Cerebral Neurons in *Rattus norvegicus* Following a Mild Impact to the Skull: Equivalence of Modulation by Post-Impact Pregnancy or Exposure to Physiologically-Patterned Magnetic Fields

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Abstract: The aim of the study was to discern if one natural (pregnancy) and one man made (patterned magnetic field) treatment could reduce the incidence of neuronal anomalies following very mild head trauma. For several weeks following an impact of mechanical energy to the skull that was sufficient to stun about 50% of female rats, conspicuous shrunken, darkly stained neuronal somas remained scattered within the cerebral cortices and lateral limbic region on the side of the impact. Rats that became pregnant and cared for young following the injury or that were exposed for a comparable duration to a 5 to 500 nT physiologically-patterned magnetic field that has been shown to reduce depression following closed head injuries in human patients showed half the numbers of areas containing these abnormal neuronal somas compared to females that remained virgins or were exposed to sham fields or to stronger (1.5 μT) intensities. The results support previous observations that oestrogen compounds and some patterns of magnetic fields can be neuroprotective following minor closed head injury and indicate their efficacy is similar in magnitude.

Key words: Mechanical impact, neuronal morphology, pregnancy, physiologically patterned magnetic fields, recovery

INTRODUCTION

Mild closed head injuries and correlated alterations in brain micromorphology and function produce permanent changes in the behavior and physiology of humans and rats (Parker, 1990; Persinger, 1995; Shaw, 2002). These changes can occur following mechanical impacts to the skull of magnitudes that are encountered routinely within ecologically-relevant contexts such as predator-prey or habitat interaction. Lado and Persinger (2003, 2008) have found that a single impact of a force (equivalent to 15 km h⁻¹) from dropping a 200 g weight from a distance of 0.9 m upon the dorsal skull resulted in the appearance of neurons with conspicuously shrunken somas scattered in the cerebral cortices beneath the impact site and within the ventral limbic area (primarily amygdala and entorhinal cortices) even though the rats displayed no evidence of transient neurological signs such as stunning or loss of consciousness. The anomalous neuronal soma persisted for several weeks.

The numbers of microscopic areas containing these neurons were correlated positively with mild but clinically relevant and statistically significant behavioral deficits that are traditionally associated with cerebral areas beneath the site of impact. Like the effects from hypoglycaemia the shrunken soma were found scattered among normal neurons within a healthy extracellular matrix. They were unlike the shrunken soma grossly correlated with ultimately necrotic or apoptotic degradation (Liou et al., 2003). Temporal cross-sectional studies indicated this type of neuronal anomaly induced by this weight drop method was present for more than a month (Lado and Persinger, 2003, 2008) after the impact.

This impact force had been selected because its magnitude of energy per somatic volume would be within the same order of magnitude as the energy generated from glucose metabolism (about 1 picoJoule). We had calculated that the passage of this quantum of mechanical energy or pressure wave through the rat brain was about 2 to 3 m sec. We reasoned that passage through the

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cytoplasmic volume would disrupt but not destroy the functions of the organelles and produce a variant of dormancy of interneurons that is seen in rats in which epileptic seizures have been induced (Bekenstein and Lothman, 1993). This dormancy can occur for several months and could render these neurons sensitive to either subsequent necrosis or positive treatments that might foster recovery.

In the present study, we investigated the efficacy of two post-impact treatments: pregnancy and exposure to physiologically-patterned weak magnetic fields. We predicted both treatments would reduce the numbers and areas of these anomalous neurons. Both treatments were based upon our clinical experiences with mild closed head injuries. In the first case, we have observed that women who become pregnant after what would be classified as a mild closed head injury (no loss of consciousness or mild stunning), actually showed less quantitative neuropsychological impairment one to two years after the incident compared to women who sustained apparently similar mechanical impacts but did not become pregnant.

