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Microdissection of different brain regions

Enhancer identification by differential ChIP-seq

EDGE-rAAV preparation

Injection of EDGE-rAAV
Enhancer-Driven Gene Expression (EDGE) enables the generation of viral vectors specific to neuronal subtypes.

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Summary

While a variety of remarkable molecular tools for studying neural circuits have recently been developed, the ability to deploy them in particular neuronal subtypes is limited by the fact that native promoters are almost never specific enough. We recently showed that one can generate transgenic mice with anatomical specificity surpassing that of native promoters by combining enhancers uniquely active in particular brain regions with a heterologous minimal promoter, an approach we call EDGE (Enhancer-Driven Gene Expression). Here we extend this strategy to the generation of viral (rAAV) vectors, showing that some EDGE rAAVs can recapitulate the specificity of the corresponding transgenic lines in wild-type animals, even of another species. This approach thus holds the promise of enabling circuit-specific manipulations in wild-type animals, not only enhancing our understanding of brain function, but perhaps one day even providing novel therapeutic avenues to approach disorders of the brain.
Introduction

The mammalian brain is the most complex biological structure known, with innumerable distinct cell types differing in cytoarchitecture, electrophysiological properties, gene expression and connectivity (Luo et al., 2008, Zeng and Sanes, 2017). Understanding brain function requires understanding neural circuits at the level of specificity at which they operate. Recent years have seen the development of truly revolutionary molecular tools that allow neuroscientists to elucidate precise neural connectivity (Callaway and Luo, 2015) and monitor (Chen et al., 2013) and manipulate (Roth, 2016, Sternson and Roth, 2014, Boyden et al., 2005) neural activity. However, optimal use of these tools to examine the functional circuitry of the brain requires the ability to deliver them specifically to particular elements of neural circuits (i.e. neuronal cell types), rather than as a nonspecific bolus affecting all of the neurons in a brain area. The use of molecular genetics is the only method by which one can perform truly cell-type specific manipulations, as evidenced by a variety of studies using transgenic animals expressing transgenes from neuronal promoters (genomic regions just upstream of the transcriptional start site) (Kanter et al., 2017, Miao et al., 2017). However, such approaches are limited by the fact that because individual genes are expressed in a variety of cell types in the brain, so promoters are not specific to a single neuronal cell type. While estimates vary (Consortium, 2012), there are at least an order of magnitude more cis-regulatory elements (i.e. enhancers and repressors, distal genomic regions which help regulate where and when promoters transcribe DNA) than promoters, suggesting that enhancers may be more specific. This led us to take an approach to the generation of molecular genetic tools that we call Enhancer-Driven Gene Expression (EDGE), based upon identifying the cis-regulatory elements uniquely active in particular brain
regions and combining them with a heterologous minimal promoter. When we used this strategy to make transgenic mice, they were indeed significantly more specific than the presumed parent gene, often driving expression primarily in particular sets of neurons in the brain region they were derived from (Blankvoort et al., 2018).

However, while transgenic animals are powerful tools for the analysis of neural circuits, they have limitations. They are costly in both time and resources, can be subject to insertional effects (Matthaei, 2007, Feng et al., 2000), and are most practical in a limited number of species. Moreover, while they are often excellent models of disease, transgenic technologies are far from therapeutic applications. Recombinant adeno-associated viral vectors (rAAVs) can overcome many of the above issues. They can be made relatively quickly, generally do not insert into the genome or replicate, and can be used in a variety of species (Watakabe et al., 2015) including humans and therefore have clinical potential as well (Bouard et al., 2009, Dias et al., 2017, Kotterman and Schaffer, 2014, Mendell et al., 2017). However, efforts to generate cell-specific viral vectors by capsid modifications (Koerber et al., 2008, Koerber et al., 2009, Klimczak et al., 2009) or using promoters (Delzor et al., 2012, Kugler et al., 2003, Shevtsova et al., 2005) have been largely unsuccessful to date to address a particular cell-type, with a few notable exceptions (Dimidschstein et al., 2016, Hartl et al., 2017), and even those are likely to have multiple subclasses. This is in large part because the relatively small payload size of rAAVs puts most native promoters out of reach. However, most enhancers are much smaller than promoters, raising the intriguing possibility of targeting specific neuronal cell types in any species by adapting EDGE to viral vectors, provided the background expression of the viral backbone and promoter can be minimized. Towards this end, we present results demonstrating enhancer-based viral
vectors which specifically express in particular neurons of the entorhinal cortex (EC) in two different species of wild-type animals.
Results

