SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

THE EFFECT OF PRENATAL ULTRASOUND ON THE MORPHOLOGY OF DEVELOPING NEURONS

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DEBRECEN, 2025

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, April 24, 2025, at 1:00 PM

1. Background and objectives of the doctoral thesis

Ultrasound (UH) is a mechanical wave with a frequency exceeding 20 kHz, the upper limit of human auditory perception. The frequency range used in medical diagnostics is usually between 1 and 15 MHz.

The use of ultrasound is particularly important in the field of obstetrics and gynaecology, where it is an indispensable tool for monitoring foetal development and for the early detection of possible anatomical anomalies. The timing of ultrasound examinations during pregnancy is of paramount importance, as the development of the foetus requires different examinations at different stages of pregnancy.

Two types of transducers are usually used during obstetric-gynaecological examinations: a vaginal transducer in the first trimester and a convex transducer in the later periods. These transducers operate in different frequency ranges: convex transducers usually operate at 3-5 MHz, while transvaginal transducers operate at 5-7.5 MHz.

In ultrasound examinations, it is essential to take into account the thermal index (TI) and the mechanical index (MI), which are indicators of the most important biological effects and are visible in real time during the examination. The MI measures the ability to induce biological effects related to cavitation phenomena, while the TI indicates the internal temperature rise of the tissue. In order to avoid undesirable effects, international protocols recommend that both TI and MI should remain below 1 throughout the test.

Although these safety limits help to minimise the risk, it is important to bear in mind that even when these criteria are met, ultrasound exposure can still have the potential to cause cellular changes. For example, ultrasound has the potential to alter neuronal morphology and may affect dendritic tree development both *in vitro* and *in vivo*.

Results from an *in vitro* study showed that pulsatile ultrasound exposure can induce transient morphological changes in the nervous system, such as retraction of neurites and shrinkage of the cell body.

In an *in vivo* experiment, when adult mice brains were exposed to a repetitive 1 MHz ultrasound stimulus, it was found that after 3 months, while apical dendrites in the control group showed a reduction in the number of nodes, total dendrite length and 3D dendrite volume, this reduction was not observed in CA1 pyramidal cells of the ultrasound-treated mice. Thus, the results of this experiment suggest that ultrasound is effective in preventing age-related degradation of the apical dendritic tree. These effects are enhanced with repeated

ultrasound exposure, which may induce a significant increase in the average length of neurites and proliferation of neuroblasts.

Ultrasound scans usually last 10-15 minutes, but a 3D or 4D "baby movie" can add an additional 30-40 minutes to the procedure. Although diagnostic ultrasound is generally considered safe at the population level, some publications suggest that it may have mild side effects, such as delayed speech development, increased incidence of left-handedness and increased birth weight.

It is important to note that data from a large review of studies show that ultrasound scans during pregnancy have not been associated with adverse maternal or perinatal outcomes, impaired physical or neurological development, or increased risk of childhood malignancies, lower than normal intellectual performance or mental illness.

During the second and third trimesters of pregnancy, obstetric ultrasound is thought to mechanically stimulate neurons in the developing brain, particularly in the cortex and hippocampus. This period is of particular importance for the formation of neuronal networks and axo-dendritic growth, for example in the limbic system, which plays a key role in learning, memory, motivation and emotion processing.

The process of neurogenesis follows a well-defined temporal and spatial pattern. It has been known for more than a century that the mammalian cerebral cortex consists of six layers. The neocortex is characterised by an inside-out arrangement of projecting neurons, with the oldest neurons in the deepest layers and the youngest neurons in the most superficial layers, with layer 2 containing the youngest neurons and layer 6 the youngest. The inside-out stratification means that all newly born excitatory neurons must migrate out of the periventricular space and then migrate through the developing white matter to the earlier born neurons. It settles according to its destination, then undergoes terminal differentiation and establishes synaptic connections. The first layer of migrating neurons is called the subplate (SP, layer 6b), while the subsequent layers (6, 5, 4, etc.) accumulate above it. While the stimulating neurons are born periventricularly and subsequently build up the cortex sequentially from the inside out by radial migration, the progenitor cells of the cortical inhibitory neurons are located in the medial ganglion eminence, and the postmitotic daughter cells migrate tangentially in the subventricular zone or towards the cortical plate below the pia mater.

Ultrasound has been shown to promote the proliferation of neuroprogenitor cells while limiting the migration of pyramidal cells in the developing cerebral cortex. In an *in vitro* experiment, using a frequency slightly higher (6.7 MHz) than the diagnostic ultrasound range,

they found that neurons marked at the onset of migration and then exposed to ultrasound stimulation a day later slowed down, with some remaining in the white matter or becoming stuck in the deeper cortical layers. It should be noted that several different durations of ultrasound stimulation were used in the experiment, but significant differences were only observed for stimulation lasting 30 minutes or longer. In addition, low-intensity (<3 W/cm²) ultrasound treatment has been shown to promote the differentiation and survival of neural stem cells (progenitors) both *in vitro* and *in vivo*.

