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Ultrasonic Vocalization Changes and FOXP2 expression after Experimental Stroke

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Abstract

Speech impairments affect one in four stroke survivors. However, animal models of post-ischemic vocalization deficits are limited. Male mice vocalize at ultrasonic frequencies when exposed to an estrous female mouse. In this study we assessed vocalization patterns and quantity in male mice after cerebral ischemia. FOXP2, a gene associated with verbal dyspraxia in humans, with known roles in neurogenesis and synaptic plasticity, was also examined after injury. Using a transient middle cerebral artery occlusion (MCAO) model, we assessed correlates of vocal impairment at several time-points after stroke. Further, to identify possible lateralization of vocalization deficits induced by left and right hemispheric strokes were compared. Significant differences in vocalization quantity were observed between stroke and sham animals that persisted for a month after injury. Injury to the left hemisphere reduced early vocalizations more profoundly than those to the right hemisphere. Nuclear expression of Foxp2 was elevated early after stroke (at 6 hours), but significantly decreased 24 hours after injury in both the nucleus and the cytoplasm. Neuronal Foxp2 expression increased in stroke mice compared to sham animals 4 weeks after injury. This study demonstrates that quantifiable deficits in ultrasonic vocalizations (USVs) are seen after stroke. USV may be a useful tool to assess chronic behavioral recovery in murine models of stroke.

Keywords

Ultrasonic vocalization; middle cerebral artery occlusion; *FOXP2/Foxp2* gene (in humans and other mammals, respectively)

Introduction

Aphasia is one of the most common deficits seen in stroke survivors. Characterized by difficulty with speech production and language processing, aphasia affects 21–38% of stroke

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patients (1). Speech disorders after stroke have been associated with a disruption in white matter tracts such as the arcuate fasciculus, in addition to other cortical areas (2). For the vast majority of humans the arcuate fasciculus and other areas involved in language production or comprehension localize to the left hemisphere (3, 4). Recent studies have shown that loss of function of the arcuate fasciculus manifests in speech deficits (2). Essential to the development of this pathway is the transcription factor forkhead box P2 (FOXP2), a key mediator of expressive speech development (5–8). FOXP2 is highly conserved and its absence is associated with verbal dyspraxia in humans (5–8). Patients with heterozygous mutations in FOXP2 exhibit critical articulation deficits (8) and dyslexia (9). Foxp2 has also been implicated in the development of vocalization production in animals (9). Absence of FOXP2 in mice manifests as motor impairment, abnormal cerebellar development and an absence of vocalization when exposed to stressors (10). Foxp2 also plays an important role in neurogenesis and neuroplasticity, impacting neurite outgrowth as well as white matter organization in the brain (11).

While current brain imaging using diffusion tensor tractography (DTT) can identify the arcuate fasciculus and damage associated with this neural tract, aphasia treatments continue to range widely based on the severity of clinical symptoms (12, 13). Recently, a positive correlation between clinical improvement in motor function and language ability after stroke was seen, suggesting that motor outcome after stroke plays a large role in predicting language outcome (12). Interestingly, mice with insertion of humanized FOXP2 had enhanced learning and significant physiological changes in corticostriatal circuits that are known to be involved in motor control and speech (14).

One of the biggest challenges for the development of efficacious therapies for aphasia is the lack of an appropriate pre-clinical model of aphasia. Currently there are no well accepted techniques to model speech or expressive deficits in rodents after experimental stroke. While previous studies have shown deficits in rapid auditory processing after stroke that may serve as a useful model for receptive language deficits (15), less is known about expressive deficits in rodent models following ischemic injury. A recent report showed abnormal vocalization production in female mice after injury but mice were assessed at short time points after stroke and females have very low baseline vocalization rates (16). Although rodents do not produce speech per se, male mice vocalize at ultrasonic frequencies (40–120kHz) when presented with a female mouse (17, 18, 19, 20). These courtship songs have been analyzed for spectrogram patterns and quantity in normal mice, but analysis of chronic changes in vocalization patterns and quantity in males after stroke has not been investigated (19, 20, 21, 22, 23). USVs have also been well characterized in neonatal mice, which vocalize robustly when removed from the dam (24). Similar to the language deficits seen in humans, pups with heterozygous mutations of FOXP2 show deficits in vocalization production (25, 26, 27). This study evaluated vocalization production deficits after stroke, examined potential laterality of language after injury, and assessed the response of FOXP2 to ischemic injury.