Optimization of rAAV design for Enhancer-Driven Gene Expression

Because one can obtain some degree of apparent specificity with rAAVs by means other than transcriptional regulation, we took steps to ensure that any observed specificity comes from the enhancer element used. Most notably, AAV serotypes exhibit distinct tropisms for different cell types: for instance, AAV8 is most efficient for oligodendrocytes and astrocytes (Aschauer et al., 2013, Hutson et al., 2012), AAV 1, 2, 5, 7, 8, 9 prefer neurons (During et al., 2003, Davidson et al., 2000, Aschauer et al., 2013, Castle et al., 2016) (though are by no means exclusive to them), while rAAV9 appears well suited for cortical neurons (Aschauer et al., 2013), and a variety of AAVs with engineered capsids show specific tropisms (Deverman et al., 2016, Tervo et al., 2016). We therefore used a single serotype (AAV2/1), with a wide tropism for neurons (Hauck et al., 2003) for the vast majority of our efforts towards engineering rAAVs transcriptionally specific to particular subtypes of neurons. We selected AAV 2/1, a chimera between capsid-1 (less efficient neuronal transduction(Castle et al., 2016)) and capsid-2 (vast tropism (Wang et al., 2003)), because of its broad transduction efficiency (Hauck et al., 2003) and to prepare viruses with high purity (During et al., 2003, McClure et al., 2011) via heparin columns (see Transparent methods).

Because injections of small volumes of rAAVs can appear specific because of the specific parcellation around the injection site, we used an medial entorhinal cortex (MEC) enhancer (MEC13-53) known to be specific to a particular subset neurons in the entorhinal cortex (Blankvoort et al., 2018) in transgenic animals so we knew what to look for. Figure 1A shows the expression pattern obtained from crossing one of the MEC13-53 tTA driver lines to a payload line expressing the helper transgenes for the ΔG-rabies monosynaptic tracing
system (Blankvoort et al., 2018). Expression in this cross was limited to Reelin-positive (RE+), Calbindin-negative (CB-) excitatory projection neurons in layer (LII) of the EC (Kitamura et al., 2014, Varga et al., 2010, Witter et al., 2017). Finally, we injected the same large (400 nl in mice, as opposed to the ~50 nl injections typically used with nonspecific rAAVs) volume of each virus into multiple animals using the same EC coordinates, and only compared green fluorescent protein (GFP)-expressing rAAVs of similar titer (see Table S1 and Transparent methods). For the purposes of comparison, Figure 1B shows the widespread strong expression throughout the various layers of the entorhinal cortex (as well as subiculum and parasubiculum) resulting from injecting a control AAV with a relatively (it has been shown to prefer neurons) nonspecific cytomegalovirus promoter (CMV-rAAV) of the same serotype and similar titer.

The initial step in obtaining viruses capable of driving expression as specific as the EDGE transgenic animals in wild-type brains is to find a minimal viral promoter which is capable of robust expression only when paired with a heterologous enhancer. This is complicated by the fact that the viral inverted terminal repeats (ITRs) themselves have transcriptional activity (Carter et al., 1993, Flotte et al., 1993, Haberman et al., 2000), as can be seen by the very weak (but still above autofluorescence) nonspecific expression obtained from a viral construct with neither a promoter nor an enhancer (Figure 1C). Note that the expression levels in 1C are far below those seen with the other viruses: each panel in Figure 1 has been differentially post-acquisition processed to aid visualization, the “background” expression seen in 1C would otherwise be imperceptible (see Figure S1 for comparison of each image with the same processing). To minimize this issue, we reversed the orientation of the expression cassette relative to the ITRs such that the sense strand was under the influence of the 3’ ITR, which we attenuated by putting WPRE (Zufferey et al., 1999) between the
3’ITR and the enhancer (see schematics in 1C, D). The substantial reduction in background expression enabled us to recapitulate MEC LII-specific expression in a wild-type mouse (Fig 1D) with a mutated minimal CMV promoter (CMV*)(Loew et al., 2010). Roughly similar results varying in amount and specificity were obtained with other minimal promoters (Figure S2) but we selected CMV* for all subsequent experiments (and hereafter simply refer to the enhancer) as it was the smallest one that worked well. The specificity of the expression of this virus as compared to a nonspecific CMV-rAAV virus is quantified in Figures 2. While still clearly far more specific than the CMV-rAAV, the quantification of MEC13-53 rAAV does not seem as specific as they look in the figure panels because in our counts we did not distinguish between weak “background” label (such as that seen in Figure 1C, S1 without a promoter) and the strong specific labelling (see below).

