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Periventricular leukomalaci (PVL) is critically dependent upon the presence of extracellular ions (Ca^{2+} and Na^{+})

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ABSTRACT

This work is clarifying the role of extracellular ions in PVL in neonates. Ischaemic injury of adult brain was critically dependent upon the presence of extracellular Ca^{2+} as revealed by a range of studies (Stys et al., 1992). Along with they found that two extracellular ions were essential for the development of permanent loss of function during ischaemia in adult brain: Ca^{2+} and Na^{+}. The dependence of ischaemic injury in adult upon both Ca^{2+} and Na^{+} indicates that these ions are potentially toxic to cell viability by causing permanent loss to axonal function. Therefore, the effects of these two ions during 90 min oxygen-glucose deprivation (OGD), (the practical model of ischaemia) in neonatal brain were investigated using electrophysiology techniques for compound action potential (CAP) recording. This produced the interesting finding that removal of either Ca^{2+} or Na^{+} from the extracellular space protected the neonatal brain from ischaemic injury during OGD. When Ca^{2+} or Na^{+} were removed from the extracellular space during 90 min OGD, the CAP declined at the end of OGD. Afterward, the CAP recovered following restoration of normal conditions. *The results of this study indicated that,* extracellular Ca^{2+} and Na^{+} are essential for the development of permanent loss of function during OGD in neonatal brain.

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2. The aim of the study:

To establish that ionic channels play very important role in the pathogenesis of PVL, in addition, to open the door for the researchers in further research about the role of ionic channel blockers, they might have a protective role against ischaemia in neonatal brain.

3. Methodology:

3.1. Animals and tissue dissection

Neonatal Wistar rats were used at age of (P0), an age when the optic nerve is non-myelinated (Hildebrand and Waxman, 1984; Fern et al., 1998). The optic nerve is translucent at this age because the axons lack a myelin sheath. Therefore, the dissection was difficult compared to adult nerves and typically took 5-10 min. The perinatal rats were decapitated and dissected following the same procedures used for adult rodents. All dissected nerves were placed in an interface perfusion chamber (Harvard Apparatus Inc.) Where they were allowed to equilibrate for an hour before being inserted into the electrodes. This was done to give the nerve time to recover from the trauma of the dissection and so to regain homeostatic control over the intracellular and extracellular ionic environment. When oxygen-glucose deprivation was required (OGD), glucose was omitted from solutions, and bubbled with 95% N2, 5% CO2. Osmolarity was periodically measured and maintained at 310-320 mOsm using NaCl or sucrose as required. Electrophysiology is used to record the action potential from the nerves. The RONs were perfused with artificial cerebro-spinal fluid (aCSF) at a rate of 1-2 ml/min during normal conditions. The temperature was maintained at 37°C throughout the time of the experiment (101 C temp controller, Warner etc.), and the RONs oxygenated with 95%O2/5%CO2, which was passed through aCSF and through a water chamber for warming and humidification.

3.2. Data analysis:

The amplitude of the biphasic CAP, calculated from the maxima of the positively deflected peak minus the minima of the negatively deflected peak, was used as a measure of the functional integrity of neonatal RONs (Fern et al., 1998). Data are expressed as mean ± SEM, significance determined by Bonferroni`s Multiple Comparison Test or ANOVA as appropriate.

4. Results:

In the zero-Na^{+} experiments, Na^{+} was omitted and choline or[N-methyl-d-glutamine] ($NMDG^{+}$) was added. In zero-Ca^{2+} OGD experiments, CaCl2 was omitted from the artificial cerebrospinal fluid (aCSF) and (50μM) ethylene glycol-bis [β-aminoethyl ether]-N, N, N', N'-tetra acetic acid (EGTA) was added. Nerves were first exposed to 10 min normal conditions, followed by 10 min zero-Ca^{2+} aCSF. This was followed by 90 min OGD in zero-Ca^{2+}, and then the nerves were re-perfused with zero-Ca^{2+} aCSF for 20 min before re-instatement of normal conditions for 70 min. When Ca^{2+} was removed from the extracellular space during 90 min OGD, the CAP fell to 50.4±10.01% of the initial value at the end of OGD. Afterward, the CAP recovered to 74.3±1.3% of the initial value, (n=6; $P<$ 0.0001 vs OGD) following restoration of normal conditions. The recovery in the CAP following 90 min OGD is greatly enhanced by removal of Ca^{2+} from the perfusate. Note that under control conditions, zero-Ca^{2+} evoked a transient decline in the CAP after an initial increase within the first 10 min to 67.4±7.8% of the initial value, and recovered following restoration of normal conditions to 92.8±2.01% of the initial value, (n=6). In a separate set of experiments in which Ca^{2+} is not removed from the perfusate, removing Na^{+} from the perfusion has

