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Stroke

BASIC AND TRANSLATIONAL SCIENCES

Long Noncoding RNA Fos Downstream Transcript Is Developmentally Dispensable but Vital for Shaping the Poststroke Functional Outcome

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BACKGROUND AND PURPOSE: Stroke induces the expression of several long noncoding RNAs in the brain. However, their functional significance in poststroke outcome is poorly understood. We recently observed that a brain-specific long noncoding RNA called Fos downstream transcript (FosDT) is induced rapidly in the rodent brain following focal ischemia. Using FosDT knockout rats, we presently evaluated the role of FosDT in poststroke brain damage.

METHODS: FosDT knockout rats were generated using CRISPR-Cas9 genome editing on a Sprague-Dawley background. Male and female FosDT$^{-/-}$ and FosDT$^{+/+}$ cohorts were subjected to transient middle cerebral artery occlusion. Postischemic sensorimotor deficits were evaluated between days 1 and 7 and lesion volume on day 7 of reperfusion. The developmental expression profile of FosDT was determined with real-time polymerase chain reaction and mechanistic implications of FosDT in the ischemic brain were conducted with RNA-sequencing analysis and immunostaining of pathological markers.

RESULTS: FosDT expression is developmentally regulated, with the adult cerebral cortex showing significantly higher FosDT expression than neonates. FosDT$^{-/-}$ rats did not show any anomalies in growth and development, fertility, brain cytoarchitecture, and cerebral vasculature. However, when subjected to transient focal ischemia, FosDT$^{-/-}$ rats of both sexes showed enhanced sensorimotor recovery and reduced brain damage. RNA-sequencing analysis showed that improved poststroke functional outcome in FosDT$^{-/-}$ rats is partially associated with curtailed induction of inflammatory genes, reduced apoptosis, mitochondrial dysfunction, and oxidative stress.

CONCLUSIONS: Our study shows that FosDT is developmentally dispensable, mechanistically important, and a functionally promising target to reduce ischemic brain damage and facilitate neurological recovery.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: apoptosis ■ brain ■ development ■ ischemic stroke ■ lncRNA ■ motor function ■ reperfusion

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Stroke alters the profiles of various classes of noncoding RNAs (ncRNAs), including microRNAs, long ncRNAs (lncRNAs), circular RNAs, and transcribed ultraconserved regions.[1–5] The lncRNAs (200 nucleotides to >100 kb) are the largest class of ncRNAs involved in transcriptional and translational regulation in a developmental-stage and a disease-specific manner.[6–8] Many lncRNAs are organ and cell type specific. It was reported that <10% of the lncRNAs in humans ubiquitously express in different cell types, whereas ≈30% are seen in only one cell type.[9] Furthermore, ≈40% of the lncRNAs are expressed specifically in the brain.[6] Particularly, lncRNAs transcribed within the vicinity of protein-coding genes are preferentially expressed in the brain and share overlapping transcription factor binding sites with protein-coding genes.[2,10]

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Nonstandard Abbreviations and Acronyms

FosDT	Fos downstream transcript
lncRNAs	long noncoding RNAs
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
P	postnatal day
pDrp1	phosphorylated dynamin-related protein-1
REST	RE1-silencing transcription factor

We previously showed that induction of a highly conserved brain-enriched lncRNA called Fos downstream transcript (FosDT, MRAK159688) promotes ischemic brain damage by interacting with REST (RE1-silencing transcription factor)-associated chromatin-modifying proteins.[2,11] *FosDT* gene is cogenic to the *Fos* gene, which is a marker of cellular stress.[12] *FosDT* and *Fos* genes are located within a gene desert of ≈240 000 nucleotides on chromosome 6 in rats (chromosome 14 in humans).[11] We currently evaluated the functional significance of FosDT in brain development and post-ischemic outcome by using FosDT$^{-/-}$ rats developed by CRISPR-Cas9 genome editing.

We also evaluated the reproducibility of our data by analyzing the role of FosDT in ischemic brain damage by another lab. We further conducted RNA-sequencing analysis of FosDT$^{-/-}$ and FosDT$^{+/+}$ rats following transient focal ischemia to understand the mechanistic implications of FosDT in the ischemic brain.

METHODS

The data supporting results are available within the article and its Data Supplement. Experimental protocols using animals were approved by the University of Wisconsin Research Animal Resources and Care Committee and Center for Laboratory Animal Medicine and Care at the University of Texas Health Science Center at Houston in accordance with the Institutional Animal Care and Use Committee guidelines. Animals were cared in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services publication no. 86–23 [revised]). Experiments were conducted in compliance with the Animal Research: Reporting of In Vivo Experiments guidelines.[13] Animals were randomly assigned to study groups and outcome parameters were evaluated blindly.

FosDT Knockout Rats

FosDT$^{-/-}$ rats were generated using CRISPR-cas9 genome editing on the Sprague-Dawley background by deleting 539 nucleotides that do not overlap with the *Fos* gene. The target sequence having no perfect matches elsewhere in the genome within the protospacer adjacent motif (PAM)-proximal 12 bp seed region and >3 mismatches in the distal 8 bp region on each flanking end was selected. A single-stranded DNA donor was designed to integrate 75 bp of homology across both sides of the region targeted for excision. Two purified guide RNAs (50 ng/μL), a single-stranded DNA donor (50 ng/μL), and a Cas9 protein (40 ng/μL) were microinjected into the pronucleus of fertilized one-cell Sprague-Dawley rat embryos and surgically implanted into the oviducts of 2 pseudopregnant Sprague-Dawley females. Offspring were genotyped and further bred to obtain homozygous FosDT knockouts. Tail DNA was genotyped using the primer set (5′-to-3′) ACTCCAGTCCTCACCTCTTC (sense) and AATACCCTGAACTAGCGTTTC (antisense).

Cerebral Vasculature and Histological Evaluation

FosDT$^{+/+}$ and FosDT$^{-/-}$ cohorts were observed for any signs of disability at regular intervals and euthanized at 3 months of age (300±20 g; n=3/group) by transcardiac paraformaldehyde perfusion. Brain, lung, heart, liver, kidney, spleen, and muscle were embedded in paraffin, sectioned (10 μm), and stained with hematoxylin and eosin, luxol fast blue, and silver stain. Sections were analyzed for histological changes by a trained pathologist.[3] To study the cerebral vasculature, cohorts of FosDT$^{-/-}$ and FosDT$^{+/+}$ rats (3 months; 300±20 g; n=3/group) were transcardially perfused, injected with 25% India ink in PBS containing 6% gelatin, postfixed, and cerebral vasculature was observed.[14]

Focal Ischemia

Rats were subjected to 90 minutes of transient middle cerebral artery occlusion (MCAO) using a silicone-coated nylon monofilament (4-0 Doccol) under isoflurane anesthesia as described earlier.[3,11,15] Rats were euthanized at various reperfusion time points between 12 hours and 7 days as needed. Sham-operated rats underwent the same surgical procedure, except for MCAO. Physiological parameters (pH, PaO_2, and $PaCO_2$) were monitored, and rectal temperature was maintained at 37.0±0.5 °C during surgery. The impact of the estrous cycle on functional outcomes in females was minimized by randomly assigning to groups. Rats that showed no signs of neurological deficits during the acute phase after MCAO and or a hemorrhage after euthanasia were excluded.