**MEC13-53 EDGE rAAVs express specifically in layer II stellate cells in wild-type mice and rats**

The neuron-specific stain NeuN (Boccara et al., 2015) confirms the robust LII-specific expression of the MEC13-53 rAAV (Fig S3A, S3C) in neurons (100% of labeled cells were NeuN+, data not shown). Weak, “background” GFP expression was observed in other layers as well in both this virus (Figure S3A, inset) and in the rAAV backbone (i.e. the same virus lacking the enhancer, Fig S3B, inset) which in contrast did not strongly label any cells. Within LII of MEC there are two major classes of excitatory principal neurons, RE+ stellate cells and CB+ pyramidal cells (Rowland et al., 2018, Witter et al., 2017), with RE label providing a sharp boundary between MEC and parasubiculum (Varga et al., 2010, Witter et al., 2017) (see arrows in Figure 2A, 2E inset). We therefore performed immunohistochemical analysis comparing these markers to viral GFP and found that for the MEC13-53 rAAV, 96% (2300/2406) of GFP+ cells in layer II were RE+ (Figure 2A, D), while 4% (74/1668) of them
were CB+ (Figure 2B, D). In contrast, for injections of roughly equal amounts of the ubiquitous CMV-rAAV, only 34% (319/929) of GFP+ LII cells were RE+ while 10.5% (142/1353) were CB+. Thus, the MEC13-53 rAAV drives transgene expression specifically in a particular subset of excitatory neurons in EC of wild-type mice, i.e. RE+ EC LII neurons (stellate cells in MEC), avoiding the adjacent CB+ pyramidal cells, like the transgenic lines based upon the same enhancer.

While this nicely illustrates the specificity of this EDGE rAAV, perhaps the greatest utility of EDGE rAAVs is that because enhancers are highly conserved (Cotney et al., 2013) and can be obtained from any tissue sample, they have the potential to work across species. As seen in Figure 2 E-G, S3C, S4C, the MEC13-53 rAAV derived from mouse EC is, if anything, more specific in the rat. Figure 2E, S3C shows GFP expression almost exclusively in MEC LII (as quantified in Figure 2G, S6B), while the few labelled neurons in the virus with no enhancer have no layer specificity (Figure S3D), just as in mouse (Figure S3B). Similarly, 100% (2332/2332) of MEC LII GFP+ neurons in rats injected with MEC13-53 rAAVs were RE+ (Figure 2E, H), while only 1.4% (25/1799) were CB+ (Figure 2F, H), even though the two excitatory subtypes are intermingled (Witter et al., 2017). This, and the presence of LII-specific label throughout the dorso-ventral and medio-lateral axes of the MEC (Figure S4C), provides compelling evidence for cellular specificity. Note that with the nonspecific CMV-rAAV, 35% (189/518) of GFP+ LII cells were RE+ while 46% (285/613) were CB+ (Figure S5). It is interesting to note that while these two markers are largely mutually exclusive, there are reports of a very small subpopulation of RE+ neurons that are also CB+ (Fuchs et al., 2016, Varga et al., 2010), so the single-digits label with the MEC13-53 virus may be those cells. Clearly, though, the two rAAVs with the same serotype have very different expression patterns, both in terms of layer and cellular specificity.
Systemic administration of blood-brain barrier crossing MEC13-53 EDGE recapitulates MEC layer II stellate cell expression

While we are mainly interested in developing tools to be used in analysis of the EC, it is interesting to ask whether this enhancer would express in other brain regions if it were systemically administered. We therefore packaged the MEC13-53 EDGE enhancer (shown with the 2/1 serotype in Figures 1 and 2) into the blood-brain barrier crossing PHP (Deverman et al., 2016) serotype and performed noninvasive intravenous injections via the tail vein. Systemic injections of MEC13-53 EDGE PHP resulted in much sparser GFP+ cells overall, but they are also mostly confined to layer II of MEC throughout the caudal forebrain (Figure 3A, S7). However, we also noticed sparse expression of the transgene in regions other than MEC, typically also in brain regions we would sometimes see transgene expression in MEC13-53 transgenic lines (Figure 3B, C, Table S2). Curiously, we did not see expression in LII of the piriform cortex, the major site of non-EC expression in the MEC13-53 transgenic lines, possibly due to the particular tropism of the PHP capsid. Furthermore, we confirmed that these GFP+ cells in MEC are RE+ (Figure 3D). These results suggest that EDGE rAAV can retain its particular cell-type specificity, even when assembled in a serotype with a different innate tropism.