a similar protective effect. As a result, the CAP declined at the end of 90 min OGD to 45.1 ± 4.03% of the initial value, and the recovery in the CAP following restoration of normal conditions was enhanced by zero-Na^+ conditions to 74.3±2.0% of the initial value, (n=6; P<0.0001 vs OGD). Note that under control conditions, zero-Na^+ evoked a large decline in the CAP to 20.9±1.12% of the initial value, and recovered to 75.4±5.2% of the initial value, (n=6) following restoration of normal aCSF. The dependence of ischaemic injury upon both Ca^{2+} and Na^+ indicates that these ions are potentially toxic to cell viability by causing permanent loss to axonal function (Siesjo, 1986; Choi, 1994). It is therefore clear that, extracellular Ca^{2+} and Na^+ are essential for the development of permanent loss of function during OGD in pre-myelinated axons, (Figs. 1-6).

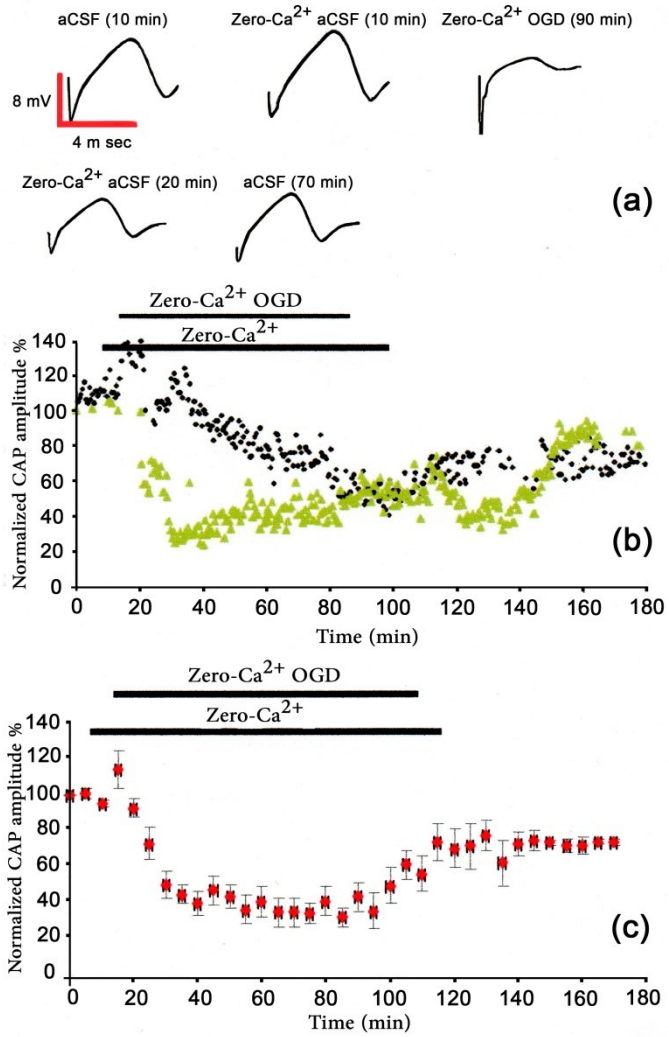

Fig. 1. Exposure to 90 min OGD after initiating zero-Ca^{2+} condition for 10 min resulted in similar changes in the CAP amplitude of zero-Ca^{2+} control.

In Fig.1.: *a*, representative CAPs during 10 min normal conditions, 10 min of zero-Ca^{2+} aCSF, 90 min zero-Ca^{2+} OGD, during reperfusion of the nerve with zero-Ca^{2+} aCSF for 20 min period and during re-introduction of normal conditions for 70 min, respectively; *b*, plots of two individual experiments against time; *c*, Mean data plotted showing the protective effect of Ca^{2+} removal during 90 min OGD in neonatal optic nerves. CAP is normalized to 100% at zero time, CAP at the end of zero-Ca^{2+} OGD is (50.4±10.01), and at the end of the experiment after re-introduction of normal conditions is (74.3±1.3), n=6. Error bars are SEM.