Following migration and layer formation, it is crucial that neuronal dendrites grow and branch properly, without which the proper formation and functioning of neuronal networks would not be possible. Dendrite arborisation, which is influenced by both intrinsic and environmental factors, significantly affects the synaptic input and membrane properties of neurons. At the subcellular level, ultrasound can induce transient and rapid changes in neurite outgrowth via neuronal cell cultures, mechanosensitive receptors and ion channels, as discussed above.

Transient receptor potential channels (TRPCs) are widely present in the developing mouse brain, particularly in the cortical plate. Previous studies have shown that TRPC4 can be activated by ultrasound at frequencies higher than 2 MHz, suggesting that it may be involved in ultrasound-mediated signaling in developing neurons. This hypothesis is further supported by the observation that animals lacking the TRPC4 mechanosensitive ion channel show significantly reduced sensitivity to ultrasound stimulation, indicating that mechanosensitive ion channels play an important role in the mechanism of ultrasound stimulation. In a previous experiment in which low-pressure ultrasound-sensitized neurons were activated in nematode (C. elegans), wild-type animals were found to be insensitive to low-pressure ultrasound, whereas in contrast, higher than normal expression of the pore-forming subunit of the mechanotransduction channel TRPC-4 was found to sensitize neurons to ultrasound stimulation, resulting in behavioral responses.

As a non-selective cation channel, located primarily in the endoplasmic reticulum membrane, TRPC4 functions to increase cytoplasmic Ca^{2+} levels upon activation. TRPC4 is highly expressed in excitable cells and is involved in the response to neuronal injury, neurite outgrowth and regulation of neuronal exocytosis, and is co-expressed with TRPC5 in hippocampal CA1 pyramidal cells, which plays an important role in neuronal Ca^{2+} homeostasis.

It is noteworthy that local intracellular Ca^{2+} signaling plays a role in influencing dendrite growth and branching, probably through pathways involving CREB and CaM kinase.

The target genes of CaM kinase and CREB that mediate dendritic growth are not well understood, but an important potential target is brain-derived neurotrophic factor (BDNF). It has been shown that CaM kinase can induce BDNF expression through a CREB-dependent mechanism, which may lead to enhanced dendrite growth in cortical neurons. Thus, Ca^{2+} and CaM kinase-induced dendrite growth may be mediated by BDNF.

The activation of TRPCs in addition to BDNF leads to elevated levels of the early response gene c-Fos, indicating increased neuronal activity. The molecular basis of the regulation of early response genes by transsynaptic stimulation is only partially understood. The influx of Ca^{2+} through voltage-dependent Ca^{2+} channels and the formation of an active Ca^{2+}-calmodulin complex is probably the first step in a process triggered by depolarizing stimuli. Notably, c-fos induction is blocked by external Ca^{2+} chelation, specific inhibitors of Ca^{2+} channels and calmodulin antagonists.

Available publications suggest that diagnostic prenatal ultrasound scanning is safe for both mother and fetus. However, the currently available results should be interpreted with caution. Existing publications have not necessarily evaluated all possible biological effects of prenatal ultrasound. Future studies may reveal effects that have not yet been recognised. Therefore, as stated in the ALARA (As Low As Reasonably Achievable) principle, the current evidence available suggests that it is prudent to consider exposing patients to the minimum ultrasound effects necessary to obtain diagnostic information.

Aims

Our aim was to investigate *in vivo* in mice the expression of the mechanosensitive TRPC4 receptor in developing cortical neurons and to characterize the effects of low-intensity ultrasound at 3 MHz frequency applied in obstetric practice on neurons of the retrosplenial cortex pyramidal layer 5. In the second phase of our study, we investigated the effects of repetitive fetal ultrasound on the morphology of CA1 pyramidal cells and, in the long term, on whole brain volume, weight and hippocampal structure.

2. Materials and methods

1. Animals, collection of samples

CD1 (ICR, Charles River, Germany) mice were used in the experiments.

Central nervous system samples from both the ultrasound-treated (see details in the section "Ultrasound exposure") and control groups were collected that showed strong GFP labeling. The use of cytoplasmic GFP facilitated labelling, allowing the evaluation of neurons (see details of GFP labelling in "Plasmids" below). The control group consisted of animals that had undergone the same procedures as the ultrasound-treated animals, but were not exposed to ultrasound stimuli. In both the treated and control groups, only female animals were examined.

2. Plasmids

Our laboratory designed and constructed an expression vector that encodes the EGFP sequence and expresses a cre-recombinase deletable GFP under a CAG promoter.

In addition, in the first study we also employed Cytbow vector, a multicistronic piggyback plasmid. This vector enables the expression of three fluorescent reporters, each regulated by different lox sequences.