EDGE rAAVs recapitulate the expression pattern of their respective transgenic lines

To examine whether this is a general strategy, we created EDGE rAAVs with several other enhancers with known specificity (Blankvoort et al., 2018). While not all enhancers that worked as transgenic lines worked in rAAVs, roughly half (Figure 4, left column) did indeed appear to recapitulate the specificity (or relative lack thereof, 4A, B) of the corresponding EDGE lines (Figure 4, right column). The MEC13-104 rAAV (Fig 4A) recapitulates the
relatively sparse labeling of a subset of LIII neurons (arrows) seen in the MEC13-104 line (Figure 4B), while the converse is true for the mainly LIII-specific LEC13-8 (compare 4C to 4D) line. Thus, the relative densities of the layer-specific label appear to be enhancer-specific, suggesting that the minority of cells which strongly express outside of their primary layer may not be “noise”. Ongoing experiments explore the functional distinctions between the cells labeled by the various enhancers, which may label distinct subsets of what has been considered a single neuronal cell type, e.g. stellate cells.
Discussion

Our prior work showed that identification of *cis*-regulatory elements uniquely active in finely dissected cortical subregions allows one to generate genetic tools specific to cells in that subregion, an approach we call EDGE (Blankvoort et al., 2018). Here we show that one can use the same approach to make rAAVs with similar specificity in both mouse and rat, provided the vector and minimal promoter’s innate transcriptional activity is minimized.

This clearly cross-validates the initial identification of enhancers in our prior work (Blankvoort et al., 2018): while transgenic lines might show highly specific expression patterns purely due to insertional effects (though not the same pattern in multiple founders, as we saw), rAAVs typically do not insert into the genome (McCarty et al., 2004), so cannot show such effects. In other words, while the precise functional significance of the enhancers presented here remains unknown, they clearly are “true” enhancers, reflecting some genetic subgroup of excitatory neurons in the entorhinal cortex of wild-type mice and rats.

Taken together, these data lead to two very interesting conclusions: 1) given that the numbers of enhancers may run into the millions (as opposed to ~44,000 promoters) (Consortium, 2012), they may provide access to the ever-growing number of neuronal cell types than promoters, which may be far greater than generally assumed (Zeisel et al., 2015, Cembrowski et al., 2016, Tasic et al., 2018, Saunders et al., 2018); and 2) although we do not do so here, one could conceivably take this approach towards generating neuronal subtype-specific transgene expression in species other than the traditional genetic models of mouse, zebrafish, fly, and worm, because one can do the required epigenomic analyses on any tissue sample.
There has been a lot of effort over the years towards making celltype-specific viral vectors, but even in those cases when a minimal native promoter is useful (i.e. when a single marker defines the cells, e.g. TH-AAV (Gompf et al., 2015) and CaMKII-AAV (Nathanson et al., 2009) the AAVs are not fully restricted to cells expressing the gene. We have previously shown that using single, uniquely-active enhancers can lead to far greater specificity than that of native parent promoters (Blankvoort et al., 2018), at least in transgenesis. That enhancers drive expression similarly in both transgenic lines and viruses is not a particularly surprising result. It has been known for decades that enhancers drive cell-specific expression (Grosveld et al., 1987, Noonan and McCallion, 2010, Shen et al., 2016) in a variety of species. Enhancers for the 6 homeobox genes related to the fly \textit{distal-less} gene (Cohen and Jurgens, 1989) (\textit{Dll} in fly, Dlx in vertebrates) have been shown to play a crucial role in morphogenesis in many species (Anderson et al., 1997, Ghanem et al., 2003, Miyoshi et al., 2010, Panganiban and Rubenstein, 2002, Zerucha et al., 2000). One such enhancer in the Dlx 5/6 gene cluster has been shown to be critical to the development of interneurons in particular (Stenman et al., 2003), and a recent paper (Dimidschstein et al., 2016) used this enhancer element in a viral vector to obtain interneuron-specific expression in a variety of species, nicely showing that enhancers can be used to drive expression in viral vectors. However, as is true for most genetically-defined enhancers active early in development, Dlx5/6 drives expression across broad classes of neurons (e.g. interneurons in general) throughout the brain, rather than to particular interneuronal subclasses and/or subregions. More recently, several groups have begun to incorporate \textit{cis-regulatory} elements into their strategies for creating viral vectors specific to neuronal subtypes. Such efforts are likely furthest along in the retina, where Juttner and colleagues (2019) created a broad rAAV resource targeting subtypes of retinal neurons using strategies based upon genes of interest.
(GOIs) identified in *a priori* transcriptomal analysis (Siegert et al., 2012) and epigenetic analysis (Hartl et al., 2017) of known retinal cell types. While most of these constructs are simply the minimal promoters of the GOIs, some also are based upon the local epigenetic landscape, using such strategies as conservation, methylation patterns, and transcription factor binding sites to identify likely cis-elements for GOIs. While the results in retina can be quite impressive, little is known how specific such vectors would be in the rest of the brain.

