



Myth Versus Measurement: Direct CNS Comparison of Two miRNA-Based Regulated Gene Therapies in a Mosaic Model of Rett Syndrome



P0531

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INTRODUCTION

- Rett syndrome (RTT) is a rare neurodevelopmental disorder caused by loss-of-function *MECP2* variants resulting in deficiency of functional MeCP2 protein.¹
- Precise regulation of the X-linked dosage-sensitive *MECP2* gene is a central challenge for gene therapy approaches in RTT,¹ and several strategies have been proposed to mitigate toxicity risk associated with excessive MeCP2 protein levels.^{1,2}
- Two miRNA-based strategies to regulate transgenic *MECP2* have advanced into the clinic:
 - NGN-401 includes EXACT™, a self-contained miRNA regulatory circuit delivered alongside full-length *MECP2* transgene and packaged in a single-stranded AAV9 genome (ssAAV9; Figure 1A). EXACT mitigates against toxic overexpression of the transgene via the self-contained miRNA regulatory circuit¹.
 - TSHA-102 embeds miRARE, which leverages endogenous cellular miRNA binding sites to dampen expression, and delivers a hypomorphic miniMECP2 (Δ NIC) transgene in a self-complementary AAV9 genome (scAAV; Figure 1B)².
- Selective expression of miRARE-regulated transgene in the mosaic brain (i.e. “MeCP2 sensing”) has been proposed but not directly demonstrated³. Here, we performed an *in vivo* study to directly test this claim.

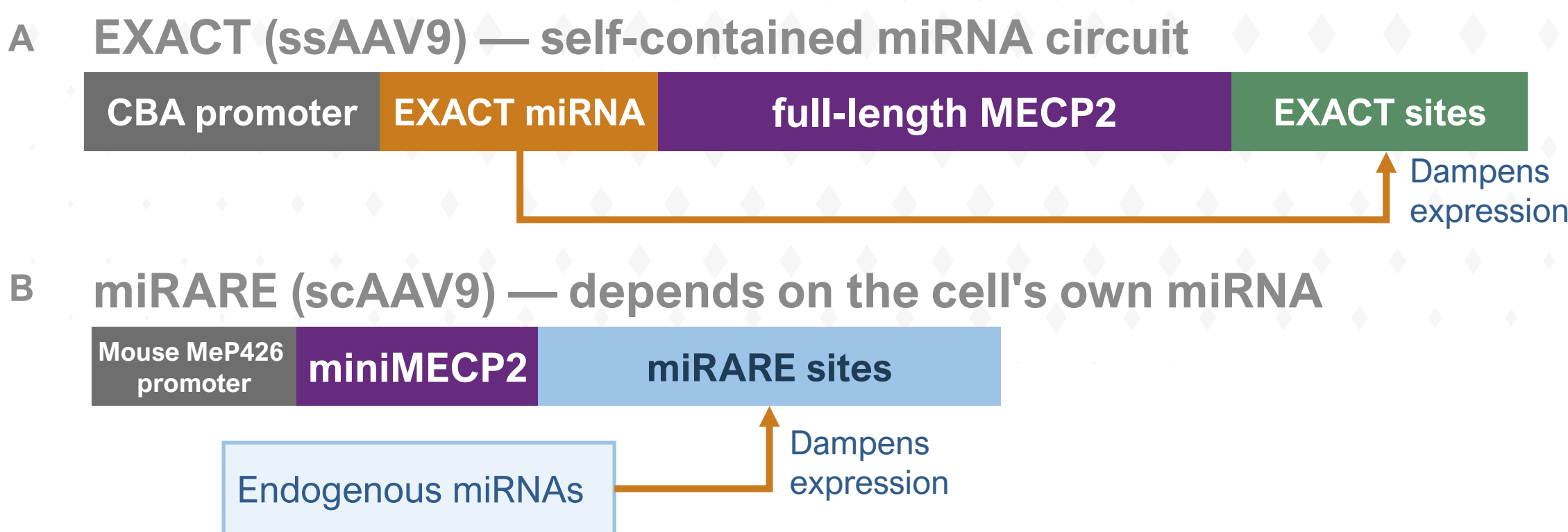


Figure 1. Mechanisms to regulate *MECP2* transgene expression by (A) EXACT and (B) miRARE miRNA-based strategies.