3. *In utero* electroporation

In our first study the impact of ultrasound exposure on neuronal morphology was evaluated in GFP and Cytbow-labeled layer 5 pyramidal neurons in the murine retrosplenial cortex. In the second study morphological changes of GFP-labeled CA1 pyramidal neurons was evaluated using the same methodology. To minimize the influence of inherent variability in pyramidal cell morphology, we compared cells that were born within the same time frame, migrated to the same layer, and likely shared a similar differentiation state. To achieve this, we utilized *in utero* electroporation of plasmids containing fluorescent reporter genes as the electroporated plasmid DNA facilitates stable gene expression only in postmitotic neurons that exited the cell cycle concurrently with the electroporation. To label layer 5 pyramidal neurons in the retrosplenial cortex, we conducted *in utero* electroporation at E14.5. At embryonic day 14.5, pregnant mice were deeply anesthetized with sodium pentobarbital and their uterine horns were exposed via an abdominal incision. Embryos underwent identification followed by the injection of a plasmid solution into their right lateral ventricle.

Subsequently, the embryos were electroporated using an Electro Square Porator following injection. Subsequently, the uterus underwent a saline rinse and was repositioned inside the abdominal cavity, which was then closed using two-layer sutures.

4. Ultrasound exposure

In the case of both experiments four days following *in utero* electroporation (on E18.5), the mice were thoroughly anesthetized using Na-pentobarbital and subsequently exposed to a 10-minute ultrasound stimulus. The GE Logiq V2 ultrasound device operated at a consistent frequency of 3 MHz, maintaining both mechanical and thermal indexes below 1.0. For *in utero* ultrasound exposure, the gel was delicately administered to the abdomen of the pregnant animals and for postnatal treatments, it was directly applied to the skull of the mice. The initial prenatal stimulus was administered on embryonic day 18.5, followed by four additional stimuli postnatally during the first four weeks after birth, each administered once a week. On E18.5 during the treatment, the focus was maintained on the midline of the pregnant animals to ensure uniform exposure for all embryos. In the postnatal treatments, non-targeted ultrasound was administered to the small craniums of the subjects. The animals in the control group were subjected to identical procedures, including anesthesia and *in utero* electroporation, with the exception of the samples used for qRT-PCR. The ultrasound transducer was applied to the abdomen of the control animals without actual exposure to ultrasound, mirroring the conditions of the ultrasound-stimulated group.

During our second experiment, for the micro-CT analysis the animals of both the US-treated and the control groups were observed over a 12-month period before being humanely euthanized under deep anesthesia. Subsequently, we transcardially perfused the animals with 4% PFA followed by saline. Finally, we isolated their brains from their skull.

5. Immunohistochemistry

Immunofluorescent labeling was utilized to capture the GFP signal and identify TRPC4, c-Fos, and BDNF in mouse brains subjected to ultrasound treatment, as well as in control samples. In the first study E18.5 embryos and P3 and P30 mouse pups, in the second experiment P28 mice were euthanized by decapitation, and their entire brains were meticulously dissected and then fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Afterward, the brain samples were washed with PBS, embedded in 4% agarose, and sliced into 100 μm thick free-floating coronal sections using a

vibratome. Sections were exposed to primary antibody for 2 days at 4°C and then to secondary antibody overnight at 4°C. The antibodies were diluted in PBS. Following the incubation, the sections underwent three 15-minute washes in PBS.

In the final step of the procedures, the sections were treated with cell nucleus-specific DAPI for 2 hours at room temperature to aid in identifying layer boundaries. Subsequently, the sections were affixed with Hydromount medium and confocal images were captured.

During the second study, following the collection of fluorescent images, the immunofluorescent signal was transformed into DAB-labeled samples. The coverslips were removed in PBS, followed by overnight incubation of the sections with IgG, and subsequent incubation with avidin–biotin complex for 2 hours. Visualization was achieved using the DAB peroxidase substrate kit as per the supplier's protocol.

6. Neuron reconstruction and morphometry

In our first study, we conducted 3D reconstruction of layer 5 pyramidal cells in the retrosplenial cortex using Neurolucida from confocal image stacks. The confocal images were captured at 0.5-μm intervals with a × 40 oil immersion objective on an FV3000 microscope. Manual 3D reconstructions were carried out on GFP- and Brainbow-labeled neurons from control and ultrasound-treated animals. Cell body contours were meticulously delineated at all Z levels in the image stacks. Neuronal dendrites were accurately reconstructed, with attention to the diameter of the processes at each reconstructed point. In the retrosplenial cortex, the pyramidal cells in the deepest part of layer 5 were specifically chosen to ensure that only cells within layer 5 were being compared. Data from pyramidal cells labeled with GFP and Brainbow were combined, as there were no observed differences between the pyramidal neurons labeled with the different plasmids. The morphometric parameters derived from the reconstructions included the total number of neurites, total length, number of branch points (nodes), and dendritic segment length. When selecting sampling cells, certain criteria were applied, including the following: 1) localization within the same layer of the labeled region, 2) strong GFP labeling, 3) sparse and relevant labeling for the reconstruction process, and 4) the possibility to trace the dendrites in the neighboring slices.