Methods

Animals

All animal experimental work was approved by the Institutional Animal Care and Use Committee (IACUC) at University of Connecticut Health Center and was performed in accordance with National Institutes of Health guidelines. Male C57BL/6 black mice were obtained from Charles River (Wilmington, MA, USA) and allowed to acclimate for two weeks. All mice were housed 5 per cage (11"L, 6"W, 6"H) with a 12-hour light/dark schedule in a temperature- and humidity-controlled vivarium, with *ad libitum* access to food and water.

Two cohorts of mice were used for USV analysis. One cohort was used for short-term analysis and mice were sacrificed 3 days after stroke. These animals were subjected to right hemispheric stroke (RMCAO), left hemispheric stroke (LMCAO), or sham surgery (initial n=20/gp). Brains were assessed for infarct size after testing. A second cohort (initial n=12/gp) was assessed for 28 days after injury to assess USV recovery. Brains were taken for immunohistochemistry analysis at sacrifice.

Two further cohorts of mice were used for Western blot analysis at 6-hour and 24-hour endpoints for FOXP2 expression (n=10/gp; stroke vs. sham). Samples were pooled to obtain adequate nuclear protein for a final n of 4 per group.

Ultrasonic vocalization recording

Mice were prescreened one week prior to stroke and high-vocalizers were used in subsequent studies one. High-vocalizers were defined as mice with 35 or more USVs within 120 seconds when presented with a mature female. Therefore each animal had an assessment of baseline vocalization prior to random assignment into stroke vs. sham groups. USV was recorded as previously described (16, 17, 18, 20, 28) with minor modifications. Experimental subjects were placed in an empty cage for two minutes to acclimate at the same time each day by a blinded investigator. After two minutes, a female mouse was introduced to the cage. Two successive 120 second trials were recorded with a sampling frequency of 300,000 Hz. As described by others (19, 23, 28), females did not produce vocalizations when exposed to a male, thus all vocalizations that were recorded were produced by the male mouse. Animals were returned to their home cage and the apparatus was cleaned with 70% ethanol to remove olfactory cues before the next subject was tested. Vocalizations were recorded using a condenser ultrasound microphone (Avisoft Bioacoustics, Germany), sensitive to frequencies of 10–200 kHz. The microphone was placed on a mini tripod 12 cm above the floor of the cage and connected to computer via Avisoft-UltraSoundGate turn-key system (Avisoft Bioacoustics, Germany) with an extension cable provided by the manufacturer. The Avisoft-RECORDER USGH software allows the investigator to record and visualize the real-time acoustic data as spectrogram display. This data was then analyzed using Sound Analysis and Synthesis Software (Avisoft-SASLab Pro, Avisoft Bioacoustics, Germany). An experienced investigator blinded to the animal surgical conditions counted the total number of USVs for each independent animal and analyzed the spectrogram of each vocalization based on call duration and frequency

range. For the short-term cohort sacrificed at post-stroke day (psd) 3, USVs were recorded one day prior to stroke (baseline), post-stroke day (psd) 1, and psd3. In the long-term cohort sacrificed one month after stroke, USVs were recorded one day prior to stroke (baseline) and at psd1, psd3, psd7 and psd28. A naïve group was not used as the mice were compared to their own baseline vocalization rate to provide a ratio of “change from pre-stroke baseline.” Data are presented as fold change in calls. Investigators performing testing and analysis were blinded.

