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TITLE: The effect of Mecp2 mutation on cortical projections revealed by correlated single-cell transcriptomics and projectomics

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14. ABSTRACT
Rett Syndrome is caused by mutations in *Mecp2*, which result in a constellation of language, cognitive, motor, and autonomic deficits later in life. Although changes in long-range neuronal connectivity likely underlie the behavioral defects in Rett syndrome, it is unclear how long-range axonal projections are disrupted. Here we develop and apply high-throughput single-cell techniques to identify cell type-specific changes in projections in *Mecp2* animals. Initial analysis indicates that corticothalamic neurons are reduced in both the visual cortex and anterior cingulate cortex in *Mecp2* animals. This result is the first step in identifying the long-range circuitry changes associated with *Mecp2* mutation. Furthermore, our approach is generally applicable to other brain areas and disease models to reveal cell type-specific changes in projections that are difficult to detect using conventional methods.

15. SUBJECT TERMS
Rett syndrome, Mecp2, Long-range projections, high throughput, single cell, MAPseq, BARseq, barcode sequencing, in situ sequencing

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Rett syndrome is a neuropsychiatric disease caused by mutations in the gene *Mecp2*. Although aberrations in long-range neuronal connectivity have been implicated in many neuropsychiatric diseases, such circuitry basis for Rett syndrome is unclear. Such changes in neuronal connectivity likely underlie the behavioral defects in Rett syndrome. In this study, we use high-throughput single-cell techniques to identify changes in cortical projections in a cell type-specific manner. We further identify the developmental process that results in such defects in projections.

2. **KEYWORDS:**

Rett syndrome, *Mecp2*, Long-range projections, high throughput, single cell, MAPseq, BARseq, barcode sequencing, in situ sequencing

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Major goal 1: Identification of long-range projections disrupted by Mecp2 mutation using MAPseq,

Target dates: 7 months

Actual completion dates: 7 months

Major goal 2.1: Combining MAPseq and scRNAseq

Target dates: 9 months

Actual completion dates: 11 months. See the next sub-section for details.

Major goal 2.2: Using MAPseq and scRNAseq to find cell type-specific changes in projections caused by Mecp2 mutation

Target dates: 18 months.

Percentage of completion: Subtask 1 is under way. Subtask 2 and 3 were scheduled for months 12-18.

Major goal 3: Identification of the effect of Mecp2 on the developmental refinement of cortical projections

Target dates: 18 months.

Percentage of completion: 0%, all work is scheduled for months 12-18.

What was accomplished under these goals?

Major goal 1: Identifying long-range projection defects in Mecp2 mutants

To identify projection defects caused by a lack of *Mecp2*, we used a high-throughput approach, MAPseq, to map and compare the projection patterns of single neurons in *Mecp2 -/y* and wild-type animals. We mapped the projections of 8,937 neurons from four *Mecp2 -/y* animals and 8,463 neurons from five WT animals of matching age. These neurons included 2,660 *Mecp2 -/y* neurons and 6,910 WT neurons in the visual cortex, and 6,277 *Mecp2 -/y* neurons and 2,454 WT neurons in the anterior cingulate cortex (Fig. 1A, B). Although neurons in mutant animals project to the same

sets of brain areas that WT neurons do at a population level, we found that corticothalamic (CT) neurons, defined as having projections only to the thalamus and no projections to the midbrain or the striatum, were reduced in *Mecp2* mutants (Fig. 1C, D). This change in the fraction of CT population was found in both visual cortex and anterior cingulate cortex and was consistent across animals. **Our results thus suggest a general reduction in CT neurons, as defined by projections, across cortical areas.**