As for the brain, Hrvatin et al. (2019) recently published an interesting screening strategy called PESCA (Paralleled Enhancer Single Cell Assay), in which multiple rAAVs containing barcoded putative enhancers (they use the term Gene Regulatory Elements, or GREs) are screened via single-cell transcriptomics (scRNAseq) rather than the more traditional one-at-a-time anatomical techniques shown here. While scRNAseq does not always reflect actual viral expression, this technique nevertheless promises to greatly increase the throughput involved in first-pass screening of rAAVs. In a very interesting study, Graybuck and colleagues compare scRNAseq data to the epigenetic single-cell Assay for Transposase-Accessible Chromatin with Sequencing (scATACseq) data from layer-specific transgenic mice. Hits that co-register in both the transcriptomic and epigenetic clusters are then cloned into PHP.B Cre-rAAVs and systemically (retro-orbitally) injected into a Cre-reporter mouse for anatomical characterization.

The overwhelming similarity of these various approaches is the idea that individual cis-regulatory elements may be more specific than promoters. Each strategy has two stages: identifying likely cis-regulatory elements, and then making and screening the resulting rAAVs. The major difference clearly comes at the identification stage: each of these other GRE (as opposed to promoter) -based approaches have been based upon *a priori* knowledge of the transcriptomics of whatever cell type one is looking for, often even taking advantage...
of transgenic animals, while EDGE simply looks for regionally-specific chromatin marks in reproducibly-dissected bulk tissue. The advantage of the former is resolution: by a “deep dive” into subtypes of what we had originally thought were cell types, one both gets at the scale of neuronal diversity and immediately puts the cell types in context, whereas with pure differential screens of bulk tissue such as EDGE you really do not know what cell types you will get, you just know that they are more or less specific to your tissue of interest. However, the flexibility (one simply needs ChIP of an ROI), ease of doing EDGE in other species, and ability to discover truly new cell types, counterbalance this disadvantage. A more purely technical difference is between bulk ChIPseq and ATACseq. While the latter can be done with much less tissue (even single cells), the former’s use of particular histone marks may provide greater specificity for active enhancers rather than other forms of open chromatin. At the screening level, systemic viral injections (Graybuck, 2019) with AAV serotypes which cross the blood-brain-barrier clearly give you the best idea of where a particular enhancer can express throughout the brain. We regret that we are as of yet unable to obtain permission to perform retro-orbital AAV injections from our local regulators, so our systemic injections were with a less effective technique (tail vein), lowering the effective titer. If PESCA (Hrvatin et al., 2019) can reliably be done on bulk tissue, however, it may end up as a better screen for our purposes. All in all, there are advantages to each approach which make them largely complementary, suggesting that combinations of these techniques, and comparisons between the resulting datasets (ChIP vs. ATAC, bulk vs. single cell) may well end up being the best overall approach.

