

<https://doi.org/10.1038/s44384-026-00050-z>

Optimized ultrasound neurostimulation of ventromedial prefrontal cortex reverses depression behaviours and normalizes brain metabolism



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Ultrasound neurostimulation (USNS) offers non-invasive access to deep mood-regulating circuits, but most protocols rely on arbitrary parameters and lack mechanistic evaluation. We first optimised single-pulse USNS in healthy mice by mapping pressure–response curves during motor cortex stimulation with behavioural scoring, electromyography and hydrophone measurements to define a focal, reproducible setting. We then applied repeated USNS to the infralimbic cortex, the rodent homologue of ventromedial prefrontal cortex, for five days during unpredictable chronic mild stress. Behavioural outcomes (nest-building, open-field exploration), whole-brain ¹⁸F-FDG microPET and targeted metabolomics in infralimbic cortex, amygdala and hippocampus were assessed. Optimised infralimbic USNS selectively activated local neurons, reversed self-care and anxiety-like deficits to near baseline and outperformed chronic fluoxetine. PET revealed sustained hypermetabolism in vmPFC, dorsal hippocampus, periaqueductal grey and raphe nuclei, while metabolomics showed coordinated down-regulation of glutamate-centred pathways, supporting USNS as a precise, non-destructive antidepressant-like intervention.

Major depression (MD) is a leading global cause of disability and a major contributor to disease burden^{1,2} worldwide. The mainstay of treatment remains pharmacotherapy, particularly selective serotonin reuptake inhibitors (SSRIs). However, first-line SSRI therapy is often inadequate: nearly half of patients fail two trials and meet criteria for treatment-resistant depression (TRD)³. Comorbid anxiety further reduces the effectiveness of antidepressants⁴. These limitations highlight the urgent need for innovative, mechanistically targeted therapies for TRD.

Brain stimulation techniques provide alternatives to pharmacotherapy for depression. Key targets include the dorsolateral prefrontal cortex (dlPFC), ventromedial prefrontal cortex (vmPFC), nucleus accumbens (NAc), and lateral habenula (LHb)⁵. Non-invasive approaches such as transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) can access superficial regions but lack the spatial resolution and depth needed for deeper nodes. Deep brain stimulation (DBS) achieves precise modulation but is invasive and reserved for refractory cases.

This gap underscores the need for non-invasive, focal methods capable of reaching deep mood-regulating circuits.

Ultrasound (US) has recently gained attention as a versatile tool for non-invasive brain interventions. Depending on acoustic parameters, it can achieve tissue ablation^{6–8}, transient blood–brain barrier opening^{9–11}, or functional modulation of neural circuits^{12–15}. Of particular interest is low-intensity focused ultrasound (LIFU), which can stimulate or inhibit neural activity in a spatially precise and non-destructive manner.

In psychiatric research, US neurostimulation (USNS) has begun to show promise for modulating affective brain circuits implicated in mood disorders. Preclinical studies report that repeated pulsed US applied to the prefrontal cortex of stressed rodents increases hippocampal brain-derived neurotrophic factor (BDNF) and improves behaviour¹⁶. Other reports confirm antidepressant-like effects of medial prefrontal cortex stimulation without histological damage¹⁷, as well as

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reduced depressive-like behaviours and pro-inflammatory cytokines in inflammation-induced models¹⁸. Extending to other circuits, hippocampal sonication improves forced swim and open-field performance while downregulating Notch1-mediated neuroinflammation¹⁹. Moreover, prefrontal stimulation reduces immobility in forced swim and tail suspension tests while blunting IL-6, IL-1 β , and TNF- α upregulation¹⁸. In non-human primates, repetitive low-intensity US of the dorsal striatum enhanced motivation and decision-making over several weeks with no detectable neuroimaging deficits²⁰. Together, these findings establish the safety and efficacy of USNS across species, but they also highlight that applications to depression remain exploratory.

Despite encouraging preclinical demonstrations, the application of USNS to depression remains highly exploratory. Most studies rely on one or two behavioural assays, typically forced swim or sucrose preference, without assessing domains such as daily living, anxiety-related exploration, or motivational drive. Molecular endpoints are likewise limited, often restricted to single markers such as BDNF or pro-inflammatory cytokines, providing little insight into neural network dynamics or neurotransmitter balance. A further weakness is methodological: sonication parameters are frequently chosen by precedent, effectively as “magic numbers,” rather than derived from systematic dose-response optimisation, limiting reproducibility and mechanistic interpretation.

Crucially, very few studies have targeted the ventromedial prefrontal cortex (vmPFC), a millimetre-scale hub central to mood regulation and a validated target for neuromodulation. Its rodent homologue, the infralimbic cortex (IL), the homologue of the human vmPFC²¹, is strongly implicated in depression, altered in imaging studies of patients^{22,23}, and successfully modulated by DBS in TRD²⁴. In the unpredictable chronic mild stress (UCMS) model, depression-associated molecular changes are reversed by fluoxetine²⁵, and both DBS²⁶ and rTMS²⁷ of the IL restore behaviour.

Transcranial ultrasound stimulation (TUS) has recently entered early clinical testing for depression, with initial trials demonstrating promising antidepressant effects in humans²⁸. For example, an open-label pilot in severe TRD reported nearly 60% reduction in symptom severity after five days of focused US sessions²⁹, and a sham-controlled study targeting the subcallosal cingulate (a deep limbic region implicated in mood regulation) found significantly greater mood improvements with real vs. sham TUS after a single session²⁸. Similarly, a recent randomised trial applying low-intensity TUS to the prefrontal cortex observed meaningful clinical improvement alongside modulation of fronto-limbic network connectivity³⁰. While these early trials demonstrate the feasibility and preliminary efficacy of TUS in mood disorders, their small scale and limited mechanistic scope underscore the need for continued preclinical work to elucidate underlying neurobiological mechanisms and optimise stimulation strategies.