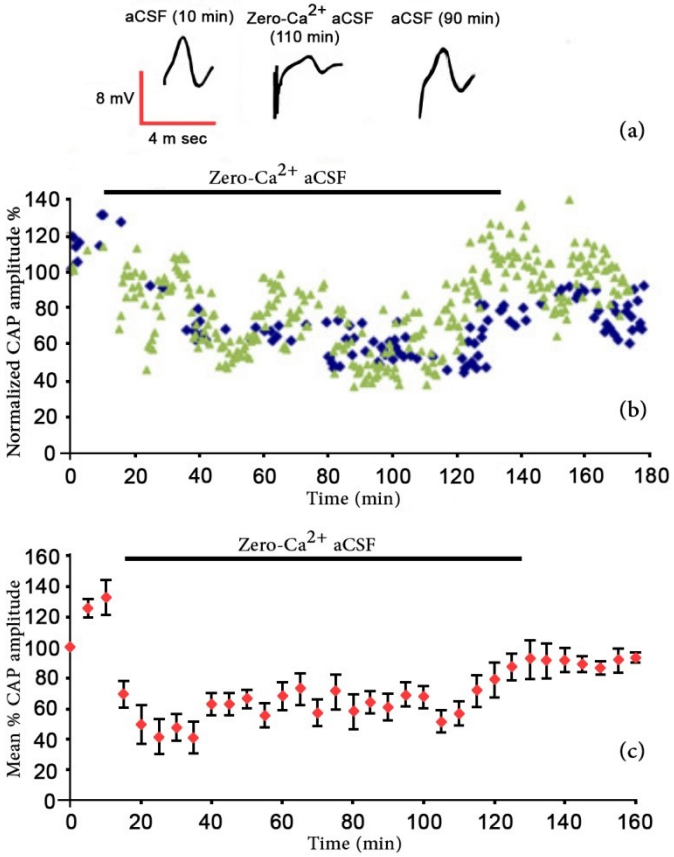

Fig. 2. Removal of Ca^{2+} from control conditions evokes a transient decline in the CAP in P0 RONs.

In Fig. 2.: *a*, representative CAPs recorded during normal conditions, during zero-Ca^{2+} aCSF for 110 min, and during re-introduction of normal conditions for 90 min; *b*, Plots of two individual experiments against time; *c*, Mean data plotted against time showed that removal of Ca^{2+} from the perfusion evoked a transient decline in the CAP followed by a large recovery after re-institution of normal conditions, n=6. CAP at zero time is normalized to 100 %, CAP at the end of zero-Ca^{2+} aCSF is (67.4±7.8), and at the end of the experiment after re-instruction of normal conditions is (92.8 ± 2.01). Error bars are SEM.

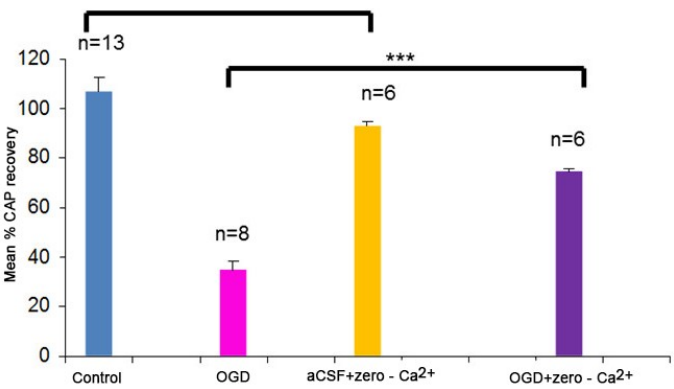

Fig. 3. Histogram summarizing the effect of Ca^{2+} removal from the perfusate on the CAPs recovery in P0 RONs.

The values shown represent the mean ± SEM of 6-13 investigations. * Denotes a statistically difference between zero-Ca^{2+} OGD and OGD alone by t-test (p<0.0001). There was no significant comparison in the CAP recovery between zero-Ca^{2+} aCSF and control.