RNA-Sequencing and Real-Time Polymerase Chain Reaction

Total RNA was extracted using the mirVana miRNA Isolation Kit (ThermoFisher Scientific). Following ribosomal RNA depletion, samples were fragmented, DNA library was prepared and sequenced on Illumina's NovaSeq 6000 system. The differentially expressed mRNAs were selected with log2 (fold change) ≥1 or log2 (fold change) ≤-1, and with P<0.05. FosDT and Fos expression was estimated with real-time polymerase chain reaction with SYBR Green and TaqMan methods. Relative gene expression was normalized to 18s rRNA by the comparative Ct method ($2^{-\Delta\Delta Ct}$). See Data Supplement for additional details.

Sensorimotor Function Testing

Sensory and motor functions were evaluated on days 1, 3, 5, and 7 of reperfusion by rotarod test (cylinder rotating at 8

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rpm for 4 minutes), beam walk test (foot faults while crossing a tapered 120 cm long beam), and adhesive sticker removal test as described earlier.[3,11,15] Animals were pretrained in these tasks for 3 days. The spontaneous locomotor activity was evaluated by an open field test.[16] Rats were prescreened for baseline spontaneous motor activity and poststroke motor behavior was evaluated on days 3 and 7 of reperfusion. Each rat was placed in a separate arena (16"×16"); locomotion was tracked for 20 minutes and automatically analyzed by video tracking and analysis software (Noldus Information Technology, Inc).

Lesion Volume Analysis

Coronal brain sections (40 μm) from each rat were stained with cresyl violet and scanned with National Institutes of Health ImageJ software. Ischemic lesion volume was calculated by numeric integration of data from 6 serial coronal sections with respect to sectional interval and corrected for edema and differential shrinkage.[3,11,15,17]

Immunostaining

Brain sections from FosDT$^{-/-}$ and FosDT$^{+/+}$ rats subjected to transient MCAO and 1 day of reperfusion were stained with antibodies against cleaved caspase-3, pDrp1 (phosphorylated dynamin-related protein-1), 3-nitrotyrosine, and NeuN (neuronal nuclei) followed by suitable secondary antibodies and homologous areas were used to analyze immunostaining as described earlier.[3] See Data Supplement for details.

RESULTS

FosDT Is Highly Localized in the Brain and Regulated Developmentally

In postnatal day (P) 7 rats, FosDT expression was observed in all brain regions tested (cerebral cortex, striatum, hippocampus, cerebellum, and thalamus) with the highest level in the striatum and the lowest level in the cerebral cortex (Figure 1A). In the adult rats, the cerebral cortex and cerebellum showed a significant increase in FosDT levels over P7 by ≈20 fold and ≈8 fold, respectively (Figure 1B and 1C). Fos expression mirrored FosDT expression in all brain areas in P7 and adult rats (Figure 1A–1C). Although the intrarelative abundance of FosDT and Fos were higher in muscle, spleen, and lung in comparison to the liver, only the heart showed a significant age-dependent increase in FosDT and Fos abundance (Figure I in the Data Supplement).

FosDT Deletion Had No Effect on Development, Cytoarchitecture, and Cerebral Vasculature

Tail DNA genotyping showed a 821 nucleotides band for FosDT$^{+/+}$ and a 282 nucleotides band for FosDT$^{-/-}$ cohorts confirming the knockout (Figure 2A2). Polymerase chain reaction analysis showed a 142 nucleotides band of FosDT RNA in the brains of the FosDT$^{+/+}$ cohort, which was absent in the FosDT$^{-/-}$ cohort (Figure 2A3). However, both the cohorts showed similar expression of Fos mRNA (Figure 2A3). FosDT$^{+/+}$ and FosDT$^{-/-}$ rats showed no visible phenotypic defects at the P2 or adult (2 months) stage (Figure 2B). The growth pattern and fertility of FosDT$^{-/-}$ rats are similar to FosDT$^{+/+}$ rats (Figure 2B). Adult FosDT$^{-/-}$ rats showed no neuronal degeneration in the gray matter (in the cerebral cortex and hippocampus; Figure 2C) or white matter (corpus callosum), compared with the FosDT$^{+/+}$ rats (Figure IIA and Figure IIB in the Data Supplement). No noticeable difference was observed between FosDT$^{+/+}$ and FosDT$^{-/-}$ rats in major cerebral blood vessel structures, including MCA, anterior cerebral artery, and posterior cerebral artery (Figure 2D). None of the peripheral organs evaluated (lung, liver, heart, spleen, muscle, and kidney) showed any cytoarchitectural differences between the adult FosDT$^{+/+}$ and FosDT$^{-/-}$ rats (Figure IIC in the Data Supplement). When subjected to transient MCAO, the FosDT$^{+/+}$ cohort showed significant induction of both FosDT and Fos expression in the cerebral cortex (Figure III in the Data Supplement). In contrast, FosDT$^{-/-}$ cohort subjected to transient MCAO showed no induction of FosDT expression and a significantly curtailed induction of Fos (≈60% lower) in the cerebral cortex compared with FosDT$^{+/+}$ (Figure III in the Data Supplement).

FosDT Deletion Ameliorated Poststroke Functional Outcome in Both Sexes

Following transient MCAO, poststroke motor function recovery was significantly faster and higher in adult male and female FosDT$^{-/-}$ rats compared with the sex-matched FosDT$^{+/+}$ rats assessed by rotarod test, beam walk test, and adhesive removal test between reperfusion days 1 and 7 (Figure 3A and 3B). Postischemic lesion volume was also significantly smaller at 7 days of reperfusion in both male and female FosDT$^{-/-}$ rats compared with the sex-matched FosDT$^{+/+}$ rats (by ≈42%; $P<0.05$; n=8–10/group; Figure 3C and 3D).

