



Neural stem cells influenced by ultrasound: Frequency and energy density dependencies



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ABSTRACT

Therapeutic ultrasound is a widely used application in clinics. The response to ultrasound is tissue specific and the parameters of ultrasonic treatments are decisive. Here, the responses of primary enteric (ENS) and central nervous system (CNS) neural stem cells were investigated to different frequency – energy density – combination treatments between 510 kHz–3 W s/cm² and 4.36 MHz–25 W s/cm². Responses to ultrasonic treatments were both frequency and energy density dependent. Ultrasound enhanced the expansion and showed enlarged neurospheres for both ENS and CNS neural stem cell cultures. Differentiation was not impaired in regard to neuronal and glial cell number as well as neurite and glial fiber outgrowth. Ultrasound is a promising tool to expand neural stem cells *in vitro* neither influencing neurogenesis nor gliogenesis by applying specific frequency – energy density – combinations.

1. Introduction

Ultrasound is a form of mechanical energy and is widely used in diagnostic and therapeutic approaches. It is defined as acoustic signal with frequencies between 20 kHz and 1 GHz with longitudinal waves in solutions and gas. Ultrasound waves are distributions of pressure and density fluctuations. Medical ultrasound is used without or in combination with drugs. Research fields of treatments are cancer [9,11], stroke [7,21,26] and thrombosis [3,38]. The non-invasive technique of focused ultrasound surgery is a combination of high-intensity focused ultrasound (HIFU) and imaging methods. The thermal effect of HIFU leads to tumor cell destruction due to coagulation necrosis and is already used as a treatment for a variety of tumors [9]. Applying pulsed transcranial ultrasound stimulation to rats directly after ischemia causes a reduction of neutrophils, an increase of the cerebral blood flow and can be neuroprotective [7]. In addition, ultrasound treatment of rats after intracerebral haemorrhage increases the expression of collagens and integrins of extracellular matrix-related proteins, it promotes the formation of blood vessels and forces the recovery of neurological functions [21]. Another approach is to use ultrasound to induce sonothrombolysis by infused microbubbles that dissolve coronary arterial and microvascular thrombi [38]. Ultrasound acts as an acoustic pressure wave and applies indirect mechanical stress to the

tissue. Several studies are describing different tissue responses and the effect of ultrasound on cell level. It increases ossification [24,41], protein synthesis [6,8] and calcium influx [10,20]. The molecular mechanisms behind are widely unknown. Although the cell response of different cell types to ultrasound is dependent on frequency and energy density [34], studies considering these parameters are rare. For instance, the proliferation rates of endothelial and epithelial cells are increased after application of low energy densities and frequencies, whereas malignant cells indicated a decelerated proliferation rate. For these cells an energy density of 600 W s/cm² was needed to increase proliferation rates [34], which is 12 times higher than the tissue compatible threshold (50 W s/cm²) [25,31]. Ultrasound applications of 50 W s/cm² on developing mouse embryos during neocortex formation resulted in neuron migration beyond their determination point [2]. Influencing the central nervous system (CNS), possibly the enteric nervous system (ENS) could also be affected by ultrasound. The main functions of the ENS are the control of gut motility, secretion, absorption and permeability, regulation of fluid exchange and local blood flow. It is organized in several interconnected neuronal networks, whereas the two major ganglionated networks are the myenteric and the submucous plexus. Neurons, supporting glial cells and neuronal stem cells are located within the ganglia of these plexus. To respond to altering conditions as dietary habits or inflammation the ENS maintains

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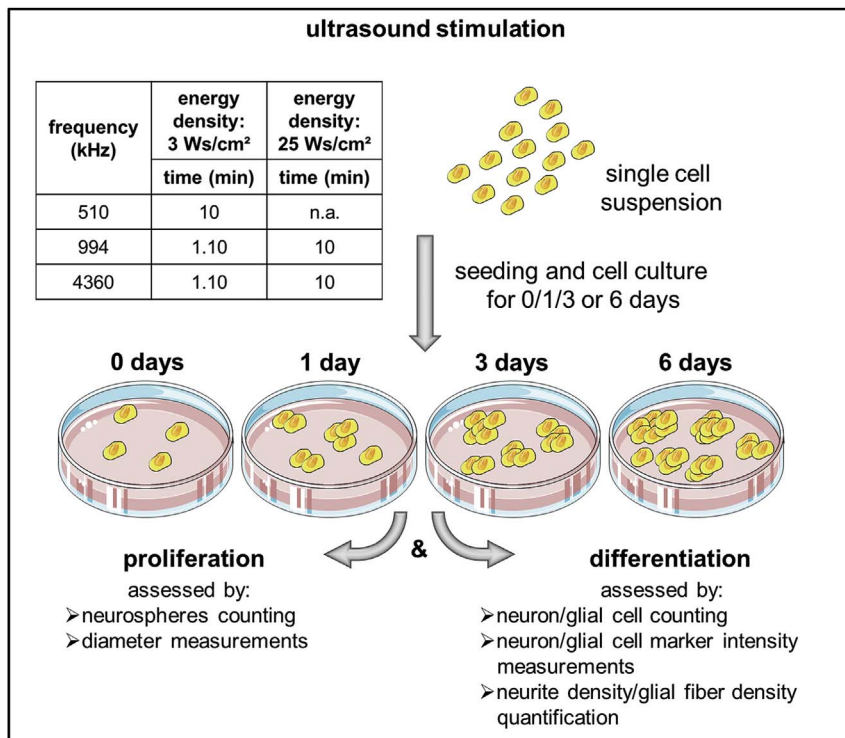


Fig. 1. Schematic illustration of experimental approaches in this study. The different frequency – energy density – combinations are displayed in the table. After initial ultrasound stimulation upon single cell suspension, cells were cultured for 0, 1, 3 and 6 days. Ultrasound effects on proliferation and differentiation were assessed using different methods.

lifelong plasticity [29]. Neural stem cells located in the ganglia are found forming the enteric stem cell niche [23]. Within the ENS neural stem cells undergo neurogenesis and gliogenesis to generate new neurons and glial cells continuously. The aim of this study is to elucidate the response of neural stem cells from the CNS and ENS to ultrasonic treatments using different frequency – energy density – combinations with respect to expansion and differentiation behaviour.