Middle Cerebral Artery Occlusion Model

Baseline pre-stroke vocalizations were recorded one week after high-vocalization screening and analyzed prior to surgery. Mice were then randomized using SPSS software such that each group displayed comparable baseline vocalization production ability for each of the surgical conditions. Focal transient cerebral ischemia was induced by 60 minutes of reversible unilateral middle cerebral artery occlusion (MCAO) under Isofluorane anesthesia followed by reperfusion as described previously (29, 30, 31). In brief, a midline ventral neck incision was made, and unilateral right MCAO (RMCAO (end n=16 for short-term cohorts, n=8 for long-term cohorts) or left MCAO (LMCAO) (end n=19 for short-term, n=9 for long-term) was performed by advancing a 6.0 silicone-coated nylon monofilament (Doccol Corporation, CA) to block the blood flow to MCA via an external carotid artery stump. Blood flow measurements by laser Doppler flowmetry (DRT 4/Moor Instruments Ltd, Devon, UK) confirmed MCAO (reduction to 80% of baseline flow). Rectal temperatures were monitored with a Monotherm system (VWR LabShop, Batavia, IL, USA) and temperature was maintained with a heating pad at ~37°C during surgery. Sham mice were subjected to similar surgical conditions except the suture was not inserted into the MCA (end n=18 for short-term cohorts, n=11 for long-term cohorts). All animals were administered 0.2 mL saline subcutaneously psd 0–7 and had free access to wet mashed food. Subjects were excluded if they died over the span of the testing period, if there was evidence of surgical failure, including the presence of subarachnoid hemorrhage or no histological evidence of stroke.

Neurological Deficit Score

Neurological deficit scoring (NDS) was performed prior to USV recording on test days as previously described in Liu et al, 2009 (32). Neurological deficits were scored using the following rubric on a scale from 0–5: 0=no deficit, 1=forelimb weakness and torso turning to ipsilateral side when held by the tail, 2, circling to affected side; 3, unable to bear weight on affected side and circling immediately when placed on a bench, 4=no spontaneous locomotor activity or barrel rolling, and 5=immobile/dead.

Histology

Mice assessed at short-term endpoints were sacrificed at 3 days after stroke with avertin (0.1mL/10g, Sigma) followed by cervical dislocation. The brains were extracted and cut into five 2-mm coronal sections and stained with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 38 °C. These sections were then transferred into 4% formalin over-night for fixation. Infarct volumes were analyzed (Sigma Scan Pro, San Jose, CA, USA) from the digitalized images as previously described (33). The final infarct volumes are presented as a

percentage of the volume of the contralateral structure (with correction for edema) as in (34).

Mice in the long-term cohort were similarly anesthetized at 28 days after stroke and transcardially perfused using ice-cold sodium phosphate-buffered saline followed by 4% paraformaldehyde. Brains were then extracted and post-fixed for 24 hours in paraformaldehyde and then cryoprotected in 30% sucrose solution. Brains were cut into 30 micron sections using a microtome and then stored in antifreeze at -20°C until use. Sections were mounted on Fisherbrand Superfrost Plus charged slides (Fisher scientific, Hampton, NH) and stained with cresyl violet for infarct analysis as in (34). Infarct volumes were quantified from digitalized section images using Sigma Scan Pro software by a blinded investigator.

Laryngeal nerve segments were removed from animals in the Western cohort, 24 hours after stroke onset. These were fixed in 4% paraformaldehyde for 24 hours, transferred to phosphate buffered saline for 30 minutes and then to 70% ethanol. Samples were then embedded in paraffin, sliced transversely into 30 micron sections and stained with Hematoxylin and Eosin (H&E) (n=5 per surgical condition). Cell damage was quantified by density of nerve fibers using Sigma Scan Pro (San Jose, CA, USA) and is represented as a percentage of nerve fibers of the section area.