STUDY DESIGN

- To directly assess cells with different MeCP2 status in the same brain, we generated heterozygous female *Mecp2*^{GFP/+} mice⁴ that are mosaic for GFP-positive neurons expressing endogenous MeCP2 (WT cells) or GFP-negative neurons, which are MeCP2 deficient (null cells).
- Constructs were designed to mimic the clinical products with the inclusion of a C-terminal FLAG tag on the transgene. The FLAG tag enabled robust cross-cohort comparisons, allowing transgene-derived MeCP2 to be distinguished from endogenous MeCP2, and enabled transgene-specific quantification in neuronal populations by single-nucleus flow cytometry and in bulk DNA- and protein-level analyses.
- As a control group, the miRARE seed sequences were scrambled in the ‘inactive miRARE’ construct, while all other genetic elements were identical.
- Constructs were packaged in AAV9 capsids using HEK or Sf9 processes, aligning with production used for clinical manufacturing. Quantity and quality of AAV9 material was similar across constructs when assessed by digital droplet PCR, AAV genome sequencing, CDMS and VP1 protein gel.
- All constructs were delivered by neonatal ICV dosing (P1; 1×10¹¹ vg/mouse, n=4-5) to eliminate confounding effects of divergent delivery and to maximize detection in various brain tissues, which were collected at 6 weeks post treatment (Figure 2).

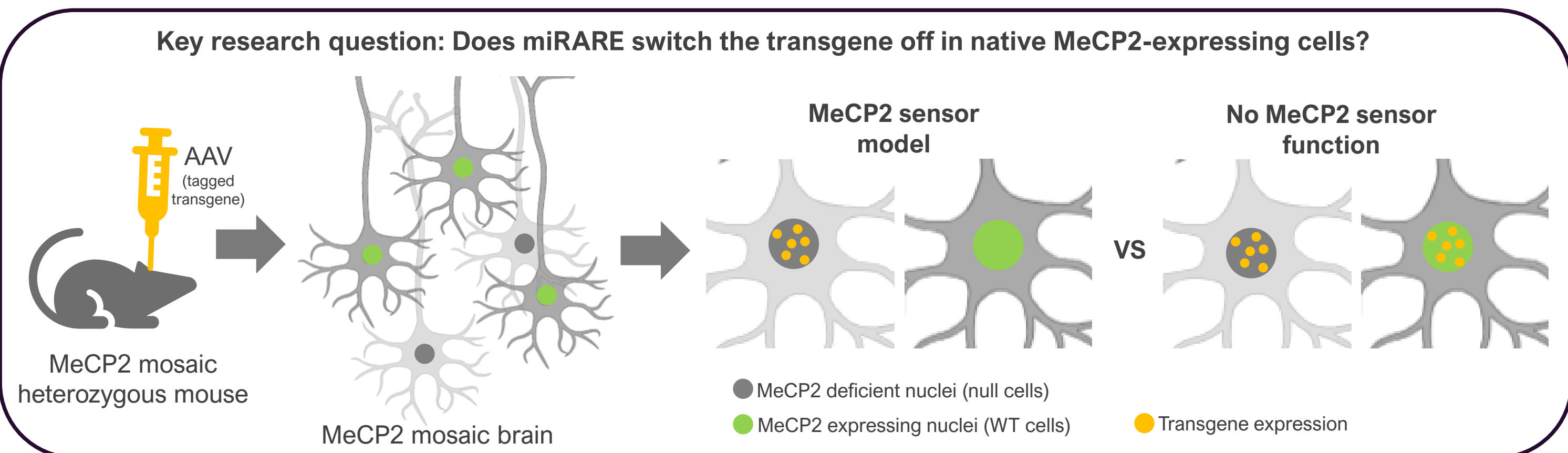


Figure 2. Experimental Concept. Illustrative model of a gene therapy vector containing miRARE cassette delivered to a MeCP2 mosaic mouse. If miRARE functions as a MeCP2 sensor, transgene expression is expected to be switched off in WT cells and expressed in MeCP2-null cells.