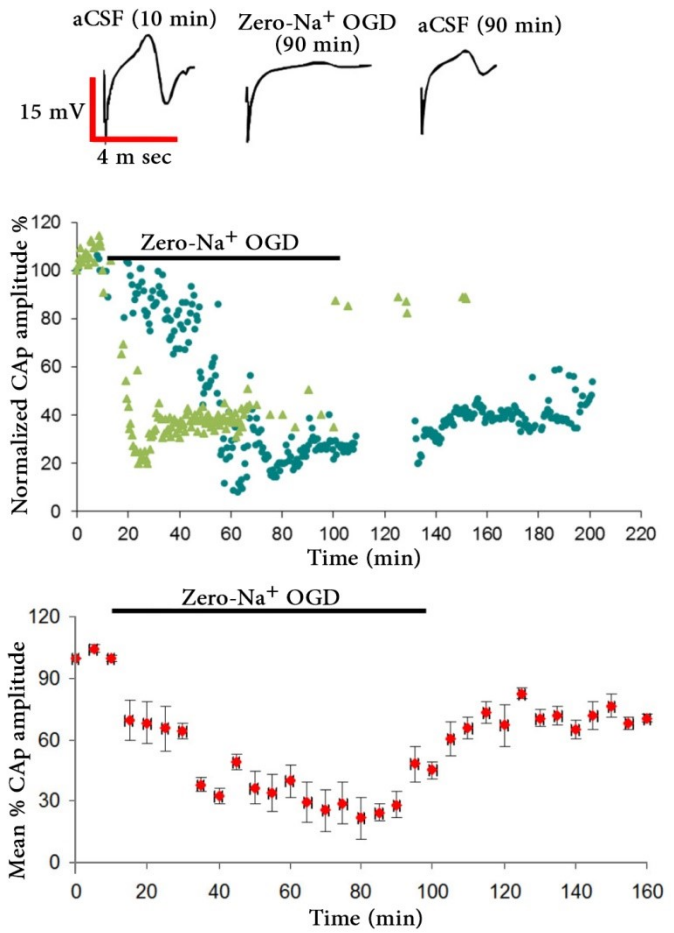

Fig. 4. Removal of Na+ during 90 min OGD was protective in P0 RONs.

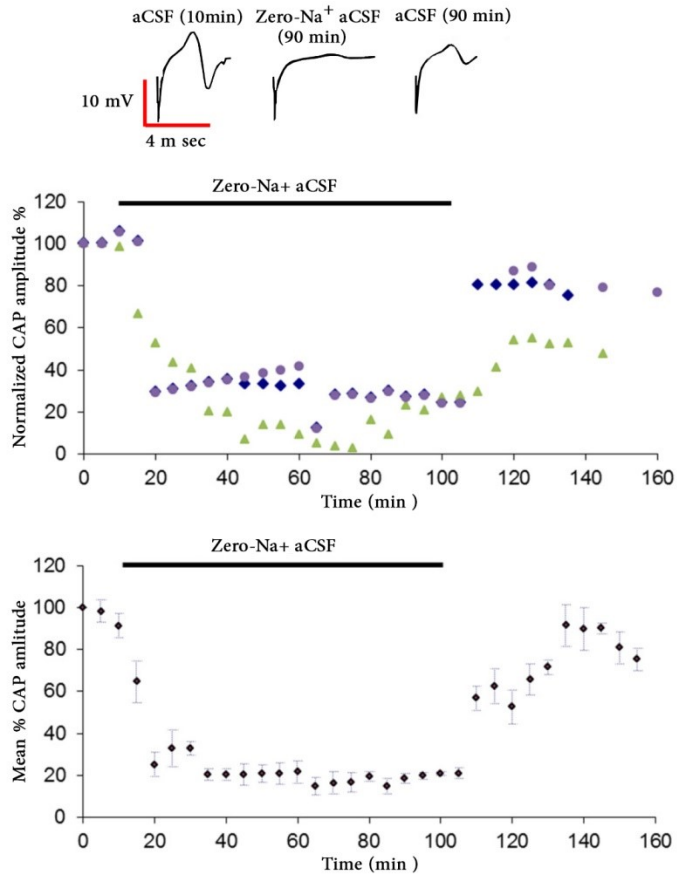

Fig. 5. CAPs recorded under Zero-Na+ aCSF in P0 RONs.

In Fig. 4.: *a*, representative CAPs amplitude during normal conditions, during exposure of P0 RON to 90 min zero-Na+ OGD, and after restoring the normal conditions. *b*, Plots of two individual experiments against time. *c*, Mean data showing the changes in CAPs amplitude during and after exposure to 90 min Zero-Na+ OGD, n=6. CAP at zero time is normalized to 100%, CAP at the end of zero-Na+ OGD is (45.1±4.03), CAP at the end of the experiment after restoring of normal conditions is (70.3±2.0). Error bars are SEM.