Neuroprotection in FosDT Knockouts Is Reproducible

We validated the improved functional recovery and smaller infarcts in FosDT$^{-/-}$ rats independently in a second laboratory at the University of Texas-Houston. All experiments were performed blinded to genotype. Focal ischemia led to a similar magnitude of weight loss between FosDT$^{-/-}$ and FosDT$^{+/+}$ rats at 3 days of reperfusion in both sexes (Figure 4A1 and 4B1). However, at 7 days of reperfusion, FosDT$^{-/-}$ rats of both sexes showed significantly better recovery of body weight compared with FosDT$^{+/+}$ rats (Figure 4A1 and Figure 4B1). In the FosDT$^{-/-}$ rats of both sexes, somatosensory dysfunction evaluated by the adhesive removal test was significantly lower (Figure 4A2 and 4B2) and the spontaneous locomotor activity

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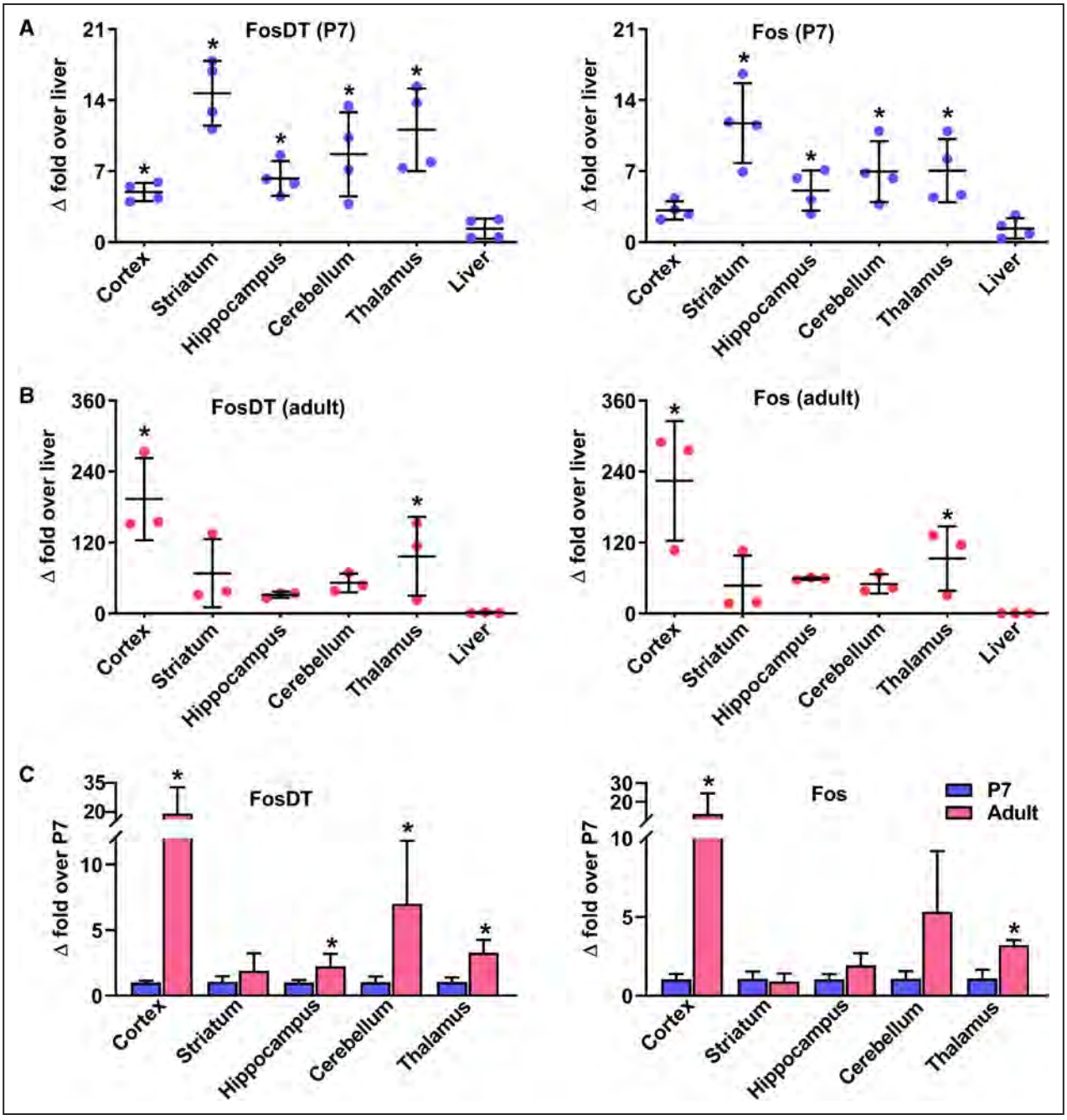

Figure 1. Developmental expression pattern of Fos downstream transcript (FosDT) and Fos in the rat brain.
At postnatal day (P) 7, all brain regions (cerebral cortex, striatum, hippocampus, cerebellum, and thalamus) showed significant FosDT expression (**A**). At the adult stage, the cerebral cortex and thalamus showed a significant increase in the FosDT expression compared to P7 (**B**). Fos expression mirrored FosDT expression at both P7 and adult stages (**A** and **B**). As the liver showed the lowest expression of FosDT and Fos, we presented the values as a fold-over liver. FosDT expression is significantly higher in the adult cerebral cortex compared to P7 (**C**). Fos expression mirrored the developmental pattern seen for FosDT in the brain (**A**–**C**). Values are mean±SD (n=3–4/group). *P<0.05 compared with liver or P7 by Mann-Whitney U test.

assessed by the open field test was significantly higher (Figure 4A3 and 4B3) compared with the FosDT$^{+/+}$ rats at days 3 and 7 of reperfusion following transient MCAO. Both male and female FosDT$^{-/-}$ rats showed significantly smaller infarcts at 7 days of reperfusion compared with FosDT$^{+/+}$ rat (by ≈28%; P<0.05; n=6 to 8/group; Figure 4C and 4D).

FosDT Deletion Altered Postischemic Cerebral Transcriptome

RNA-sequencing analysis showed that 288 protein-coding transcripts were differentially expressed in the cerebral cortex of the adult FosDT$^{-/-}$ rats compared with the FosDT$^{+/+}$ rats (122 upregulated >3-fold and 166