RESULTS

Single cell resolution analysis of expression in the mosaic brain reveals no selectivity between MeCP2-null and MeCP2-positive cells for all constructs

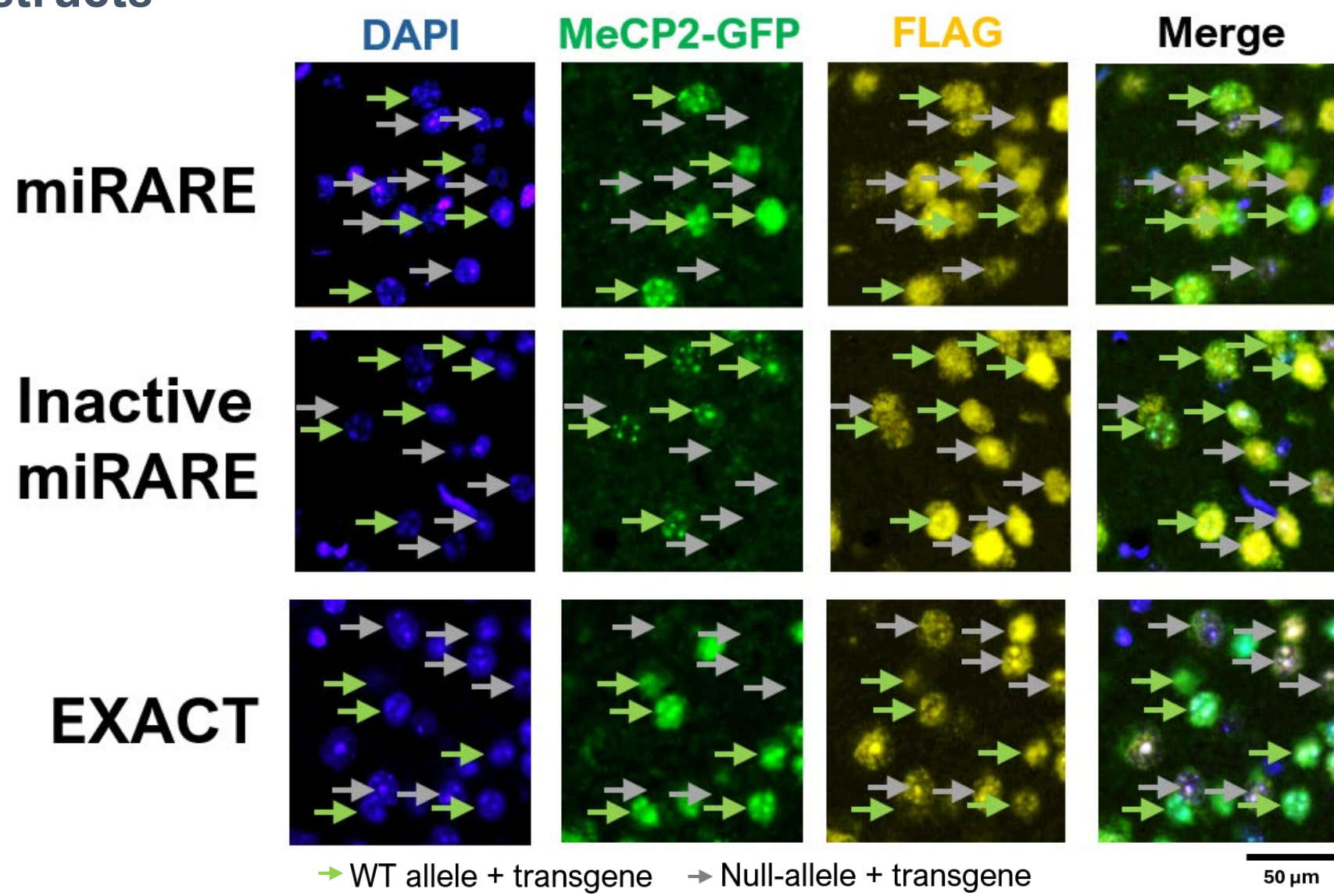


Figure 3. Immunolabeling of native MeCP2 status and transgene expression in the mosaic brain. Representative images of neocortex from treated mice stained for DAPI (all nuclei), anti-GFP (nuclei expressing endogenous MeCP2) and anti-FLAG (transgene expression). Green arrows and grey arrows indicate transgene positive ‘WT’ and ‘null’ cells, respectively. Vehicle-treated mice showed no FLAG signal. Scale bar, 50 μ m.