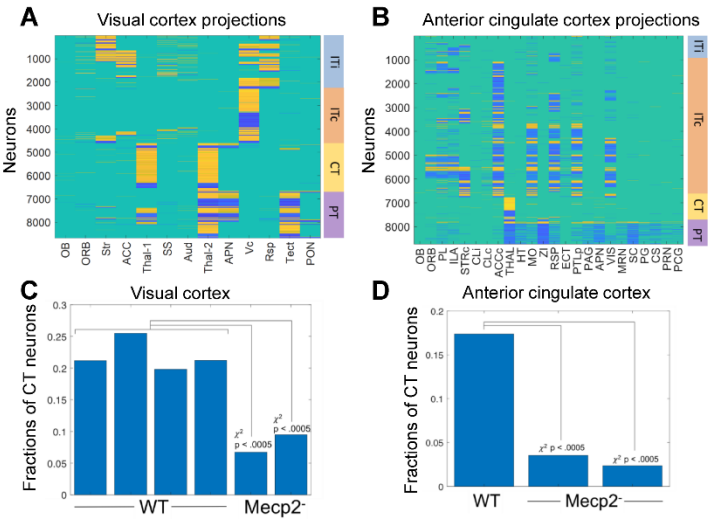

Fig. 1. Projection mapping in Mecp2 -/y and WT animals. (A)(B) Projection matrix of neurons mapped in the visual cortex (A) and anterior cingulate cortex (B). Columns represent projection areas and rows indicate single neurons. Yellow and deep blue bars indicate projections by WT (yellow) and *Mecp2 -/y* (blue) neurons. Neurons are sorted by major classes defined by clustering the projections and color coded by class labels on the right: two classes of intratelencephalic (IT) neurons with (ITc) or without (ITi) contralateral projections, pyramidal tract (PT) neurons, and corticothalamic (CT) neurons. (C)(D) Fractions of CT neurons in each animal in the visual cortex (C) and the anterior cingulate cortex (D), $p < 0.0005$ comparing each *Mecp2 -/y* brain to each WT brain using Chi-square test.

Major goal 2.1: Combining single-cell RNAseq with MAPseq

In this section I first describe the combination of single-cell RNAseq and MAPseq as originally proposed. I then describe an alternative approach based on *in situ* sequencing, which achieves higher throughput and additionally preserves spatial organization of neurons with similar costs.

We combined single-cell RNAseq with MAPseq using a similar pipeline as CITE-seq. To achieve this, we re-engineered the barcode cassette used for MAPseq so that the barcodes can be sequenced simultaneously with endogenous genes during single-cell RNAseq using 10x Genomics Chromium. We showed that gene expression was generally reduced in barcoded cells, but relative gene expression was unchanged (Fig. 2). **Therefore, gene expression in barcoded neurons reflect gene expression in non-barcoded neurons.**

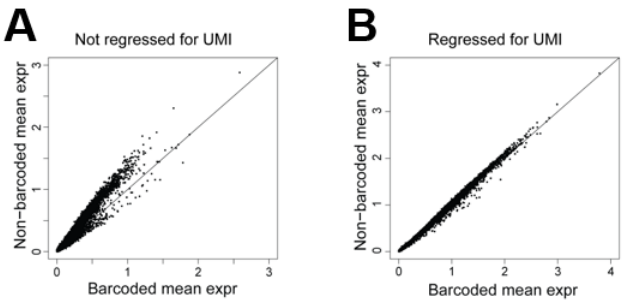

Fig. 2. Gene expression in barcoded neurons. (A)(B) mean gene expression in non-barcoded (y-axis) and barcoded (x-axis) neurons with (B) or without (A) regression for differences in endogenous read counts per cell.

One limitation of this approach is that the throughput is about 50× lower than MAPseq alone due to the low survival rate of neurons during single-cell dissociation. To solve this problem, we switched to an alternative technique, BARseq, which we have recently published (Chen et al., 2019). BARseq uses *in situ* sequencing to read out barcodes in somata, and thus can resolve barcoded somata with high spatial resolution during projection mapping (Fig. 3A, B). We further optimized *in situ* sequencing

to achieve efficient and multiplexed read out of endogenous genes and to allow calling transcriptionally defined cell types (Fig. 3C, D). Combining *in situ* sequencing of both endogenous genes and barcodes allowed us to correlate projections, gene expression, and laminar positions with similar throughput as MAPseq. As a proof-of-concept, we correlated cadherin expression to projections in 1,342 projection neurons in mouse auditory cortex and motor cortex (Fig. 3E, F). **BARseq thus achieves our proposed goal of correlating projections to gene expression with high throughput.**