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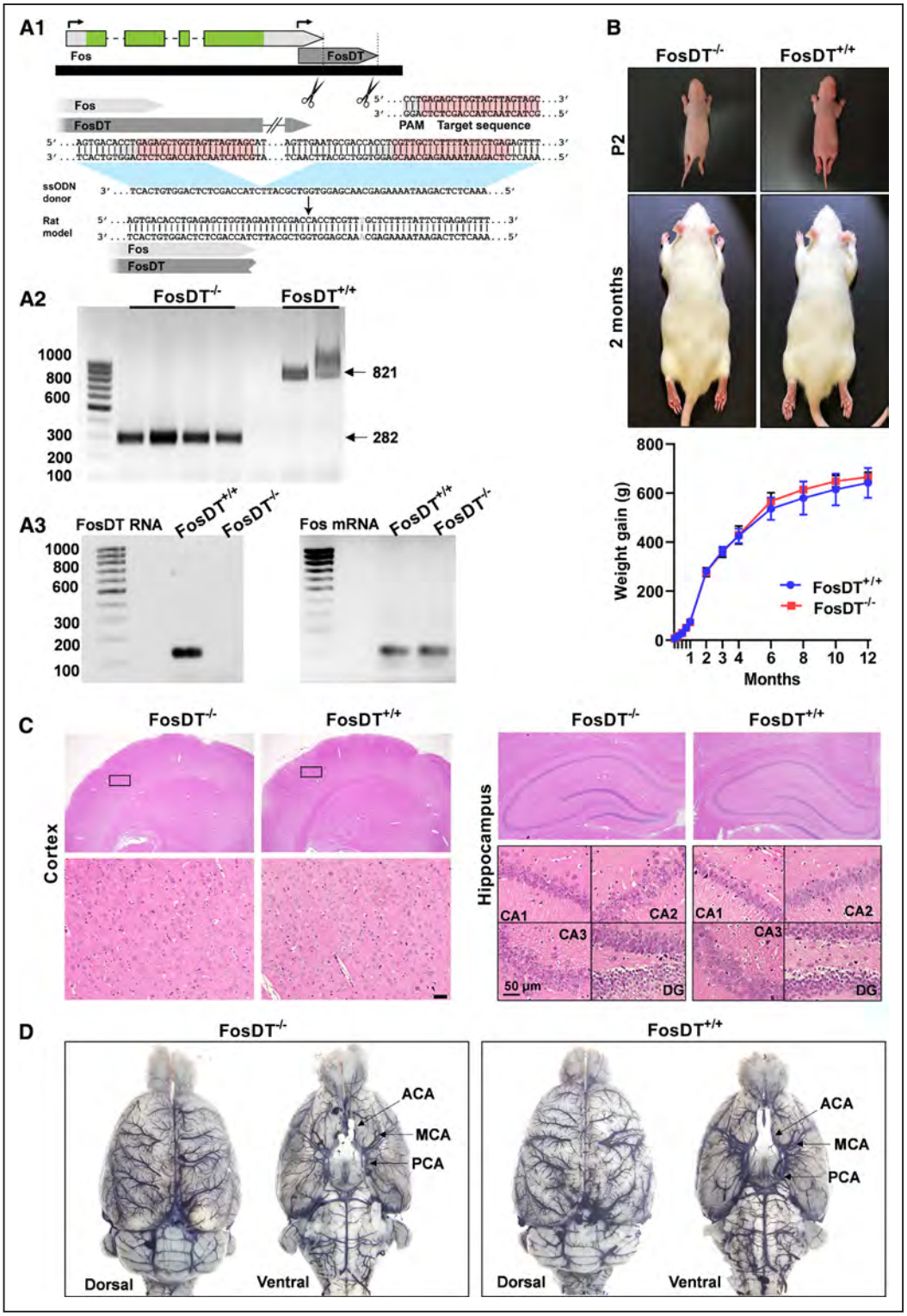

Figure 2. Fos downstream transcript (FosDT) deletion had no effect on development, brain cytoarchitecture, or vasculature. FosDT$^{-/-}$ rats were generated using CRISPR-Cas9 genome editing (**A**1). Scissors represent Cas9 cutting sites. The transcription start site for FosDT is located within the Fos 3′ untranslated region (**A**1). A portion of FosDT that does not overlap with Fos was selected for precise excision. Cas9 target sites at the regions flanking the excision region were selected (**A**1). Tail DNA genotyping showed 821 bp and 282 bp polymerase chain reaction products in FosDT$^{+/+}$ and FosDT$^{-/-}$ rats, respectively (**A**2). FosDT deletion abolished FosDT without affecting Fos (**A**3). FosDT$^{+/+}$ and FosDT$^{-/-}$ rats showed similar phenotype and growth up to 1 y (**B**). Hematoxylin and eosin–stained brain sections showed no structural and cellular differences between adult male FosDT$^{-/-}$ and FosDT$^{+/+}$ cohorts in the cerebral cortex and hippocampal cornu ammonis (CA1, CA2 and CA3), and dentate gyrus (DG) layers (**C**). Cerebral vasculature demarcated by India ink shows no observable differences between FosDT$^{-/-}$ and FosDT$^{+/+}$ rats in the major arterial organization, including MCA, anterior cerebral artery (ACA), and posterior cerebral artery (PCA; **D**). n=3/group. Scale bar=50 µm. P2 indicates postnatal day 2; PAM, protospacer adjacent motif; and ssODN, single-stranded DNA donor.

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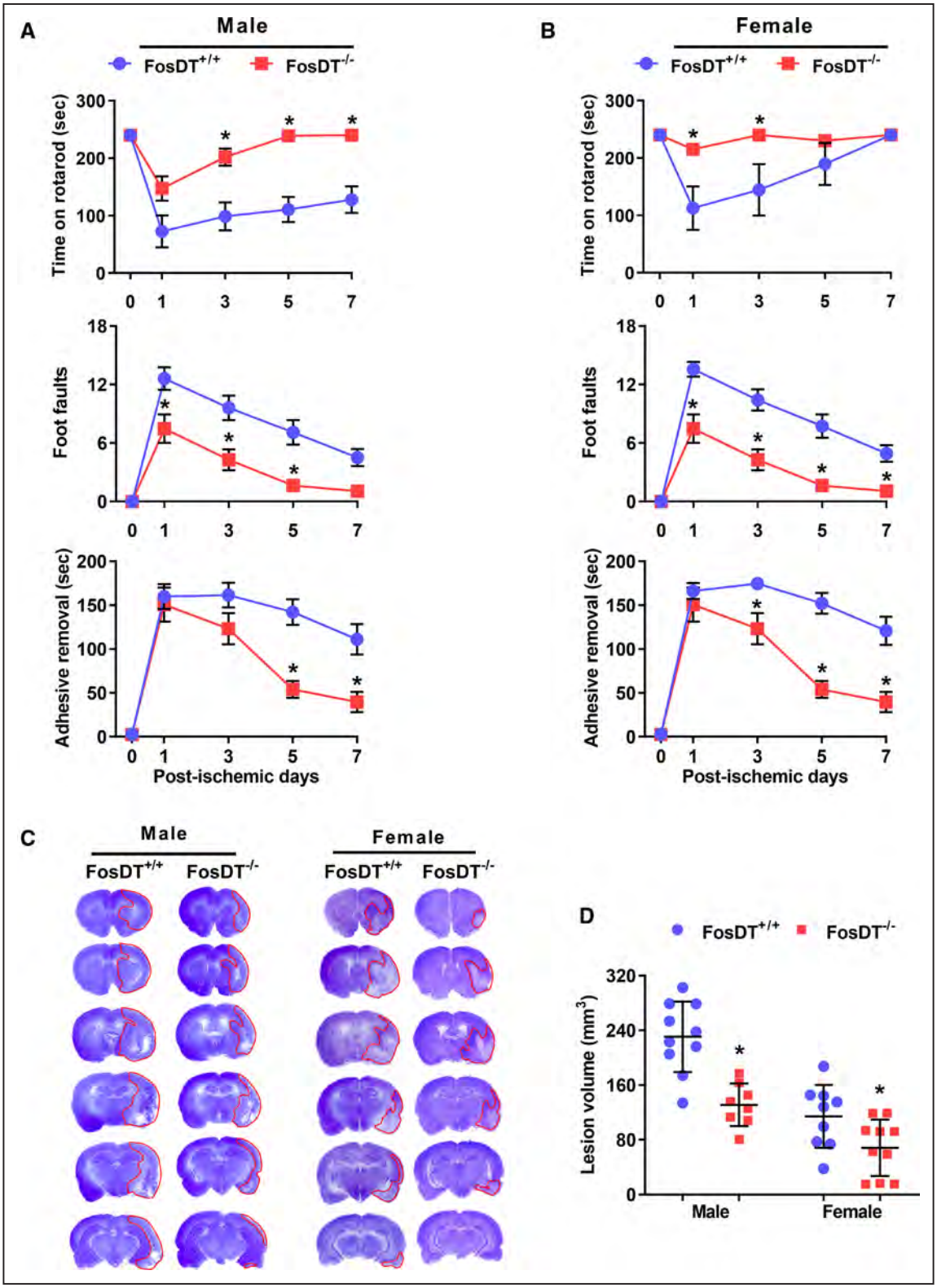