2. Materials and methods

2.1. Animals

All animal procedures were performed under the guidelines of the local ethic committee and according to the animal protection laws in Rhineland-Palatinate, Germany.

2.2. Isolation of neural stem cells from the enteric nervous system and the subventricular zone

Postnatal (balb/c) mice 1–4 days old were sacrificed by decapitation. The smooth muscle of the small intestine was stripped from the mucous layer and incubated in digestion medium composed of Hank's balanced salt solution (PAN, Aidenbach, Germany), 50 ng/ml trypsin-chymotrypsin inhibitor (Sigma-Aldrich, Taufkirchen, Germany), 1 mg/ml collagenase type 2 (Worthington, Lakewood, USA) and 200 µg/ml DNase (Roche, Mannheim, Germany). Incubation time at 37 °C was between 90 and 120 min depending on the age of the animal. For expansion assay smooth muscle were dissociated after digestion. Following a centrifugation step at 115 g for 5 min, supernatant was removed and cells were seeded in cell culture flask for 24 h with a cell density of 1 million per 5 ml. For differentiation assays the dissection of small intestine and stem cell cultivation were performed according to previously described protocols [27,28]. Briefly, myenteric nets were collected and digested in accutase (PAA, Cölbe, Germany) for 10 min. After the digestion, cells were gently titrated using a 27G needle. Centrifugation and seeding were done as described above. The preparation yield per animal was approximately 500,000 cells. The number

of animals used per experiment was chosen accordingly.

The subventricular zones (SVZs) were dissected from the same animals as the ENS. The skull was opened and the brain was transferred into ice-cold MEM-Hepes (PAN). After opening each hemisphere, the individual SVZ was dissected. Tissue was digested for 20 min at 37 °C in accutase (PAA). After the dissociation by aspiration through a 27G needle, cells were centrifuged at 115 g for 5 min and supernatant was removed. Stem cells were seeded with a density of 1 million per 5 ml in cell culture flask. One animal delivered approximately 1.5 million cells.

The isolated cells were cultured in proliferation medium based on Neurobase AD (PAA) with 2% B27 without retinoic acid (Invitrogen, Darmstadt, Germany), 1% bovine serum albumin (Sigma-Aldrich), 0.1% β-mercaptoethanol (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 0.25% L-glutamine (Sigma-Aldrich) or differentiation medium containing 2% B27 supplement (Invitrogen) instead of B27 without retinoic acid. For proliferation, enteric cultures needed a cocktail of 10 ng/ml EGF (epidermal growth factor, ImmunoTools, Friesoythe, Germany), 20 ng/ml b-FGF (fibroblast growth factor, ImmunoTools) and 10 ng/ml GDNF (glial cell derived neurotrophic factor, ImmunoTools), whereas cultures of the subventricular zone were supplemented with 10 ng/ml EGF and 20 ng/ml b-FGF. Differentiation medium for enteric cultures needed 10 ng/ml GDNF [27].

2.3. Ultrasound stimulation

Ultrasound stimulation was performed as described before [34]. After an initial ultrasonic treatment, cells were seeded according to the experiments described below. Ultrasound with following frequency – energy density – combinations were used for initial treatments: 510 kHz – 3 W s/cm², 994 kHz – 3 W s/cm², 4.36 MHz – 3 W s/cm², 994 kHz–25 W s/cm² and 4.36 MHz–25 W s/cm². The time was adapted due to the required energy density (Fig. 1). The frequency – energy density – combination 510 kHz–25 W s/cm² could not be applied due to technical limitations of the device.

2.4. Neurosphere numbers

For neurosphere number determinations and expansion measurements neural stem cells from the ENS and SVZ were seeded with a density of 5000 cells per well in a 24 well format after initial ultrasound treatment. The expansion of cells was quantified by the number of neurospheres which were assessed after 1, 3 and 6 days in culture using an Olympus CKX 41 microscope (Olympus, Hamburg, Germany) with a Moticam 2500 and the Motic Images Plus 2.0 software (Motic, Wetzlar, Germany). Diameters of neurospheres were quantified after 6 days cultivation period. The diameters of 150 neurospheres were measured with the image processing software ImageJ. During culturing neurospheres attach to the bottom of the flask and start to differentiate into neurons and glial cells due to an unknown stimulus. After ultrasonic treatment and 6 days culturing, the attached neurospheres were fixed with 4% formaldehyde (Applichem, Darmstadt, Germany) for 20 min and immunostaining was performed. This spontaneous differentiation was analysed using cell observer Z1 (Zeiss, Jena, Germany). Neurospheres arise from the SVZ attached to a much lesser extent.