Immunohistochemistry

Ten 30 micron sections were generated for each surgical group and were slide mounted and incubated in blocking solution as previously described (33). Sections were incubated overnight with rabbit anti-FOXP2 (1:200, abcam), mouse anti-NeuN (1:200, Millipore), and then incubated for 60 min with anti-rabbit and anti-mouse secondary antibody conjugated with a fluorophore (1:1000, FluorAlexa) and DAPI nuclear stain solution (1:1000, Invitrogen) as previously described in O'Keefe et al, 2013 (34). Images were obtained with an inverted light Zeiss-Axiovert fluorescence microscope.

Subcellular fractionation

For western analysis cerebral hemispheres were extracted from mice at 6 and 24 hours after surgery and flash frozen in 2-methyl-butane. Samples were pooled (2 stroke hemispheres, 2 sham hemispheres) to achieve a high enough protein concentration to assess protein in the nuclear fraction. Samples were homogenized with Dounce homogenizers in cold lysis solution (10mM Tris-HCl pH 7.5, 5mM MgCl_2 , 0.1mM EDTA, 1.5mM CaCl_2 , 0.25mM sucrose, 1M DDT, 10% Triton X-100, 1:50 protease inhibitor cocktail). These samples were then centrifuged at 800g for 10 min at 4°C , with the pellet containing the nuclear fraction. The supernatant was centrifuged again at 14000g for 30 minutes for the cytosolic fraction. The nuclear pellet was re-suspended in lysis buffer and was run through a sucrose gradient composed of 1.8M and 2.3M sucrose with ultra-centrifugation at 30,000g for 45 min. The subsequent pellets were collected with Nuclei pure storage buffer (Sigma-Aldrich, St. Louis, MO), and centrifuged at 2300rpm for 10min. The pellets were dissolved in Extraction Buffer (Sigma-Aldrich, St. Louis, MO), sonicated 10 seconds three times, and stored at -80°C as the final nuclear fraction (32).

Western Blot

To achieve equal protein loading of 10µg/well of our nuclear and cytosolic fractions, the protein concentration of each independent sample was determined by BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and subjected to Western blotting as in (32). Nuclear and cytosolic fractions were run on a 4–12% TGX Criterion electrophoresis gels (BioRad, Hercules, CA) and transferred to a polyvinylidenedifluoride membrane (BioRad, Hercules, CA). All blots were blocked with 5% milk and incubated overnight with FOXP2 (1:1000) (abcam), actin (1:5000) (Sigma), and macrophage migration inhibitory factor (MIF) (1:1000) (abcam) in TBS with 4% bovine serum albumin and 0.1% TWEEN. Secondary antibodies (goat anti-rabbit IgG 1:5000 for FOXP2, anti-mouse 1:10,000 for Actin and MIF) (GE Healthcare) were diluted, and ECL detection kit (Amersham Biosciences, Buckinghamshire, UK) was used for signal detection. Densitometry was performed with ImageJ software (NIH) as in (32).

Statistical Analysis

Statistics are presented as means $\pm$ SEM for all experiments except the NDS which is represented as medians (interquartile range). Statistics were performed using GraphPad Prism. One-way ANOVAs with repeated measures was used for all vocalization analysis with a Tukey post hoc analysis for multiple comparisons, unless otherwise noted. A probability value of $P < 0.05$ was considered statistically significant. Behavioral testing, USV recording, data analysis and infarct quantification were performed by an investigator blinded to the surgery condition (stroke vs. sham).

Results

USV

On the day prior to stroke (baseline) all vocalizations exhibited similar spectrogram patterns to those previously described by others (16, 17, 18, 20, 28). Calls were within the range of 30–120 kHz and had a duration of less than 0.05s with no differences between groups over days tested. However, on psd1 and psd3, the calls of stroke mice were too distorted to allow for classification by pattern type. This abnormal vocalization spectrogram pattern was seen in the majority of stroke mice, regardless of the hemisphere affected (figure 1). The ability to produce a clear spectrogram pattern of vocalization was restored in all mice by psd7.