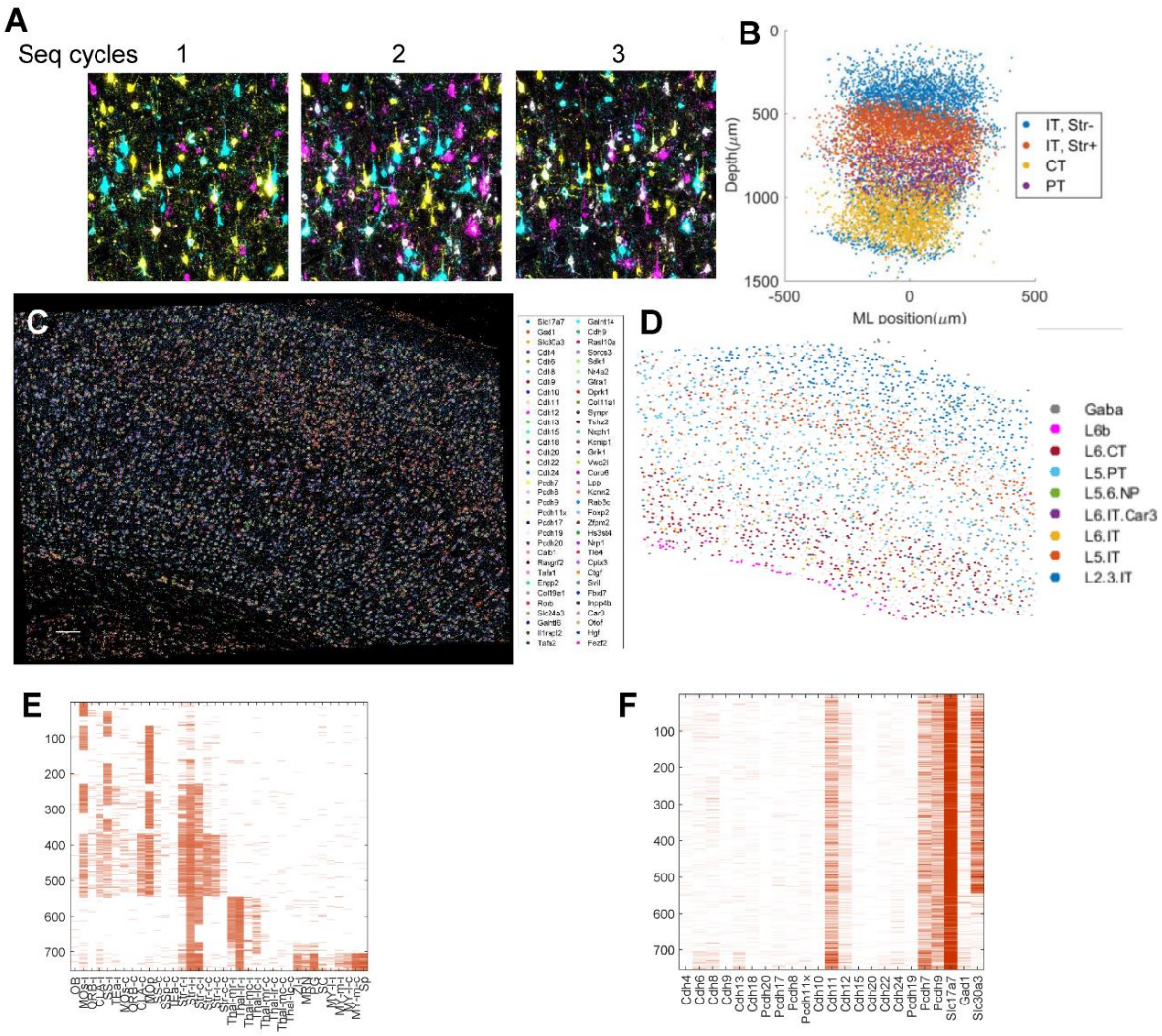

Fig. 3. BARseq correlates projections with gene expression and spatial organization of neurons with high throughput. (A) Three cycles of in situ sequencing images of barcoded neurons in a cortical brain slice. Colors (cyan, yellow, magenta, and white) correspond to the four bases (G, T, A, and C) being read out. (B) the locations of ~10,000 projection neurons in the motor cortex mapped by BARseq. Neurons are color-coded by major clusters of projection patterns. (C) In situ sequencing of 46 endogenous genes that are differentially expressed across excitatory neuronal types in the motor cortex. (D) Transcriptionally defined neuronal types of the same neurons in (C) determined by gene expression. (E)(F) Long-range projections (E) and gene expression (F) of the same neurons in the motor cortex determined by BARseq. Columns indicate projection areas or genes, and rows indicate neurons. Corresponding rows in (E) and (F) represent the same neuron. Neurons are sorted by projection patterns.