2.5. In vitro differentiation

For induced differentiation freshly isolated neural stem cells from the ENS were treated with 4.36 MHz–25 W s/cm² (10min) prior differentiation with B27 supplement (Invitrogen). After 2 h and 6 days differentiated cultures were fixed and stained for immunofluorescence, respectively. In a second approach freshly isolated neural stem cells were cultured for 1, 3 and 6 days with and without an initial ultrasound treatment of 4.36 MHz–25 W s/cm² (10min). After double digestion with accutase (PAA) at 37 °C for 10 min cells were plated in a density of 30,000 cells per well in a 24 well format on poly-L-lysine/laminin coated coverslips. Differentiation occurred for 2 h and 6 days, respectively. Cells were fixed and stained for immunofluorescence. Pictures were taken using cell observer Z1. The whole cell number per picture was counted and neuron-glial cell ratio was assessed. In addition, the mean fluorescence intensity (MFI) per picture was measured using the image processing software ImageJ. Quantification was done of in total 7200 pictures in three independent experiments. Neurite density as well as glial fiber density was quantified of 150 pictures using ImageJ, respectively. Therefore pictures were overlaid with a grid of 2,500 µm² containing 63 fields. Averages of neurite density and glial fiber density were quantified for each image and normalized to control.

2.6. Immunofluorescence

Cells were fixed with 4% formaldehyde (Applichem, Darmstadt, Germany) for 20 min at room temperature and were permeabilized with 0.5% triton prior to immunostaining. After a blocking step with 10% normal goat serum (DAKO, Hamburg, Germany) in PBS, the samples were stained with anti-βIII-tubulin (1:200, MAB1637, Millipore) and anti-GFAP antibody (1:500, Z0334, DAKO) for 1 h. Samples were visualized with alexa-488 or alexa-594 secondary antibodies (1:1000, Invitrogen), which were incubated for 1 h at RT. All cultures were finally counterstained with DAPI (1:1000, Sigma-Aldrich) and mounted with fluorescent mounting medium (DAKO). Stainings were examined using a cell observer Z1.

2.7. Statistical analysis

All experiments were achieved at least in three biological replicates with three technical replicates, respectively. Statistical analysis was performed utilizing Kruskal-Wallis test for nonparametric tests by operating with SYSTAT 12 software (SYSTAT software, Chicago, USA) with p-value ≤ 0.05: *. All variances are displayed as standard error of the mean if not indicated otherwise.

3. Results

Different aspects were investigated in this study: 1) the effect of ultrasound with different frequency – energy density – combinations on the cultivation behaviour of neural stem cells derived from ENS and SVZ, 2) the effect of ultrasound with different frequency – energy density – combinations on the expansion of neural stem cells, 3) the effect of ultrasound on the differentiation and 4) the effect of ultrasound on the outgrowth density of enteric neurons and glial cells. A schematic illustration of used ultrasound approaches in this study is displayed in Fig. 1.

3.1. Ultrasound effect on cultivation of neural stem cells

Neural stem cells from both ENS and CNS form neurospheres in culture [17,27]. Some of these neurospheres start to attach to the bottom of the flask due to an unknown stimulus. After an initial ultrasonic treatment cells were cultured for 6 days, where the number of free floating neurospheres was assessed after 1, 3 and 6 days. ENS neural stem cells showed a significant decrease of free floating neurospheres in control and treated cultures over time. The ratio of free floating neurospheres between the culturing days was comparable in control and treated cultures (3 W s/cm²) and frequency independent. 25 W s/cm² showed a shifted ratio. Here the attachment of neurospheres was reduced. However, a higher number of free floating neurospheres was recorded compared to cultures treated with 3 W s/cm². Longer culture durations after 25 W s/cm² treatments slightly compensated the deceleration of attachment and the differences between day 3 and 6 decreased (Fig. 2A). In control and treated cultures (510 kHz – 3 W s/cm²) derived from the SVZ, the number of neurospheres increased during culturing. Stimulations with other frequency – energy density combinations showed no differences in neurosphere number during culturing indicating either a certain amount of neurospheres attached or the number of neurospheres was not enhanced during culturing (Fig. 2B). As adhering neurosphere numbers did not differ in treated and untreated cultures, the investigation of a proliferative effect was assessed only considering free floating neurospheres.

3.2. Ultrasound effect on expansion of neural stem cells

The effect of ultrasound on the expansion of ENS neurospheres was dependent on frequency and energy density. As 3 W s/cm² showed no effect on expansion rate, 25 W s/cm² displayed an increased number of neurospheres. Ultrasonic stimulations of 4.36 MHz–25 W s/cm² resulted consistently in a higher number of neurospheres compared to 994 kHz–25 W s/cm² indicating a frequency dependency. Comparison of effects on expansion after ultrasonic treatments with energy densities of 3 W s/cm² and 25 W s/cm² demonstrates the dependency on energy densities of the proliferative behaviour upon ENS neural stem cells (Fig. 2C). The expansion rates of SVZ cultures indicated an increase after stimulation with 994 kHz – 3 W s/cm² and 4.36 MHz – 3 W s/cm², respectively. These effects were no longer detectable after 6 days in culture. Applications of 994 kHz–25 W s/cm² and 4.36 MHz–25 W s/cm² resulted repeatedly in an increase of the expansion rates. After 6 days the positive effect on expansion was still detectable in 4.36 MHz–25 W s/cm² treated cultures representing that the expansion of SVZ neural stem cells is depending on frequency and energy density (Fig. 2D).