Figure 3. Fos downstream transcript (FosDT) deletion is neuroprotective in both sexes.
Rotarod test (**upper**), beam walk test (**middle**), and adhesive removal test (**lower**) show better motor function recovery between days 1 and 7 following transient middle cerebral artery occlusion (MCAO) in the male (**A**) and female (**B**) FosDT$^{-/-}$ rats compared to FosDT$^{+/+}$ rats. FosDT$^{-/-}$ rats showed smaller infarcts following transient MCAO in both males and females compared with sex-matched FosDT$^{+/+}$ rats (**C** and **D**). Cresyl violet-stained sections are from representative FosDT$^{-/-}$ and FosDT$^{+/+}$ rats subjected to transient MCAO and 7 d of reperfusion (**C**). Lesion volume was computed from the numerical integration of the infarct area and distance between coronal sections (**D**). Values are mean±SEM (**A** and **B**) and mean±SD (**D**) of n=8–10/group. *P<0.05 compared with respective reperfusion time point by repeated-measures ANOVA followed by Bonferroni multiple comparisons test (multiple groups) or by Mann-Whitney U test (2 groups).

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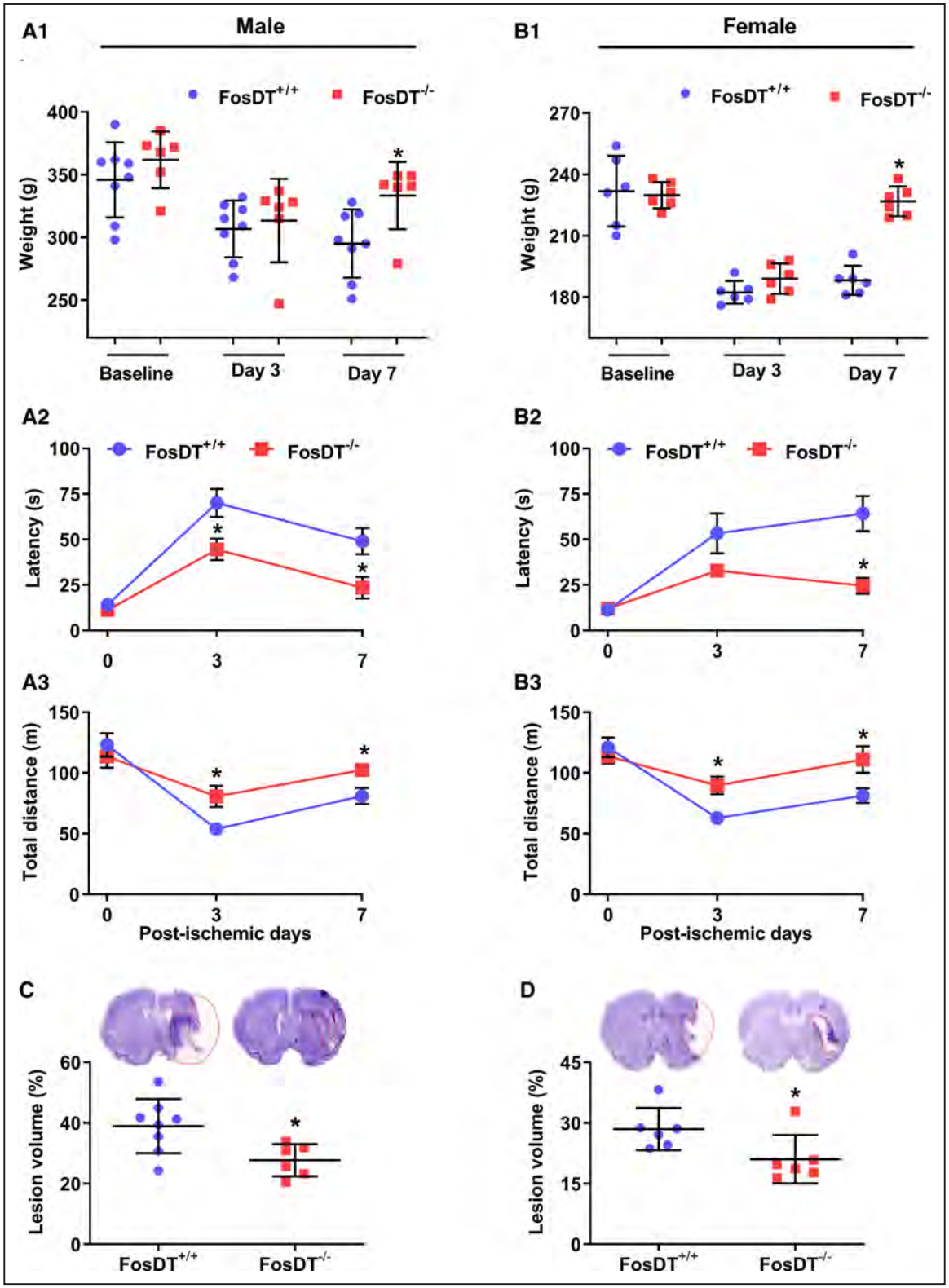

Figure 4. Poststroke neuroprotection in Fos downstream transcript (FosDT)$^{-/-}$ rats was independently validated by a second lab. Male and female adult FosDT$^{-/-}$ rats showed better poststroke weight gain on day 7 (**A**1 and **B**1), improved motor function recovery assessed with adhesive removal test (latency; **A**2 and **B**2), and open field test (total distance; **A**3 and **B**3) on day 3 and day 7 of reperfusion following transient middle cerebral artery occlusion (MCAO) compared with FosDT$^{+/+}$ rats. FosDT$^{-/-}$ rats of both sexes also showed decreased lesion volume compared with FosDT$^{+/+}$ rats estimated at day 7 of reperfusion following transient MCAO (**C** and **D**). Cresyl violet-stained sections are from representative rats of each group. Values are mean±SD (**A**1, **B**1, **C**, and **D**) and mean±SEM (**A**2, **B**2, **A**3, and **B**3) of n=6–8/group. *P<0.05 compared with respective reperfusion time point by repeated-measures ANOVA followed by Bonferroni test (multiple groups) or by Mann-Whitney U test (2 groups).

