there was a rapid increase in the rate of discharges,in the number of electrodes exhibiting discharges and also in the voltage of discharges.

Panel A shows voltage traces obtained through Multiple Electrodes Arrays from slices perfused with a medium containing 0.1 mM 4-AP, zero Mg and 30 mM Mannitol. Data are from a selection of 51 channels out of the 60 recorded. Red arrows point to the time of occurrence of discharges. Discharges occur in several rows of electrodes. Calibration bars are 0.5 sec and 300 μV

Panel B shows voltage traces recorded after the switch to a solution with 0.1 mM 4-AP, zero Mg but without Mannitol. Data show a spread of discharges into a larger number of electrodes and also an increase in the amplitude of discharges.

Panel C is a plot of the time course of discharge rates when switching from a solution with Mannitol (red circles and lines) into a solution without Mannitol (blue circles and lines). Bars are SE values. Within 5 min after the switch, the rate of discharge doubles.

Panels D and E show that switching from a solution with Mannitol (red columns) into a solution without Mannitol (blue columns) results in an increase in the voltage and in the number of electrodes with discharges, that is, in a broader spatial spread. Data are means ± SE. * $p < 0.05$ paired Student's t test. ** $p < 0.01$ paired Student's t test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recruitment into synchronized firing is driven by *synaptic* mechanisms. For example, in hippocampal slices picrotoxin-induced block of GABA-A receptors unmasks latent polysynaptic pathways (Wong et al., 1986): excitation of a critical number of neurons becoming simultaneously active may lead the recruitment through cascade excitation of a broader number of neurons. Non-synaptic mechanisms are likely playing an important role, too: epileptiform activity can still occur even when chemical synaptic transmission is depressed (Roper et al., 1992; Dudek et al., 1990, 1998; Haglund and Hochman, 2005; Seigneur and Timifeev, 2011; Hochman, 2012). Swelling of neurons with consequent shrinking of the extracellular space can facilitate epileptic synchrony through non synaptic mechanisms.

Indeed, it is established that treatments aimed at reducing cytotoxic edema inhibit epileptiform activity: hypertonic solutions do exert an anti-seizure effect in experimental model (Reed and Woodbury, 1964; Baran et al., 1987; Baxter et al., 1986; Roper et al., 1992) and can exert an anticonvulsant effect even in human subjects (Haglund and Hochman, 2005).

4.4. Extracellular space shrinking may specifically influence discharge initiation rather than their spread

The process of epileptic synchronization is thought to involve a cascade of at least two different classes of mechanisms. *First*, an epileptic discharge must be *initiated* by neurons with a spontaneous bursting activity and *then* may recruit and spread to adjacent neurons into synchronous firing. The increases in physical proximity between adjacent neurons due to extracellular shrinking, heightens excitatory non synaptic electrical interactions between neurons. The effect of extracellular ionic changes occurring in tinier volumes would be likely enhanced. It is unclear whether this would affect discharge initiation, recruitment-spread or both. The specific mechanisms of Mannitol, inhibiting the rate of discharges without affecting significantly the voltage of discharges, can provide novel insight. The data suggest that cytotoxic swelling and extracellular shrinking are critical for *the initiation* of discharges rather than or more than for the subsequent propagation and recruitment into epileptic hypersynchrony.

4.5. Mannitol vs Phenytoin anticonvulsant mechanisms: clinical correlations

Brain slices' recordings demonstrate a difference in the anticonvulsant mechanism of action between Phenytoin and Mannitol. Mannitol anticonvulsant effect is due mostly to a decrease in the rate of discharges. In contrast, Phenytoin does not decrease the rate of discharges but rather decreases the duration of rhythmic repetitive ictal-like discharges as well as their spatial spread. These differences in anticonvulsant mechanism observed in brain slices

do match observations in human subjects. For example, in four human subjects (Haglund and Hochman, 2005) IV Mannitol exerted a specific anticonvulsant effect by decreasing the rate of interictal discharges by 60%. In contrast, in localization-related epilepsy anticonvulsant drugs do inhibit seizures without decreasing the rate of interictal epileptiform discharges (Gotman and Marciani, 1985). Thus, the differences recorded in brain slices between Mannitol and Phenytoin anticonvulsant mechanisms do exhibit a similarity to human epilepsy observations.

Much clinical interest has been growing towards rational polypharmacy, that is, therapeutic combinations of anticonvulsant drugs with different mechanism of action (Brodie and Sills, 2011). Rational polypharmacy, may allow improvement of refractory drug-resistant seizures in cases where no other therapeutic options are available. The clinical availability of anticonvulsant agents with distinct mechanisms allows a wider range of possible combinations.

Mannitol mechanism, is different than those of established anticonvulsant drugs currently prescribed by clinicians: in the perspective of a possible therapeutic use it may therefore represent a helpful therapeutic agent to be combined with established anticonvulsant agents.

4.6. Mannitol hyperosmolar solutions are already utilized routinely in neurocritical care and might be utilized as anticonvulsant agents, too

Hypertonic solutions containing Mannitol or hypertonic saline are used in patients with large strokes or traumatic head injuries (Qureshi et al., 1999; White et al., 2006; Todd, 2013; Jeon et al., 2014; Surani et al., 2015). In clinical practice the dose of Mannitol is adjusted so as maintain serum osmolarity below 320 mosm/l. Yet, this threshold of 320 mosm/l is frequently crossed without adverse consequences (Diringer and Zazulia, 2004) and its clinical relevance has been disputed. In our study the concentrations of Mannitol inhibiting epileptiform activity allow for an enhancement of osmolarity by 30 mosm/l and, as such, are close to those already utilized in neurocritical care.

Mannitol anticonvulsant effect might not be applied routinely to a *clinical outpatient setting*. Yet, it might be applied in neurocritical care as it is already utilized in this setting, though not for seizures. For example, Mannitol anticonvulsant effect could be utilized for status epilepticus. Uncontrolled status epilepticus often leads to refractoriness to pharmacological treatment and to high rates of mortality (Drislane, 2005). Mannitol, working as an anti-convulsant agent *novel under a clinical standpoint* and with a mechanism of action different than currently available anticonvulsant drugs may improve the outcomes for this condition.

5. Summary and conclusions

An extracellular Mannitol concentration in the range of that utilized in neurocritical care can inhibit epileptiform activity as effectively as Phenytoin. Seizures-induced extracellular shrinking likely plays a role in furthering epileptic hyperexcitability. Furthermore hyperosmolar solutions do exert an anti-seizure effect in experimental models of epilepsy. Because hyperosmolar solutions are already been used routinely in critical care, these findings may pave the way for a novel therapeutic approach for the management of seizures in such clinical setting.

Acknowledgments

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