The laryngeal nerve, which runs parallel to the internal carotid artery, plays a role in the motor pathway of vocalization production in many animals, such as in frogs and cats (35, 36). As the MCAO surgery occurs in close proximity to the laryngeal nerve, possible damage to the nerve was investigated. H&E staining showed no statistically significant difference in nerve fiber density over all surgery conditions (quantification not shown), (figure 2), indicating that changes seen in USV production are not due to local nerve damage.

The short-term cohort (figure 3) showed similar baseline vocalization production between animals assigned to LMCAO (n=19), RMCAO (n=16), or Sham (n=18), with no significant differences between groups prior to injury [$F(2,49) = 0.135$, $p = 0.87$]. On psd1, all groups

showed a decrease in USV production, suggesting a potential effect of surgery or anesthesia. However, LMCAO mice, showed a significant decrease in USVs compared to sham mice on psd1 [$F(2,49) = 3.480$, $p = 0.04$] whereas RMCAO mice did not. On psd3, sham mice recovered to baseline levels, while RMCAO and LMCAO mice continued to have significant vocalization deficits relative to shams [$F(2,49) = 7.880$, $p = 0.001$].

Similar results were seen in the long term cohort (figure 4) on psd1 and psd3. All groups showed a decrease in USV production on psd1, with a significant difference in mice subjected to LMCAO compared to sham [$F(2,25) = 3.804$, $p = 0.036$]. Again, by psd3 sham mice returned to baseline whereas LMCAO and RMCAO mice had continued significant deficits in vocalization on psd3 [$F(2,25) = 5.757$, $p = 0.008$]. On psd7, RMCAO and LMCAO continued to vocalize significantly less than sham mice [$F(2,25) = 5.386$, $p = 0.01$]. On psd28, a trend of continued deficits in vocalization in the MCAO mice compared to sham was seen [$F(2,25) = 2.461$, $p = 0.10$], but this was not significant. While no differences between LMCAO and RMCAO were seen on later days, and both were significantly reduced compared to sham, LMCAO mice again showed significantly decreased vocalization production on psd1 when compared to sham while RMCAO mice did not.

When comparing baseline calls to number of vocalizations in the long-term cohort over time, mice subjected to LMCAO showed a significant drop in vocalization production on psd1 [$F(1,36) = 5.048$, $p = 0.031$] and psd3 [$F(1,36) = 5.361$, $p = 0.027$] when compared to sham mice; again this deficit was not seen in RMCAO mice on either psd1 [$F(1,34) = 0.435$, $p = 0.51$] or psd3 [$F(1,34) = 1.722$, $p = 0.199$]. Interestingly, when animals were used as their own baseline control to individualize vocalization production, a significant decrease in call production was seen in stroke mice (LMCAO and RMCAO combined vs. sham) at both day 8 [$F(1,29) = 8.608$, $p = 0.007$] and day 28 [$F(1,29) = 5.424$, $p = 0.028$] compared to shams (figure 5).

NDS

No significant difference in the median NDS between MCAO groups ($p > 0.05$) were seen on any day tested, showing that gross motor deficits do not differ based on the hemisphere damaged (data not shown). Both MCAO groups had a NDS of less than 1 by psd7, demonstrating the rapid recovery of gross neurological deficits in this model (36).

Infarct analysis

No difference in infarct size was seen between LMCAO and RMCAO mice 3 days after stroke ($p = 0.63$). No significant difference in cerebral atrophy between LMCAO and RMCAO mice was seen at psd28 ($p = 0.81$) (data not shown).