The diameters of 150 neurospheres were measured for all ultrasonic stimulations after 6 days in culture. The enteric ones were significantly larger after an ultrasonic treatment (69 µm ± 9.2 µm) compared to control (40 µm ± 1.3 µm) except when stimulated with 510 kHz – 3 W s/cm². The effect was not dependent on frequency but marginally dependent on the energy density. A higher energy density indicated a less strong effect on neurosphere diameters (Fig. 2E). An increase in

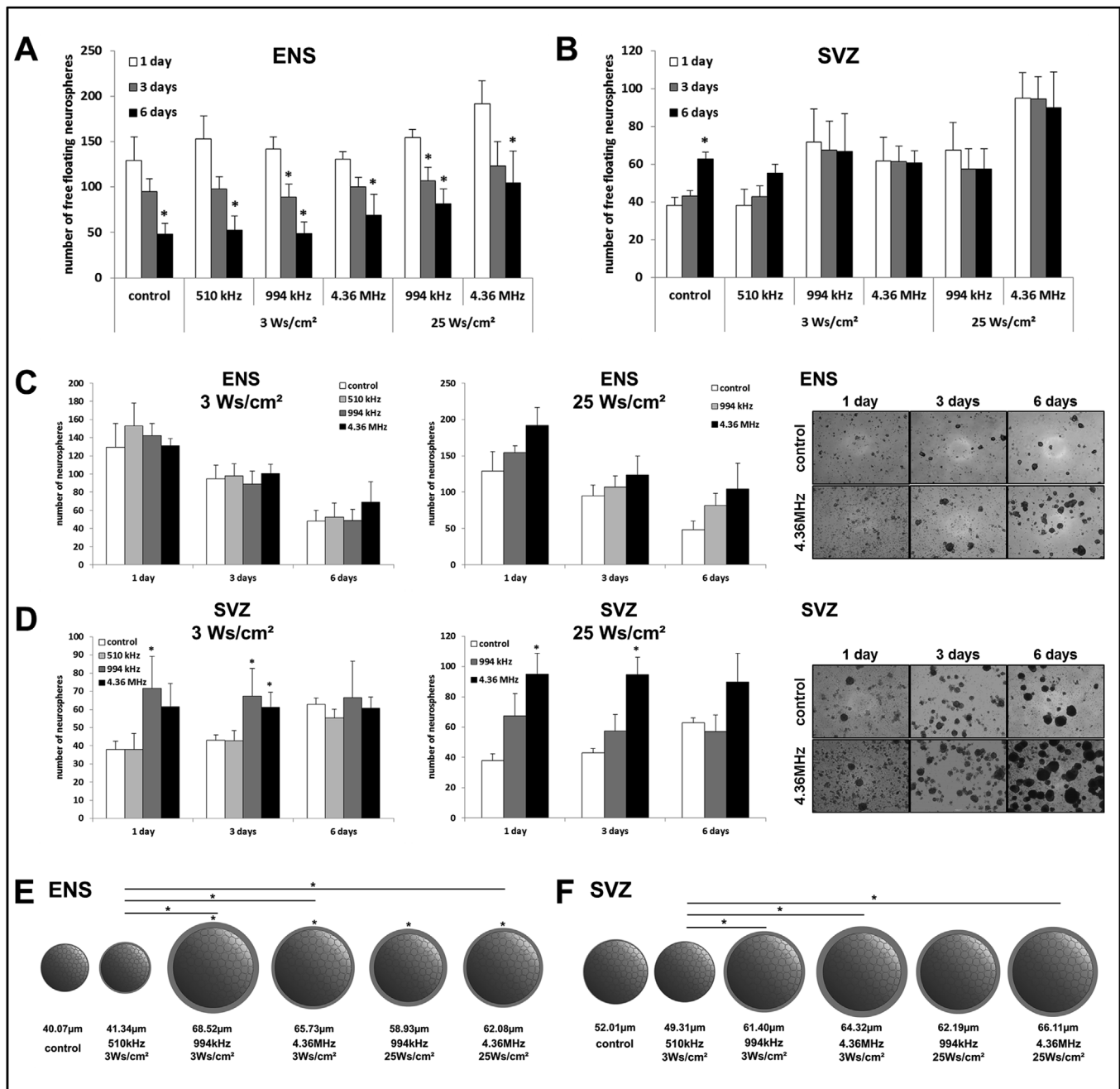


Fig. 2. Ultrasound effect on cultivation and expansion of neural stem cells. A) B) Number of free floating neurospheres derived from ENS and SVZ after initial ultrasound treatment was quantified. Culturing occurred for 6 days. The number of free floating ENS neurospheres decreased significantly during culturing. SVZ control neurospheres as well as after 510 kHz – 3 W s/cm² treatment showed an increase in number. Other frequency – energy density – combinations had no influence on the number of free floating neurospheres. C) D) The expansion of neural stem cells derived from ENS and SVZ after initial ultrasound treatment were quantified during a culture period of 6 days. The positive effect on the expansion rate after 3 W s/cm² application was regulated over time. Stimulation with 4.36 MHz–25 W s/cm² still indicated an increased expansion after 6 days. A frequency dependent increase was only detectable after application of 25 W s/cm². Light microscopic pictures reflect the increased number of neurospheres treated with 4.36 MHz–25 W s/cm² after 1, 3 and 6 days compared to control cultures. Bars: 100 μm. E) F) Diameters of neurospheres derived from ENS and SVZ were measured after initial ultrasound stimulation and 6 days culturing (n = 150 for each ultrasound stimulus). The diameters were significantly increased except after a stimulation with 510 kHz – 3 W s/cm². Variance displayed as standard error of the mean by surrounding circles. Significance compared to control is shown directly above the neurosphere. Significance compared to other stimulation is indicated by lines.

diameters of SVZ neurospheres was also observed for all stimulation conditions. Exception was as well the stimulation with 510 kHz – 3 W s/cm² showing no effect. Control neurospheres displayed a diameter of 52 μm ± 1.6 μm whereas after ultrasonic treatment largest diameter was 66 μm ± 7.8 μm. Here the effect was again slightly energy density dependent, whereas a higher energy density indicated enhanced effects on diameters of neurospheres (Fig. 2F). The following differentiation experiments are focussed on ENS neural stem cells.