Thus, the most important aspect of these and other papers is not that enhancers can work in viral vectors, it is illustrating the promise of applying modern genomic techniques to the study of the precise neural circuitry of the vertebrate brain. The striking diversity of
enhancers found in these tiny subregions of cortex (numbers comparable to those found for entire organs) may indicate a similar diversity of neuronal cell types in the brain. However, the relationship between enhancers and cell types remains unclear. Indeed, the expression patterns we obtain are arguably more specific than our current understanding of neuronal cell type (Zeng and Sanes, 2017, Luo et al., 2008). For instance, stellate cells are a generally accepted excitatory neuronal cell type of the medial entorhinal cortex (Rowland et al., 2016, Varga et al., 2010, Witter et al., 2017). However, we show that distinct enhancers drive expression in EC LI stellate cells to different degrees in both transgenics and rAAVs. The question becomes whether these enhancer-driven expression patterns reflect functionally distinct stellate cells, or states of stellate cells, or just random subsets of the same indivisible cell type. In the specific case of stellate cells, a recent paper used optogenetic tagging to show that stellate cells of the MEC exhibit a variety of quite distinct receptive field properties (i.e. they can be grid cells or spatial cells or border cells, etc), suggesting that there are many functional subtypes of stellate cells (Rowland et al., 2018). More generally, the relationship between differential enhancer usage and neuronal cell types is a highly non-trivial question, not least because there is not even complete agreement even as to how to define neuronal cell types (though there are notable exceptions) (Tremblay et al., 2016, Tasic et al., 2018, Cembrowski et al., 2016), let alone how many there are. There are several other interesting explanations for differential enhancer usage beyond cell type, for instance it could dictate distinct states of a single cell type. In support of this, neural activity drastically changes the chromatin landscape of the brain, including which enhancers are active (Gallegos et al., 2018, Malik et al., 2014). It will likely take years of anatomical, molecular, and physiological characterization of these tools to disentangle such questions, so for our current purposes the most important consideration is that these enhancer-based
molecular genetic tools remain true to type, as appears to largely be the case, comparing
the virus to the transgenic.

It should be noted, however, that specificity is almost never absolute, especially with viral
vectors. While we obtain neuronal subtype-specific results with large injections into the
entorhinal cortex (Figures 2, S4), it is likely that any cell type in other brain regions which
express the transcription factor(s) appropriate for a particular enhancer would be labeled as
well, as can be seen with the systemic injections shown in Figure 3. Thus, we do not claim
that the rAAVs shown here are necessarily 100% regionally specific—indeed, it is hard to
imagine that a particular enhancer is only used once in development. Rather, we
demonstrate clear cell-type specificity when the MEC13-53 rAAV is injected into a particular
brain region, which is nevertheless good enough for the study of neural circuitry. Moreover,
many more cells are infected than show strong GFP label, and there is a baseline level of
transcription from other elements in the viral construct (i.e. the minimal promoter and the
ITRs). This implies that superfection of enough rAAVs could lead to discernible nonspecific
transgene expression in any cell regardless of the promoter, something that is shown most
clearly by making viruses containing no exogenous promoter whatsoever (Figure 1C). Viral
expression is thus not all-or-nothing, but the difference between background and enhancer-
driven expression levels can be quite marked (Figure S1). This background expression
inherent to rAAVs can be quite problematic when a little bit of expression can have a large
effect. This is true when expressing enzymes such as recombinases or when complementing
replication-competent viruses (e.g. pseudotyped ΔG-rabies (Weible et al., 2010)), but is
likely not an issue with transgenes whose effects vary roughly linearly with their expression
levels, such as the chemogenetic (Sternson and Roth, 2014) and/or optogenetic tools
(Boyden et al., 2005) used to study neural circuits.
Thus, identification of the active enhancers of a mere four cortical subregions of the mouse brain has led to a variety of transgenic, and now viral tools for circuit analysis that appear to work across species, at least in rodents. Since in principle, one can do this on any reasonably well-annotated genome, one could conceivably develop tools for anatomically specific “circuit-breaking” tools in any species, even our own. Thus, not only will circuit-specific tools greatly facilitate our understanding of normal and pathological brain function, they could in time possibly provide circuit-specific therapeutic avenues. For example, it has been known for decades that preclinical stages of Alzheimer’s disease (AD) are characterized by neuronal loss and accumulation of neurofibrillary tangles in the superficial layers of trans-entorhinal cortex (Braak and Braak, 1991), a region roughly equivalent to rodent MEC layer II. In addition, intracellular amyloid-β is found specifically in MEC layer II RE+ neurons in human AD pathology and rodent disease models (Kobro-Flatmoen et al., 2016). Given the emerging consensus that AD may progress trans-synaptically (Spires-Jones and Hyman, 2014, de Calignon et al., 2012), it is conceivable that one could use something like a MEC13-53 rAAV to deliver therapeutic agents directly to the presumed pre-α cells, thereby stopping AD before it starts. More generally, it is possible that the reason that many neurological and neuropsychiatric disorders are resistant to drug therapy is that they are imbalances in particular neural circuits, not diseases of the entire brain. A drug having tropism for multiple circuits (as most do) would then by definition produce unwanted side effects: it may do the right thing in the right circuit, but it does the wrong thing to normal circuits. Results like those presented here allow the hope that investigators may one day be able to design interventions with the specificity required to treat the complex diseases of the brain.
Limitations of the study.

