Transcriptional regulatory elements, including promoters and enhancers, play a key role in the cell-type specific regulation of the transcriptome. Application of rapidly evolving genetic tools, such as optogenetic/chemogenetic actuators and fluorescent reporters, to elucidate the function of cell subtypes in vivo necessitates cell-type specific promoters or enhancers. In this context, methods for genome-wide functional screening of cis-regulatory elements, including enhancers, are of utmost importance. In this study, we describe a novel method for genome-wide functional screening of enhancer activity in vivo with minimal handling. Application of the method to cells from different brain structures and subsequent differential analysis allow identification of active enhancers in the target tissue or brain structures. To demonstrate proof of concept, we applied this method to samples from the dorsal raphe nucleus (DRN) and the medial prefrontal cortex of the mouse brain and successfully identified six enhancers with highly biased activity towards the dorsal raphe nucleus. Considering that these two structures consist of largely similar cell types whereas serotonin and dopamine neurons exist only in the DRN, our results confirm the validity of this method in identifying cell-type specific and brain-structure specific enhancers. Overall, this method will be helpful in identifying cis-regulatory elements suitable for cell-type specific manipulations.

**Key words** enhancer; dorsal raphe nucleus; viral vector

INTRODUCTION

Transcriptional regulatory elements, including promoters and enhancers, play a key role in development, tissue homeostasis, and disease. Moreover, application of rapidly evolving genetic tools, such as optogenetic/chemogenetic actuators and fluorescent reporters, to elucidate the function of cell subtypes in vivo necessitates cell-type specific promoters or enhancers. Although an ample amount of Cre-driver rodents are being widely used for cell-type specific expression of transgenes, promoters are often active in multiple neuronal cell types, which hampers specific manipulations of the neurons-of-interest. To address this issue, recent studies have highlighted the critical role of cis-regulatory elements, including enhancers and repressors, in cell-type specific transgene expression. In this context, methods for the functional screening of enhancer activity on a massive scale have been developed. Massively parallel reporter assays utilize plasmids composed of a minimal promoter, green fluorescent protein (GFP) sequence, enhancer-of-interest, and barcode sequence, appended in this order in a 5’ to 3’ direction. This design allows the estimation of enhancer activity by quantifying the expression of the barcode sequence; however, the method is limited by the time and cost required to clone enhancer sequences conjugated with barcode sequences from millions of Escherichia (E.) coli colonies. Additionally, high-throughput next generation sequencing (NGS) of the libraries to relate each of the barcode sequences to enhancers is required. To circumvent NGS of libraries, self-transcribing active regulatory region sequencing (STARR-seq) utilizes a similar design without barcode sequences. However, obtaining a STARR-seq library necessitates high cell input and redundancies of inactive enhancers (a result of using DNA fragments from sonicated genomic DNA) cannot be prevented. In variants of STARR-seq including assay for transposase-accessible chromatin (ATAC)-STARR-seq and CapStarr-seq, putative regulatory regions are enriched by preferential tagmentation by Tn5 transposase (ATAC-STARR-seq) or capture of DNA fragments through hybridization on the custom-made microarray (CapStarr-seq). Nonetheless, these methods still require analysis of large cell input (tens of millions of cells) and their application has been limited to in vitro studies. Overall, a method for the functional screening of enhancers in vivo with minimal handling is still lacking.

In this study, we reported a method for genome-wide functional screening of enhancer activity in vivo. ATAC reaction was applied to cell nuclei prepared from the target brain region to enrich cis-regulatory elements active in the region-of-interest with minimal handling. PCR-amplified fragments were incorporated into reporter plasmid capable of direct packaging into AAV. RNA-seq analysis of reporter AAV expression and subsequent differential expression analysis between multiple brain nuclei made it possible to isolate enhancers highly active in the target brain nuclei (Diff-ATAC-STARR-seq). As a proof-of-concept, we applied Diff-ATAC-STARR-seq in samples from two brain structures, the dorsal raphe nucleus (DRN) and the medial prefrontal cortex (mPFC). The method allowed detection of six enhancers and discriminated between high activity in the DRN and minimal activity in the mPFC.
MATERIALS AND METHODS

Animals The experiments were conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee (Approval Codes: 13-41-2, 19-41-1, 23) and those of the Japanese Pharmacological Society. 8- to 12-week-old male and female C57BL/6J mice (Japan SLC, Shizuoka, Japan), maintained in our laboratory, were used in this study. The animals were housed in groups (no more than six mice per cage) in a plastic cage with wooden bedding and free access to food (MF, Oriental Yeast, Tokyo, Japan) and water. They were kept at a constant ambient temperature of 22 ± 2 °C under a 12:12 h light–dark cycle; stereotaxic surgeries were conducted during the light phase. Mice were randomly assigned to each experimental group.