3.3. Ultrasound effect on differentiation of enteric neural stem cells

Spontaneous differentiation of ENS neurospheres was observed for two third of the neurospheres after 6 days of cultivation. The amount of attached neurospheres was comparable in treated and non-treated cultures. Stimulations with an energy density of 3 W s/cm² indicated a frequency dependent increase of neuronal and glial outgrowth. The energy density of 25 W s/cm² showed this increase of neuronal and glial outgrowth as well, but it was frequency independent (Fig. 3A). Whether

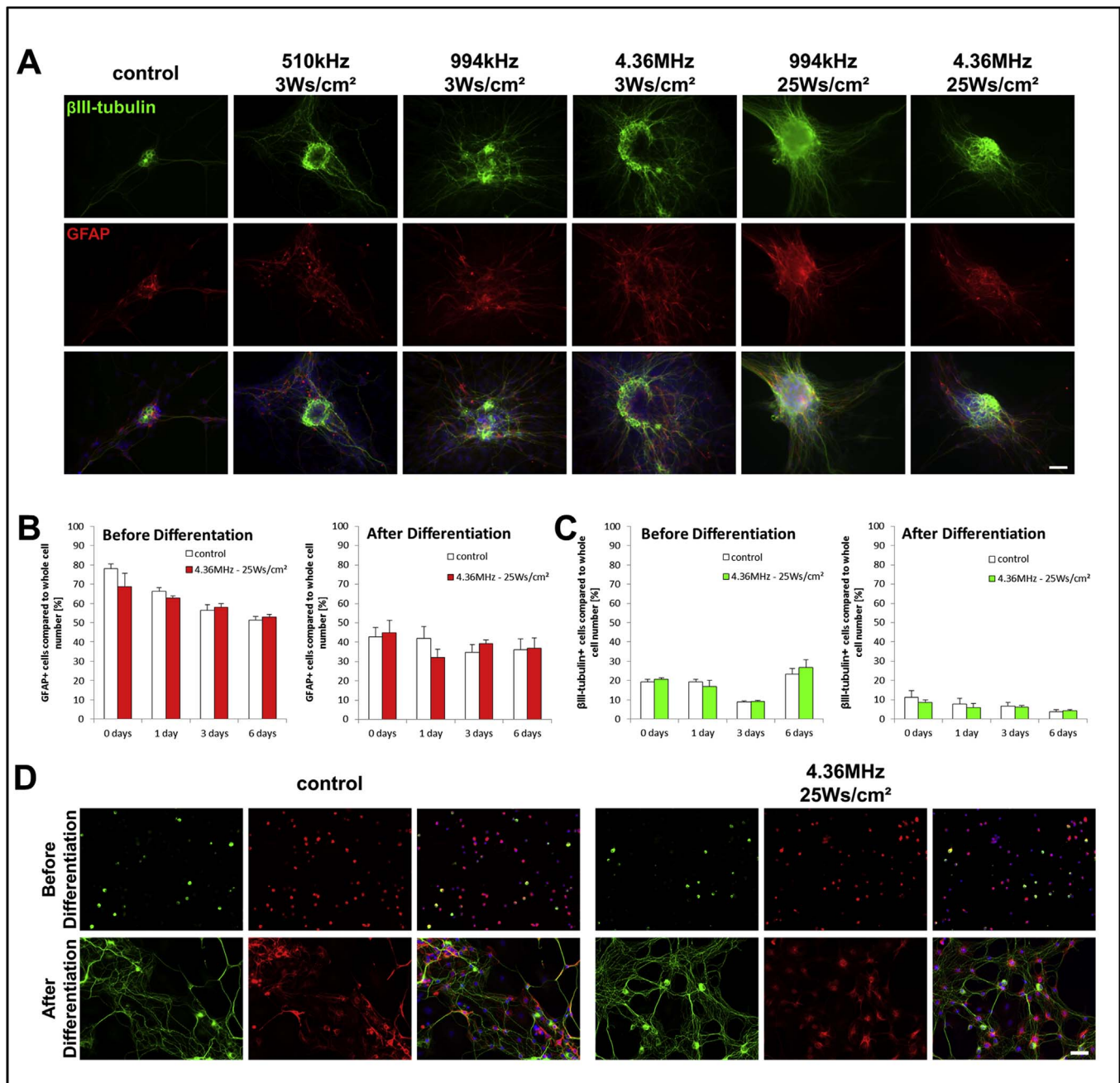


Fig. 3. Ultrasound effect on differentiation of enteric neural stem cells. A) ENS neurospheres were stimulated with different frequency – energy density – combinations, cultured for 6 days and stained against the neuronal marker β III-tubulin (green), the glial cell marker GFAP (red) and the nucleus marker DAPI (blue). Neurospheres attached and started to outgrow. Each frequency – energy density – combination indicated an increased neuronal and glial outgrowth compared to control cultures. Within the different ultrasonic stimuli no difference could be obtained. Bar: 50 μ m. B) Number of GFAP positive cells were assessed before and after induced differentiation. The proliferation periods before differentiation indicated no differences in the number of GFAP positive cells in treated and untreated cultures, but decreased treatment independent over time. After induced differentiation the amount of GFAP positive cells remained comparable in untreated and treated cultures. C) The amount of β III-tubulin positive neurons showed no major differences during 6 days culture period before differentiation. After induced differentiation the number of β III-tubulin positive neurons decreased in control and treated cultures indicating the independency on ultrasonic applications. D) Representative pictures of treated and untreated cultures before and after induced differentiation for 6 days. Bar: 50 μ m.

the increased outgrowth was impacted by ultrasonic treatments or as a result of already larger neurospheres after ultrasonic stimulation was examined in further experiments.