During the second experiment, confocal images were acquired at 0.5 μm intervals utilizing a 40× oil immersion objective. Every automatic reconstruction underwent verification by an expert, with manual correction performed when necessary, such as when the software misidentified dendritic crossings as spines. In the case of the DAB-stained

samples we specifically selected CA1 pyramidal cells for 3D reconstruction using Neurolucida software. The cell bodies of the neurons were meticulously delineated across all Z levels in the images, and particular attention was given to the reconstruction of neural dendrites, with a special focus on process diameter. Morphometric parameters derived from the reconstructions included the total number of dendrites, highest order, number of nodes, average segment length, average segment tortuosity, average segment diameter, dendrite length, average terminal distance, and mean dendritic length. It is important to note that the person performing the analysis was blind to the evaluation.

7. RNA Isolation and cDNA Transcription and qRT-PCR

The mouse brain samples were collected 60 minutes after exposure to ultrasound at embryonic day 18.5 (E18.5). Control animals were subjected to the same handling and treatment procedures as the ultrasound-stimulated group but were not exposed to actual ultrasound. Total RNA extraction from 30 mg of mouse brain tissue was carried out with an additional DNA digestion step. In the course of the standard isolation protocol augmented with the DNA digestion step, 30 mg of mouse brain tissue underwent isolation and subsequent quantification and qualification with a spectrophotometer. Subsequently, 1,000 ng of total RNA underwent reverse transcription to cDNA, that was followed by PCR amplification. Gene expression quantification was conducted through the 40-Ct method, with BDNF expression normalized to the housekeeping genes of HPRT1 and GAPDH. Notably, no template controls (NTC, RT-NTC, NRT) were employed to detect potential contaminants at each stage of the reaction.

8. Whole mouse brain sample preparation for contrast-enhanced computed tomography and morphological measurements

8-8 animals (subjected to 5 instances of ultrasound treatment compared to a control group), demonstrated one year of survival before being euthanized under deep anesthesia with Na-pentobarbital. Following euthanasia, the animals underwent transcardial perfusion with 4% paraformaldehyde (PFA) and saline, after which their brains were postfixed in 4% PFA at 4°C for one week. Subsequently, the brain samples underwent two cycles of washing in phosphate-buffered saline (PBS), followed by dehydration in a series of graded alcohols ranging from 70% ethanol. For staining, a 1% iodine solution in concentrated ethanol was utilized. In order to rehydrate the samples, a descending series of graded alcohol was utilized,

followed by rinsing in distilled water. Subsequently, the iodine-stained whole brain samples were subjected to scanning using micro-CT system. The scan duration was 120 minutes. Post-processing of the image data involved alignment, beam-hardening and ring artifact correction, and smoothing. The output formats obtained were .bmp and DICOM images. The 3D Volume rendering tool utilized in this study was provided by DataViewer software. On coronal sections, measurements were taken for the longest latero-lateral diameter of the third ventricle at the level of the posterior commissure, as well as the diameter of the short axis of the hippocampal formation at its highest point. It is worth noting that the resolution of the micro-CT used was insufficient to detect the layers of the hippocampus. The total volume of the brain was calculated utilizing the DataViewer software.

9. Statistical evaluation

During the first experiment, the morphometric and qPCR data from control and ultrasound-treated animals were analyzed using the Mann–Whitney U-test in OriginPro for comparison.

During the second experiment, the morphometric data from control and ultrasound-treated animals were subjected to statistical comparison using the Mann–Whitney U-test or Two Sample T-test in OriginPro and PAST software. To account for a large number of statistical comparisons on small sample sizes, a Bonferroni correction was applied, adjusting the significance level from $p \leq 0.05$ to $p \leq 0.017$. Additionally, MANOVA analysis was conducted using PAST software to compare the morphometric data, indicating a significance level of $p \leq 0.05$.

3. New scientific results of the thesis

1. Labeling pattern of the *in utero* electroporation at E14.5

The pyramidal neurons found in the cerebral cortex and hippocampus originate from the progenitors located in the ventricular zone of the cortical plate. Our initial investigation focused on the distribution pattern of the labeled neurons at the time of the ultrasound stimulation (E18.5) and at the time of sampling (P3) using *in utero* electroporation at E14.5.

Following electroporation, four days later (at E18.5), the labeled pyramidal cells had migrated to their anticipated locations in layer 5 of the retrosplenial cortex and the pyramidal layer in the CA1 region of the hippocampus. The GFP-positive cells exhibited a polarized morphology, characterized by the migratory leading process transforming into a branching apical dendrite. Some of the GFP-labeled neurons displayed identifiable basal dendritic processes, indicating their stage of morphological differentiation, while others did not, suggesting that most labeled neurons were still undergoing morphological differentiation. At postnatal day 3 (P3), the frontal and retrosplenial cortices on the injected side, as well as the contralateral somatosensory area, exhibited GFP positivity. Within the injected hemisphere, the majority of GFP-labeled cortical neurons were pyramidal cells situated in layer 5. Correspondingly, the ipsilateral hippocampus displayed primarily labeled pyramidal cells in the pyramidal layer of the CA1 region. In certain instances, the injected DNA disseminated into the contralateral hemisphere and the third ventricle during electroporation, resulting in the labeling of the contralateral hemisphere and diencephalon with GFP. It is essential to note that the GFP-labeled neurons were born after E14.5 within a narrow timeframe, and they attained their final presumptive layer destinations, culminating in an identifiable cortical layer by P3. Apart from the cortical layers, the pyramidal cell morphology, characterized by the two distinct dendritic domains comprising long and branching apical dendrites terminating in layer 1, along with basal dendrites, was observable. However, subclassification of the layer 5 pyramidal cells was not feasible at this early stage.