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downregulated <0.3-fold; n=4/group; Figure 5A–5C; Figure IV and Table I in the Data Supplement). RNA expression profiles were evaluated subacutely at 12 hours of reperfusion following transient MCAO when poststroke transcription changes are high. The peri-infarct cortex of adult male FosDT$^{+/+}$ rats at 12 hours of reperfusion following transient MCAO showed altered expression of 810 transcripts (526 upregulated >3-fold and 284 downregulated <0.3-fold) compared with sham control (n=4/group; Figure 5A–5C; Figure IV and Table II in the Data Supplement). Whereas, in the FosDT$^{-/-}$ rats subjected to 12-hour of reperfusion following transient MCAO, of the 526 transcripts induced in the FosDT$^{+/+}$ cohort, 294 showed either curtailed induction or no induction and one showed downregulation (Figure 5A–5C; Figure IV and Table II in the Data Supplement). Furthermore, of the 284 transcripts downregulated in the postischemic FosDT$^{+/+}$ cohort, 9 showed upregulation, 36 downregulation, and 239 showed reduced or no downregulation in the FosDT$^{-/-}$ cohort (Figure 5A–5C; Figure IV and Table II in the Data Supplement). In addition, FosDT$^{-/-}$ rats showed altered expression of 409 transcripts (235 upregulated >3-fold and 174 downregulated <0.3-fold; n=4/group) that were not altered in FosDT$^{+/+}$ rats after transient MCAO and 12-hour of reperfusion (Figure 5A–5C; Figure IV and Table III in the Data Supplement). FosDT$^{-/-}$ rats subjected to sham surgery or transient MCAO showed switch-on or switch-off of specific genes. Figure 5 shows 15 upregulated (Figure 5D1 and 5D2, upper) and 15 downregulated (Figure 5D1 and 5D2, lower) unique transcripts in sham control (Figure 5D1) and transient MCAO (Figure 5D2) groups of FosDT$^{-/-}$ compared to respective FosDT$^{+/+}$ rats.

Gene Set Enrichment Analysis using the set of transcripts that were differentially expressed after stroke showed that inflammation mediated by cytokines and interleukins is the major downregulated category, and metabolism, transport, neuronal system, post-translational protein modification, and adaptive immune system were the major upregulated categories in the FosDT$^{-/-}$ compared with the FosDT$^{+/+}$ rats following focal ischemia (Figure 6A). Gene ontology analysis indicated that the neutrophil chemotaxis, inflammatory response, cytokine production, and extracellular exosome functions were significantly changed in FosDT$^{-/-}$ rats compared to FosDT$^{+/+}$ rats following focal ischemia (Figure 6B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis also showed that inflammation and metabolism are the significant functions altered due to FosDT deletion following focal ischemia (Figure 6B).

FosDT Deletion Curtailed Postischemic Pathological Changes

Following transient MCAO and 1 day of reperfusion, FosDT$^{-/-}$ rats showed curtailed immunostaining of cleaved caspase-3 (apoptosis), pDrp1 (mitochondrial dysfunction), and 3-nitrotyrosine (oxidative stress) compared with the FosDT$^{+/+}$ rats (Figure 6C).

DISCUSSION

Focal ischemia is known to extensively alter the cerebral lncRNAome.[2,18] However, the functional significance of lncRNAs in promoting ischemic brain damage is yet to be evaluated in detail. We previously showed that lncRNA FosDT is significantly induced in the postischemic brain and its knockdown is neuroprotective.[11] FosDT is primarily a brain-specific lncRNA. In adult rats, except the heart, other peripheral organs (spleen, liver, kidney, muscle, and lung) showed low FosDT expression. Although FosDT is expressed in all areas of the brain analyzed (cerebral cortex, striatum, hippocampus, cerebellum, and thalamus), the cerebral cortex showed >20× more expression than other brain regions of adult rats. Furthermore, FosDT$^{-/-}$ rats showed normal growth and no observable phenotypic changes or any discernible differences in the cytoarchitecture of the brain or peripheral organs compared with the FosDT$^{+/+}$ rats. The FosDT$^{-/-}$ rats also showed no changes in the cerebral vasculature. It is intriguing that a highly expressed lncRNA had no noticeable function in the adult body. However, FosDT function might be masked by compensatory mechanisms during normal physiological states but activated when neurons are stressed. In support of this notion, FosDT$^{-/-}$ rats showed a higher level of recovery in motor function and acquired smaller infarcts than FosDT$^{+/+}$ rats when subjected to focal ischemia. Although this effect was observed in both sexes, there were overall smaller infarcts in the females, which is consistent with the literature and may explain (ceiling effects) why females had no significant differences between FosDT$^{+/+}$ and FosDT$^{-/-}$ at day 5 or 7 of reperfusion in the rotarod test.[19] As data reproducibility is of paramount importance, we confirmed our results independently in a second laboratory at the University of Texas. They also observed that FosDT$^{-/-}$ rats subjected to transient focal ischemia show smaller infarcts and better neurological functional outcome in both sexes.

To understand the molecular mechanisms that might be responsible for the poststroke neuroprotection observed in the FosDT$^{-/-}$ rats, RNA-sequencing analysis was conducted using the ipsilateral cortical tissue from adult rats subjected to transient MCAO and 12 hours of reperfusion. As both sexes showed improved poststroke functional outcome and recovery, we used only males for this. Many studies showed that the dominant neurotoxic mechanisms, including inflammation and apoptosis, are activated within 24 hours of transient MCAO.[20] Inflammatory genes constitute a significant category of genes induced during the acute phase following focal ischemia.[21–23] Uncontrolled inflammation is also a known precipitator of secondary