The second treatment was based upon a physiologically patterned magnetic field (St-Pierre et al., 2008) which, when presented once per week for four weeks across the temporal lobes of patients with recalcitrant depression following a closed head injury, produced a remarkable improvement of cerebral function as defined by both electroencephalographic and psychometric criteria (Baker-Price and Persinger, 1996, 2003). In fact the effect size, the amount of variance explained in the change in psychometric depression scores, was comparable to that found following treatment with Transcranial Magnetic Stimulation (TMS) which involved simpler patterns but intensities about a million times stronger.

As summarized by Behl (2002), oestrogen can be a powerful neuroprotective hormone, protecting cultured neurons against a wide range of insults that include L-glutamate, the viral protein gp 120, glutathione depletions, oxidation by H,O_2, staurosporine-induced apoptosis and Fe^{2+}-induced peroxidation. In addition to the classic genomic pathway involving nuclear translocation and gene transcription, oestrogen can reduce apoptosis by inducing MAP-K (mitogen activating protein kinase) and by the phosphorylation of ERK-1/2. Physiological concentrations of oestradiol reduce ischemic injury by 50% (Wise, 2005) with a delay and attenuation of injury-mediated DNA fragmentation 8 h after middle artery occlusion in female rats (Rau et al., 2003). Pregnancy, through a combination of progesterone and prolactin, stimulates neurogenic processes (Shingo et al., 2003) and improves learning and memory.

A thorough review of the effects of extremely low frequency magnetic fields upon cell protection and repair by Robertson et al. (2006) has shown that exposure to biofrequency (1 to 100 Hz) pulsed magnetic fields reduced the severity of tissue damage in organs and cell cultures with intensities within the microTesla range. These ranges are often found in the vicinity of modern habitats. As indicated by Vladimirskii and Temryants (1996) the impact of a magnetic field depends upon the form of the signal and the presence of different types of modulation. Consequently, like pharmacology, where the specific structure of the molecule determines the biological efficacy, the temporal pattern of the applied field determines the biological response.

Martin et al. (2004a) showed that physiologically-patterned magnetic fields can be bioeffective within the 5 to 500 nT range and can produce long-term changes in blood chemistry (St-Pierre et al., 2008). In addition, a component of this complex field, a frequency-modulated pulse (Thomas et al., 1997) known to produce powerful analgesia, has recently been shown to affect ERK 1/2 in cancer cell lines (Martin et al., 2004b). It is further demonstrated that the inhibition of ERK but not PKC, PI3 kinase, or calcineurin, partially blocked this frequency-modulated magnetic field's inhibition of cell growth. A similar effect has been shown for oestrogen (Behl, 2002).

By far the most elegant series of studies to show magnetic fields could stimulate functional recovery was reported by Ahmed and Wierszko (2008). They found that daily exposures (7.5 min) for 24 consecutive days of mice with experimentally-induced spinal injuries to 450 pulses of 1.5 T delivered at a frequency of 1 Hz (pulse duration 450 μsec; rise time 60 μsec) resulted in remarkable recovery when combined with acrobatic exercise. The combination of the exercise and the magnetic stimulation upon the percent improvement of locomotor recovery after the spinal cord injury was equivalent to the summation of both treatments applied separately. That altered states within the neuromatrix (Fournier and Persinger, 2004) can increase the brain's susceptibility to physiologically-patterned magnetic fields has been shown experimentally (McKay and Persinger, 2006).

To test these hypothesis female rats sustained the impact of 200 g to the tops of their right skulls. One group either became pregnant within the following week and engaged in normal motherhood or remained nulliparous. A second group was exposed immediately for the same period to one of four intensities of computer-generated magnetic fields composed of physiologically-relevant patterns known to affect blood chemistry (St-Pierre et al.,
2008) and subtle hippocampal morphology in prenatally exposed rats (Whissell et al., 2008) or to a sham condition. We predicted that both treatments, pregnancy and continuous whole body exposure for 50 days to this specifically-patterned magnetic field, should reduce the numbers of anomalous neurons below the impact site.