2. The number of basal dendrites of layer 5 pyramidal cells is increased by ultrasound stimulus

The pyramidal cells exhibited a similar labeling pattern when employing a Brainbow vector for labeling, as opposed to GFP. The high density of labeled layer 5 retrosplenial pyramidal cells posed challenges in reconstructing single cells and processes, which were

overcome by employing multicolor fluorescent imaging through the Brainbow system. The two-dimensional representations of the randomly selected reconstructed cells from layer 5 exhibited remarkably similar morphological characteristics in terms of the branching patterns of the apical and basal dendrites. Notably, a single ultrasound stimulation did not induce significant changes in pyramidal cell morphology, except for an increase in the number of dendrites. In all reconstructed pyramidal neurons, a single apical dendrite was present. Consequently, the application of ultrasound stimulation resulted in an increase in the number of basal dendrites by 1-2. The analysis of the branching tree indicated that ultrasound treatment did not produce significant effects on the branching numbers. Additionally, the dendrites displayed a similar shape to those in the non-stimulated control group. The Brainbow vector-labeled pyramidal cells, present in layer 5 at P3, displayed distinct colors representing different cell lines, providing a comparable neuron population for statistical analysis. Furthermore, the arborization of the neurons was well developed, as evidenced by the reconstructed neurons in 3D. To assess the impact of prenatal ultrasound exposure on neuronal morphology, labeled pyramidal cells of layer 5 were manually reconstructed using Neurolucida software. The analysis revealed no significant changes in the average segment tortuosity, average segment length, number of nodes, and dendrite length of treated and control pyramidal neurons, except for an elevated number of dendrites in the ultrasound-stimulated group.

3. Neurons are rapidly activated after a single ultrasound stimulus

Neuronal activation within the central nervous system can be identified through a general increase in the expression of early response genes, with particular emphasis on c-Fos. An earlier study demonstrated that c-Fos levels increased by 2–3-fold upon ultrasound stimulation in a frequency-dependent manner. This finding led to the hypothesis that the higher relative expression of c-Fos may elevate the expression of downstream morphogenetic molecules such as BDNF. It is suggested that this increase could facilitate the outgrowth of dendrites in maturing neurons in the retrosplenial cortex.

In order to investigate this hypothesis, we conducted an analysis of c-Fos expression following a single ultrasound stimulation at E18.5. The non-stimulated control brain did not exhibit a significant immunofluorescent reaction for c-Fos, whereas the immunoreactive signal notably increased following ultrasound stimulation. The transient c-Fos signal reverted to baseline levels 30 days following the stimulation, in comparison to the non-stimulated

control mice. Repeated stimulation resulted in the activation of c-Fos expression in nearly all cell nuclei within the cortex, which was not limited to GFP-labeled neurons.

At E18.5, BDNF immunoreactivity was predominantly localized to the meninges and blood vessels. A single ultrasound stimulation did not result in a change in BDNF immunoreactivity in the cortex, unlike the observed effect on c-Fos, and after 60 minutes of stimulation, there were no significant differences observed in the levels of BDNF mRNA. Conversely, the application of repetitive ultrasound stimulation resulted in heightened BDNF immunoreactivity within the retrosplenial cortex.

Intrauterine ultrasound stimulation might have an impact on the migratory properties of immature cortical neurons. However, during our experiment we found that neither single nor repetitive ultrasound stimulation led to any discernible changes in the layered distribution and basic morphology of the layer 5 pyramidal cells. We hypothesized that the absence of this phenomenon could be attributed to the termination of migration of layer 5 neurons by the commencement of the first ultrasound stimulation at E18.5.

4. GFP-labeled pyramidal neurons are positive for TRPC4

Following the adjustment of the parameters, the frequency of diagnostic ultrasound is below the threshold required for cell membrane cavitation, which can directly elevate c-Fos levels in the cells. Our hypothesis suggests that receptor-mediated signaling may be responsible for the increased c-Fos levels following ultrasound stimulation. In order to gain a better understanding of the potential signaling mechanisms behind ultrasound-stimulus-dependent c-Fos activation, our focus was directed toward the examination of mechanosensitive receptors at E18.5. Our findings revealed a punctate distribution of TRPC4 immunoreactivity in GFP-labeled (at E14.5) pyramidal cells within the retrosplenial cortex at E18.5. Additionally, we also observed TRPC4-immunopositive signals within the processes of GFP-labeled pyramidal cells, where the presence of endoplasmic reticulum was equivocal. Given the positivity of TRPC4 in the GFP-labeled neurons, it is plausible that ultrasound stimulation may lead to an increase in cytoplasmic Ca^{2+} concentration in these neurons.