Single nuclei level analysis reveals similar transgene expression in MeCP2-null and MeCP2-positive neurons across all brain regions and spinal cord for all constructs

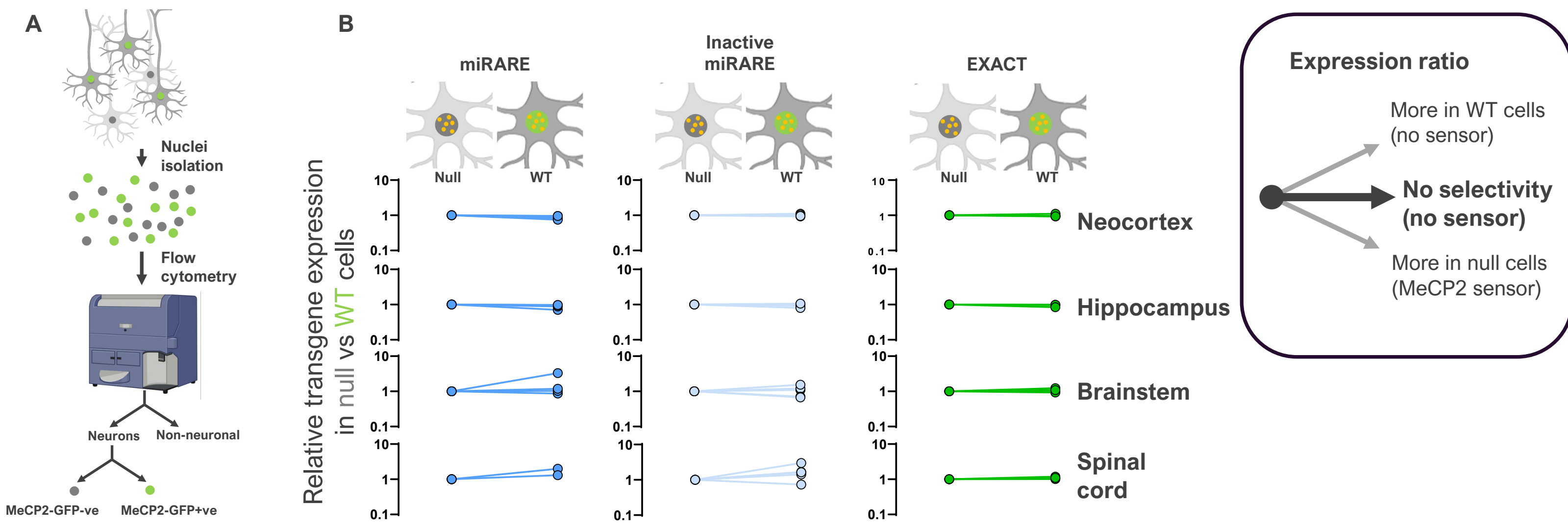


Figure 4. Flow cytometric analysis of transgene expression in MeCP2-positive and MeCP2-deficient neuronal nuclei. (A) Nuclei were isolated from brain regions and transgene expression (anti-FLAG signal) quantitated in GFP +ve (endogenous MeCP2-expressing) and GFP -ve (MeCP2-deficient) neurons. (B) Transgene expression (FLAG fluorescence intensity; shown relative to expression in null cells) is similar in both null and WT cells in cortex, hippocampus, brainstem and spinal cord with no apparent sensing of endogenous MeCP2 status.