Western blot analysis

An increase in nuclear FOXP2 expression was seen 6 hours after stroke compared to sham mice ($p = 0.04$). However, 24 hours after stroke a significant decrease in nuclear FOXP2 was seen in stroke cohorts when compared to sham ($p = 0.02$). There was a decrease in FOXP2 expression when compared to sham in the cytosolic fraction but this was not significant

($p=0.07$). Similar to nuclear changes, cytosolic FOXP2 was significantly decreased by 24 hours when compared to sham ($p=0.01$) (figure 6), which could represent overall cell death.

Immunohistochemistry

To assess chronic changes in FOXP2 when acute cell death is complete, brains were assessed one month after injury. FOXP2 expression was seen in the nucleus (stained with DAPI) and co-localized to neurons (NeuN). In both RMCAO and LMCAO higher levels of nuclear FOXP2 were seen in the ipsilateral hemisphere, especially within the infarct region (figure 7).

Discussion

In this study we systematically investigated how stroke impacts vocalizations in male mice. First, our findings suggest that surgery can lead to early impairments in vocalization. Further, to identify possible lateralization effects in vocalization we compared strokes in the left versus the right hemisphere. Mice subjected to LMCAO showed significantly reduced vocalizations at early time points compared to sham mice, an effect not seen in RMCAO mice. Significant differences in vocalization quantity were observed between stroke and sham animals that persisted after gross neurological deficits had resolved. Vocalization deficits continued to measurable 28 days after stroke. Our study design included multiple recordings of the same animal, as the frequency of baseline vocalizations was used to control for between animal variability. Repeated testing may have led to accommodation or learning and thus may have altered subsequent USV measurement specifically in brain injured animals. We cannot fully exclude the possibility that the impaired vocalization production seen after stroke is due to desensitization to the USV task itself. Nevertheless, the lasting effect of vocalization impairments provides strong evidence that stroke-induced vocalization deficits could be used to assess long-term recovery.

As recent reports suggest that FOXP2 is an important upstream regulator of vocalization, is involved in neuroplasticity, and influences cortical-striatal circuitry, we further explored short and long-term changes in cerebral FOXP2 levels. Early increases in nuclear FOXP2 levels were seen 6 hours after injury. As FOXP2 is an important transcription factor, translocation to the nucleus would be the first step in initiating gene transcription which is consistent with the corresponding but non-significant decrease in cytosolic FOXP2 levels. Both nuclear and cytosolic FOXP2 levels were reduced by 24 hours, likely reflecting overall cell death which is maximal at this time point in this model (37). However, nuclear FOXP2 levels were also increased one month after injury, primarily in neurons around the area of infarct. As FOXP2 has been implicated in neurogenesis, further studies are needed to determine the potential role of FOXP2 in recovery of stroke-induced vocalization and motor deficits.

Vocalization production across many species requires an interaction between the cortex and the basal ganglia in addition to other fine-motor pathways (6, 38, 39). FOXP2 has been shown to influence not only vocalization production under stress but also cerebellar organization of white matter fine-motor tracts (39, 40). As this study shows that the laryngeal nerve is not damaged during experimental stroke, cortical or upper motor neurons

are likely affected leading to deficits in vocalization production and possibly providing a model that could be used to explore expressive speech deficits. While only expressive deficits were investigated in this study, auditory processing deficits have also been shown to occur in mice after stroke (15); expressive and receptive language abilities should be studied concurrently to determine whether these deficits are localized to specific areas of the murine brain as they are in humans.