5. The morphological characteristics of CA1 neurons are changed by repeated ultrasound exposure

Based on the morphometric analysis, it was observed that the mean length of the dendrites remained consistent. This reaffirms our previous findings, the exposure to

ultrasound resulted in an increase in the number of basal dendrites but did not affect the number of dendrites on the apical dendritic tree. Furthermore, for the basal dendrites, the average segment length of the dendrites and the dendrite length displayed a significant increase ($p \leq 0.01$) as a consequence of ultrasound treatment. Conversely, the properties of the apical dendritic tree did not exhibit significant changes. Through MANOVA testing, significant differences in the properties of the investigated dendritic trees between the groups were identified overall; p: 0.004.

6. Morphological analysis of the hippocampal region with micro-CT

The brains of one-year-old mice underwent micro-CT examination to assess total whole brain volume and weight. Measurements were taken on coronal brain sections at the level of the posterior commissure (as an identifiable anatomical landmark) including the largest horizontal diameter of the III. ventricle and the diameter of the short axis of the hippocampal formation. These measurements were independently conducted by three expert examiners. A significance level of $p \leq 0.01$ was applied to total brain volume and weight due to potential dissection-related injuries on the basal surface, while a significance level of $p \leq 0.017$ was used for all other measurements. The study identified a notable difference in the thickness of the hippocampal formation between the control and ultrasound-treated groups, with the diameter being larger in the ultrasound-treated group.

4. Discussion

Our findings indicate that even a single short-term diagnostic ultrasound procedure has a minor yet enduring impact on neuronal morphology. This impact is characterized by an increase in the number of basal dendrites of layer 5 pyramidal cells in the retrosplenial cortex, one of the key regions within the limbic system. The limbic area plays a crucial role in various functions such as learning and spatial navigation in rodents.

It is important to highlight that it induced morphological changes in both layer 5 and CA1 pyramidal cells. In the CA1 region, there was an observed increase in the length of the basal dendrites and their segments. In addition, we observed that after repeated exposure to ultrasound, the hippocampal formation of one-year-old mice exhibited greater thickness compared to the control group. This led us to conclude that it exerts a long-term protective influence on the brain.

Notably, repetitive ultrasound exposure led to not only morphological, but also biochemical changes in neurons, characterized by elevated expression of the early response gene c-Fos and the secreted morphogenetic molecule BDNF.

1. Neuronal differentiation following ultrasound exposure

The 14th day of mouse embryonic cortical development corresponds to the 12th week of human cortical development, specifically the birth of layer 5 neurons. According to research, ultrasound exposure at E18.5 in mice has similar effects on neurons in the nervous system as ultrasound exposure in human pregnancy after the 30th week. Exposure to pre- and perinatal ultrasound has been found to influence various aspects of brain development, especially in relation to neuronal migration. In a study involving a 30-minute, 6.7-MHz pulsed ultrasound stimulation, it was observed that the migration of neurons populating the neocortex was significantly slowed down, leading to the misplacement of some pyramidal cells in improper layers or even their entrapment in the white matter. Given that we administered the ultrasound stimulation at E18.5, a time when the layer 5 neurons had already reached their destination, investigating the potential impact on neuronal migration in the developing limbic areas was not pertinent within the scope of our experimental conditions. Nevertheless, the impact of the 3-MHz frequency ultrasound exposure used in the study on neuronal and glial cell migration cannot be discounted. Pyramidal neurons undergoing migration exhibit polarity, with a leading process that differentiates into the apical dendrite,

while the axon originates from the trailing process. Our research findings indicate that exposure to the ultrasound increased the number of basal dendrites in the layer 5 pyramidal cells. These dendrites were observed to be newly formed rather than resulting from the differentiation of existing migratory processes. This is attributable to the development of dendritic arbor in a highly coordinated manner, involving dynamic processes that necessitate rearrangements of the cytoskeleton. This results in the continual growth and retraction of newly formed dendritic processes, which respond to electrical, microenvironmental, or intrinsic signals.

2. The influence of mechanosensitivity on the growth of neuronal processes

The specific mechanisms by which ultrasound influences the growth of neuronal processes during neuronal polarization and differentiation are yet to be fully understood. *In vitro* studies have shown that mechanical stimulation (tension) promotes neural stem cell differentiation and increases the complexity of their dendritic arbor. In our experiments, it is plausible that the increased number of dendrites resulted from the destabilization of growing neurites due to ultrasound stimuli, leading to cytoskeletal rearrangements. After exposure to ultrasound (0.5 MHz for 10 minutes), it was found that rapid reorganization of the cytoskeleton led to a reduction in the length of primary cilia on hippocampal CA1 neurons in rat brain slices that were cultured *in vitro*. Conversely, exposure to ultrasound stabilized the growth of dendrites and axons that had already formed synaptic contacts. The application of mechanical tension at a cellular level can trigger molecular signalization. Various mechanosensitive channels, such as Piezo1, TRPA1, TRPC4, TRPV4, MEC-4, two-pore domain potassium channels (K2Ps), and voltage-gated sodium and Ca^{2+} channels were reported to respond to ultrasound. The exact mechanism by which ultrasound-mediated activation of these ion channels influences dendritic growth is not fully understood. However, it is known that exposure to ultrasound facilitates dendritic growth through the activation of MAPK or ERK1/2 pathways, attributed to the increased intracellular Ca^{2+} levels mediated by mechanosensitive ion channels such as TRPV4 and Piezo1. Notably, the majority of these channels in the embryonic mouse brain exhibit low or negative expression levels, with the exception of TRPC4. The expression of TRPC4 initiates prominently in the subpallial regions at E13.5, gradually extending to the cortex, including the limbic area, by E18.5 and TRPC4 continues to be expressed by cortical neurons throughout adult life. Given that the application of ultrasound exposure was at E18.5 or later, there is a considerable likelihood that this