Bulk brain tissue analysis reveals miRARE acts as a strong transgene dampener

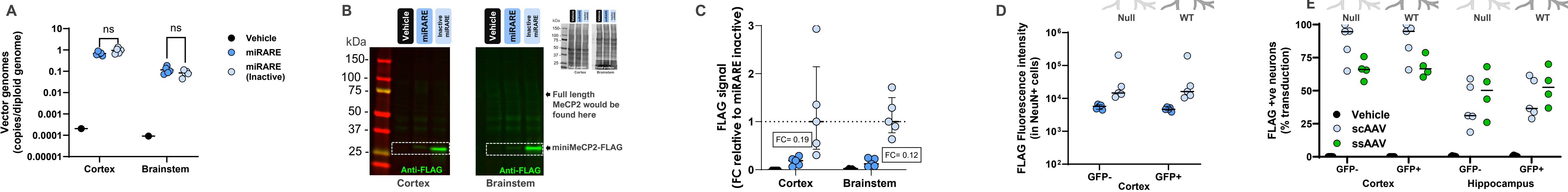
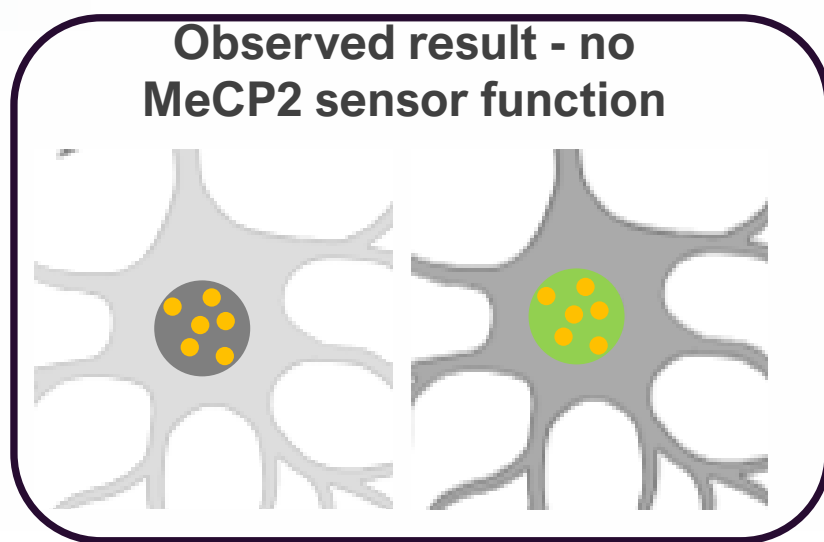


Figure 5. In vivo characterization of expression following direct brain delivery in mice. (A) Vector genomes quantified in cortex and brainstem. (B) Representative immunoblots of cortical and brainstem lysates probed with anti-FLAG antibody to detect transgene protein. Insert: total protein staining served as loading control. (C) Quantification of tissue transgene expression using FLAG signal, shown relative to inactive miRARE treatment group. (D) Quantification of transgene expression at single nuclei level using flow cytometry. (E) Flag positive neurons for scAAV (inactive miRARE) and ssAAV (EXACT) shows similar transduction in cortex and hippocampus at 6 weeks post dosing. miRARE was evaluated but not plotted due to poor accuracy associated with low levels of expression. In each plot, points represent an individual animal; n=4-5 per group. Data shown is mean $\pm$ S.E.M. in (A); data shown as median in (C-E). Two-way ANOVA with Tukey's post-hoc comparison where shown.

KEY CONCLUSIONS

- miRARE-containing vectors produced comparable transgene expression in MeCP2-null and MeCP2-expressing neurons, providing *in vivo* evidence that miRARE is not regulated by endogenous MeCP2 levels, and is not an MeCP2 sensor.
- miRARE reduced miniMeCP2 expression by ~85% relative to the inactive miRARE control in WT and MeCP2-null cells equivalently, consistent with a mechanism of global transgene dampening.
- Single-stranded and self-complementary AAV constructs demonstrate comparable transduction efficiency of brain tissue *in vivo* at 6 weeks. This data is consistent with rapid single-stranded vector expression kinetics [see poster P0110].



References

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Acknowledgements and Funding

We thank Dr. Mark Bailey for helpful comments and advice on study design. This study was supported by Neurogene Inc.