These emerging human studies, commonly referred to as TUS, highlight the therapeutic promise of US-based neuromodulation. Building on this, we developed and optimised an USNS protocol in rodents to address the methodological and mechanistic gaps left unresolved in clinical work. Specifically, we tested whether a five-day course of empirically optimised, focal USNS of the IL could reverse the behavioural and neurobiological consequences of UCMS. We first established stimulation parameters in healthy mice using motor assays, electromyography (EMG), and c-Fos mapping to achieve millimetre-scale precision. Using this optimised protocol, we then applied repeated IL-USNS during UCMS and conducted a multimodal evaluation: validated behavioural readouts spanning daily living and anxiety domains, whole-brain ¹⁸F-FDG microPET to capture network-wide metabolic remodelling, and region-specific metabolomics in IL, amygdala, and hippocampus to uncover biochemical mechanisms. Demonstrating that repeated, non-invasive IL-USNS can produce durable behavioural rescue alongside brain-wide metabolic and neurochemical normalisation would establish US as a translationally relevant, next-generation neuromodulation strategy.

Results

Optimising single pulse USNS parameters

To establish a reliable and focal stimulation paradigm, we first quantified the pressure–response characteristics of single pulse USNS applied to the M1 (Fig. 1A, B). Thirty mice were assessed in two cohorts:

- Visible motor screening ($n = 20$). Under near-awake anaesthesia (0.1% isoflurane)³¹, animals received ten 160 ms pulses at each pressure step from 50 kPa to 500 kPa in 50 kPa increments (10 s inter-stimulus interval) (Fig. 1A). Motor success, contralateral forepaw twitch, was plotted against peak negative pressure, yielding a sigmoidal fit with a motor threshold (MT, 50% success) of 250 kPa (Fig. 1B). Success rose to $89 \pm 2.7\%$ at 400 kPa and plateaued at $98.5 \pm 0.8\%$ at 500 kPa.
- EMG ($n = 10$). A separate cohort, implanted with subdermal EMG electrodes in the right brachioradialis muscle group³², was tested under surgical anaesthesia (1.5% isoflurane). Mean motor-evoked potential (MEP) amplitude increased sharply with pressure (repeated-measures ANOVA $F(2, 18) = 13$, $p = 0.0003$). Post-hoc Tukey showed the maximal and least-variable response at 400 kPa ($63.7 \pm 6.5 \mu\text{Vpp}$; RSD 32%; $p < 0.0001$ vs 250, 300 kPa; $p < 0.01$ vs 350, 450 kPa) (Fig. 1B; Supplementary B, Table SII).

Hydrophone mapping verified a lateral focal span of roughly 1–2 mm, the zone sustaining 350–400 kPa pressures at the focus (Fig. 1C), supporting millimetre-scale targeting under our conditions. Because the *in vivo* field can still vary with skull-related aberration and positioning, we do not claim that free-field mapping alone ‘guarantees’ intracranial focality. Consequently, 160 ms pulses at 400 kPa repeated at 0.1 Hz were selected for all subsequent IL experiments.

This optimisation step provided (i) a quantitative MT, (ii) a pressure that maximised success and MEP amplitude while minimising variability, and (iii) precise spatial resolution, key prerequisites for the behavioural, imaging and metabolomic investigations that follow.

Single-session IL-USNS increases c-Fos expression in IL

Guided by the single-pulse optimisation data and previous neuromodulation work³³, we devised a repeated-pulse protocol consisting of 60 pulses (160 ms, 400 kPa) delivered at 0.1 Hz over 10 min. The collimator centre was stereotactically positioned at bregma +2.0 mm (AP), midline (ML = 0)³⁴. IL boundaries were defined according to the Paxinos & Franklin mouse brain atlas, using coordinates centred at AP +2.0 mm nd segmented along the anterior–posterior axis for consistency across animals. Region definitions and abbreviations follow the Paxinos & Franklin mouse brain atlas (IL, PrL, Cg, M1, M2)³⁴. The beamwidth encompassed the IL but spared neighbouring PrL and Cg areas (Fig. 1D). Four mice received active USNS; four sham controls underwent identical anaesthesia and positioning without US.

Ninety minutes post-stimulation, brains were processed for c-Fos immunohistochemistry. Quantitative cell counts revealed a robust, segment-specific increase in neuronal activity centred on the IL: Segment-wise analysis confirmed significant increases at six IL levels (Supplementary B, Table SIII for full statistics), peaking at $+96 \pm 27$ cells mm^{-2} at bregma +2.10 mm ($t = 3.6$, $p = 0.0061$) and $+82 \pm 26$ cells mm^{-2} at +1.40 mm ($t = 3.1$, $p = 0.021$). To facilitate interpretation given the small sample size ($n = 4/\text{group}$), we report standardised effect sizes (Hedges’ g) with 95% confidence intervals for IL and control regions in the Supplementary Information. These estimates support a large IL-specific effect and substantially smaller/uncertain effects in neighbouring mPFC regions and auditory cortex.

Importantly, no statistically significant c-Fos elevation was detected in neighbouring medial prefrontal areas (PrL, Cg, M1/M2) or in auditory cortex (Supplementary C). Effect-size estimates provided in the Supplementary Information indicate substantially smaller and/or uncertain effects in these regions compared with IL, so modest off-target effects cannot be excluded with $n = 4/\text{group}$.