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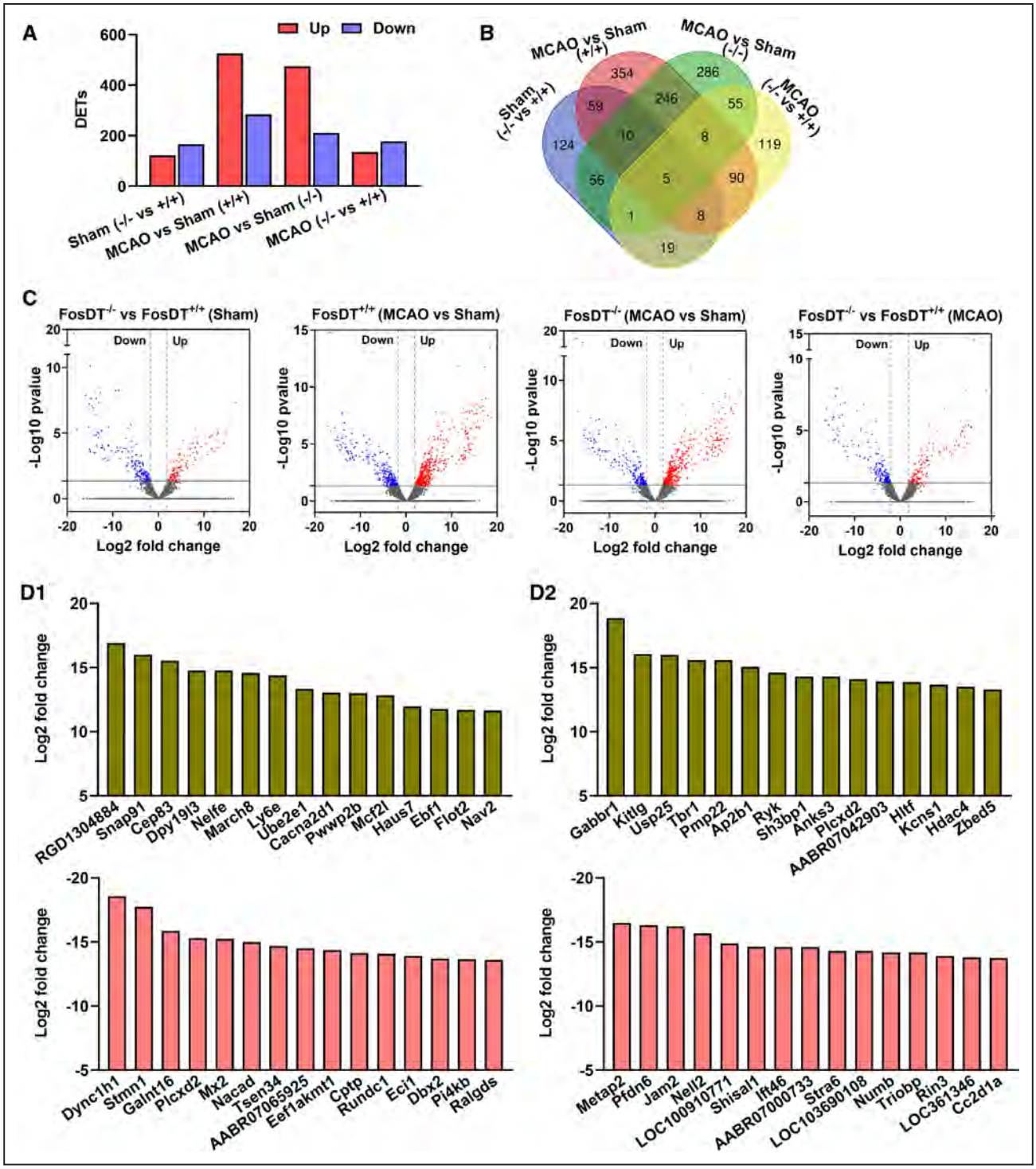

Figure 5. Fos downstream transcript (FosDT) knockdown altered the cerebral RNA expression profiles.
Total RNA isolated from peri-infarct cortical tissue of adult male FosDT$^{-/-}$ and FosDT$^{+/+}$ rats subjected to 90 min of transient middle cerebral artery occlusion (MCAO) and 12 h of reperfusion or equivalent area in sham groups was analyzed by RNA-sequencing. When the 4 groups were compared in different permutations, FosDT$^{+/+}$ (MCAO vs sham) group showed highest numbers (810) of differentially expressed transcripts (DETs) followed by FosDT$^{-/-}$ (MCAO vs sham, 686), FosDT$^{-/-}$ vs FosDT$^{+/+}$ (MCAO, 312), and FosDT$^{-/-}$ and FosDT$^{+/+}$ (sham, 288; **A**). Venn diagrams show the distribution of common and independent DETs among the 4 groups (**B**). Volcano plots show the upregulated (red dots) and downregulated (blue dots) DETs between different comparisons (**C**). The plots are represented as log2 fold change against -log10 of the *P* value (n=4/group). FosDT$^{-/-}$ sham showed induction or suppression of several transcripts compared with FosDT$^{+/+}$ sham (**D**1). FosDT$^{-/-}$ MCAO also showed induction or suppression of many transcripts compared with FosDT$^{+/+}$ MCAO (**D**2; n=4/group).

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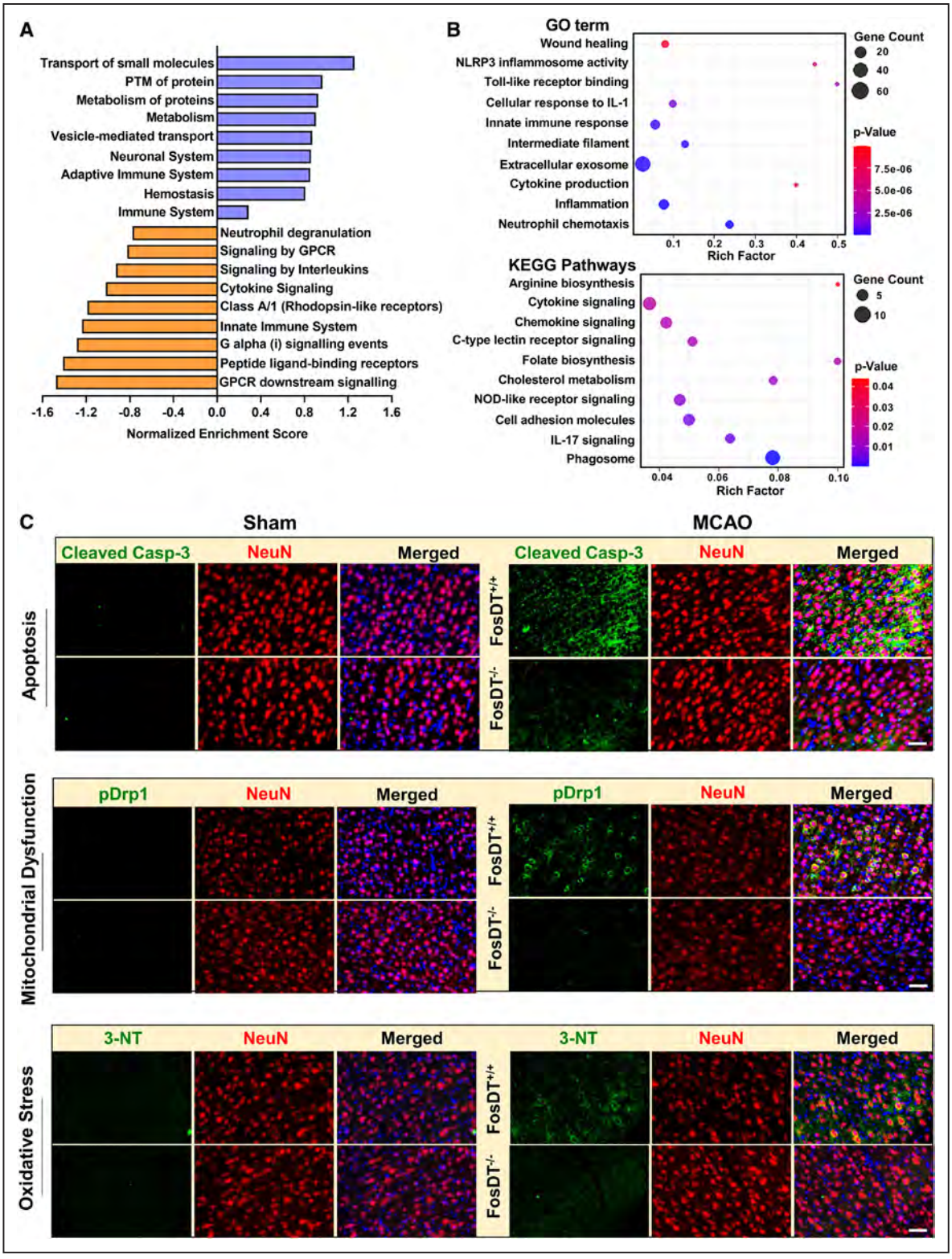