In Melodic Intonation Therapy (MIT), a speech and language therapy used in the clinical setting, the patient sings sentences instead of saying them (41). This relatively successful therapy has been shown to engage both sides of the brain, (41). Recent studies have shown that speech recovery after left hemisphere infarcts involves recruitment of the right hemisphere in auditory tasks (42). While all groups in this study show a decrease in vocalization production on psd1, LMCAO mice vocalize significantly less than shams while RMCAO mice do not exhibit this difference. This suggests that while murine vocalization production is decreased when either hemisphere is damaged, vocalization production may be more sensitive to lesions in the left hemisphere, especially at early time points. While most aphasia patients show some improvement in speech production over time, many patients never regain full speech abilities (13). This study shows that even one month after stroke, mice had quantifiable decreases in vocalization production compared to shams. Mice show dramatic recovery in many deficits within days after injury especially with shorter ischemia times such as the 60 minutes used in this study (37). This can limit the utility of recovery studies especially when screening neuroprotective agents, as maximal deficits are seen only in the acute phase. The assessment of USV may represent a useful tool to study chronic behavioral recovery of a functionally relevant task. This could potentially be used to test new therapeutic agents that specifically target stroke-induced speech disorders. Speech recovery in humans is a slow process that involves activation of distant, contralateral areas of the brain (42). Whether similar bilateral activation occurs in the recovery of rodent vocalization production remains undetermined.

FOXP2 has been implicated as a key transcription factor in neurogenesis and synaptic growth (6, 11, 26, 39), while its role in recovery remains unexplored. While FOXP2 is expressed by many cell types in the brain (6), we found that FOXP2 co-localized with neurons four weeks after injury, possibly indicating increased long-term neuronal growth. Expression of FOXP2 is known to be essential for auditory-motor learning as well as cortical-striatal integration (6, 11, 40). This study shows that one month after injury, when mice show improved vocalization abilities, FOXP2 expression is markedly increased in the damaged hemisphere when compared to sham. While vocalization has been linked to white motor tract and cortical integration (11), this study implies that FOXP2 may also play a role in establishing and maintaining neuronal pathway integrity. Very recent work has shown that introduction of the human FOXP2 gene (by inducing the same amino acid changes that occurred during human evolution into the mouse *Foxp2* gene) improved learning and changed striatal neuroplasticity (14). These changes in corticostriatal systems may contribute to the adaptations seen in humans that allow for speech and language acquisition. Our findings suggest that FOXP2 may also play a role in language recovery.

Conclusion

Significant differences in vocalization production are present after stroke and persist for 4 weeks after injury. USVs may be a sensitive and quantifiable test for stroke-induced deficits. Mice with left hemispheric damage have a more profound reduction in early vocalization than mice with right hemispheric lesions. FOXP2 expression is up-regulated in the nucleus early after stroke and subsequently decreases in both the nucleus and the cytosol 24 hours after injury. Chronically, FOXP2 expression is selectively increased in neurons in the damaged hemisphere, and its expression corresponds with vocalization recovery. FOXP2 could be a potential target for speech recovery.

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Abbreviations

MCAO	Middle cerebral artery occlusion
RMCAO	right middle cerebral artery occlusion
LMCAO	left middle cerebral artery occlusion
ST	Stroke surgery
SH	Sham surgery
USV	Ultrasonic vocalization
FOXP2	forkhead box protein 2
psd	post stroke day

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Highlights

- Stroke mice vocalize significantly less than sham mice four weeks after experimental stroke.
- Mice subjected to left hemisphere strokes show significantly less vocalization production compared to sham mice.
- Nuclear FoxP2 levels were significantly increased 6 hours after stroke, but decreased by 24 hours in stroke compared to sham mice.
- FoxP2 expression increased in neurons of stroke mice 28 days after injury as deficits in vocalization improved.

Figure 1.

A depicts the vocalization pattern of a MCAO mouse prior to surgery (pre-stroke). **1B** shows that the ability to form an organized patterned vocalization is completely lost at psd1. The ability to structure and pattern a vocalization is partially restored at psd7 (**1C**).

Figure 2.

A shows the laryngeal nerve (white arrow) and the residual external carotid stump (black arrow) after RMCAO surgery. **2B** shows the same after LMCAO surgery. **2C** shows the nerve and the common carotid after sham surgery. **2D** shows a H&E stained longitudinal section of the laryngeal nerve. The amount of fiber damage was similar across all surgical conditions.