In the latter case, we predicted that the most significant effects would occur with the weaker intensities, particularly within the 5 to 50 nT range. This prediction was based upon our previous experiments that suggest a sensitivity of organisms during extreme physiological states, such as seizures or rapid cell growth (development), to physiological frequencies and patterns (St-Pierre et al., 2008; Lagace et al., 2009). This range was also congruent with a possible resonance recognition model, first described by Cosic (1994), who calculated the maximum velocity of electrons caused by the difference of the free electron potentials at the N and C terminals of a protein. If indeed the peak voltage gradient within the umbra of the impact site is within the range along a wound edge, in the order of 140 mV mm⁻¹ [Song et al., 2004], then the optimal magnetic field strength would be [140 × 10⁻⁹ V mm⁻¹] / [10⁶ mm sec⁻¹] or in the order of 1 to 10 nT.

**MATERIALS AND METHODS**

**Animals:** All subjects were female albino Wistar rats that had been obtained from Charles river, Quebec breeders at about 60 days of age. All experiments were conducted in the Paul Field Animal Facility during the years 2004 and 2005. They were adapted to the standard colony light conditions (12:12 h L:D, with onset at 08:00 h) and controlled temperature (21°C) for 30 days before the experiments began. All procedures had been approved by the University’s Animal Care Committee. All methods have been described previously (Lado and Persinger, 2003, 2008).

**Weight drop method:** The present study was designed to discern if pregnancy (and motherhood) or exposure to a specific, physiologically-patterned magnetic field could reduce the numbers of areas occupied by neurons with shrunken neuronal somas that emerge after a mechanical impact to the skull. An application of the weight drop (Lado and Persinger, 2003, 2008) was employed. Essentially a 200 g (analytical balance) weight was dropped through a 0.9 m (holiday wrapping paper) tube onto the top right of the caudal skull of the rat while it was being held and its head rested on the top of a Formica table.

This procedure results in stunning, defined as a discernable immobilization or partial immobilization of ambulatory, without anomalous or attenuated reflexes, for about 20 sec following the impact in about 50% of the rats. The other rats, receiving the same impact show almost immediately mobility. None of the rats ever vocalized or displayed any equivalent of loss of consciousness. This procedure produces reliable morphological changes in neurons within the cerebral cortices within the right hemisphere while neurons within the left hemisphere are spared.

**Post-impact treatments:** For the mothering study, 6 rats were placed immediately with males (pairs) while another 6 were placed with females (pairs). About 4 days before anticipated birth all 12 rats were housed singly in plastic shoebox cages. The pregnant females delivered and cared for their young in that setting. Given the effect size and reliability of the neural anomalies observed beneath the impact site, we reasoned that this sample size was sufficient and would minimize the requirement for usage of large numbers of rats.

Another 19 rats were involved with the field exposure study. In the first block pairs of female rats (n = 8) that had sustained the impact were exposed immediately to one of four plastic cages where the average magnetic field intensities from the applied physiologically-patterned magnetic field were 5 nT (1 to 10 nT), 50 nT (30 to 60 nT), 500 nT (400 to 600 nT) and 1,500 nT (1200 to 1600 nT). In the second block an age-matched normal rat was house with each rat that has sustained the impact. Consequently there was a total of 12 rats (3/intensity). In a third block, pairs of female rats (one that had sustained the impact, one normal) were exposed to the compartments but no field was applied even though the equipment was operating. These 4 rats constituted the sham field group. Finally, 3 rats, randomly selected from our colony stock of rats that had received the impact (never exposed to the magnetic field room) served as additional colony controls. Again, we estimated this sample size would be sufficient to clearly show any clinically or environmentally relevant alterations.

The physiologically patterned field, described elsewhere (St-Pierre et al., 2008) was presented continuously for 50 days. The field was generated between two coils (only one activated for any given block) that generated the decreasing intensities over distance within the four compartments. Each of the two coils was composed of wire wrapped around plastic containers and has been described by St-Pierre et al. (1998). During the second block the other coil was activated.
The pattern was generated by transforming each of 10,000 serial values between 1 and 256 to between -5 to +5 V (127 = neutral) through a custom constructed digital-to-analogue converter. The programmable (Complex) software was operated through a Zenith XT computer. The duration of each point was 1 msec and the port time, which might be considered a rise time, was 150 µsec. Verification of the shape of the applied field as well as other parameters have been described elsewhere (Mach and Persinger, 2009).