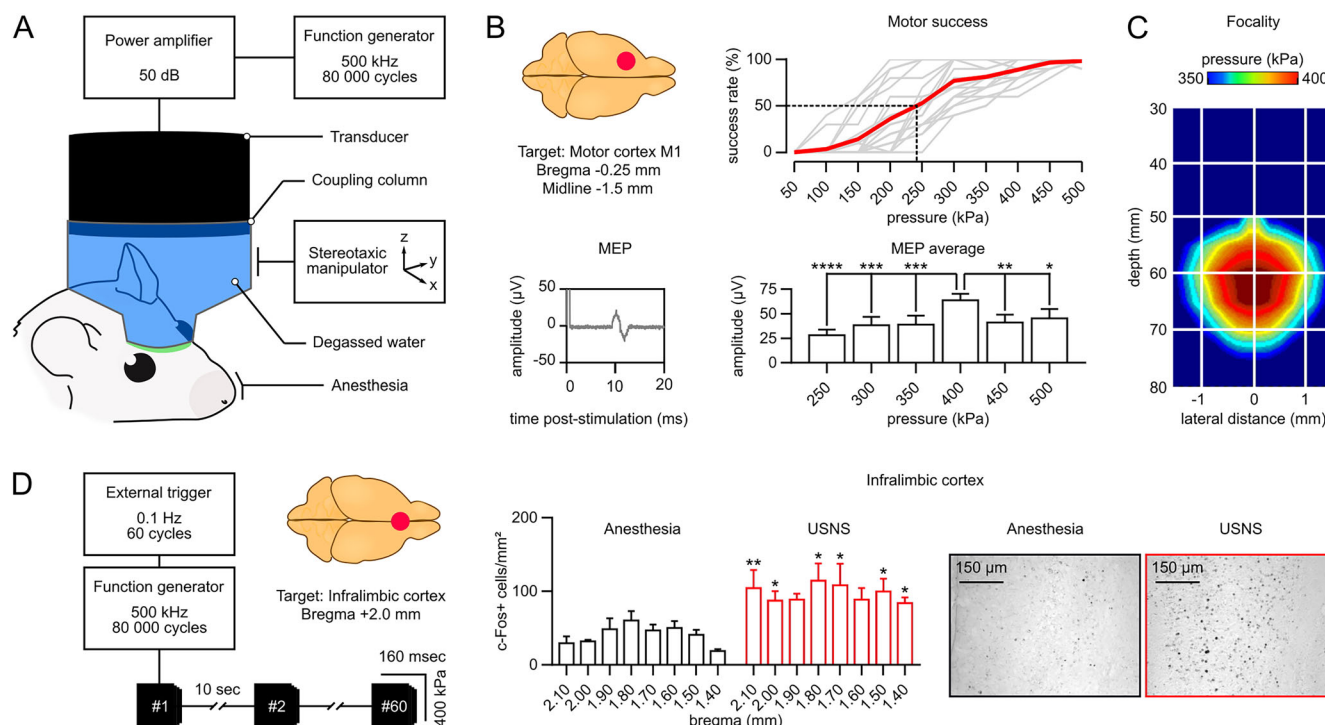


Fig. 1 | USNS targeting, motor response, and c-Fos activation. **A** Schematic view of the system for single-pulse USNS and brain targeting of the primary motor cortex. **B** Top: electrode-free recordings (0.1% isoflurane) of motor responses (ten trials for each pressure step) expressed as a success rate (%); the dashed grey line shows the projection of the motor threshold on the average curve in red. Bottom: Electromyographic recordings (1.5% isoflurane) of pressure steps above the motor threshold up to 500 kPa; the shape of a motor evoked potential (MEP) is given on the

left. **C** Focalty of the US beam at peak negative pressure 400 kPa. **D** The application of one session of repeated USNS increases c-Fos activity along the antero-posterior axis of the infralimbic cortex (IL) when compared to anaesthesia alone. Data is expressed as mean $\pm$ SEM. Statistics: repeated-measures one-way ANOVA followed by Tukey's HSD post-hoc test (MEP/pressure-step experiment). Two-by-two comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Representative micrographs illustrate dense c-Fos staining in IL after USNS compared with sparse labelling under anaesthesia alone.

Together, these data demonstrate that a single 10 min train of optimised pulses induces a focused increase in c-Fos expression in the IL, the rodent homologue of the human vmPFC, with no statistically significant increases detected in neighbouring cortical regions or auditory cortex under these conditions, supporting target engagement for chronic therapeutic experiments.

Repeated IL-USNS reverses UCMS-induced behavioural deficits

Five weeks of UCMS produced the expected deterioration in self-care and anxiety-related behaviours. To quantify therapeutic efficacy, we compared four UCMS groups: IL-USNS ($n = 15$), Sham-USNS ($n = 14$), vehicle-treated ($n = 12$) and fluoxetine-treated ($n = 12$) with a non-stressed baseline group ($n = 12$). Vehicle-treated refers to UCMS mice that received the vehicle (saline) only, under the same administration schedule as the fluoxetine group. All behavioural testing was conducted during the dark phase, 5–10 days after the final USNS session (Fig. 2A–C).

Nest-building (day 38). Construction of a cotton nest is a sensitive index of daily-living motivation disrupted by chronic stress³⁵. One-way ANOVA followed by Fisher's post-hoc tests showed a strong treatment effect ($F(3, 49) = 9.4$, $p < 0.0001$). UCMS vehicle mice built markedly poorer nests than non-stressed controls ($p = 0.0043$), confirming the depressive-like phenotype. IL-USNS completely normalised this deficit: nests in the USNS group were significantly superior to those of Sham-USNS mice ($p < 0.0001$) and statistically indistinguishable from non-stressed levels. Fluoxetine did not improve nest quality versus vehicle ($p > 0.05$). Thus, five brief IL-USNS sessions restored a complex goal-directed behaviour that pharmacological treatment left unchanged (Fig. 2B).

Open-field exploration (day 43). Anxiety-like behaviour and general activity were assessed in a brightly lit circular arena. Anxiety-related outcomes (latency to centre) and locomotor activity (total ambulatory time) were analysed as independent behavioural dimensions.

Latency to centre. UCMS vehicle mice displayed long hesitation before first entering the anxiogenic centre zone. Treatment significantly altered this measure ($F(3, 49) = 4.1$, $p = 0.011$). IL-USNS mice reached the centre almost three-fold faster than Sham-USNS controls ($p = 0.0031$) and at latencies comparable to non-stressed animals, indicating an anxiolytic-like effect. Neither vehicle nor fluoxetine differed significantly ($p > 0.05$), suggesting limited impact of chronic SSRI on this endpoint (Fig. 2C).