Preparation of the AAV Library Recombinant Tn5 was prepared according to a previous report using plasmid encoding Tn5 (Addgene #60240). The details of Tn5 preparation are described in the Supplementary Methods. ATAC reaction of cell nuclei prepared from the mouse DRN was performed according to previous reports. The generated ATAC library was PCR-amplified and digested with BsaI (New England Biolabs, Ipswich, MA, U.S.A.). Purified fragments were ligated with a plasmid bearing inverted terminal repeats, a minimal promoter, Venus, the BsaI site, poly-A signal, and inverted terminal repeats, appended in this order by T4 ligase (TOYOBO, Osaka, Japan). Competent cells were transformed with ligation products and were subsequently cultured overnight in LB medium supplemented with antibiotics. AAV plasmids were purified from cultured cells.

Preparation and Stereotoxic Injection of AAV and Immunohistochemistry Preparation and stereotoxic injections of AAV and immunohistochemistry were performed as described previously. The detailed process is described in Supplementary Methods.

Quantification of Enhancer Activity After recovery of mice, tissue blocks containing the mPFC and DRN were harvested. Total RNA was purified from the tissue and treated with deoxyribonuclease (DNase). The resultant total RNA was then purified and used for reverse transcription with oligo dT primer (SuperScript IV, Thermofisher Scientific, Waltham, MA, U.S.A.). Determination of the optimal PCR cycle for library preparation was performed according to previous reports. The library was sequenced in the Illumina platform (Novaseq 6000, Illumina, San Diego, CA, U.S.A.). The detailed computational analysis of the resultant reads is described in Supplementary Methods.

RESULTS

Motif Analysis of the AAV Bearing ATAC-STARR-Seq Library Prepared from the DRN Although ATAC-STARR-seq can detect enhancer activity in open chromatin regions, differences in the amplification efficiency during library preparation can lead to apparent differences in enhancer activity. Moreover, analysis focused on a single brain region cannot discriminate ubiquitous but less active enhancer with enhancers highly active only in rare cell populations; consequently, ATAC-STARR-seq becomes suboptimal for the screening of cell-type specific enhancers. To overcome these limitations, we opted to focus on the differences in ATAC-STARR-seq signals between two distinct brain structures. The selected structures comprise largely similar cell populations; however, the presence of distinct cell subtypes exclusively in one of the structures, allowed us to identify highly active enhancers in these cell subtypes (Figs. 1A, B). As proof of concept, we focused our study on the mPFC and DRN. Both structures contain neurons that express glutamate and...
gamma-aminobutyric acid as well as glial cells, whereas serotonergic and dopaminergic neurons are present only in the DRN.21,22)

ATA C reaction was applied to the DRN cell nuclei preparation to enrich cis-regulatory elements active in this nucleus. The PCR-amplified ATA C library was cloned into BsaI sites of AAV plasmids bearing minimal promoter, Venus sequence, BsaI sites, and poly-A signal assembled in this order (Fig. 1A). To confirm enrichment of the cis-regulatory elements active in the DRN, we performed motif analysis of the cloned putative cis-regulatory sequences (Fig. 1C). Previous single-nuclei chromatin profiling of midbrain where the DRN exists has shown that motifs of cell-type-specific transcription factors, such as CTCF, Nfil3, and Rfx1, are enriched in putative cis-regulatory sequences from neurons of this brain region.23) Consistent with the earlier data, we found that motifs of CTCF (p = 1 × 10^{-69}), Nfil3 (p = 1 × 10^{-29}), and Rfx1 (p = 1 × 10^{-21}) were significantly enriched in the AAV plasmids (Fig. 1C).

ATAC-STARR-Seq Signals in mPFC and DRN Were Highly Similar Each AAV theoretically induces transcription of mRNA which consists of open reading frame of Venus, cloned candidate sequences, and poly-A. Expression level of resultant mRNA depends on enhancer potency of cloned candidate sequences. For this reason, occurrence of each of cloned candidate sequences in RNA-seq analysis can be considered as a proxy of its enhancer potency. Therefore, comparison of occurrence of each of cloned sequences in mPFC and DRN makes it possible to estimate bias of enhancer activity to one of these nuclei. Although efficiency of ATAC/PCR reaction (i.e., occurrence of cloned sequence in AAV library) naturally affects occurrence of cloned candidate sequences, the effect of ATAC/PCR reaction efficiency on estimation of enhancer potency can be also mitigated by comparison of the same AAV solution in two different brain regions. Purified AAV was injected into the mPFC and DRN of mouse brains. Four weeks after injection, total RNA was harvested from the target structures and reverse-transcribed into cDNA. Because enhancer sequences were flanked by adapter sequences for NGS in the Illumina platform, a sequence-ready library could be prepared by PCR amplification of cDNA with an index primer. The amplified library was purified and then sequenced. The optimal PCR cycles were determined by quantitative PCR through a process similar to that for library preparation for ATAC-seq.15,16) The clean reads were mapped to the mouse genome (Fig. 1D). Correlation analysis revealed that the resultant reads from the mPFC and DRN samples were highly similar (Pearson’s correlation coefficient; 0.443, p = 3.85 × 10^{-120}; 0.446, p = 9.77 × 10^{-77}; 0.751, p < 1 × 10^{-323}, for replicates 1, 2, and 3, respectively).