The ultrasound effect on ENS neural stem cells was investigated before and after induced differentiation with B27 supplement. Analyses were performed using immunocytochemical methods. As the frequency – energy density – combination 4.36 MHz–25 W s/cm² showed the strongest effect on cell expansion, cell differentiation experiments were performed using only this combination. First, the amount of glial cells and neurons were assessed after 1, 3 and 6 days in culture before and

after differentiation. The amount of GFAP positive cells showed no difference in untreated and treated cultures before and after differentiation. A decrease of GFAP positive cells was observed over culture time before differentiation and was treatment independent. In general, the amount of GFAP positive cells was higher before differentiation compared to cultures after induced differentiation (Fig. 3B, D). The amount of β III-tubulin positive neurons remained comparable during cultivation time before differentiation. But the number of β III-tubulin positive neurons decreased after induced differentiation. This decrease was observed in control as well as in treated cultures and therefore

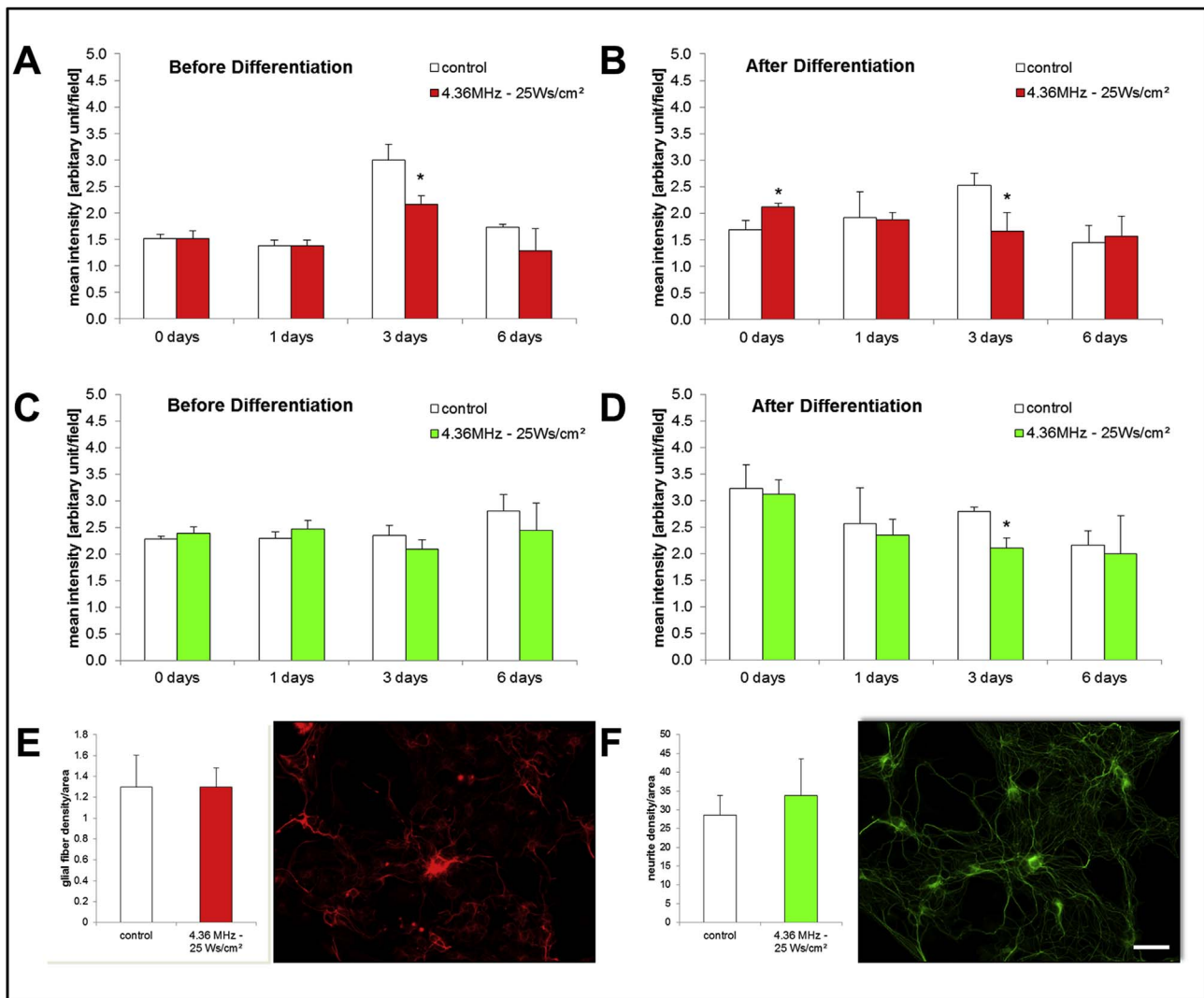


Fig. 4. Ultrasound effect on expression and outgrowth of enteric neural stem cells. After ultrasonic treatment (4.36 MHz–25 W s/cm²) ENS neural stem cells were cultured for 0, 1, 3 or 6 days prior induced differentiation for 6 days. Cultures were fixed and stained against neuronal and glial cell marker. A) Quantification of the mean fluorescence intensity (MFI) per field of GFAP showed a decrease in treated cultures after 3 and 6 days culturing before differentiation and B) after induced differentiation with a previous proliferation period of 3 days. C) Quantification of MFI per field of βIII-tubulin indicated no differences between stimulated and control cultures before differentiation. D) After induced differentiation the MFI decreased in treated cultures with a previous proliferation period of 3 days. E) F) Freshly isolated ENS neural stem cells were stimulated with 4.36 MHz–25 W s/cm² and differentiated for 6 days. Quantification of glial fiber density (E) and neurite density (F) indicated no differences after ultrasonic application. Staining was performed against the glial cell marker GFAP (red) and the neuronal marker βIII-tubulin (green). Representative pictures of the glial fiber density and neurite density are shown. Bar: 50 μm.