**Histology:** Termination, 50 days after the impact, involved decapitation within 20 sec of being removed from the home cage. Because exposure to carbon dioxide before decapitation produces an increased number of anomalous soma diffusely throughout the cortex, this procedure was not employed. The brain was removed within 4 min and fixed in ethanol formalin acetic acid. After 4 days the brains were processed, paraffin embedded and cut at 10 microns with a microtome. Ten sections equally spaced between the posterior and anterior commissures, as described by Paxinos and Watson (1986) were stained with toludine blue O.

The numbers of sections within a 6×6 grid that contained at least one neuron with the criteria of conspicuously shrunken and deeply stained soma at 400 x equivalent within the left and right cerebral cortices and amygdala (where the vast majority of these cells occur) was counted and converted to mm². This method has been described elsewhere (Lado and Persinger, 2008). The measurements were completed blind with respect to experimental conditions.

**Statistical analysis:** Analysis of variance were completed as a function of treatment and the affected area containing shrunken cells within the upper and lower and left and right sides of the brain. Post hoc tests involved combinations of Tukey's and paired t-tests. All analysis were completed with SPSS software on a VAX 4000 computer.

**RESULTS**

Figure 1 shows the typical morphology of neurons with shrunken soma that appeared and remained present for about 50 days after the mechanical impact. The means and SEMs for the mean values of shrunken neurons within the left and right ventral limbic areas for groups of rats exposed to these conditions are shown in Fig. 2. The numbers and distributions are similar in magnitude to our previous studies (Lado and Persinger, 2003, 2008). The pattern was similar within the dorsal cortices immediately beneath the impact site. Analysis of variance for the numbers of these anomalous cells between mother and nulliparous rats within the left vs. right or upper vs. lower
Fig. 3: Examples of the conspicuously shrunken soma of neurons in the cortices of a rat that sustained the minor impact of mechanical energy to the skull. The soma reduction of about 50% is dispersed amongst normal-looking neurons. (a) 400x and (b) 1000x

regions of the brains after mechanical impact to the right dorsal skull showed the expected greater number of anomalous cells in the right hemisphere compared to the left [$F(1, 10) = 20.84, p<0.001$; eta-squared $= 70%$].

The significant interaction [$F(1,10) = 4.35, p<0.05$; eta-squared $= 33%$] between hemisphere and treatment was due to the fewer numbers of areas of anomalous cells in the right side (impact) area of the brain for the group who had become mothers compared to those who had not. The interaction between dorsal vs. ventral brain and treatment was marginally significant [$F(1, 10) = 3.25, p<0.05; 27%$] and was due to the fewer numbers of areas of anomalous cells in the limbic region (left and right side) for the rats who had been mothers compared to those who had not. There were no other statistically significant effects.

There was a significant [$F(1, 18) = 12.99, p<0.001$; eta-squared $= 0.45$] difference in the total numbers of areas of anomalous cells between the groups exposed to the various intensity magnetic fields. Post hoc analysis indicated that the rats exposed to the 5, 50 and 500 nT intensities showed fewer numbers of areas of anomalous cells than those exposed to the 1,500 nT (15 mg) or sham fields (Fig. 3a, b). The interactions between group and upper/lower region of the brain [$F(1, 18) = 14.20, p<0.001$] and left vs. right side of the brain [$F(1, 16) = 10.20, p<0.001$] was due to the fewer numbers of areas of anomalous cells in the lower right limbic region for those intensity groups compared to the other groups (sham field and 1.5 nT).

For direct comparison of all treatments four, one-way analysis of variance for each quadrant were completed for the groups that had been mothers, not mothers, exposed to the magnetic field or not exposed after the brain injury. Only the numbers of areas of anomalous cells within the left and right somatic regions displayed group differences; the left and right dorsal cortices did not. Post hoc analysis indicated that the areas of anomalous cells within the brains of rats receiving the post-impact exposure to the effects of pregnancy and mothering or to the physiologically-patterned magnetic field did not differ from each other but were significantly lower than non-mothers or sham-field rats that also did not differ from each other.