Differential Analysis of ATAC-STARR-Seq Signals Identified cis-Regulatory Elements Overrepresented in the DRN As expected from the design, bulk analysis of ATA C-STARR-seq signals in mPFC and DRN resulted in apparently similar patterns. Considering that serotonin and dopamine neurons are present only in the DRN,21,22) enhancers which are active in these cell populations will show higher expression in the DRN than that in the mPFC. Indeed, differential analysis of peak signals consistently identified six cis-regulatory elements which show more than 30-fold enrichment in DRN samples consistently in the three biological replicates (Fig. 2, Supplementary Table S1).

We examined whether identified enhancers are capable of transgene expression such as fluorescent proteins in the DRN cells especially serotonergic neurons. The identified enhancers were cloned into AAV backbone which is the same as one used for library preparation. Purified AAVs were injected into the mouse DRN. After recovery, we performed immunohistochemical analyses for Venus and Tph2, a marker for serotonergic neurons, in the DRN. We found that all six AAVs induced Venus expression in the DRN cells including serotonergic neurons (Fig. 3). These results indicate that identified enhancers have sufficient activity to drive transgene in the mouse
DISCUSSION

In the present study, we present a method for genome-wide functional screening of enhancers applicable for in vivo tissue. As proof of concept, we applied this to the two brain structures, the DRN and mPFC, and identified cis-regulatory elements showing highly biased activity towards the DRN. Although our method was tested in the mouse brain, it could theoretically be applied to any tissue and species capable of AAV infection.

Manipulation of specific cell types in multicellular organisms is fundamental to better understand the molecular mechanisms underlying function of cells-of-interest. The explosive growth of methods for single-cell analysis has revealed a spectrum of cellular heterogeneity. The intersectional approach using Cre and Flpo is widely used to manipulate newly identified cell types characterized by a combination of genes. However, this approach is difficult, although not impossible, to be applied to subpopulation characterized by more than three marker genes such as striatal medium spiny neurons characterized by the presence of Drd1a, Foxp1, and Dner and those characterized by Drd2, Synpr, and Cartpt. In these cases, using viral vectors bearing enhancers and methods for the functional screening of enhancers in vivo is crucial. More importantly, cell-type specific manipulation by viral vectors can be easily applied to non-human primates to demonstrate the role of cells-of-interest in complex functions.

Enrichment of active enhancers in the library is a critical step for the functional screening of enhancer activity. Here, we performed ATAC reaction on our samples to isolate and amplify putative cis-regulatory elements active in the target structures, according to a previous report; it has been demonstrated that binding of transcription factors to active enhancers induces nucleosomal eviction and results in hypersensitivity to ATAC reaction. Indeed, motif analysis of the screened enhancers revealed significant enrichment of binding motifs of transcription factors involved in the differentiation of midbrain neurons, including DRN neurons. Because we used AAV as a platform for ATAC-STARR-seq, it is possible that viral tropism to a certain brain cell types may lead to apparently biased expression. However, we and others have shown that AAV-DJ, which we used in this study, can efficiently infect a broad range of brain cells including serotonergic neurons, dopaminergic neurons, glutamatergic neurons, γ-aminobutyric acid (GABA)ergic neurons, and astrocytes, indicating that possible confounding of AAV-DJ tropism in the result is relatively small. We found that ATAC-STARR-seq signals in the DRN and mPFC samples were highly similar. This high similarity could be explained by the fact that these two brain regions consist of largely similar cell types, espe-
cially a large number of glial cells, leading to the similar po
tency of each enhancer as a whole. Indeed, we found neuron-
like morphology of Venus expression driven by six enhancers showing biased potency in the DRN, indicating that enhancers active in glial cells commonly present in both brain regions were effectively depleted by differential analyses of ATAC-
STARR-seq signals. We found that the identified six enhancer-
s induced Venus expression in DRN cells including seroto
nergic neurons (Fig. 3). Although these results were consistent
with ATAC-STARR-seq signals in the DRN, further investiga-
tion to reveal whether these AAVs induced minimal transgene
expression in the mouse mPFC is necessary to strengthen possible usefulness of identified enhancers. Although we
successfully detected several DRN-biased enhancers, further
enrichment of putative cis-regulatory elements active in se
rotoninergic and dopaminergic neurons could result in the
identification of more enhancers; in this context, fluorescence
activated cell sorting and subsequent ATAC reaction or cleav-
age under targets and tagmentation (CUT&Tag) for specific tran
scription factors or histone modifications should be con
sidered. Indeed, none of the identified enhancers were found
to be selective for serotonergic neurons, indicating that further
refinement of the method is required for identification of cell-
type specific enhancers by incorporating the above-mentioned
techniques.

In summary, we developed a method for the functional screen-
ing of enhancers in vivo and assessed its applicability to cell populations from two brain structures. Differential analy-
ysis successfully identified six enhancers active in the DRN with weak activity in the mPFC. This method has the po
tential to assist researchers in identifying cis-regulatory elements, a useful step for cell-type specific manipulations.

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Conflicts of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supple
mentary materials.

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