Total ambulatory time. Locomotor activity also varied across groups ($F(3, 49) = 9.0$, $p < 0.0001$). UCMS reduced mobility relative to non-stressed controls ($p = 0.0024$). IL-USNS reversed this suppression, increasing ambulatory time by ~30% versus Sham-USNS ($p = 0.0002$). Fluoxetine produced a non-significant trend toward greater activity but did not reach statistical significance ($p > 0.05$) (Fig. 2C). Heat map exploration patterns (Fig. 2C) showed structured trajectories including centre exploration rather than a generalised hyperlocomotor pattern, supporting an anxiolytic-like effect beyond increased activity alone. Heatmaps are shown as representative trajectories; anxiety-related behaviour was quantified primarily by latency to first centre entry (Fig. 2C), a commonly used open-field readout of centre avoidance³⁶.

Interpretation and controls. The behavioural improvements were not attributable to brief isoflurane exposure during stimulation. Sham-anaesthetised mice performed equivalently to fully untreated UCMS mice, consistent with reports that sub-anaesthetic isoflurane does not confer lasting antidepressant benefit under these conditions³⁷. In addition, sham anaesthetised mice (anaesthesia without USNS) showed no

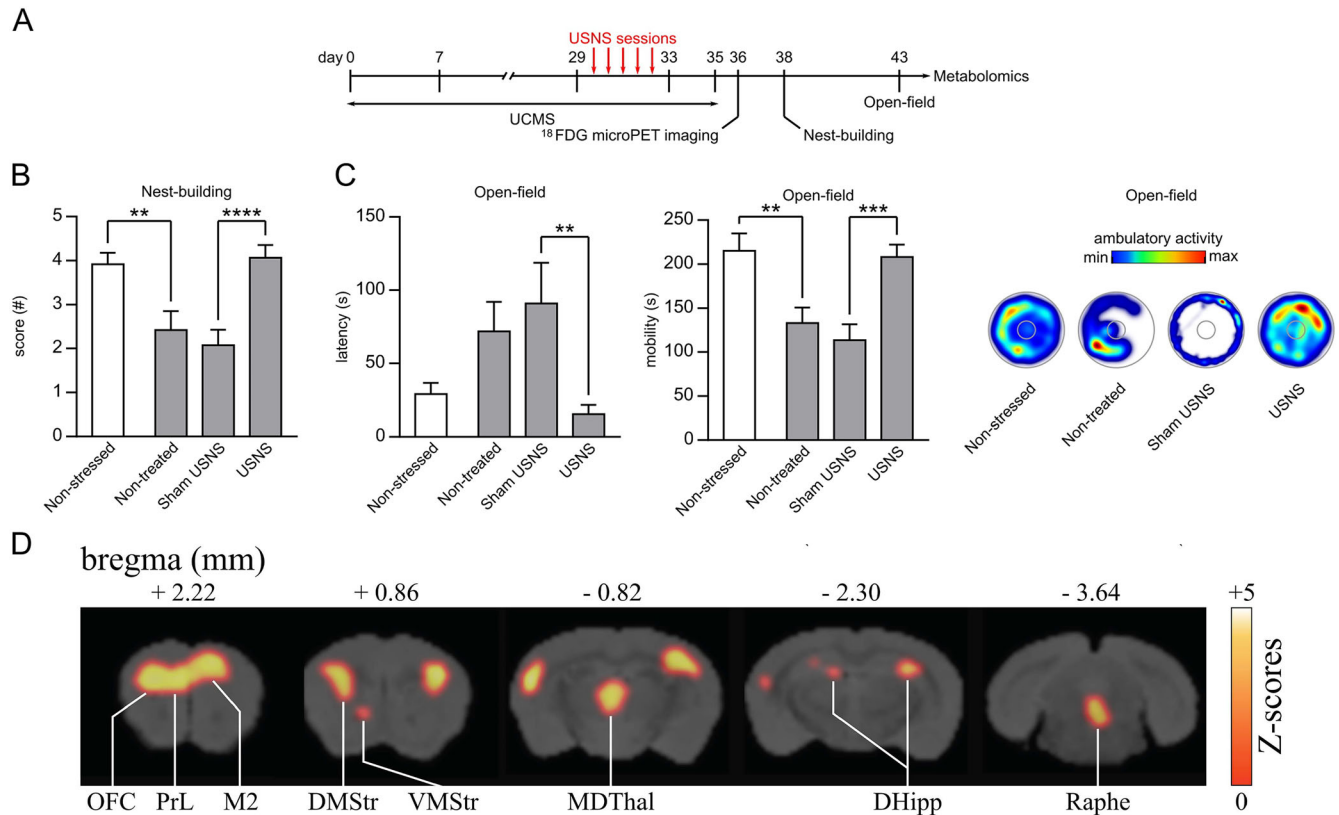


Fig. 2 | Experimental protocol and behavioral/metabolic effects of repeated USNS. **A** Experimental timeline for the main cohort ($n = 53$). **B** Nest-building scores (scaling from 1 to 5). **C** Open-field task (5-min duration); behavioural measures are the latency to reach the centre and the time spent mobile in the apparatus; heatmaps of the cumulative positions in the field are extracted from one representative individual of each group. **D** ^{18}F -FDG microPET imaging; OFC orbitofrontal cortex, PrL prelimbic cortex, M2 secondary motor area, DStr dorsal

striatum, VMStr ventro-medial striatum, MDThal medio-dorsal thalamus, dHPC dorsal hippocampus, Raphe raphe nuclei. USNS ultrasound neurostimulation. UCMS unpredictable chronic mild stress. Data is expressed as mean $\pm$ SEM. Statistics: one-way ANOVA followed by Fisher's LSD post-hoc tests for behavioural group comparisons (**B**, **C**). Two-by-two comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

increase in c-Fos expression in IL, PrL, Cg or auditory cortex, indicating that isoflurane alone does not induce modifications under our conditions.

Taken together, the data show that a short, non-invasive IL-USNS regimen robustly ameliorates UCMS-induced deficits in self-care and anxiety-like exploration, outperforming chronic fluoxetine on these dimensions and bringing performance close to baseline non-stressed levels.

Repeated IL-USNS induces region-specific metabolic up-regulation

^{18}F -FDG microPET acquired three days after the final USNS session revealed a distinct pattern of metabolic facilitation in IL-USNS mice versus sham controls (Fig. 2D). Z-score maps were computed by standardising whole-brain-normalised uptake values across animals within the group.