Figure 6. Fos downstream transcript (FosDT) deletion regulated poststroke pathological changes.
Gene Set Enrichment Analysis of differentially expressed transcripts (DETs) between FosDT$^{-/-}$ middle cerebral artery occlusion (MCAO) vs FosDT$^{+/+}$ MCAO groups showed various upregulated and downregulated pathways (**A**). Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis also showed the response of various pathways, including inflammation in FosDT$^{-/-}$ rats compared with FosDT$^{+/+}$ rats after focal ischemia (**B**). Immunostaining decreased apoptosis (cleaved casp-3 [caspase-3]), mitochondrial dysfunction (pDrp1 [phosphorylated dynamin-related protein-1]), and oxidative stress (3-nitrotyrosine [3-NT]) in the neuronal nuclei (NeuN)$^{+}$ cells in the cortical peri-infarct region of FosDT$^{-/-}$ and FosDT$^{+/+}$ rats at 24 h of reperfusion after 90 min of transient MCAO (n=3/group; **C**). Blue is DAPI for nuclei. Scale bar=50 μm. GPCR indicates G protein-coupled receptor; IL, interleukin; NLRP, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing; NOD, nucleotide-binding and oligomerization domain; and PTM, post-translation modification.

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brain damage after stroke.[23] Interestingly, FosDT$^{-/-}$ rats showed curtailed postischemic induction of many inflammatory genes compared with FosDT$^{+/+}$ rats. Specifically, genes that regulate neutrophil chemotaxis, inflammatory response, cytokine production, and exosome function were significantly downregulated in the FosDT$^{-/-}$ rats compared with FosDT$^{+/+}$ rats following focal ischemia. In addition, following transient MCAO, FosDT$^{-/-}$ rats showed significant induction of several genes that modulate metabolism, transport, post-translational protein modifications, and adaptive immune response compared with FosDT$^{+/+}$ rats. Thus, FosDT significantly influences a set of genes that might be responsible for the pathological events that lead to secondary brain damage after stroke.

In mammals, lncRNAs are the largest class of noncoding RNAs discovered so far.[24] Importantly, many lncRNAs are brain-specific.[6,25] They are functionally diverse and control cell fate during neural differentiation and synaptogenesis, neurite outgrowth, and synapse maturation by mechanisms including scaffolding of epigenetic machinery, recruiting translational repression machinery, and controlling transcription factors and splicing regulators.[25,26] Poststroke outcome in rodents was shown to be altered by modulating several lncRNA, including CAMK2D-associated transcript 1 (C2dat1), metastasis associated lung adenocarcinoma transcript 1 (Malat1), macrophage contained LCP1 related pro-inflammatory lncRNA (Maclpil), maternally expressed 3 (MEG3), and Nespas.[27–31] *FosDT* gene is located in a gene desert of nearly a quarter-million nucleotides with only the *Fos* gene in its upstream proximity.[11] We observed that the expression pattern of FosDT mirrors that of the Fos in various organs of both P7 and adult rats. Fos, which is a marker of cellular stress and is induced rapidly after brain injury, forms a complex with Jun/AP1 (activator protein 1) transcription factor and regulates cell development by modulating signal transduction, cell proliferation, and differentiation.[11,12,32,33] We previously showed that both FosDT and Fos are induced after focal ischemia in the rat brain.[11] Both FosDT$^{-/-}$ and FosDT$^{+/+}$ rats showed induction of Fos expression following focal ischemia, but the fold induction was much lower in FosDT$^{-/-}$ than FosDT$^{+/+}$ rats indicating that FosDT controls Fos expression. Many genes observed to be dysregulated in lncRNA Malat1 knockout mice are its neighboring genes and hence Malat1 is thought to play a cis-regulatory role.[34] Similarly, FosDT might control postischemic induction of Fos, indicating that intragenic lncRNAs such as FosDT can modulate the expression of their host genes.[11] Similar to FosDT, Malat1 is also developmentally and functionally dispensable in the normal brain.[34] However, some lncRNA knockouts (Gomafu, Fendrr, Peril, and Mdgt) are developmentally normal but behaviorally impaired and show postnatal lethality.[35,36]

FosDT interacts with transcription factor REST-associated chromatin-modifying proteins to modulate the transcription of downstream genes, suggesting that FosDT acts as a scaffold and guides protein complexes to target genomic loci.[11,37] Our previous studies showed that knockdown of FosDT allows the expression of the nuclear factor κB2 (*NFκB2*) gene.[11] NFκB2 (p100/p52) protein is known to prevent the nuclear translocation of NFκB RelA/p65 owing to its IκB property and thereby minimizes inflammation and cell death after ischemia.[38,39] FosDT shares the characteristic of controlling genes by collaborating with transcription factors with other lncRNAs. For example, lncRNA HOX antisense intergenic RNA (HOTAIR) regulates chromatin dynamics and silencing of epigenetic target genes/loci by recruiting polycomb repressive complex 2 and the LSD1/coREST (Lysine-specific demethylase 1/REST corepressor 1)/REST complex.[40,41] This implies that lncRNAs like FosDT and HOTAIR coordinate multilevel gene regulation in a cell.

Overall, we show that the lncRNA FosDT is not essential for the development of the brain and peripheral organs, but its induction after stroke promotes ischemic brain damage. This is confirmed by the observation that FosDT$^{-/-}$ rats show reduced brain damage and better recovery of motor function following focal ischemia. We further showed that FosDT promotes ischemic brain damage by inducing the expression of many inflammatory genes. Future studies will establish FosDT as a novel therapeutic target for stroke.

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Disclosures

None.

Supplemental Materials

Online Figures I–V
Online Tables I–III

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