**DISCUSSION**

These results support the observations and inferences by others (Parker, 1990; Shaw, 2002) that following impacts of mechanical energies not sufficient to produce unconsciousness there is a population of neurons that undergo specific and protracted morphological changes. Rats that sustained the impact from the fall of a 200 g mass from 0.9 m to the right dorsal skull showed more areas of anomalous cells beneath the impact site (right hemisphere). Such morphologies were rarely seen in control rats. Because these cells were still evident 50 days after the impact, it is unlikely they were manifestations of either necrosis or apoptosis (Liu et al., 2003).

Our unpublished studies involving the total numbers of cells within each cortical layer indicated that the shrunken anomalous cells emerge about 1 day after impact and are persistent for at least a month without any conspicuous change in numbers. When rats were killed with carbon dioxide before they were decapitated similar shrunken cells were noted in the left (non-impacted) hemisphere. The effects were similar to the injection of carbon dioxide (Wilson and Boxer, 2002) except vacuolation of neuropil was not apparent. These results suggest that the manner in which the rat is killed may confound or mask the significance of neuropathological changes from mild mechanical impacts that were not associated with loss of consciousness.
As predicted, the females who had been placed with males immediately after the impact, became pregnant, delivered and cared for their young until they were killed 50 days later showed a reduction by more than half of the numbers of microscopic fields with anomalous cells. The effect was particularly evident within the limbic region in the right hemisphere. These results would be consistent with the protective effects of oestrogen (Behl, 2002; Kinsley et al., 1999; Rau et al., 2003; Shingo et al., 2003). A preferential effect of oestrogen or motherhood-related chemistry would be congruent with the enhanced spatial memory reported following motherhood (Kinsley et al., 1999).

Also as predicted, the immediate and continued application of a physiologically-patterned magnetic field known to affect neuronal development (St-Pierre et al., 2008; Whissell et al., 2008) for 50 days following the impact reduced the area of anomalous cells within the limbic region. The right limbic region was also more positively influenced than the left for the optimal intensities. That the right hemisphere is more sensitive to weak, complex patterned magnetic fields has been indicated by both correlational studies for human beings (Belishcheva et al., 1995) and experimental studies for rats (Cook et al., 2000).

The specific targeting of neuronal subpopulations by whole brain (and body) exposure to magnetic fields whose temporal patterns are resonant with these neurons has empirical support. Extrapolating from Yun et al. (2002) observation that different patterns of electrical stimulation produced more effective long term potentiation or LTP in different regions of the entorhinal-hippocampal region, Lagace et al. (2009) exposed young rats to various types of LTP-patterned magnetic fields for about 2 h during and after the initiation of lithium/pilocarpine- precipitated limbic epilepsy. When the brains of these rats were examined as adults specific populations of neurons in the right temporal lobe had been saved. Continuous whole body exposure for two weeks to either 7 or 40 Hz amplitude-modulated magnetic fields presented for 7 min once per hour during the nocturnal phase with intensities of either 50 or 500 nT also produced remarkably different patterns of infiltration of mononuclear cells throughout the brain of rats in which experimental allergic encephalomyelitis had been induced (Persinger, 2009).

There were two results which may appear counterintuitive. First, the effects of exposure to the magnetic fields for reducing the area of cellular anomalies were comparable in size to that of pregnancy and motherhood. Secondly, the largest effect was for the weaker intensities between 5 and 500 nT. These intensities are equivalent to .05 to 5 mG, a range of values that occurs frequently within the human and animal habitat (St-Pierre et al., 1998). That the effect was not spurious is suggested by that fact that the brains from the sham field, 1,500 nT and colony-maintained rats did not differ significantly from each other. We found similar nonlinear effects for this intensity range for the ameliorating effects of amplitude modulated magnetic fields when treating experimental allergic encephalomyelitis (Cook and Persinger, 2000; Cook et al., 2000; Kinoshima and Persinger, 2004).