- Prefrontal cortex. ^{18}F -FDG metabolism measurements revealed increased activities in frontal cortical regions including the prelimbic/M2 area but also in orbitofrontal regions.
- In addition, chronic repeated USNS increased metabolic activity in distant subcortical areas such as the hippocampus, periaqueductal grey matter (PAG) and raphe nuclei (lower Z-scores). Regions underneath the target (anterior olfactory regions and dorsal peduncular cortex) and adjacent regions (somatosensory cortices) did not show modified metabolic activity.

Together, these findings show increased glucose utilisation (normalised ^{18}F -FDG uptake) measured 72 h after five sessions of focal IL-USNS in medial prefrontal regions (including IL/PrL/M2 and orbitofrontal

cortex) and interconnected limbic/brainstem nodes, while sparing neighbouring cortex. Because PET was acquired 72 h after the final session, this signal is interpreted as a sustained network-level consequence of treatment rather than an acute, direct neuromodulatory effect.

IL-USNS elicits long-lasting, glutamate-centred metabolomic reprogramming

Targeted profiling performed ten days after the final stimulation revealed enduring biochemical alterations within cortico-limbic structures involved in the UCMS model³⁸ and in the pathophysiology of MD³⁹ (Table I).

- **Infralimbic / prelimbic cortex.** Six metabolites met the significance and fold-change criteria (Mann-Whitney $p < 0.05$; FC > 1.25 or < 0.75), including a pronounced decline in glutamic acid (FC 0.67). Pathway enrichment identified five significantly impacted routes, headed by alanine-aspartate-glutamate metabolism (FDR < 0.05).
- **Amygdala.** Ten metabolites were differentially expressed; nine pathways were enriched, again converging on glutamate turnover and associated amino-acid cycling.
- **Hippocampus.** Four metabolites changed significantly, influencing five pathways, most prominently alanine-aspartate-glutamate metabolism, similar to the cortical and amygdala pattern.

Across all three regions, the metabolite-level changes and pathway-enrichment outputs repeatedly implicated glutamate- and amino-acid-related metabolism (Table 1). These enrichment results should be interpreted as associative and hypothesis-generating, because pathway analysis does not provide a direct readout of neurotransmitter turnover or

Table 1 | Metabolic pathways and metabolite expressions modified by chronic repeated-pulse USNS

	PrL/IL	AMY	HPC
Metabolic pathways	• Arginine and proline metabolism, • Alanine, aspartate and glutamate metabolism, • Glutathione metabolism, • Glycine, serine and threonine metabolism, • Aminoacyl-tRNA biosynthesis	• Aminoacyl-tRNA biosynthesis, • Arginine and proline metabolism, • Nitrogen metabolism, • β-Alanine metabolism, • Alanine, aspartate and glutamate metabolism, • Histidine metabolism, • Glutathione metabolism, • Glycine, serine and threonine metabolism, • Ubiquinone and other terpenoid-quinone biosynthesis, • Lysine biosynthesis	• Aminoacyl-tRNA biosynthesis, • Alanine, aspartate and glutamate metabolism, • Nitrogen metabolism, • Arginine and proline metabolism, • Glutathione metabolism
Metabolite expression	Trans-aconitate↑ 4-hydroxy-D-proline↑ Homovanillate↓ Glutamic acid↓ Hypoxanthine↓ 1-NH2-cyclopropane-1-carboxylate↑ Trans-aconinate↑	Malate↑ Glutamine↑ Uridine↓ N-acetylneuraminate↓ β-Alanine↓ Alanine↓ Adipic acid↓	Phenylalanine↑ Ornithine↑ Homoserine↓ Urocanate↓

Arrows indicate the direction of the significant fold changes in metabolite expression. PrL/IL, prelimbic/intralimbic cortex, AMY amygdala, HPC hippocampus.

metabolic flux. Because metabolomics was acquired 10 days after the final session, these shifts may reflect downstream adaptation or behavioural normalisation rather than a direct effect of stimulation. Together with the FDG-PET findings obtained 72 h after the last stimulation, these data indicate persistent, region-specific metabolic adaptations in IL/PrL-associated medial prefrontal and connected limbic circuits following repeated IL-USNS, while the underlying causal sequence will require dedicated time-course and functional assays.

Discussion

It is hypothesised that MD involves persistent dysregulation within extended cortico-limbic networks, which current pharmacotherapies and surface neuromodulation techniques may only partially normalise^{5,40}. Here we show that five brief sessions of low-intensity, focally targeted USNS of the IL, the rodent homologue of the human vmPFC, robustly reverse stress-induced behavioural deficits. Specifically, nest-building and anxiety-related avoidance were restored to near-baseline levels, an effect not reproduced by chronic fluoxetine. Chronic fluoxetine did not reproduce the behavioural rescue observed with IL USNS. Strain dependent and inter individual variability in SSRI responsiveness is well documented in chronic stress models, and our findings are consistent with this established heterogeneity. Convergent multimodal evidence revealed that IL-USNS produced localised increases in c-Fos expression, network-wide metabolic facilitation, and coordinated glutamate rebalancing. These findings position optimised USNS as a precise, non-invasive candidate therapy for mood disorders.

A central limitation in prior US neuromodulation studies has been the reliance on arbitrary “magic numbers”, acoustic parameters adopted without physiological justification. We directly addressed this by performing systematic single-pulse mapping, establishing a steep sigmoidal pressure-response curve with a motor threshold near 250 kPa and a stable plateau at 400 kPa. Higher pressures increased response variability and risk of cortical spreading depolarisation, consistent with earlier reports using US and high-frequency rTMS^{41–43}. Using 400 kPa secured a lateral focal diameter of approximately 1–2 mm, defined by the region that maintained pressures within the effective 350–400 kPa range, narrow enough to spare the neighbouring PrL and cingulate cortices while still delivering a stable drive for chronic treatment. Importantly, parameter optimisation was performed under the same 1.5% isoflurane level used during therapy, ensuring translational validity. This strategy replaced guesswork with reproducible rules for effective focal sonication. Future work should systematically compare additional acoustic parameter sets and dosing schedules to determine generalisability and potential further gains.