Major goal 2.2: Identifying projection defects specific to cell types defined by gene expression in Mecp2 mutants

Because BARseq achieves much higher throughput than MAPseq combined with single-cell RNAseq, we have switched to using BARseq-based strategies for projection mapping of transcriptionally defined cell types for Goal 2.2. and Goal 3. We have begun BARseq experiments on 4 *Mecp2 -/y* animals and 3 WT littermates.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Major goal 2.2

We will use BARseq to map cell type-specific changes in projections in *Mecp2 -/y* animals compared to WT animals. We expect to confirm the reduction of CT neurons defined by projections in BARseq experiments and determine whether this reduction is caused by a reduction of the number of CT neurons or a change in projection patterns. Similar experiments will also be performed in *Mecp2^{loxp}* animals to determine whether *Mecp2* acts cell-autonomously to affect projections.

Major goal 3

We will perform BARseq in juvenile *Mecp2 -/y* and WT animals to identify the changes in projections over post-natal development.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The finding that corticothalamic neurons are reduced in *Mecp2 -/y* animals provides a possible neuroanatomical substrate for behavioral defects seen in Rett syndrome. This finding can guide future research in the circuitry mechanisms that lead to these behavioral defects.

The development of BARseq, which allows correlation of gene expression, long-range projections, and spatial organization of neurons, can be further applied to other brain areas to identify changes in gene expression and/or projections associated with *Mecp2* mutation.

What was the impact on other disciplines?

BARseq is generally applicable to other brain areas and disease models. The high throughput and relative low cost of BARseq makes it an attractive tool to screen for connectivity defects in disease models, and to interrogate the organization of nervous systems in general.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We plan to switch to BARseq for cell type-specific projection mapping instead of using single-cell RNAseq and MAPseq. BARseq has higher throughput and can additionally identify the laminar positions of neurons, which is also indicative of cell types. The cost of BARseq per mapped neuron is similar to the original approach of combining single-cell RNAseq and MAPseq. We thus think BARseq is more suitable for achieving the goals of this project.

Actual or anticipated problems or delays and actions or plans to resolve them

Work during the last month of the reporting period was delayed by COVID-19 related shutdowns, and work in the first few months of the next reporting period will likely be affected too. We will request no-cost extension if necessary.

We have switched to using BARseq to correlate projections with gene expression. This switch delayed the completion of Goal 2.1 due to extra work in optimizing BARseq, but the higher throughput of BARseq compared to the originally proposed technique should allow the remaining goals to proceed on schedule.

Changes that had a significant impact on expenditures

Work during the last month of the reporting period was impacted by COVID-19 related shutdowns. This has led to less spending during this period than expected. The spending should resume to a normal level after the COVID-19 related shutdowns are resolved.

We have switched from using single-cell RNAseq and MAPseq to using BARseq for mapping projections of specific transcriptionally defined cell types. However, this change in approach should not significantly impact the overall cost.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications. Nothing to report

  Books or other non-periodical, one-time publications. Nothing to report

  Other publications, conference papers and presentations. Nothing to report

- **Website(s) or other Internet site(s)** Nothing to report

- **Technologies or techniques**

  We have improved BARseq, which can correlate gene expression, long-range projections, and spatial organization of neurons with high throughput and cellular resolution. The manuscript describing the improved version of BARseq is in preparation. The protocol for BARseq is publicly available at protocol.io: dx.doi.org/10.17504/protocols.io.bdedi3a6

- **Inventions, patent applications, and/or licenses** Nothing to report

- **Other Products**

  We have produced projection data for thousands of neurons at cellular resolution in both wild-type and *Mecp2 -/y* animals. These projection data allow further analysis to understand the organization of cortical projections, which could provide additional insights in connectivity changes in neurological diseases.

  BARseq can be applied to other disease models to understand changes in gene expression and/or long-range projections associated with neurological diseases.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Xiaoyin Chen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2807-6125
Nearest person month worked:	12
Contribution to Project:	Dr. Chen performed and analyzed experiments.
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

The Quad Chart is submitted in the attachment.

9. APPENDICES:

The Quad Chart is attached.