Figure 3.

A shows the average number of vocalizations in a 2 minute trial in our short-term experiment over three groups: sham (n=18), LMCAO (n=19), and RMCAO (n=16). While there are no differences at baseline (pre-stroke) as seen with ANOVA (3B), at psd1 we see that LMCAO mice vocalize significantly less than sham mice [$F(2,49) = 3.480$, $p = 0.04$] (3C). At psd3 both MCAO groups vocalize significantly less than shams [$F(2,49) = 7.880$, $p = 0.001$] (3D).

Figure 4.

A shows the average number of vocalizations made in two minutes chronically over a series of time-points. Similar to the acute cohorts ANOVA revealed no differences at baseline between groups (4B); yet at psd1 LMCAO vocalize significantly less than sham mice [$F(2,25) = 3.804$, $p = 0.036$] (4C). At psd3 LMCAO and RMCAO vocalize significantly less than sham mice [$F(2,25) = 5.757$, $p = 0.008$] (4D). At psd7 we see that both LMCAO and RMCAO continue to vocalize at significantly lower rate than sham mice [$F(2,25) = 5.386$, $p = 0.01$] (4E). At psd28 this significant difference between stroke and sham is now absent [$F(2,25) = 2.461$, $p = 0.10$] (4F).

Figure 5.

shows the fold change in vocalizations in sham (n=11), LMCAO (n=8), and RMCAO (n=9) tested over 4 weeks. Fold change is defined as the number of baseline calls (pre-stroke)/ calls recorded on each post stroke test day. An overall effect of stroke on fold change in calls was significant across days psd3 [$F(1,51) = 4.133$, $p = 0.047$], psd8 [$F(1,29) = 8.608$, $p = 0.007$] and psd28 [$F(1,29) = 5.424$, $p = 0.028$] compared to shams. Mice with a LMCAO show significantly less vocalizations on psd1 [$F(1,36) = 5.048$, $p = 0.031$] and on psd3 [$F(1,36) = 5.361$, $p = 0.027$] when compared to sham mice. This deficit was not seen in RMCAO psd1 [$F(1,34) = 0.435$, $p = 0.51$] and psd3 [$F(1,34) = 1.722$, $p = 0.199$].

Figure 6.

A shows nuclear and cytosolic FOXP2 levels. Histone and Macrophage migration inhibitory factor (MIF) were used as the nuclear and cytoplasmic loading controls, respectively.

Nuclear FOXP2 levels were significantly higher in stroke animals compared to shams 6hrs after stroke. Nuclear FOXP2 levels drop at 24 hours in stroke animals compared to sham. **6B** shows cytosolic FOXP2 levels at 6hrs and 24hrs after stroke with MIF as the loading control. Quantification of the nuclear fraction is shown in **6C** showing that there is a statistical differences in FOXP2/histone ratio in stroke compared to sham at both 6hrs and 24hrs ($p<0.04$ and $p<0.02$ respectively) ($n=4$ per group). Quantification of the cytosolic fraction is shown in **6D** shows a non-significant decrease in FoxP2/MIF ratios in stroke brain compared to sham at 6hrs and a significant decrease in FOXP2/MIF ratio at 24hrs after stroke when compared with sham ($p=0.07$ and $p<0.01$ respectively)

Figure 7.

A shows an area of cortex in the ischemic hemisphere stained with FOXP2 (red) which co-localizes with nuclear DAPI (blue). **7B** shows an identical stain in the same area of sham cortex, showing low levels of cortical FOXP2. **7C** and **7D** show similar levels of FOXP2 in the striatum of mice subjected to RMCAO and LMCAO respectively. **7E** shows that FOXP2 colocalizes with both NeuN (green/neuronal) and DAPI (blue/nuclear). **7F** is a high magnification showing nuclear co-localization of FOXP2 and NeuN.