A single 10-minute train of optimised pulses drove robust c-Fos expression selectively within IL, with no detectable activity in adjacent prefrontal or auditory cortices. This confirms that the functional resolution of USNS matches its geometric footprint and supports limited recruitment of auditory regions under our parameters, but we did not perform ABR or hearing-blockade/deafening controls. Therefore, an indirect auditory contribution cannot be fully excluded.

Pulsed neuromodulation is thought to preferentially engage axons rather than neuronal somata⁴⁴. This has been well described for rTMS, where efficacy depends on fibre orientation and stimulation frequency. A similar axonal sensitivity may contribute to the spatial pattern of c-Fos observed after IL-USNS^{45,46}. Accordingly, the observed c-Fos mosaic likely reflects direct activation of IL projection fibres located within the acoustic focus rather than widespread somatic depolarisation. Establishing this spatial precision was essential before therapeutic testing, as uncontrolled off-target activation would compromise mechanistic interpretation and translational potential.

The brain-wide consequences of the five-day IL-USNS regimen align closely with our imaging and metabolomic read-outs. ¹⁸F-FDG-PET showed sustained hypermetabolism not only in IL but also in dorsal hippocampus, PAG and raphe nuclei, regions functionally connected to IL and pivotal for stress resilience and behavioural control^{40,47}.

In parallel, targeted LC-HRMS identified delayed changes in metabolites and pathway-enrichment outputs involving glutamate-related metabolism across regions. Glutamate-related pathways have been implicated in depression pathophysiology and antidepressant response, which provides a rationale for further targeted functional assays in future work^{48–51}. These findings are consistent with persistent metabolic adaptations after IL-USNS, but they do not by themselves establish a specific neurotransmission mechanism. Future work using time-course sampling and functional assays will be required to determine whether these associations reflect changes in glutamatergic signalling versus downstream normalisation processes.

Relative to existing neuromodulation modalities, IL-USNS combines the depth reach of DBS with the non-invasiveness of repetitive transcranial magnetic stimulation (rTMS). Unlike high-frequency rTMS, which can spread activation via volume conduction, or higher-intensity US, which risks auditory-mediated artefacts^{52,53}, our 400 kPa protocol elicited no c-Fos activity in auditory cortex, underscoring its spatial selectivity. Furthermore, metabolomic analysis revealed no increase in inflammatory or excitotoxic markers typically associated with tissue damage^{54,55}, supporting an excellent safety profile. Advances in phased-array technology and skull aberration correction now enable millimetre-scale targeting in humans, raising the prospect of safe, focal IL sonication in clinical trials.

Several early clinical studies have already demonstrated the feasibility of TUS in depression, with encouraging reductions in symptom severity after stimulation of subcallosal cingulate, dorsolateral prefrontal, or limbic targets. However, these trials remain small and methodologically heterogeneous, with limited mechanistic endpoints. By systematically optimising stimulation parameters, confirming focal engagement of the IL, and combining behavioural, metabolic, and neurochemical readouts, our study provides a mechanistic and methodological framework that directly complements these emerging clinical efforts and helps guide future trial design.

Several limitations merit consideration. First, stimulation and imaging were performed under light isoflurane, which can subtly alter brain activity. However, sham-anaesthetised mice retained full UCMS deficits, indicating that anaesthesia alone did not drive the therapeutic effect. Second, the study employed only male BALB/c mice (young adults at study start); potential sex-dependent effects should be evaluated in females, and strain-dependent generalisability should be assessed in future work. Third, behavioural testing occurred 5–10 days post stimulation, with PET at 72 h and metabolomics at 10 days; this timing was identical across groups and dictated by imaging logistics and recovery/habituation requirements. Because shipping and facility transfer can transiently affect anxiety-like behaviour, future studies should include baseline behavioural testing after acclimation to quantify any transfer-related effects before treatment. Moreover, future work should include earlier time points to clarify the temporal sequence of circuit and biochemical changes and should systematically compare additional acoustic parameter sets and dosing schedules to determine generalisability and potential further gains. Finally, a baseline (pre-treatment) ¹⁸F-FDG scan was not acquired. Therefore, PET effects are based on between-group comparisons rather than within-subject longitudinal changes, and future studies should include pre/post imaging within the same animals.

This study shows that infralimbic ultrasound neurostimulation (IL-USNS) can reverse depression-like symptoms in a robust mouse model after only a short treatment. The improvement in behaviour was linked to changes in brain metabolism and glutamate balance across key circuits, without signs of side effects or tissue damage. By combining the ability of DBS to reach deep brain regions with the safety and precision of US, IL-USNS appears as a promising new neuromodulation approach. The next important steps will be to confirm the results in both sexes and other animal models, to use real-time functional imaging during stimulation, and to connect these preclinical findings with ongoing clinical protocols in TRD.

Methods

Animals and experimental layout

Eighty-three male BALB/cByJrJ mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France), aged 9 weeks (29 ± 1.5 g) at the start of

experiments. Upon arrival, mice were allowed to habituate to the animal facility and the 12:12 light–dark cycle for one week before the start of experimental procedures. Housing conditions were $22 \pm 1^\circ\text{C}$, $50 \pm 10\%$ relative humidity, food and water ad libitum. Cages contained corn cob bedding; no enrichment during UCMS to preserve the protocol's face validity.

All procedures were approved by the ethical committee (Comité d'Éthique en Expérimentation Animale Val de Loire) and by the French Ministry of Higher Education and Research under approval number APAFIS#7290-2016101817023246. They complied with Directive 2010/63/EU on the protection of animals used for scientific purposes. Thirty mice were used for setting-up US stimulation parameters with (i) motor cortex stimulation using single-pulse USNS and (ii) one session of repeated-pulse USNS targeted to the IL, while 41 mice underwent the UCMS to assess the efficacy. Twelve non-stressed mice were added to provide ground values for behaviours (details in Supplementary A).