While we think that we have made a substantive contribution towards the generation of circuit-specific tools that could be used outside of traditional genetic models, we freely acknowledge the limitations of our data. While it is indeed true that active enhancers can be identified in any tissue sample of reasonable size from any species and used to make EDGE-rAAVs in ways similar to presented here, we have only showed the same specificity for stellate cells in two rodent species-larger animals such as primates pose significant challenges with viral vectors. In addition, while we can see remarkable cellular specificity when EDGE rAAVs are injected into the region they were designed for, systemic administration suggests that the enhancer may also express in other cell types if injected in other regions. Regardless, we feel that these are quite useful tools for the analysis of neural circuits.
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Author contributions

C.K., R.R.N. and S.B. conceptualized the study. R.R.N did the data curation. Formal analyses were done by R.R.N., M.J.L. and S.B. C.K did the funding acquisition. Investigation by R.R.N., M.J.L. and S.B. Methodology by R.R.N., S.B. and C.K. Project administration and supervision were done by C.K. and R.R.N. Resources for the study were from R.R.N., M.J.L. and S.B. Validation by R.R.N., S.B., M.J.L., C.K. Original draft was prepared by R.R.N. and C.K. Review and editing by C.K., R.R.N., S.B. and M.J.L.
Declaration of Interests

C.K., S.B., and R.R.N. are inventors on US Patent Application no. 62/584,282, Appl. Norwegian University of Science and Technology (NTNU), which is related to this work. The authors have no other competing interests to declare.
**Legends**

**Figure 1. Optimization of rAAV constructs for Enhancer-Dependent Gene Expression.** (A) Transgene expression in a MEC13-53 tTA X tetO-TVAG transgenic cross visualized by anti-2A immunostaining is restricted to RE+ LII projection neurons in EC (Blankvoort et al., 2018). Since this is a different antibody, this is purely a qualitative comparison. (B) Injection of a nonspecific (CMV-rAAV) virus into the EC shows broad label throughout the entire region, including all layers of EC, as well as subiculum (S) and parasubiculum (PaS); (C) The same construct without a minimal promoter shows weak nonspecific expression throughout the region which would not be visible at normal image settings (see Figure S1). (D) Changing the orientation of the expression cassette leads to a marked reduction in nonspecific expression of MEC13-53 rAAV (see inset in C and D, note that most of the LIII label in D is not cellular, unlike C, and when it is, it is very light, i.e. from baseline transcription). All murine injections were 400nl. NB: images were differentially modified to best visualize the GFP expression pattern in each panel, comparisons of these images with the same post-acquisition settings are shown in Figure S1 (see Transparent methods). Note that all label above background auto-fluorescence was treated as positive, even though there were two markedly distinct intensities of label. See also related supplemental Figures S1 and S2. Schematics of the viral designs are depicted on top of the corresponding image. Inverted terminal repeat (ITR), woodchuck hepatitis virus post-transcriptional regulatory element (W), human growth hormone polyadenylation signal (pA), enhancer (E), Green fluorescent protein (G), cytomegalovirus promoter (C), mutated minimal cytomegalovirus promoter (CMV*). Scale bar = 100 µm.
Figure 2. MEC13-53 EDGE rAAVs recapitulates the cell-type specificity seen in the MEC13-53 EDGE transgenic crosses in WT mice and rats. Equal amount of MEC13-53 rAAV was injected into MEC of wild-type mice (A-D) and rats (E-H). Insets show anti-GFP (top); marker (middle); and overlay (bottom) of box in main panel. Sections of MEC13-53 rAAV injections counterstained with anti-RE antibody (red, A, E) and anti-CB antibody (red, B, F); with a CB+ cluster (asterisks) in the insets in (B, F). Note the extensive co-localization of the RE stain with the GFP, the sharp delineation of the entorhinal/parasubicular boundary by both labels (arrows, A, E), and the exclusion of viral label from the CB clusters (asterisks, B, F). (C) Proportion of GFP expressing cells in different parahippocampal regions for both MEC13-53 and nonspecific CMV-rAAV, Each point is a section, note the large number of sections where 100% of the cells are in LII and 0% in other regions exclusively in the MEC13-53 rAAVs compared to the controls (for pictures of control injections see Figures 1B and S4, S5). 13096 and 8540 GFP+ cells were counted from 3 mice injected with CMV-rAAV and 7 mice with MEC13-53 rAAV respectively, data represented as mean ± SEM. In G), 7191 and 2831 GFP+ cells from sections were counted from MEC13-53 rAAV and CMV-rAAV respectively from 3 rats. Quantitation of results are shown in D (for mice) and H (for rats), showing overlap of GFP with cell-marker Reelin stain (green) in LII MEC of mice (96%) and rats (complete overlap). 4% overlap of GFP with Calbindin (red) was observed in mice and <2% overlap in rats, with number of cells counted in MECLII region). MEC-LII GFP+ cells were counted from separate RE and CB immunostained sections from 7 mice and 3 rats injected with MEC13-53 rAAV, data represented as mean ± SEM. See also related supplemental Figures S3-S6. Scale bar = 100 µm, all images processed identically.
Figure 3. Recapitulation of LII MEC specificity of MEC13-53 using a BBB-crossing rAAV serotype. (A) Representative image of the GFP+ neurons in horizontal brain section from a mouse injected with $10^{12}$ particles of MEC13-53 rAAV PHP, intravenously into tail vein. The boxes in A are zoomed in panel B. (C) MEC13-53 transgene expression in same regions as in B, are in the MEC13-53 tTA X tetO-TVAG transgenic cross. (D) Sections of MEC13-53 rAAV PHP injected brain counterstained with anti-RE antibody. Insets show anti-GFP (top); Reelin (middle); and overlay (bottom) of box in main panel. Label is throughout the layers of EC and sparsely in other regions (arrow heads, C). Note the extensive co-localization of the RE stain with the GFP (arrows). See also Figure S7. Scale bar = 100 µm. See also related supplemental Table S2.