stimulus led to the activation of TRPC4 channels. These mechanosensitive ion channels are pivotal in the mechanism of ultrasound stimulation and are involved in regulating neurite outgrowth. This regulation may be attributed to the augmentation of Ca^{2+} efflux from the endoplasmic reticulum, as well as the initiation of CaM kinase and CREB-mediated pathways. Animals lacking TRPC4 channels exhibited a notable decrease in responsiveness to ultrasound stimulation, consistently displaying larger wild-type reactions to ultrasound. Conversely, subjects with impaired TRPC4 function demonstrated reduced significant reversal responses to ultrasound stimulation.

3. Early response genes and the release of neurotrophins are activated by ultrasound stimuli

Exposure of neuronal mechanosensitive receptors and ion channels to ultrasound can trigger a signaling cascade, resulting in heightened expression of c-jun and c-Fos. A single 3-MHz 10-minute ultrasound exposure led to elevated c-Fos immunoreactivity in the fetal mouse brain one hour post-stimulation. The rapid and transient expression of c-Fos plays a significant role in regulating various aspects of circuit behavior, notably impacting processes such as learning and memory.

According to an earlier research, the activation of c-Fos in the rat retrosplenial cortex upon brain-derived neurotrophic factor (BDNF) release is essential for fear-motivated long-term memory. It is noteworthy that BDNF acts as an upstream signal for c-Fos, and an increase in BDNF expression in the brain elevates c-Fos expression. Conversely, our research outcomes did not validate the elevation of BDNF mRNA and protein levels following 1 hour of ultrasound stimulation in E18.5 mice. However, repetitive ultrasound stimulation significantly increased the expression of both BDNF and c-Fos. The evidence indicates that the increase in c-Fos levels in response to ultrasound stimuli is likely mediated through mechanosensitive receptor signaling pathways, potentially leading to elevated BDNF expression downstream of c-Fos activation. However, it is also plausible that BDNF is released from other sources such as blood vessels. BDNF plays a crucial role in dendritic arborization, promoting dendritic tree elaboration, while not being necessary for survival, proliferation, or migration.

4. The activity and morphology of neuronal circuits are altered by ultrasound stimuli

Exposure to ultrasound alters the activity of neurons in a manner that is dependent on frequency. This modulation can lead to long-lasting effects, resulting in morphological changes at the dendritic and synaptic levels. The modifications in dendritic branching and pattern can alter local neural circuits, potentially leading to changes in behavior and cognitive functions. Previous studies have indicated that low-dose ultrasound exposure (4 minutes, 3.5 MHz, MI: 0.1, TI: 0.1) may improve cognitive functions in rat pups based on the Morris water test. Conversely, using a higher dose of ultrasound (20 minutes, 3.5 MHz, MI: 1.4, TI: 1.0) yields the opposite effect. It is worth noting that alteration of mechanical properties in ultrasound stimulation can elicit varying effects on neural cells *in vitro*. Similar outcomes were observed in mouse pups, with a notable distinction noted in prenatal ultrasound exposure at E14.5, specifically in memory and cognitive function. However, postnatal growth and physiological characteristics remained consistent between the treated and control groups.

The previous studies did not primarily focus on the detailed molecular and morphological changes of the central nervous system (CNS); however, it was verified that prenatal ultrasound examinations do not have adverse effects on maternal or perinatal outcomes. Furthermore, these examinations do not alter normal physical or neurological development, nor do they increase the incidence of malignancies, mental diseases or subnormal intellectual performance in humans. Consequently, due to the benign effects of diagnostic ultrasound, many countries conduct mandatory ultrasound screenings multiple times before birth (at 12, 18, and 32 weeks). Additionally, upon request, ultrasound examinations can also be performed at 28 and 38 weeks, as well as a 3D or 4D „baby movie" at any point during the pregnancy (even without medical recommendation).