independent of ultrasonic applications (Fig. 3C and D). As a next step, the mean fluorescence intensities (MFI) of untreated/treated and undifferentiated/differentiated cultures were assessed, respectively. This value correlates with the protein expression of the individual samples. The MFI of GFAP indicated a decrease after ultrasonic treatment during culturing the cells for 3 and 6 days. The decrease was independent on induced differentiation indicating that the expression of GFAP was already decreased before differentiation (Fig. 4A and B). The MFI of βIII-tubulin showed similar results. After culturing the cells for 3 and 6 days, βIII-tubulin expression was reduced in treated cultures irrespective of induced differentiation (Fig. 4C and D). Ultrasound has rarely an effect on the differentiation of ENS neural stem cells in regard to the number of differentiated cells, but has an impact on the expression levels of differentiation markers.

3.4. Ultrasound effect on outgrowth density of enteric neurons and glial cells

Although ultrasonic treatment has no impact on the differentiation in terms of neuron and glial cell number, the neuronal and glial cell

outgrowth can still be affected by ultrasound. Further investigations were assessed by comparing the glial fiber density and neurite density of 4.36 MHz–25 W s/cm² treated cells to control cultures after induced differentiation. Both glial fiber density and neurite density were not affected by ultrasonic stimulation (Fig. 4E and F). Therefore the increased glial and neuronal outgrowth after spontaneous differentiation can be explained by the increased diameters of the neurospheres. This enhanced cell number resulted in increased neuronal and glial cell outgrowth.

4. Discussion

Ultrasound is an important tool in medical diagnostic. Since certain years ultrasound is also used in therapeutic approaches for instance in wound healing therapies [30], in the treatment of bone fractures [5,37] as well as the treatment of stroke [26]. Due to ultrasound plasminogen was activated [19] and artery recanalization started [1]. The ultrasound device EXOGEN2000 (Bioventus Coöperatief U.A., Hoofddorp, Netherlands) is often utilized for therapeutic applications. Published studies

demonstrated that this device enhances wound healing [30] and fracture repair [14]. EXOGEN2000 applies low-intensity pulsed ultrasound of a frequency of 1.5 MHz and intensity of 30 mW/cm² to the tissue. In comparison to the parameters of continuous ultrasound used in the present study, the intensities are similar (40 mW/cm² present study, 30 mW/cm² EXOGEN2000) but the energy densities are different (25 W s/cm² present study, 7.2 W s/cm² EXOGEN2000). The energy density of pulsed ultrasound is smaller than the one of continuous ultrasound because the absolute time of application is reduced due to pulse pauses. The tissue compatible threshold is based on intensity and time. Therefore the relevant parameter for therapeutic applications is the energy density (energy density (W s/m²) = intensity (W/m²) x time (s)). The threshold was defined at 50 W s/cm² [25,31]. In addition, recent investigations of different ultrasonic applications resulted in a frequency, intensity and energy density dependent effect on cells [34]. Effects were detected after application of 25 W s/cm² and 50 W s/cm², but an energy density of 3 W s/cm² showed still cell responses [34]. Nevertheless, studies focussing on the different effects of frequency, intensity and energy density are rare, wherefore the current study is highly relevant for the development of future therapeutic applications of ultrasound.

In the present study the ultrasound effects on primary neural stem cells derived from ENS and SVZ were assessed regarding cultivation, expansion, differentiation and outgrowth behaviour (Tab 1). Ultrasound had a frequency and energy density dependent influence upon cultivation of ENS and SVZ neurospheres. Around two third of enteric neurospheres attached independently of stimulation, which is a normal cultivation behaviour. SVZ neurospheres attached to a much lesser extent. In controls the number of free floating SVZ neurospheres increased, whereas ultrasound with higher frequencies enhanced the attachment of neurospheres. Ultrasound is described to induce extracellular matrix protein synthesis [4,39], which can explain the increased number of attached SVZ neurospheres. In contrast to the frequency dependency, the effect on the cultivation behaviour of ENS neurospheres was slightly energy density dependent. ENS and CNS share a broad range of similarities and may react in a similar way to stimulation with ultrasound. Both ENS and SVZ neural stem cells showed a response to ultrasonic treatments although the cultivation behaviour is different in regard to attachment of neurospheres. In general, the degree of the response to the same treatment can differ between ENS and SVZ neurospheres [32,33].

Regarding the effect on expansion, an energy density of 3 W s/cm² demonstrated only a short-term effect on the expansion rates of ENS and SVZ neural stem cells. In comparison, an energy density of 25 W s/cm² resulted in a persisting increase. After 6 days the effect was still detectable. The enhanced expansion rates were dependent on both frequency and energy density, where SVZ cultures were more sensitive to ultrasonic treatments compared to ENS cultures. The different sensitivities of ENS and SVZ neural stem cells to treatments were already described before [32,33]. These results on expansion confirm the necessity of the adaptation of ultrasound applications to tissue and cell type. As in this study only continuous ultrasound was applied, the effect of pulsed ultrasound even with the same frequency – energy density – combination can vary. The elicited response in regard to proliferation behaviour can also vary among tissue and cell type. The diameters of neurospheres were increased independent from the applied frequency of ultrasound but marginally dependent on the energy density. For ENS neurospheres higher energy density showed less strong effect. It might be that this high energy density induces apoptosis by inducing p53 [6]. In contrast, SVZ neurospheres displayed an increased diameter with higher energy densities indicating a certain threshold is not yet reached to induce apoptosis. In general, low frequency and low energy density have no or few effect on expansion of ENS and SVZ neurospheres meaning again a certain threshold of ultrasound is necessary to start special biological effects. A huge number of studies already demonstrated, that ultrasound has a positive effect on the proliferation rates of