US neurostimulation apparatus

US stimuli were produced by a spherically focused transducer (500 kHz, 38 mm diameter, 65 mm focus, Imasonic, Besançon, France), driven by a function generator (AFG3022, Tektronix) and 500 W amplifier (ADECE) through a 50 Ω matching network. Pressure fields were mapped in degassed water with a calibrated hydrophone (HGL-200, ONDA). Peak negative pressures were corrected using an ex vivo mouse-skull insertion-loss measurement at 500 kHz ($n = 7$; attenuation coefficient $6.32 \pm 2.18\%$, mean $\pm$ SD; Supplementary A). The reported pressures are derated intracranial estimates. The transducer was coupled to the shaved scalp by a degassed-water collimator ($\varnothing$ 10 mm) sealed with Tegaderm™ film and servo-positioned by a custom 3axis micromanipulator (Supplementary A, Fig. 1A).

Anaesthesia and physiological monitoring

Mice breathed 1.8 L min^{-1} 100% O_2 with isoflurane (2.5% induction; 1–1.5% maintenance unless otherwise stated). Each USNS or sham session involved approximately 10 min of anaesthesia at 1–1.5% isoflurane. For M1 motor-threshold screening only, isoflurane was lowered after induction (to 0.1%) because deeper anaesthesia suppresses US-evoked forelimb motor responses; exposure at 0.1% was brief (<10 min) and continuously monitored, with no signs of stress or aversive reactions observed. All IL-targeted USNS procedures (acute and chronic) were performed under 1–1.5% isoflurane, never under 0.1%. Rectal temperature and respiratory rate were continuously monitored and maintained at $37 \pm 0.5^\circ\text{C}$ and $70 \pm 5 \text{ min}^{-1}$, respectively. No analgesics were required because all procedures were fully non-invasive (no incision or tissue penetration), and no nociceptive responses (withdrawal, vocalisation, struggle) were observed under the maintained anaesthesia plane.

Optimisation of stimulation parameters

Single continuous 160 ms pulses were delivered to the left primary M1 at 50–500 kPa (50 kPa increments, repeated every 10 s (0.1 Hz; duty cycle 1.6%). Twenty mice under very light anaesthesia (0.1% isoflurane, “near-awake”) were scored for visible right-forepaw twitches. Ten additional mice under 1.5% isoflurane were implanted with subdermal EMG electrodes as described. The subdermal EMG electrodes were positioned in the right brachioradialis muscle, and a reference electrode was inserted between the 3rd and 4th carpometacarpal joints, as detailed in the EMG procedure. Signals were recorded at 2 kHz and band pass filtered (300–1000 Hz), and motor evoked potentials were averaged for each animal. A Boltzmann fit yielded a motor-threshold (MT) of 250 kPa and a plateau at 400 kPa, which provided the highest success rate ($89 \pm 2.7\%$), largest mean MEP amplitude ($63.7 \pm 6.5 \mu\text{Vpp}$) and lowest variability (RSD 32.4%) (Supplementary B, Table SII). Applying 400 kPa provided a lateral focal diameter of approximately 1–2 mm, defined by the region that maintained pressures within the effective 350–400 kPa stimulation range (Fig. 1C). At $p \approx 0.40 \text{ MPa}$, $I_{\text{SPPA}} \approx 5.33 \text{ W/cm}^2$ and $I_{\text{SPTA}} \approx 0.085 \text{ W/cm}^2$.

Focality confirmation by c-Fos immunohistochemistry

For a nested subset of eight mice (experimented upon at distance from the single-pulse procedure described above) the focality of one session of repeated-pulse USNS targeted to the IL was assessed with c-Fos activity⁵⁶. One session of USNS was defined as a 160-msec pulse at the optimal pressure repeated 60 times every 10 s (0.1 Hz). The subjects were sacrificed 90 min after either one session of repeated USNS (under anaesthesia) or one 10 min session of anaesthesia.

After a classic immunolabelling of the c-Fos protein (which reflects neuronal activity)⁵⁶ on 40 µm thick coronal sections, cellular densities (cFos⁺ cells/mm²) were quantified across the full cortical depth of the IL region of interest, without distinguishing between layers II/III and V/VI. Cell counting was performed blind to group identity.

Because all sections were collected at identical thickness (40 µm), surface-based density normalisation (cells/mm²) was appropriate, and volumetric correction was not required. Densities were also obtained in close surrounding regions (olfactory areas, OL; prelimbic cortex, PrL; anterior cingulate cortex, Cg; primary motor cortex, M1; secondary motor cortex, M2; see Supplementary A).

Six coronal sections per mouse were analysed, covering stereotaxic levels AP + 1.10 to +2.30 mm according to Paxinos & Franklin, sampled at 160 µm intervals. Cell counts were performed bilaterally, and the mean value per mouse was used for statistical analysis.

UCMS protocol

A cohort of 41 mice followed the UCMS regimen from day 0 to day 35 (Fig. 2A) (described our previous study³⁸). Briefly, mice were isolated in 24 × 11 × 12 cm cages without environmental enrichment and submitted to daily random socio-environmental stressors including exposition to another mouse bedding, removal of sawdust, contention and light-dark cycle perturbations. Daily UCMS stressors included bedding exchange, sawdust removal, restraint, wet bedding, tilted cage, light-dark cycle perturbation, and short social disruption. Stressors were presented in a pseudo random sequence to avoid predictability. At day 7, UCMS mice were significantly different from non-stressed mice in terms of coat state deterioration, a standard measure of UCMS onset and evolution³⁹ (Supplementary A, Figure SI). The full UCMS schedule was identical for all mice, with differences appearing only on days 29–33, when animals received USNS, sham anaesthesia, or no treatment. From this timepoint, UCMS mice were assigned using stratified randomisation based on coat state deterioration, ensuring balanced baseline stress levels across groups.

USNS treatment and control conditions

The chronic repeated USNS treatment ($n = 15$) was given during the fifth week of UCMS (stressors: 08:00–12:00 AM, treatment: 02:00 PM) and evaluated against anaesthesia alone (“Sham USNS”, $n = 14$). The USNS session (60 160-msec pulses at 400 kPa every 10 s) was repeated for 5 consecutive days from day 29 to day 33 to obtain a chronic, 5-session USNS treatment (Fig. 2A). Through stereotaxic framing, the collimator was positioned along the midline at bregma +2 mm, a region that comprises the IL. Twelve mice were subjected to UCMS but did not receive any kind of treatment (“Non-treated”, $n = 12$) and 12 mice were non-stressed and also non-treated (“Non-stressed”, $n = 12$).