The concept of non-linear effects from physiologically-patterned, weak magnetic fields has been suggested by many researchers (Ady, 1980; Whissell et al., 2008). Bawin and Ady (1976) found that applied (primarily 6 to 20 Hz) electric field strengths between 10 and 100 V m⁻¹, corresponding to field gradients within the tissue of about 0.1 μV cm⁻¹, affected (decreased) calcium exchanges in the isolated cerebral hemispheres of cats. Weaker or stronger field strengths were not effective.

Later Zhongqi et al. (1998) replicated both the frequency (16 Hz) and intensity (15 V m⁻¹) window effects. Independently, Weaver and Astumian (1990) showed that μV cm⁻¹ fields could be detected if the response was in a narrow range of frequencies. These values may appear small and highly negligible for any physical interaction. However, for comparison, the transfer of solar wind energy into the earth's magnetosphere (total value in the order of 10¹² J sec⁻¹) involves an electric field of only 4 μV cm⁻¹ along the lines of reconnection (Phan et al., 2000).

Similar intensity window dependence was found in the growth rate of Acetobacter suboxydans with no effect below 1 μA or above about 30 μA (Berg, 1995). According to Brocklehurst and McLaurihian (1996), the survival of free radicals also exhibited such non-linear effects. It is interesting that durations of 1000 μsec (1 μm sec), the point durations employed in our study, produced the strongest signal to noise ratio at the binding site for Ca²⁺/CaM-dependent myosin phosphorylation during neurite outgrowth from embryonic chick dorsal root ganglia (Pilla et al., 1999).

The 1 to 3 msec window effect may have neuroquantum implications (Persinger and Koren, 2007). There was also an intrinsic pulse within the magnetic field configuration employed in this study. This was the port time, the duration required to deliver the sequential values between 1 and 257 to the digital-to-analogue converter for voltage. This value was about 250 μsec and was within the range of the rise time and pulse duration of the TMS employed by Ahmed and Wieraszko (2008) to obtain the significant recovery in their animals following spinal cord injury.
Non-linear intensity effects from magnetic fields could reflect correlative neurochemical processes. In the domain of pharmacology and neurochemistry, non-linear effects for concentrations of ligands and receptor subtypes, depending upon affinity and $K_r<50$ values, are well known. Even optimal hormonal effects are known to be specific to a physiological range. For example poor sexual behaviour in rats was restored with 5 or 10 $\mu$g kg$^{-1}$ of prolactin but not with 50 $\mu$g kg$^{-1}$ or less than 1 $\mu$g kg$^{-1}$ (Drago and Liassandrello, 2000).

The convergent mechanisms for the presumed beneficial effect for motherhood and this physiologically-patterned magnetic field must still be elucidated. If these neurons are in a third state (neither necrosis nor apoptosis) of dormancy, the application of the field may have reactivated cellular function. It may be relevant that the approximately picofarad level of energy from glucose-derived sources within a soma volume (10 $\mu$m in diameter) is in the same order of magnitude as the energy passing through that volume from the mechanical impact. We propose that the quantum of mechanical energy moving through the brain within 2 m see disrupted the cellular machinery operating within a comparable biochemical quantum. The maintained exposure to either the biochemistry of motherhood or the magnetic fields following the impact reactivated the normal cellular process and hence reduced the somatic anomalies at least in a subpopulation of neurons.

**CONCLUSION**

The present study shows that casual mechanical impacts to the skull, not sufficient to produce suspension of consciousness or stunning in animals are followed by scattered concentrations of shrunken, darkly stained neuronal soma within the cerebral cortices beneath the impact site and within the limbic region. However, the numbers of these anomalous cells can be reduced by subsequent pregnancy or continuous exposure to weak intensity, physiologically-patterned magnetic fields. The results suggest that relatively weak magnetic fields afford a similar magnitude of protection as pregnancy and indicate that patterned magnetic fields may be effective at intensities frequently encountered in the bioenvironment.

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