cells such as fibroblasts [22], osteoblasts [15], chondrocytes [40] or barrier cells [34]. Most of the studies are based on pulsed ultrasound, where thermal effects are suppressed. Intensities can be increased and mechanical effects can be investigated. In comparison, continuous ultrasound is used in therapeutics because of the thermal effects or to transfer drugs into the skin. In the present approach thermal influences of continuous ultrasound were counteracted by a perfusion with constant temperature [34]. Thermal effects can thus be excluded. In general, the positive effect of ultrasound on proliferation of ENS and SVZ neural stem cells can be based on the activation of voltage-gated calcium channels [35] and L-type voltage-sensitive calcium channels. Due to the intracellular increase of calcium PI3K/Akt and NF- κ B pathways are activated and enhancing proliferation [16]. By inhibiting PI3K/Akt pathway special biological effects activated by ultrasound are suppressed [39]. In ENS myenteric neurons derived from neural stem cells L-type calcium channels were already described and can be activated by LPS [18]. Not only the activation of PI3K/Akt through stimulating L-type calcium channels can explain the enhanced proliferative effect of ultrasound, but also an induced genes expression of growth factors functioning as a positive feedback loop [12].

Neurospheres started to attach and differentiate during culturing. Adhesion was not influenced by ultrasound, but outgrowth was increased independent from the applied frequency – energy density – combination. This can be explained by larger neurospheres after ultrasonic stimulation compared to control. The increased outgrowth after ultrasound application and attachment was previously described [13]. The attachment of ENS neurospheres is rather an effect to cultivation time than to treatment. Investigations of the influences on number of neurons and glial cells were performed using immunocytochemical staining against the neuronal marker β III-tubulin, which is a decent marker for enteric neurons [23], and the glial cell marker GFAP. GFAP positive enteric glial cells respond to micro-environmental changes e.g. dietary habits or inflammation called reactive gliosis [36]. Neurogenesis was not influenced by the frequency – energy density – combination of 4.36 MHz–25 W s/cm². Neither variations in culture time nor differentiation time showed ultrasound induced differences in neurogenesis of ENS neural stem cells. Gliogenesis was impaired temporally during the first 24 h and was regulated over time. The expression of β III-tubulin and GFAP indicated decreased levels after ultrasonic treatment independent of the induction of differentiation. This effect was only detectable after a previous culture period of 3 days indicating that not the differentiation but the proliferation of neural stem cells in combination with ultrasound had an impact on the expression. One should consider that with these two widely used markers not all subpopulations of neurons and glial cells can be detected and also neural stem cells within the cultures were not considered. The glial fiber density as well as neurite density was not affected by 4.36 MHz–25 W s/cm². This frequency – energy density – combination demonstrated an enhanced expansion rate, but no influences on differentiation of neural stem cells. Although it is described that ultrasound enhances neuronal differentiation and neurons outgrowth [13], we did not observe this effect. However, Lee et al. applied ultrasound stimuli on subsequent culturing days compared to one initial ultrasound treatment in the present study. In addition, Lee et al. utilized energy densities in a range of 30–150 W s/cm², which are higher than in this study indicating that neural stem cell differentiation might be induced by tissue damaging doses of ultrasound. Ultrasound with low energy densities remain a beneficial technical resource to enrich neurospheres in culture without influencing culture and differentiation behaviour.

A next step could be to further optimize the enhancement of neural stem cell expansion by using a combinatory approach of the physical stimulation with ultrasound and a chemical one. A promising growth factor candidate is the granulocyte-colony stimulating factor (G-CSF). G-CSF enhances proliferation and induces differentiation of ENS neural stem cells [32,33]. A combinatory approach of G-CSF and ultrasound

Table 1

Summary of frequency and energy density dependencies upon different effects on neural stem cells after ultrasonic treatment.

effect on	ENS					SVZ				
	510kHz - 3Ws/cm ²	994kHz - 3Ws/cm ²	4.36MHz - 3Ws/cm ²	994kHz - 25Ws/cm ²	4.36MHz - 25Ws/cm ²	510kHz - 3Ws/cm ²	994kHz - 3Ws/cm ²	4.36MHz - 3Ws/cm ²	994kHz - 25Ws/cm ²	4.36MHz - 25Ws/cm ²
cultivation (attachment)	no effect	no effect	no effect	(-)	(-)	no effect	no effect	+	+	+
expansion	(+)	no effect	no effect	+	+	no effect	+	+	+	+
diameters	no effect	+	+	+	+	no effect	+	+	+	+
spontaneous differentiation	+	+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.
induced differentiation	n.a.	n.a.	n.a.	n.a.	no effect	n.a.	n.a.	n.a.	n.a.	n.a.
outgrowth	n.a.	n.a.	n.a.	n.a.	no effect	n.a.	n.a.	n.a.	n.a.	n.a.

frequency dependent
energy density dependent
frequency + energy density dependent

could be used to further expand neural stem cells and to drive the cells towards differentiation. With this combinatory approach neural stem cells could be primed to be used in therapeutic approaches for neurodegenerative diseases.

In conclusion, the effect of ultrasound on neural stem cells is dependent on the applied frequency and energy density (Table 1). Ultrasound treatments increased proliferation of neural stem cells, but induced no changes regarding neurogenesis and gliogenesis. Further studies could focus on ultrasound treatments with the present defined frequency – energy density – combinations *in vivo* and their effects on proliferation and differentiation of neural stem cells in the ENS as first steps towards alternative treatments of neurodegenerative diseases.

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