Fluoxetine regimen

Fluoxetine was administered chronically at 15 mg/kg, delivered in the drinking water from day 7 to day 43, consistent with standard UCMS protocols. Fluoxetine-treated mice received fluoxetine, and vehicle-treated mice received the corresponding vehicle only (same route and schedule).

Behavioural assays

All behavioural testing was conducted between day 38 and day 43, corresponding to 5–10 days after the final USNS session. This window was determined by the fixed experimental sequence (USNS on days 29–33 → ¹⁸F-FDG PET on day 36 → recovery → habituation) and was

identical across all groups, ensuring that any timing-related variability was systematic and controlled (Supplementary A).

The open field was a circular arena (Ø 33 cm, wall height 40 cm, matte floor) under bright illumination (~200 lux at centre) for 5 min. A circular arena was used to avoid corner-driven behaviour; anxiety-related exploration was quantified using centre-entry latency and centre-zone exploration. Variables extracted were latency to first centre entry (anxiety related), ambulatory time (locomotor activity), and centre crossings (EthoVision XT; thresholds and zone definitions pre-specified). Complete behavioural procedures, including nest-building scoring criteria and the open-field zone definitions/thresholds used in EthoVision XT, are provided in Supplementary A.

¹⁸F-FDG microPET imaging

Local uptake of ¹⁸FDG reflects cerebral metabolic rates of glucose utilisation and allows the investigation of regional brain metabolic statuses^{57,58}. Metabolic imaging using ¹⁸FDG was performed under basal conditions at day 36 of the procedure with the mice fasted overnight before each scan (Supplementary A). Full ¹⁸F-FDG microPET acquisition and analysis details (dose/route, uptake period, scanner/acquisition settings, reconstruction, normalisation, and Z-score processing) are provided in Supplementary Information (Supplementary A, ‘Brain imaging’).

Targeted metabolomic analysis

After brain harvest at day 43, the prelimbic/IL or medial prefrontal cortex, the amygdala and the hippocampus were dissected and assessed for metabolome analysis. This study was carried out by cerebral region analysis campaigns with a targeted metabolomic approach using LC-HRMS as an analytical platform (Q-Exactive, ThermoFisher). The brain metabolomes of Sham USNS mice were compared to those of USNS mice ($n = 8$ /group; Supplementary A).

Tissue samples were collected bilaterally and extracted in methanol:water (1:1) prior to LC-HRMS (Q Exactive, ThermoFisher) analysis in both positive and negative ESI modes using a C18 column and a 15-min water/acetonitrile gradient. Quality control samples were injected every 10 runs. Metabolites were retained only if QC RSD < 30%, and identifications required matching retention time (± 20 s), mass accuracy (± 10 ppm), and isotopic pattern. Data were normalised to total ion current. Significant metabolites required Mann-Whitney $p < 0.05$ with fold change > 1.25 or < 0.75, and pathway significance required FDR-corrected $q < 0.05$.

Statistical analysis

Analyses were performed in XLSTAT® unless otherwise stated, and differences were considered significant when $p < 0.05$.

For the comparison of MEP averages obtained with single-pulse USNS, a repeated-measures one-way ANOVA with a *post hoc* Tukey was performed.

For the c-Fos analysis, multiple sections per animal were averaged, ensuring that the statistical unit was the animal and avoiding pseudo replication. Unpaired two-tailed t-tests were used only for the coordinate-wise comparisons reported in Supplementary Table SIII, and p-values were adjusted for multiple comparisons (see Table SIII).

For behavioural analysis, independent one way ANOVAs were performed for anxiety related outcomes (centre exploration metrics) and for locomotor activity (ambulatory time), each followed by Fisher’s *post hoc* tests.

When an ANOVA was significant, pairwise comparisons were performed using the post-hoc test specified above (Tukey for the repeated-measures MEP analysis; Fisher’s LSD for behavioural one-way ANOVAs).

For metabolomic data, a Mann-Whitney univariate test was employed to select metabolites differently expressed between groups then metabolites with fold change (FC) < 0.75 or > 1.25 were selected. Pathway enrichment analysis was done with Metaboanalyst⁵⁹ and only corrected statistics (significant false discovery rate) were retained for discussion.

Data Availability

The datasets generated and/or analysed during the current study (behavioural recordings, EMG traces, c-Fos cell counts, microPET images, and metabolomics files) are not publicly available due to ethical and legal constraints associated with animal research data and institutional data governance, but are available from the corresponding author upon reasonable request.

Received: 20 November 2025; Accepted: 3 March 2026;

Published online: 18 May 2026

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Acknowledgements

The authors thank the staff of the iBrain technical platforms, particularly the Chemistry and Biomedical Analysis facilities, for their expert assistance with LC-HRMS and radiochemistry, and J. Y. Tartu for the design and machining of the stereotaxic US manipulator. This work was supported by internal resources from Inserm UMR 1253 iBrain and partially supported by the French National Research Agency (ANR; grant ANR-18-CE19-0006, DEPAAC).

Author contributions

M.L., L.G., P.E., C.B., and A.B. designed research; M.L., L.G., A.N., B.P., S.S., C.T., and B.B. performed research; B.P. performed c-Fos immunolabelling and analysed c-Fos data blinded to the conditions; T.I. characterised the transducer's properties; M.L., L.G., S.L., P.E., C.B., and A.B. analysed data; C.B. and A.B. supervised; M.L., L.G., P.E., C.B., and A.B. wrote the paper; M.L., L.G., A.N., B.B., S.L., J.M.E., W.E.-H., P.E., C.B., and A.B. reviewed the paper.

Competing interests

The authors declare no competing financial or non-financial interests. Author AB is Associate Editor of npj Acoustics journal. AB was not involved in the journal's review of, or decisions related to, this manuscript.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s44384-026-00050-z>.

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