Figure 4. EDGE rAAVs recapitulate the distinct layer-specific expression patterns seen in EDGE transgenic mice. Comparison of expression patterns obtained by injection of EDGE rAAVs (left column) with those seen in transgenic crosses made with the same enhancers (right column). Wild-type mice were injected with 400 nl of EDGE rAAVs (A) MEC13-104 and (C) LEC13-8. Transgene expression in the corresponding EDGE transgenic crosses (B, MEC13-104 tTA X tetO-TVAG) and (D, LEC13-8 tTA X tetO-HM3) visualized by ISH on horizontal sections using the respective transgene probes. The sparse expression of the transgene in minor layers is indicated by arrows both in EDGE transgenics and viruses. Scale bar = 100 µm, all sections are horizontal, and all rAAV figures underwent the same image processing. See also related supplemental Table S1 and Data S1.
Supplemental Table and Data legends

Table S1. Titers of the important rAAVs synthesized for the current study (related to Figures 1, 2 and 4). Quantitative PCR was carried out for the titration of the rAAVs. See the Transparent methods sections for detailed protocol.

Table S2. Expression pattern of the rAAVs utilized in the current study (related to Figures 1, 2, 3 and 4). Transgene expression pattern of important rAAVs, driven by various enhancers and promoters are shown in the table.

Data S1. Sequences of the minimal promoters, enhancers and EDGE-rAAVs used in the study (related to figures 1, 2, 3 and 4). (A). Several known minimal promoters were analysed while designing EDGE-rAAVs. mCMV* was the minimal promoter chosen for EDGE-rAAVs due to its smaller size and low nonspecific expression. mCMV* is a variant of CMV promoter (derived from the sequence of pTRE3G, Clontech, USA) (Loew et al., 2010), mTK was taken from HSV-TK (sequence from NEB, USA), mFGF4 was taken fibroblast growth factor 4 (FGF4) (Murtha et al., 2014) and mHSP68 was from Blankvoort et al., 2018, Cotney et al., 2013. (B). Full sequences of the rAAV constructs used. The transcription cassette of EDGE-rAAVs and enhancerless rAAV are in the reverse orientation relative to the ITRs. For the CMV-GFP and promoterless rAAV the orientation was forward with respect to the ITRs. See Transparent methods for cloning details. (C). Murine enhancers obtained from our initial enhancer screen (Blankvoort et al., 2018) such as MEC-13-53, MEC-13-104 or LEC-13-8 were used for creating EDGE-rAAVs.
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Highlights

- rAAVs with enhancers unique to a brain region specify cell types of that brain region
- This requires viral constructs optimized to express only with enhancers
- One rAAV distinguish distinct subtypes of excitatory neurons in a cortical layer
- The same specificity is seen in wild-type animals of at least 2 species