In Fig. 5.: *a*, representative CAPs recorded during normal conditions for 10 min, during exposure to 90 min zero-Na+ aCSF, and after restoring the normal conditions normal conditions. *b*, Three individual data plotted against time. *c*, Mean data showing large reversible effect of zero-Na+ on the CAP amplitude, n=6. CAP at zero time is normalized to 100 %, CAP at the end of 90 min zero-Na+ aCSF is (20.9±1.12), and at the end of the experiment after restoring the normal conditions is (75.4±5.2). Error bars are SEM.

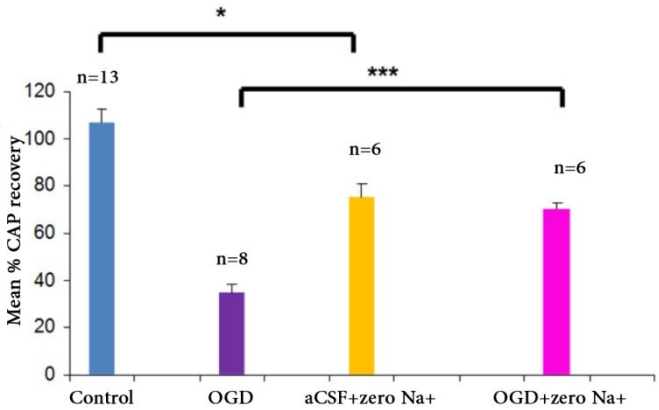

Fig. 6. Histogram summarizing the effect of zero-Na+ conditions on the CAP recovery in P0 RONs.

The values shown represent the mean ± SEM of 6-13 investigations. The recovery in the CAPs following 90 min of OGD is greatly enhanced by removal of Na+ from the perfusate. Removal of Na+ from the solutions exhibited a significant recovery in the CAPs in compare to OGD alone. ***=p<0.0001. There is a significant comparison in the recovery of the CAP during zero Na+ aCSF in compare to control conditions. *=p<0.01.

5. Discussion:

The sensitivity of neonatal brain to the withdrawal of oxygen and glucose at 37°C in isolated P0 RON axons was assessed by electrophysiological recording of CAPs. Optic nerves from this age group, when exposed to a longer period of OGD (90 min), showed a significant loss in the CAPs at the end of OGD exposure with partial recovery after re-perfusion with normal conditions. The resistance of the neonatal brain to energy deprivation is thought to be a consequence of the low metabolic rate of the immature CNS (Duffy et al., 1975; Hansen, 1985). In this age group all axons are non-myelinated; some axons begin to acquire a single layer of myelin from age P6-P7, and the proportion of axons that acquires a single layer of myelin increases from a few% at P6 to 85% at P28 – these become unsheathed completely by adulthood (Foster et al., 1982; Skoff et al., 1976). Therefore, metabolic activity is associated with the anabolic process of myelination and the degree of energy sensitivity (Azzerelli et al., 1980; Chugani et al., 1991; Kennedy et al., 1972; Rice et al., 1981). In addition to high energy reserves and low energy requirement in neonatal RONs, there are other factors that might explain the OGD tolerance at this point of development. Waxman *et al.* (1989) showed that non-myelinated optic nerves of a diameter 0.2 µm have low densities of Na+-channels. However, even a low density of Na+-channels can support action potential conduction, and would be expected to depolarize the small calibre fibres to threshold because of high input resistance of these fibres (Waxman et al., 1989). A low Na+-

channel density in these non-myelinated axons might relate to low Na^+ ion influx and weakly promote the reversal of Na^+/K^+ exchange during energy deprivation. Alix et al. (2012) showed low levels of VGCCs expression at P0 RONs which may render them less sensitive to ischaemic injury, whereas P8-P10 RONs express clusters of functional VGCCs as they prepare for myelination (Alix et al., 2008). In Optic nerves at P8-P10, undergoing initial radial expansion, ischaemic injury was mediated by Ca^{2+}-influx through VGCCs.

The effect of Ca^{2+} and Na^+ removal during OGD and normal conditions in non-myelinated optic nerves:

In 1992, Stys et al., found that Ca^{2+} and Na^+ were the two extracellular ions that were essential for the development of permanent loss of function during anoxia in adult RON. The effect of the removal of either Ca^{2+} or Na^+ from the extracellular space in P0 RONs during both OGD and normal aCSF was investigated. It was found that removal of either Ca^{2+} or Na^+ from the extracellular space protected the non-myelinated axons during OGD at this age group. The removal of Ca^{2+} from the extracellular space during 90 min OGD produced a partial fall of the CAP which recovered significantly after restoration of normal conditions. Zero-Ca^{2+} aCSF for 90 min evoked a transient decline in the CAP after an initial increase within the first 10 min, and then exhibited very partial ablation with significant full recovery after restoration of normal conditions. Recovery of the CAP occurred with the removal of Ca^{2+} during both OGD and normal aCSF, indicating that pre-myelinated axons might be directly damaged by accumulation of intracellular Ca^{2+}. The levels of Ca^{2+} increase in the postnatal period, and Ca^{2+}-channels, have been shown to contribute to injury in white matter (Lorenzon and Foehring, 1995; Fern et al., 1995). The axons of developing white matter are the focus of injury in various diseases that attack immature central white matter including periventricular leukomalacia (PVL), the main pathology associated with cerebral palsy (Back and Rivkees, 2004). PVL is thought to be partially ischaemic in origin (Alix et al., 2008), since ischaemic axonal injury is mainly Ca^{2+} -dependent (Stys et al., 1992; Fern et al., 1995), Ca^{2+}-channels may play a major role for Ca^{2+} influx into developing white matter (Alix et al., 2008). Ionic mechanisms in adult RONs that mediate toxic Ca^{2+} overload during anoxia include non-inactivating Na^+-channels (Stys et al., 1993), voltage-gated Ca^{2+}-channels (Fern et al., 1995), reverse Na^+/Ca^{2+} exchanger (Stys et al., 1992), and activation of L-type voltage-gated Ca^{2+}-channels (Brown et al., 2001). However, these channels might have a role in exacerbating Ca^{2+} overload during OGD in pre-myelinated optic nerves. Removing Na^+ from the solution in the presence of Ca^{2+} has a similar protective effect on the CAP recovery. Zero-Na^+ OGD produced a decline in the CAP with recovery after restoration of normal conditions. These results suggest the fundamental role of the ionic basis in the pathogenesis of pre-myelinated axonal injury. During energy deprivation, CNS axons can no longer make ATP and the ATP-dependent Na^+/K^+ exchange fails, causing moderate Na^+ influx through non-inactivating voltage-gated Na^+-channels and subsequent disruption of axon ion homeostasis and loss of excitability (Fern et al., 1998; Ransom et al., 1990; Stys et al., 1992). Although voltage-gated Na^+-channels are expressed somewhat equally along the non-myelinated axons (Westenbroek et al., 1989), they accumulate at high density at the AIS (Catterall, 1981; Wollner and Catterall, 1986). The density of Na^+-channels is $< 2\mu m^2$ in the neonatal optic nerve axons and increases with age until P25, then decreases in adulthood (Xia and Haddad, 1994). These Na^+-channels result in persistent Na+ currents which exacerbate white matter injury following OGD (Alzheimer et al., 1993). However, influx of both ions through voltage-gated channels and Na^+/Ca^{2+} exchanger has a significant role in the injury processes (Fern and Ransom, 1997). The data suggests that injury at P0 is more similar to that in the adult, in contrast to Alix et al. (2008) who found that developing central axons (P10) express functional voltage-gated Ca^{2+}-channels (L-type and P/Q type. These Ca^{2+}-channels represent a major pathway for ischaemic Ca^{2+} influx into developing white matter in P10 RONs (Alix et al., 2008). Therefore, blockade of voltage gated channels or Na^+/Ca^{2+} exchanger may be of clinical significance in protecting neonatal brain from ischaemic injury.

6. Conclusion:

This result raised questions about the potential role of Ca^{2+} ion in preserving the action potential conduction in the absence of Na^+ in P0 RONs?

Na^+ is an important ion in inducing the action potential conduction in both myelinated and non-myelinated white matter and consequently removal of Na^+ render the nerves useless. But in early point of development removal of Na^+ from the perfusate did not stop the conduction of action potential. Axonal Ca^{2+}-channels modulate axonal excitability via Ca^{2+}-activated K^+-channels especially under conditions of large Ca^{2+}-influx during repetitive stimulation (Lev-Ram and Grinvald, 1986). It has been shown that axonal Ca^{2+}-transients regulate the frequency and speed of action potentials, possibly by activating Ca^{2+}-dependent K^+-channels (Callewaert et al., 1996). A Ca^{2+}-dependent K^+-conductance has been reported in RON (Lev-Ram and Grinvald, 1986).

Do N-type or L-type Ca^{2+}-channels that regulate Ca^{2+}, play role in mediating axonal action potential conduction?

Therefore, another study is required to answer the question listed above regarding the type of Ca^{2+}-channels that regulate Ca^{2+} and play a significant role in mediating axonal action potential conduction.

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