To replicate the effects of ultrasound examination, we conducted a study involving the labeling of hippocampal CA1 neurons in mice at 14.5 embryonic days, which aligns with the 11th to 12th gestational weeks in the human gestation period. As a result, the ultrasound exposures used in the study were likely to affect neurons at a similar maturation state in both species. The application of ultrasound stimulation at 6.7-MHz for 30 minutes was found to potentially disturb the proliferation of neuroprogenitors, interfere with the migration of newborn neurons, and impact the lamination of the neocortex and the hippocampus. It is worth noting that ultrasound exposure may elicit varying responses in neurons of the neocortex and the hippocampus. However, exposure to ultrasound was observed to increase

the number of dendrites in layer 5 developing pyramidal cells *in vivo*. Our previous research indicated that a single scanning ultrasound exposure with the specified parameters resulted in an increase in the number of dendrites. Nevertheless, the other morphometric parameters of the dendritic tree in P3 L5 pyramidal cells exhibited consistent characteristics. These cells had a similar dendritic tree structure to the analyzed P28 CA1 pyramidal cells, particularly in terms of elaboration. The observed differences in morphological properties are likely attributed to the immaturity of the cells (P3 vs P28 age) and the distinct developmental stages and types of neurons (L5 vs CA1 neurons). Ultrasound signaling involves the activation of multiple pathways, which includes non-selective cation channels (such as TRPA1 and TRPC1), as well as direct physical effects. A study suggests that the TRPC1 receptor plays a major role and overexpression of TRPC1 results in stronger stimulation. The application of ultrasound waves induces changes in the properties of cell membranes and ion channels, leading to an increase in intracellular Na^+ and Ca^{2+} levels. This elevation in intracellular cation levels may stimulate synaptic transmission and neuronal electrical activity, consequently influencing the activity of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), and thereby affecting neural plasticity. In our first study, utilizing the same methodology and mouse strain, through quantitative polymerase chain reaction (qPCR) (testing of whole brain samples from the same age group) we found that the RNA expression level of BDNF remained unaltered, suggesting that the expression of the BDNF gene was not affected by the application of ultrasound. Our previous research has indicated that repetitive diagnostic ultrasound exposure results in elevated protein levels of BDNF and c-Fos in neurons. According to an earlier study, ultrasound prevents age-related dendritic structure loss in CA1 cells, which is attributed to increased brain-derived neurotrophic factor (BDNF) caused by ultrasound exposure. The repetitive application of ultrasound stimulation has been shown to reduce cortical atrophy in humans with Alzheimer's disease, a finding consistent with our observations in mice.

5. Limitations of the research

Since dendrite development is a lifelong process, the data presented in this study only refer to the young and immature nervous system. It is also important to note that the surgical procedure, electroporation and even GFP expression can affect the sensitivity of neurons to ultrasound, thus potentially confounding the measurement of morphometric data. It is important to acknowledge that although the selection of reconstructed neurons and dendrites was performed by experts in the field, this process may involve a degree of subjectivity. It should also be pointed out that our studies used a mouse model, which is very different from the human central nervous system.

6. Summary

In addition to the globally applied physical parameters of fetal ultrasound for screening, a single ultrasound stimulus *in vivo* directly affects the number of basal dendrites of TRPC4 positive cortical pyramidal cells.

Repeated ultrasound exposure increases the levels of BDNF and c-Fos proteins, indicating a possible molecular basis for the morphological changes described above.

Repeated ultrasound exposure alters the structural features of dendrites in retrosplenial cortex L5 and hippocampal CA1 neurons.

After repeated ultrasound exposure, the hippocampal formation of one-year-old mice shows greater thickness compared to the control group.

The finding of our study that hippocampal atrophy is less in ultrasound stimulated animals suggests that repeated ultrasound has a long-term protective effect on the brain. In addition, ultrasound may also offer an interesting perspective for regenerative medicine due to its effect on dendrite morphology, particularly for individuals with neurological disorders characterised by reduced dendrite number.

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Registry number: DEENK/550/2024.PL
Subject: PhD Publication List

Candidate: Zsuzsanna Winkler-Ferenczi
Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Winkler-Ferenczi, Z.**, Pelyvás, B., Nagy, M., Marosi, M., Béres, M., Varga, R., Bencze, J., Szűcs, P., Berényi, E., Englohner, A., Meszár, Z., Papp, T.: Repeated diagnostic ultrasound exposure modifies the structural properties of CA1 dendrites and alters the hippocampal transcriptome.
   Sci. Rep. 14 (1), 1-12, 2024.
   DOI: http://dx.doi.org/10.1038/s41598-024-62621-y
   IF: 3.8 (2023)

2. Papp, T.*, **Winkler-Ferenczi, Z.***, Szilágyi, B., Petró, M., Varga, A., Kókai, É., Berényi, E., Oláh, G., Halmos, G., Szűcs, P., Mészár, Z. M.: Ultrasound Used for Diagnostic Imaging Facilitates Dendritic Branching of Developing Neurons in the Mouse Cortex.
   Front. Neurosci. 16, 1-13, 2022.
   DOI: http://dx.doi.org/10.3389/fnins.2022.803356
   * These authors contributed equally to this work.
   IF: 4.3

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List of other publications

3. Papp, T., **Winkler-Ferenczi, Z.**, Petró, M., Mészár, Z. M., Képes, Z., Berényi, E.: Disorders of neural crest derivates in oncoradiological practice.
Transl. Cancer Res. 8 (8), 2916-2923, 2019.
DOI: http://dx.doi.org/10.21037/tcr.2019.10.38
IF: 0.986

Total IF of journals (all publications): 9,086
Total IF of journals (publications related to the dissertation): 8,1

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

05 November, 2024

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *