Norwegian University of Science and Technology (NTNU)
Kavli Institute for Systems Neuroscience

Enhanced lentiviral vectors

A comparison of enhancer driven expression of transgenes transferred by pronuclear injection or lentiviral vectors

Master's Thesis in Neuroscience
Faculty of Medicine, Department of Neuroscience

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Abstract

The brain is a vast network of different types of neurons. Interactions between these different types of neurons underlie the different kinds of neural computations the brain performs. How the different types of neurons are connected and interact is largely unexplored. However, transgenic mice allow for investigation of individual cell types in this heterogeneous network. By expressing fluorescent proteins in transgenic mice, the morphology of individual cell types can be visualized. Furthermore modified rabies viruses allow for the identification of monosynaptic inputs of specific cell types. Finally, novel techniques such as optogenetics and pharmacogenetics give control over the activity of genetically labeled neurons. In short, novel transgenic methods allow for the exploration of the brain and behavior on a cellular level.

However, these transgenic methods stand or fall by having genetic access to specific cell types. Current transgenic lines do not provide us with the specificity to get enough resolution. To increase resolution, we are using regulatory elements in the genome called enhancers to drive transgenes in specific brain regions. Transgenic lines created with enhancers have regional and cell type specificity. An alternative method to introduce the transgene to the brain is by viral vectors. In this thesis we investigate if enhancers transferred by lentiviral vectors may also show cell type specificity. The aim of this project is to compare the enhancer driven transgene expression in mice injected with lentivirus and in transgenic mice having same enhancer.

To achieve this aim, 3 enhancers which drive expression in the medial entorhinal cortex (MEC) of transgenic mice were cloned into plasmids that allow for the production of lentiviral vectors. The lentiviral vectors were stereotactically injected to the MEC of adult mice. We investigated the expression of the virally transduced transgene in comparison to the transgene expression in the transgenic mice. We did this based on anatomical location of the expression and co-expression of molecular markers.

We confirmed that in the transgenic mouse lines expression was confined to specific layers of the MEC. In contrast, in virus injected mice, the transgenes were expressed throughout layers. This suggests that enhancers did not have any specificity after gene transfer by lentiviral vectors. The molecular marker we investigated, calbindin, is not expressed in the transgene expressing cells of the transgenic lines. Neither did we find it in the cells that were transduced with the transgene by injection of viral vector. Though this may indicate specificity of the transgenic expression after transfection with the viral vector, it should be taken with care considering the unexpected staining for calbindin in the virus injected mice.

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Abbreviations

ACC	Anterior Cingulate Cortex
BAC	Bacterial Artificial Chromosome
Bp	Base pair
CB	Calbindin
ChIPseq	Chromatin Immunoprecipitation Sequencing
DAPI	4', 6-Diamidino-2-Phenylindole
GAD67	Glutamate decarboxylase 67
GFP	Green fluorescent protein
H3K27ac	Histone H3 acetylated at lysine 27
H3K4me1	Histone H3 monomethylated at lysine 4
HSP68	Heat Shock Protein 68
LEC	Lateral entorhinal cortex
LMO3	LIM domain only 3
LTRs	Long Terminal Repeats
LV	Lentivirus
MEC	Medial entorhinal cortex
NeuN	Neuronal nuclear antigen
ODZ3	Oz/ten-m homolog 3 (Drosophila)
PARA	Para subiculum
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PRE	Pre subiculum
PV	Parvalbumin
PVA-DABCO	Polyvinyl alcohol with DABCO (1, 4-diazabicyclo [2.2.2] octane)
TG	Transgenic
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloric acid
TRPS1	Trichorhinophalangeal syndrome 1
tTA	tetracycline-controlled transactivator
VIP	Vasoactive Intestinal Peptide
VSVg	Vesicular Stomatitis Virus glycoprotein
WPRE	Woodchuck Posttranscriptional Regulatory element

1 Introduction

The different types of neurons that are the elementary components of brain circuits are distinct in their morphology, physiological properties, synaptic connections, gene expression and developmental history. Before the introduction of transgenic methods, manipulations of brain circuits were carried out through surgical lesions, injections of pharmacological agents, and electrical stimulations (Lykken and Kentros, 2014) which are regional rather than cell type specific. The advent of transgenic tools make it possible to investigate and access specific cell types in a heterogeneous network. In transgenic mice, the regulation of gene expression in neurons can be used to resolve neuronal subtypes (Doyle et al., 2008). Using complementary methods morphology, connectivity and functionality of these genetically labeled neuronal subtypes can be achieved. The morphology of individual cell types can be investigated by selectively expressing fluorescent protein in transgenic mice where a subset of neurons is labeled (Feng et al., 2000). Modified rabies virus can be used to identify the monosynaptic pathway to specific cell types (Wickersham et al., 2007). Finally, novel techniques like pharmacogenetics and optogenetics have made it possible to control the activity of genetically labelled neuron (Boyden et al., 2005). The combination of cell type specificity and these novel techniques gives us insight to the cellular basis of other complex brain action.

The neuronal diversity is partly because of gene regulation guided by cis regulatory elements such as promoters and enhancers (Bird, 2002; Heintzman et al., 2007; Thurman et al., 2012). Large efforts have been made to create transgenic lines that are capable of cell type specificity. Two very common types of transgenic mice are bacterial artificial chromosome (BAC) transgenic lines and knock-in lines. In the case of creation of a BAC transgenic line, the BAC (a sequence of DNA of 150-250kbp of a mouse genome) is modified with a transgene instead of a native gene of interest (often the gene is considered of interest because of expression in a specific cell type (Heintz, 2001). The modified BAC is randomly inserted to the mouse genome by pronuclear injection. The expression of the transgene is controlled by the regulatory elements in the surrounding DNA from the BAC and from the native genome (the latter being an insertional effect). The combination of these regulatory elements give an expression profile that may give access to certain cell types. Another way to create transgenic lines is by creating knock-in mice by homologous recombination. In this case a native gene is replaced by a transgene in the mouse genome (Capecchi, 1989). Though combinatorial approaches are underway (Huang, 2014), knock-in lines generally do not allow for more specific expression than the native gene.

The limited cell type specificity of current transgenic mouse lines limits the level to which it is possible dissect a neuronal network on a cellular level. This means there is a need for a new generation of mouse lines that express transgenes in more specifically defined subtypes of neurons. Novel methods allow for the identification of individual regulatory elements such as enhancers. In the bigger context of this thesis we test the viability of using enhancers to drive expression in specific neuronal subtypes. Initial success have shown that it is possible to drive transgenes in the MEC of transgenic mice, using enhancers that are specifically active in the MEC of wild type mice.

1.1 Enhancers

Gene regulation is a tightly regulated process (Maniatis et al., 1987). The genome contains many regulatory sequences known as transcriptional enhancers that may activate gene promoters at the right time in the right cells (Visel et al., 2009). Enhancers provide a platform for binding transcription factor and promoter. Transcription factors can activate the transcription process only when the coactivator proteins are recruited (Weake and Workman, 2010). Enhancer's involved in particular biological functions are difficult to identify as they lie in noncoding and have no protein or RNA product. They are located up to hundreds of thousands of base pairs away from the promoter of the genes they regulate. Despite this large distance, enhancers may regulate the promoter, through the mechanism of DNA looping (Ptashne, 1986). Different enhancers may drive expression of the same gene in different cell types (Figure 1). The same gene can be expressed in different tissues at different time. Enhancers have been extensively studied in the context of development (Cotney et al., 2013; Pattabiraman et al., 2014; Visel et al., 2013). Recent evidence in cell cultures of different cell types (excitatory, PV+ and VIP+ neurons) has revealed the differences in DNA methylation and chromatin accessibility, suggesting enhancers may also play a role in gene regulation in the adult brain (Henikoff, 2015)

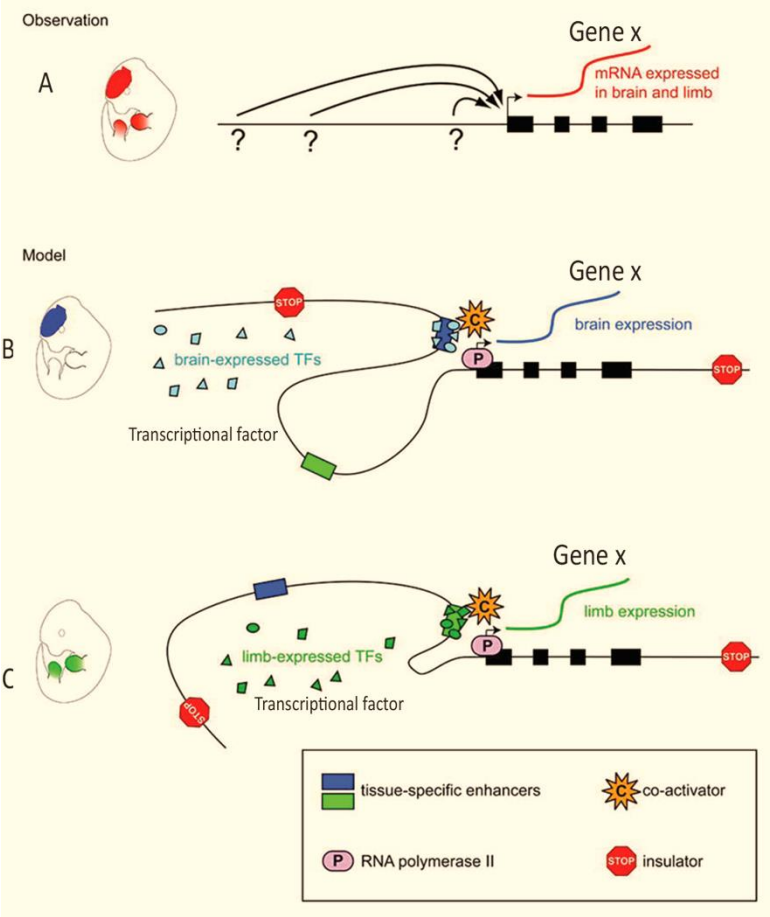


Figure 1 Model of gene regulation by enhancers. (A) Gene X is expressed in the brain and the limb. (B) Binding of blue box (enhancer) drives the expression in the brain. (C) Binding of green box (enhancer) to the promoter region drives expression in the limb. Figure adopted from (Visel et al., 2009).

1.1.1 Chromatin and ChIP seq

Though enhancers are hard to detect recently developed techniques like chromatin immunoprecipitation sequencing (ChIP seq) have made it possible to identify enhancers in specific tissue or cell populations (Cotney et al., 2013; Johnson et al., 2007; Kidder et al., 2011). Genomic DNA is condensed into chromatin of which nucleosome is the fundamental unit. Nucleosomes consist of four core histone (H3, H4, H2A and H2B) with the DNA wrapped around them. Enhancers may exist in different states, an active state or a poised ('controlled') state, these two states are accompanied by different histone signatures (Creyghton MP1, 2010). Active enhancers are usually flanked by histone H3 that is acetylated at lysine27 (H3K27ac) and H3 that is mono-methylated at lysine 4 (H3K4me1) (Heintzman et al., 2007). Poised enhancer on the other hand are associated with only H3k4me1 (Rada-Iglesias et al., 2011).

ChIP seq is a commonly used technique to identify protein-DNA interactions. First protein-DNA complexes with specific modifications are separated from the rest of the complexes. Then this is

combined with massive parallel sequencing to identify the DNA sequences associated with those protein modifications. In our case we utilize the histone H3K27ac and enhancers sequence interaction. By isolating H3K27ac and enhancer region we find the sequence of active enhancer in the brain tissue. We did ChIP seq on different brain tissues to identify brain region specific enhancers. Then we used these enhancers to create transgenic mice with region specific expression of transgenes.

1.2 Expression of enhancer driven transgenes

A gene or other genetic material that is introduced into organism through genetic manipulation is known as a transgene. The transgene may have the potential to change the phenotype of the organism. In this thesis two methods have been used to transfer transgenes: one is through the pronuclear injection into mouse oocytes and other is through a viral vector mediated method.

1.2.1 Transgenic mice

The first method to introduce the transgene is through pronuclear injection. In this process a DNA construct is injected to the nucleus of a fertilized egg cell. The injected egg is transferred into the oviducts of a pseudo pregnant foster mouse. A fraction of the resulting pups have the DNA construct randomly integrated into their genome. In our case the DNA construct consists of the putative enhancer followed by the elements form the destination vector, a Heat shock protein 68(HSP68) minimal promoter, a synthetic intron, a tetracycline transactivator (tTA) gene, an SV40 intron, a woodchuck hepatitis virus post transcriptional regulatory element (WPRE), a human growth hormone intron 5 and a polyA signal.

Expression of ‘payload’ transgenes through these ‘driver’ tTA lines is based on the tTA-tetO system. tTA-tetO is an inducible gene expression system in which the transcription is regulated in presence or absence of tetracycline or its derivative doxycycline. tTA transactivator is capable of driving expression of any transgene under the control of tetO promoter element. In tTA-tetO system, a hybrid bacterial-viral transactivator (tTA) binds with a hybrid tetracycline responsive element (TRE) that controls the expression of transgene in a Dox dependent manner. In tet-off system, in presence of Dox tTA cannot binds to TRE thus the transgene expression is inactivated, which can be activated by

removing Dox from animal diet. In case of tet-on developed later rtTA (reverse tTA) binds TRE in presence of Dox.

The protein product of our DNA construct is not directly detectible because of the absence of a reporter gene like LacZ or GFP. To be able to detect the transgene expression, we crossed our lines with tetO-TVAG (Wickersham et al., 2007). The tetO-TVAG mouse line expresses two proteins in a tTA dependent manner: TVA (Lewis et al., 2001) and rabies glycoprotein necessary for rabies virus infection (Wickersham et al., 2007). For bicistronic expression of both genes under the same promoter, a 2A sequence was used (Provost et al., 2007). In the work for this thesis we make use of antibodies against 2A to detect the transgene expression in the transgenic lines.

1.2.2 Expression of transgenes in transgenic mice

Several putative enhancers specifically active in the MEC have been tested on their potential to drive expression of transgenes in transgenic mice. The three enhancers we look at in this thesis are ODZ3, TRPS1 and LMO3. Since enhancers have no obvious name, we have named them after the gene they most likely control in a wildtype brain. The expression of native gene ODZ3 is present in all layers of the MEC and the LEC, most parts of cortex, the hippocampus and the piriform cortex (Figure 2 A). In contrast, the transgene driven by the MEC specific enhancer connected with gene Odz3 is expressed specifically in layer 2 of the MEC and the LEC (Figure 2 B) The expression of native gene TRPS1 is mainly in all layers of the MEC, the LEC, the neocortex, the piriform cortex and the CA fields of the hippocampus (Figure 2 C). While the transgene driven by the MEC specific enhancer connected with gene trps1 is specifically in the MEC layer 2 and 3 (Figure 2 D). The expression of native gene LMO3 is mainly in subiculum, deep layers of the of the MEC and the LEC and the piriform cortex(Figure 2 E) The transgene driven by the MEC specific enhancer connected with lmo3 is expressed in MEC and LEC layer 3 (Figure 2 F).

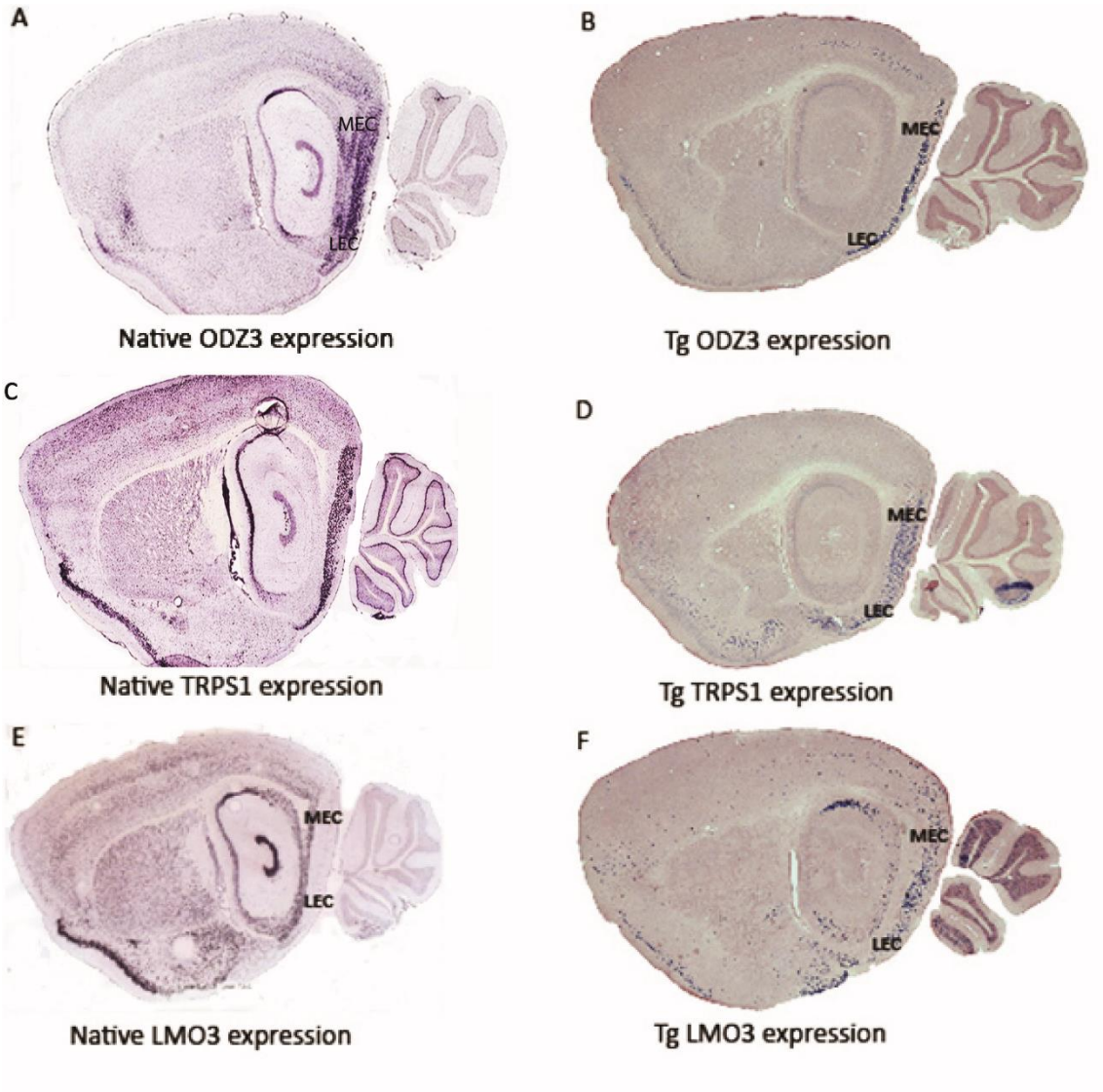


Figure 2 Gene expression of native genes ODZ3, TRPS1, LMO3 and transgenes driven by MEC specific enhancers. Expression of native genes (left panel) and expression of transgenes (right panel). Though this thesis predominantly uses horizontal sections, these are sagittal sections. Left images were taken from Allen brain atlas and the right images were on courtesy of Stefan Blankvoort.

1.2.3 The medial entorhinal cortex

Expression of the transgenes predominantly occurs in the MEC. Besides that all virus injections in this thesis were done in the MEC. Along with the perirhinal cortex, the postrhinal cortex, the pre- and parasubiculum and the lateral entorhinal cortex the MEC is part of the parahippocampal region (van Strien et al.). Neurons of layer 2 of the entorhinal cortex project to dentate gyrus and CA3, and neurons in layer 3 of the entorhinal cortex projects to CA1 and the subiculum. (Witter and Moser, 2006). MEC has clear lamination and regular organization. It is separated into superficial layer and deep layer by an acellular layer IV also known as lamina dissecans (van Groen, 2001) (Figure 3). Layer

1 contains few neurons whereas layer 2 has two principal excitatory neuron, stellate and pyramidal neurons (Canto and Witter, 2012). In layer 2 of the MEC the excitatory cells express either calbindin or reelin. Calbindin positive, reelin negative cells are grouped in patches whereas neurons outside of these patches are reelin positive, calbindin negative (Kitamura et al., 2014). Layer 3 and layer 5 and 6 are dominant with pyramidal neurons but of different sizes.

Ventrally and laterally the MEC is bordered by the LEC, the border between the LEC and the MEC is marked by changes in the cytoarchitecture of the cells in layer II of both areas. Layer 2 cells of LEC are small, is less densely packed and not as darkly stained as stellate in MEC layer 2.

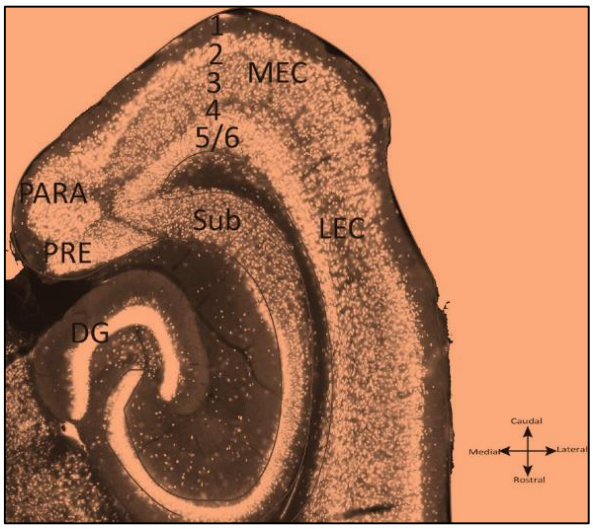


Figure 3 Horizontal section of a mouse hippocampal and parahippocampal. NeuN staining of horizontal section of mouse brain with distinct layer separation in MEC.

1.3 Viral transduction of transgenes

The second method to introduce the transgene is by a viral vector. The particular viral vector system we used is based on the HIV virus (Naldini et al., 1996). Viral vectors are delivered to the specific part of the brain regions with minimal tissue damage for genetic manipulations in local brain areas. Lentivirus, belonging to retroviridae family is used in experimental settings to deliver the transgene in both dividing and non-dividing cells. The integration of the transgene introduced through lentiviral have stable expression for a long time (Blomer et al., 1997; Dull et al., 1998; Naldini et al., 1996). Wild type HIV-1 virus has 9 different gene for viral replication and pathogenesis but these genes are reduced to three main gene gag-pol and env for making the recombinant lentivirus. These gene encode for polyprotein component of capsid, reverse transcriptase, protease, integrase and envelope protein respectively. The two regulatory genes (tat and rev) and four accessory genes (nef, vpu, vpr, vif) present in wildtype are replaced in recombinant lentivirus with LTR having a packaging signal for

viral genome replication and integration (Vigna and Naldini, 2000). For making self-inactivating lentiviral vectors deletions was made in U3 region of HIV 3'LTR (Zufferey et al., 1998). The viral enhancer and promoter for transcription of the provirus were present in the U3 region, thus deleting this region cause inactivation of both LTR. This minimizes the formation of replication competent lentiviruses and also decreased the interference from the viral promoter present in the 3'LTR (Miyoshi et al., 1998)

There have been several iterations of producing lentiviral vectors in safer ways and resulting in higher titer virus. The method used in this lab, 3rd generation, is based on a co-transfection of plasmids with the provirus with 3 helper plasmids coding for structural protein necessary for assembly of new virus (Dull et al., 1998). The packaging plasmid is split into two in 3rd generation lentivirus. pMDL.pRRE and pRSV.Rev plasmid are packaging plasmids (Figure 4) containing the gag, pol and rev genes, whereas pMD.G plasmid expresses vesicular stomatitis virus (VSV)g envelope protein. These plasmids are separated to reduce the probability of forming replicative competent cells. As there should be multiple recombination events to occur before forming replication competitive cells which is reduced because of several plasmid. Deletion of Tat gene, necessary for wild type HIV1 replication (Ulich et al., 1999), increases the safety of LV system. Only transfer plasmid with the transgene consists of packaging sequence called Psi which is deleted from all the helper plasmid so that only the plasmid of gene of interest is incorporated in the new recombinant virus. Co-transfection of 293T human embryonic kidney cells with the vector plasmid and helper plasmid is the most common way of generating LV (Figure 4). After harvesting LV from 293T cells, sterotactical injection of lentivirus is done in adult mice brain.

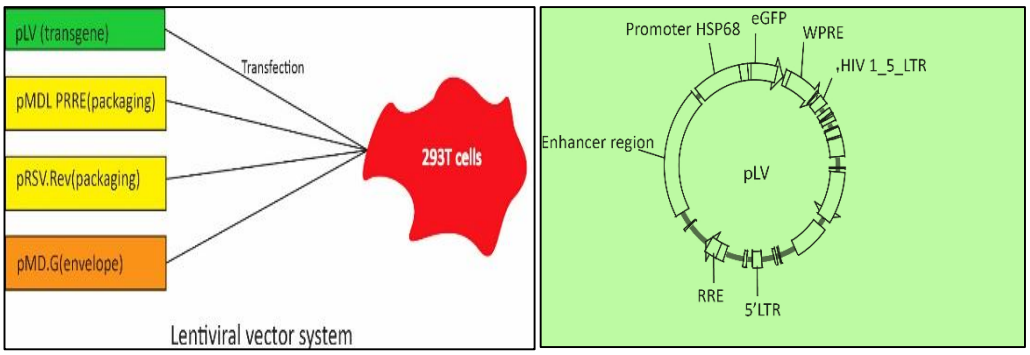


Figure 4 Schematic production of lentiviral vectors and schematic of pLV vector. (A) Cultured cells are co transfected with 4 plasmids, two of which code for structural proteins, one of which envelope proteins and a final one which contains the provirus. (B) Schematic of pLV with the transgene.

1.3.1 Use of lentiviruses with cell-type specific expression

Previous success in transgenic mice shows that enhancers can drive the expression of transgenes in a cell specific manner (Figure 2). This may allow for great advances in investigating the brain on a cellular level. However, transgenic mice have some limitations that introduction of a transgene by viral vector do not have. Some of the important application of lentivirus exhibiting enhancer driven cell specificity would be:

First to test the potential of specific enhancers before making transgenic lines. The creation of transgenic is a slow and costly process, if enhancers can be screened by virus injection first, time and money may be spared.

Another application of a cell type specific lentivirus would be in experimental settings. The lentiviral vector may be modified to carry genes that make it compatible with optogenetics or pharmacogenetics. In this setting, after virus injection the animal would be compatible with either of these techniques. This would give a platform for fast, cell specific execution of these techniques without the need for transgenic animals. A large advantage would be potential use in different species.

Another application of cell type specific transgene expression of lentivirus will be in the gene therapy (Matrai et al., 2010). The ability of lentivirus to infect non-dividing cells with high and stable transgene expression and with little to no infection has rendered lentivirus as an ideal candidate for gene therapy. Cell type specific gene transfer will be of great value as it allows for modification of specific cells within the tissue affected by disease

1.4 Aim

Three putative enhancers (ODZ3, TRPS1, and LMO3) active in MEC can drive specific expression in transgenic mice. We want to know if the enhancer driven transgene introduced through lentiviral vector can maintain this enhancer based specificity in adult mice brain. So, the main aim of this master project is to investigate if enhancer driven transgene expression in mice injected with a lentiviral vector can be as specific as enhancer driven expression in transgenic mice. To achieve this aim the 3 enhancers regions were cloned into plasmids to produce lentiviral vectors. These vectors were sterotactically injected to MEC of adult mice. Then we compare the enhancer driven transgene expression in transgenic mice and virally transduced mice based on

I. Anatomical position of transgene expression across different layers of MEC
II. Colocalization of transgene expressed cells and molecular marker specific to MEC like calbindin

The sub aim of this thesis is to know if

I. Enhancer drives similar expression in virus injected with same enhancer
II. Enhancer drives different expression in virus injected with different enhancer

2 Methods

2.1 Identification of putative enhancers

2.1.1 Micro-dissection

Brain regions of interest like the medial entorhinal cortex (MEC), the lateral entorhinal cortex (LEC), and the anterior cingulate cortex (ACC) were micro-dissected from two adult mice. Immediately after microdissection the tissue was flash frozen in liquid nitrogen and kept at -80^{0}C. The tissue was shipped on dry ice to Justin Cotney Yale for chromatin immunoprecipitation followed by sequencing (ChIP-seq).

2.1.2 ChIP-seq

In the Cotney lab the ChIP-seq was done by first isolating the chromatin (Cotney et al., 2012). After this, cross links between the histones and the DNA were made and the DNA was fragmentized by sonication. In the subsequent immunoprecipitation step, the DNA fragments were co-precipitated with histones that have a modification (H3K27ac) (Heintzman et al., 2007) that is associated with active enhancers. The precipitated DNA-histone complexes were separated by reversing the crosslinks and all DNA fragments were individually sequenced.

2.1.3 Selection of putative enhancers

The sequencing results from the ChIP-seq were aligned to a reference genome. This yielded a genome wide map of H3K27ac marked regions for each brain regions that was subjected to the ChIP-seq. uniquely active putative enhancers for individual brain regions were identified by running an algorithm that compared H3K27ac peaks between the tissues we provided and a reference signal. Based on relative strength of H3K27ac signal the putative enhancers were ranked. For each putative enhancer one or two nearby genes were given. The data we got from the ChIP-seq did not provide any information on which gene the enhancer controls, however we made the assumption that the enhancer controls a nearby gene.

We looked at gene expression profiles of the genes assumed to be under control of the putative enhancers that are uniquely active in the MEC in *in situ* hybridization experiments provided by the Allen Brain institute(AllenBrainAtlas, 2014). Based on the ISH we estimated how many cells express a gene and how highly the gene is expressed in individual cells, both of which contribute to the strength of expression of the gene in different areas of the brain. To select the most promising putative enhancers, the criteria are strength of expression in the area of interest and the specificity (level of expression in the area of interest as compared to the rest of the brain) of the expression.

2.2 Cloning of enhancer sequences to lentiviral vectors

2.2.1 Template DNA and primer design for PCR amplification of enhancers

Based on the exact genomic addresses provided by Justin Cotney, we ordered template DNA, in this case a Bacterial Artificial Chromosome (BAC). Using ensembl browser[1] we decide the particular BAC to use for each putative enhancers. After identifying the particular BAC, its availability was checked at CHORI / bacpac resource center[2] .The full sequence of the BAC was downloaded and processed further in DNA Dynamo[3].The DNA dynamo file is used to design the primers to amplify the enhancer sequence from the BAC

2.2.2 BAC maxiprep

We received BACs from CHORI as stab cultures of DH10B E.coli that were transformed with the ordered BAC. The bacteria were plated on chloramphenicol plates (BACs have a chloramphenicol antibiotic resistance gene) and transferred to 150ml LB medium with chloramphenicol (12, 5µg/ml, Qiagen, cat no.10083). After overnight incubation at 37°C with constant shaking (225RPM), BACs were isolated using a Qiagen maxiprep kit (Qiagen, cat no. k210006). The maxiprep protocol was modified to accommodate the large size (150-300KBp) of the BAC. No organic extractions or columns were used in this process. For proper settlement of the pellets after addition of P3 solution, ice was used. Later the DNA pellets formed after addition of ethyl-alchohol was subjected to dry in air then resuspended in TE buffer.

[1] http://may2012.archive.ensembl.org/Mus_musculus/Info/Index
[2] https://bacpac.chori.org/clones.htm
[3] http://www.bluetractorsoftware.co.uk/

2.2.3 PCR

BACs containing the putative enhancer of interest were used as a template for a PCR (polymerase chain reaction) to amplify the putative enhancers (table 1). These enhancers, mouse lines and viruses were referred to by name of the nearest gene in the rest of this document. The primer was designed using DNA dynamo.

The PCR was carried out using the mixture of 2µl of template DNA (BAC maxi prep product), 1,25ul of forward primer (10µM), 1,25µl of reverse primer (10µM), and 12,5µl of Q5master mix(NEB, cat.no MO5415) and 8µl of ddH2O. The PCR protocol was an initial step of 2 minutes 98°C, then 35 cycles of 10 seconds melting at 98 °C and 30 seconds annealing at 59-72°C and 120 seconds elongation at 72°C and a final elongation step of 5 minute 72 °C. The PCR product was run on a 1% agarose gel (Invitrogen agarose cat.no G800801 in TAE (0,01g/ml)) for 1 hour at 100v. The size of PCR products was determined by running a DNA ladder (Invitrogen, cat.no 239095).

Table 1.PCR amplification of putative enhancers from BACs. Primers are noted in the 5'-3' direction.

BAC	Associated gene	Annealing temperature	Product length (bp)	Left primer	Right primer
RP23-6188	ODZ3	62	2182	CACCATTATATGTATATC CTGCCTTTCTAC	GACTTAGAGAGATAAGCA ACAGTC
RP23-93K19	LMO3	69	1225	CACCAAACCAGGAAACGG GATGCTT	CATATATGTCCCAGAAAA AGCCCAGTGC
RP24-138L20	TRPS1	61	2461	GGATTTGAGAAGAGACAT GAGGTGG	CACCAGATTTTGGCTGAG ATCTTTATATT

2.2.3.1 DNA extraction

The PCR product of expected size was extracted using a gel extraction kit (Qiagen cat no 28106) and the final elution was done using 30µl of ultrapure water. DNA content of the product was measured using a spectrophotometer.

2.2.4 TOPO Cloning

TOPO cloning was performed for the PCR product. The reagents (1µl isolated DNA, 3µL H2O, 1 µl salt solution, linearized pENTR 0,5µl vector (Invitrogen cat no. K2400-20)) were mixed and incubated for 5 minutes at room temperature. 2µl of topo cloning reaction was added into a vial of one shot chemically competent TOP10 E.coli (Invitrogen, cat no 2400-20) and was mixed gently. The vial was incubated on ice for 5 minutes then a heat shock transformation was performed at 42°C for 30 seconds. 250µl SOC medium (Invitrogen, cat no. 15544034) was added to the bacteria, followed by 60 minutes incubation at 37^0C, 200 RPM horizontal shaking. About 200ul of the bacterial culture was spread over a kanamycin plate (50mg/ml kanamycin, Invitrogen cat no. 11815032) and incubated overnight at 37 °C.

2.2.5 Miniprep

Single colonies were transferred from the kanamycin plates to 3ml LB broth with kanamycin (kanamycin 50µg/ml, Invitrogen, cat no.15160-054). After overnight incubation (37^0C, 200RPM shaking) plasmids were isolated from the culture using a Qiagen miniprep kit as per the manufacturer's protocol (Qiagen, cat no. 27106). The final elution was done with 50µl of autoclaved ddH2O. The resulting plasmids are referred to as pENTR-'Enhancer'

2.2.6 Enzyme digestion

pENTR products as well as pLV products were checked by restriction enzyme digestion. The mixture consisted of 2µl of appropriate buffer (NEB cat no. B7202S), 4µl of miniprep product, 0,2µl of appropriate restriction enzyme and ddH_2O to 20µl. The mixture was incubated for 1 hour in a 37°C water bath. Then the sample was loaded on agarose gel and run for an hour at 100v. The gel was compared with an expected band pattern, as predicted by in silico digestion of the vector made by DNA dynamo. The pENTR and pLV plasmids that had a band pattern as expected were stored as a glycerol stock (500µl culture, 500µl 50% glycerol) in -80°C for future use.

2.2.7 Gateway cloning

Gateway cloning is a molecular method that is highly efficient in transferring the DNA fragments located between recombination sites named gateway att sites (attL and attR in this case). Invented

and commercialized by Invitrogen, the gateway principle is based on the site specific recombination shown by bacteriophage lambda and done in a proprietary enzyme mix, in this case LxRclonase.

In this thesis gateway cloning was done exclusively to a plasmid we named pLV, a lentivirus vector containing LTR and necessary elements for integration into virus and host genome.

For the pENTR plasmids that were verified by enzyme digestion, Gateway cloning was performed. The reaction was set up with 1, 5µl pLVdest, 1,5ul of pENTR miniprep product, 1µl TE buffer and 1ul LxRclonase (Invitrogen cat no 11791-019). Incubation was done at room temperature for 45 minutes. At the end of this incubation 1µl of proteinaseK was added and incubated for further 10 minutes at 37°C. Heat shock transformation of the 2µl of the product was done at 42 °C for 30 seconds to Stbl3 competent cells (Invitrogen, cat no.C7373-03). 250ul of S.O.C medium was added and the mixture was incubated at 37 °C under horizontal shaking (200rpm) for one hour. The mixture was spread into Ampicillin plates (100µg/ml amp, Invitrogen, cat no. 1159-019). The pLV product was checked by restriction enzyme digestion and gene sequencing.

2.2.8 Maxiprep

Single colonies were transferred from ampicillin plate to 250ml LB broth with Ampicillin (100µg/ml amp, Invitrogen, cat no. 1159-019). After overnight incubation (37°C, 200RPM shaking) plasmids were isolated from the culture using a Maxi prep kit as per the manufacturers protocol (Qiagen cat no. 12663). In short the final elution was done using 1ml of ultrapure water. The resulting plasmid are referred to as pLV-'enhancer'-GFP.

2.2.9 Sequencing

The pLV plasmids were sent for sequencing (GATC biotech[4]) to verify that the enhancer sequence of interest was present in the plasmid. The left and right primer used for initial PCR reactions of BAC were used. The left and right reads from GATC were aligned with the pLV plasmid in DNA dynamo. At the start and end of sequence the signals are poor with less distinct peaks, thus this bad signal is deleted from the sequence.

[4]https://www.gatc-biotech.com/en/index.html

2.2.10 Lentivirus production

To make lentivirus HEK293 cells were cultured. This cell line is derived from human embryonic kidney cells. The cells were co-transfected by 4 plasmids: an envelope plasmid (VSV-g), two packaging plasmids and a transfer vector plasmid.

Three days after transfection, LV was isolated from the medium. The medium collected in this process was passed through 22µm filter and the solution was ultra-centrifuged twice and the final pellet was resuspended in 100µl PBS. The resulting suspension contains lentiviral vectors with a titer of approximately 10^8-10^9 infective units per ml.

2.2.11 Stereotaxic injection to mice

Stereotaxic injection was done to medial entorhinal cortex. 8-10 week-old mice were deeply anesthetize. The head was fixed in a stereotaxic frame. The skin was disinfected with 2% iodine, and the scalp was cut exposed after an incision. Cranial windows were drilled in skull (4.5mm posterior to and 3.3mm lateral from bregma) and injections were made at depth of 1.9mm as measured from the brain surface. In both sides of brain 1µl of virus solution is injected at rate of 0.15µl per minute. The skin was sutured and mouse was returned back to its cage. The injection was performed by Stefan.

2.3 Perfusion

2 weeks after stereotaxic injection of the viral vectors, the mice were perfused by Stefan. The brain was kept in in 4% PFA for post fixation and were transferred to 30% sucrose. I was provided with the brains in 30% sucrose.

2.4 Sectioning

The brains were cut to 50µm thick horizontal sections using a microtome with the temperature set to -36°C. 30% sucrose was used to make a platform, the brain was placed upside down on the platform. Dry ice was used to freeze the brain throughout the cutting. Sections were cut and collected in 96 well plates with Tissue culture solution (TCS). We started collecting sections once the left and cerebrum start attaching with each other i.e. about -5.36mm to -1.68mm bregma (point on the top

of skull where coronal and sagittal secture intersect). The ingredient for TCS is described in appendix 3. The sections were stored at -80°C for further analysis.

2.5 Cytoarchitecture.

To make the cytoarchitectural borders of the sections visible, different methods are available such as Nissl stain, NeuN staining etc. Here we used fluorescent Nissl staining for virus injected mice and NeuN staining for transgenic mice.

2.5.1 Fluorescent Nissl staining

The sections of mice injected with lentiviral vectors were stained with fluorescent Nissl called neurotrace Blue Neurotrace (435/455, Thermofisher scientific cat no.N21479).Briefly the sections were washed with PBS 3x10 mins and subsequently incubated in 0.2% triton X-100 in PBS (Invitrogen , pH 7,4) to permeabilize the tissue. This was followed by overnight incubation in 1:1000 diluted Neurotrace. Finally the sections were incubated in 0.2% triton X-100 for 10 minutes and washed 2 x 10 mins in PBS. Then the sections were fixed in glass slides then coverslipped with PVA DABCO (2, 5%, cat no. D27802).

2.5.2 NeuN staining

To visualize the cytoarchitecture in the transgenic lines a NeuN staining was done. The sections were washed twice with PBS for 15 minutes, and permeabilized in PBS+ (PBS pH 7,4 with 1% TritonX-100). Blocking was done by preincubating with PBS++ (PBS with 1% Triton X-100 and 5% Normal donkey serum (Sigma, cat no.D9663)) for an hour at room temperature and finally incubation was done with 1:2500 Rabbit anti-2A (Invitrogen cat no.512200) and 1:500 NeuN in PBS++ for 48 hours. After this the sections were washed with PBS 6 times for 10 minutes and then incubated with 1:250 secondary antibody diluted in PBS+ for 6 hours at 4 degrees Celsius and finally washed with PBS 6 times for 10 minutes. Mounting was done using PVA DABCO (2, 5%, cat no. D27802) and then cover slipped.

2.6 Immunohistochemistry

Immunohistochemistry was performed for sections from transgenic mice and virus injected mice. For virus injected mice only calbindin was used.

The sections were washed twice with 1X PBS for 15 minutes, then premeabilized with PBS+ for 10 minutes, and after that blocked with PBS++ for an hour at room temperature. Subsequently the sections were incubated for 48 hours with primary antibody against calbindin (1:5000 mouse anti calbindin cat no. C9848) in the case of sections from virus injected mice and against calbindin and 2A (1:5000 mouse anti calbindin and 1:2500 rabbit anti-2A) in the case of transgenic mice. After incubation with primary antibody the sections were washed with PBS six times for 10 minutes, then incubated with the secondary antibody for six hours at 4 degree Celsius. For virus injected mice Donkey anti-mouse Cy3 was used whereas for transgenic mice sections donkey anti-mouse cy3 and donkey anti-rabbit 488 was used all in a 1:250 dilution. After incubation with secondary antibody the sections were washed once with DAPI (1: 10,000) for 10 minutes and subsequently washed with PBS six times for 10 minutes. Mounting was done with PVA DABCO and the slides were cover slipped.

2.7 Data collection and analysis

2.7.1 Microscopy

All the brain sections were visualized using a fluorescent microscope (Axio imager M1). The GFP expression of virus injected mice was analyzed using BP 450/490filter (Zeiss, filter set 10) and calbindin positive neurons were analyzed with BP 640/30 filter (Zeiss, filter set 50). For transgenic mice IHC slides for anti-2A the cells were analyzed with BP 450/490 filter (Zeiss, filter set 10).

2.7.2 Counting and colocalization

Counting the expression of transgenes we looked at two characteristics: anatomical location and colocalization with calbindin. Software package neurolucida was used to represent this data to a digital vector file.

First of all the general outline was drawn of the sections using transmitted light at 5X objective. Later the reflected light with 20X objective was used for counting the transgenic cells (GFP or IHC for 2A) and to verify colocalization with calbindin signal. Later neurolucida explorer was used to adjust the color and the size of the sign assigned to count.

2.7.3 Scanning

The mounted sections on slides were digitalized with the help of a fluorescence Mirax Midi fluorescence scanner (Zeiss Midi, Microimaging, Jena, Germany).The Nissl stained virus injected section were scanned through filter set 28 and BP470/40 (for GFP). The transgenic mice sections with NeuN staining were scanned through BP 545/25 (for NeuN) and BP 470/40 filter (for 2A). The IHC sections for both virus injected and transgenic mice were scanned through BP 470/40 (for GFP and 2A) filter and BP 545/25 (for calbindin).

2.7.4 Delineation and outline

The general outline of sections along with the counting from Neurolucida was overlaid with the high resolution scanned section from Mirax scanner using Adobe illustrator CS6. Then the anatomical outline from the vector file was deleted and the cells with transgene expression and colocalization was counted in illustrator. Mouse atlas was referred to define the borders of MEC with LEC and subiculum. Based on Nissl staining and NeuN staining the different layers of MEC were delineated in the scanned image. The scale bar was imported from pannoramic viewer along with the image and was overlaid over the delineated image in illustrator and the text were added to complete the figures.

2.7.5 Image processing

The figures were imported to Adobe Photoshop where the intensity and the contrast of the image were adjusted.

2.7.6 Summary of methodology

The goal of this summary is to describe how to select and clone enhancer that are predicted through ChIP seq and then how the sections were handled for the analysis after perfusion for both transgenic and lentivirus injected mice (Figure 5).

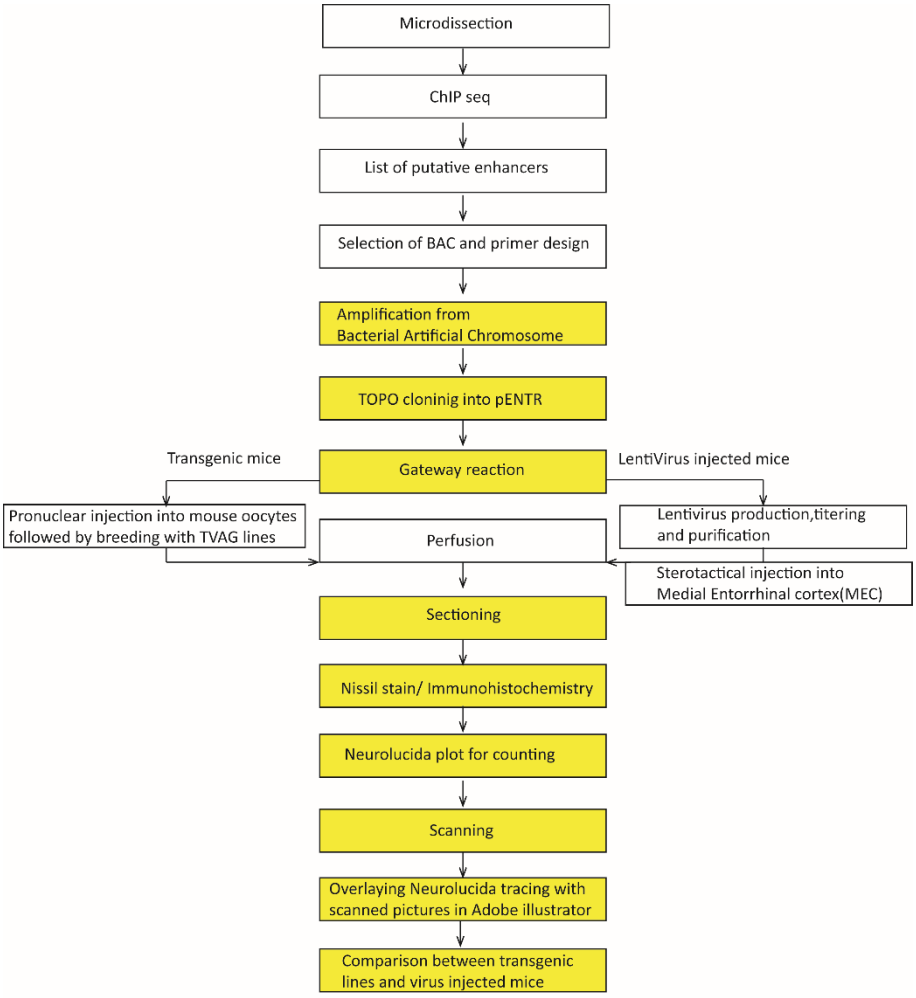


Figure 5 Schematic representation of all the steps involved in this master thesis. The yellow boxes shows the steps of experiments that I performed.

3 Results

3.1 Cloning of lentiviral plasmids

To investigate whether or not putative enhancers can drive expression in specific cell types we cloned them into plasmids. We made two different types of plasmids with different uses. The first one was made previously and used for pronuclear injection. The second one is a plasmid that can be used to make lentiviral vectors (hereafter this plasmid is referred to as pLV). This first part of the results covers the molecular biology of making the vectors required for virus production and pronuclear injection. The putative enhancers were amplified from Bacterial Artificial Chromosomes (BACs) using a polymerase chain reaction (PCR). The resulting PCR product (Figure 6, yellow box) was incorporated in a shuttle vector (pENTR) using TOPO cloning, and verified through enzyme digestion. Good clones were used in gateway reaction with a destination vector.

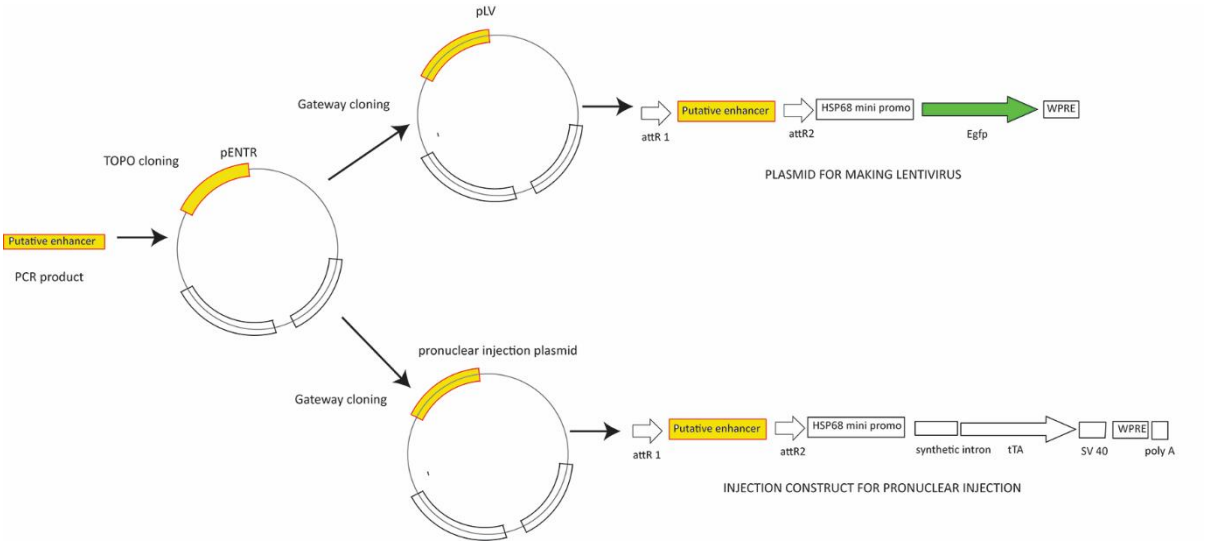
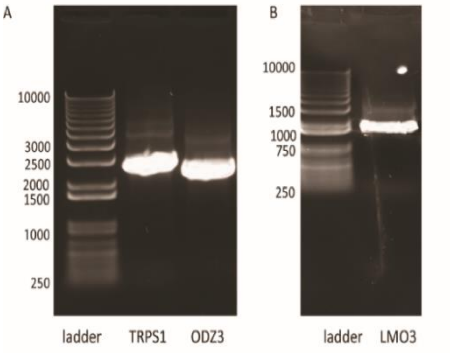


Figure 6 Cloning steps to generate plasmids. A putative enhancer (yellow box) is amplified by PCR and cloned into a pENTR plasmid using TOPO cloning. Subsequently the putative enhancer is cloned into a destination plasmid using gateway cloning. Depending on which destination plasmid is used, the resulting plasmid may be used for production of lentiviral vectors or generation of constructs for pronuclear injection.

3.1.1.1 PCR amplification of enhancers

Three enhancers that showed MEC specific expression in transgenic mice (Figure 2) were selected to be cloned into lentiviral vectors. BACs containing the enhancer of interest were obtained, grown and

used as a template for a PCR reaction to amplify the putative enhancers. Gel electrophoresis of the PCR product shows single strong bands of approximately expected sizes (Figure 7 A, B and table).



C

Gene Associated	Expected size (bp)	Observed size (bp)
TRPS1	2461	≃2400
ODZ3	2182	≃2100
LMO3	1225	≃1200

Figure 7 PCR amplification of putative enhancers.(A) 1kb+ DNA ladder (left lane) and PCR products for the enhancers TRPS1 and ODZ3 (B) 1kb+ DNA ladder and PCR product for the enhancer LMO3. (C) Comparison of expected and observed sizes for the enhancers TRPS1, ODZ3 and LM03

We transferred the PCR products into pENTR vectors using TOPO cloning. TOPO cloning is based on a 4 base homology (CACC) between the linearized pENTR vector and one of the primers used in the PCR reaction. We did an enzyme digestion to verify integration of the expected PCR product. The pENTR vectors include attL1 and attL2 sites for site-specific recombination of the PCR product into a gateway destination vector.

3.1.1.2 Gateway mediated transfer of the enhancer from pENTR vectors to pLV plasmids

Invitrogen gateway cloning was used to transfer the putative enhancers to pLV plasmids. The pLV plasmids were checked using both enzyme digestion and sequencing. Using DNA dynamo software we predicted the band sizes of resulting fragments after enzyme digestion (Figure 8). After enzyme digestion the pLV plasmids were run on an agarose gel and analyzed for the appropriate band pattern (Figure. 9 A, B, C). A comparison between expected bands and observed bands is summarized in figure 9 (D). For pLV-ODZ3-eGFP and pLV-TRPS1-eGFP the observed band pattern matched the expectation, however in the case of pLV-LMO3-eGFP the observed band pattern was different from the expectation.

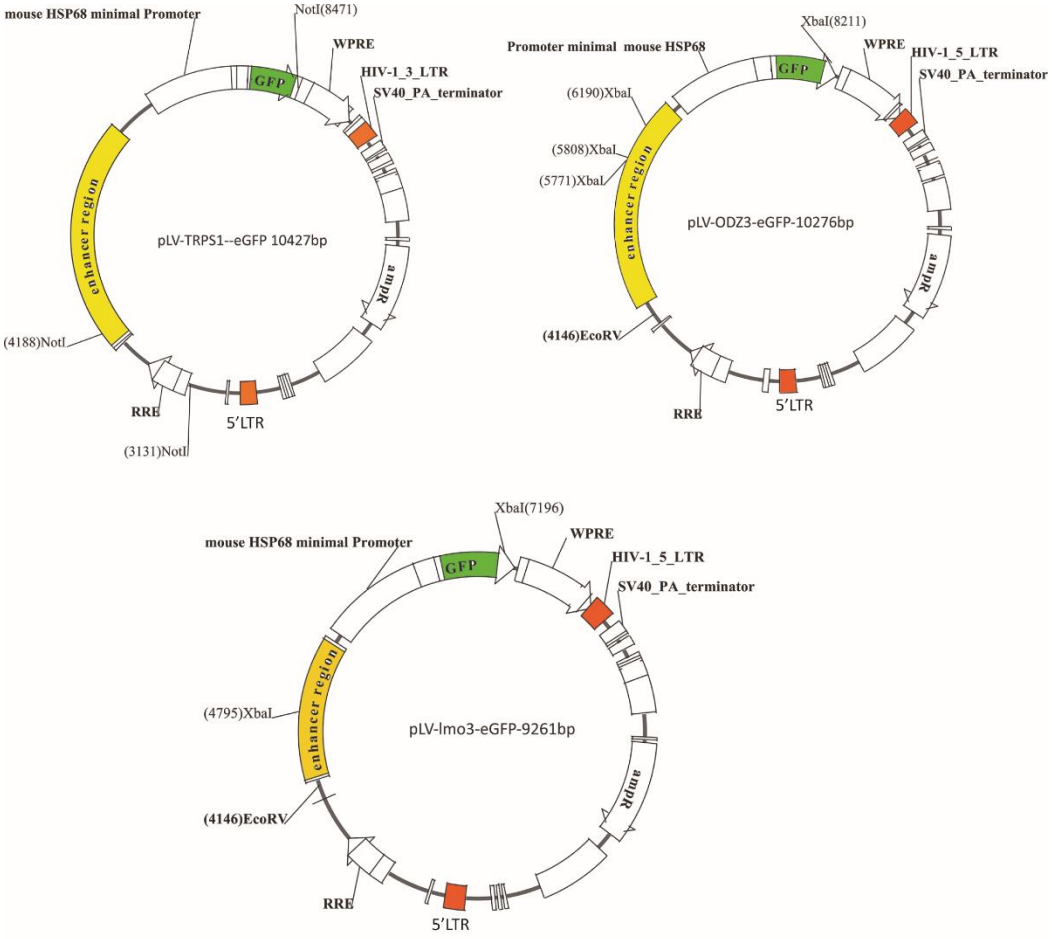
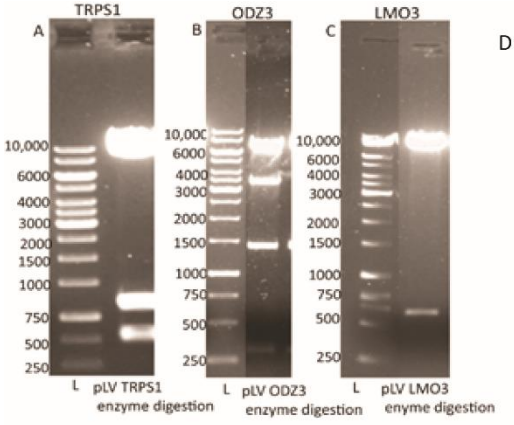


Figure 8 Circular maps of pLV plasmids. The enhancer region is marked in yellow, the eGFP gene is marked in green and the LTRs are given in red. For pLV-TRPS1-eGFP NotI enzyme was used, whereas EcorV and xbaI enzyme was used for pLV-ODZ3-eGFP and pLV-LMO3-eGFP.



D

Enhancer	Expected size of fragment(bp)	Observed size of Fragment(bp)
TRPS1	774,1145,8508	600, 900, 10,000
ODZ3	419,1625,2021,6211	300, 1400, 3500, 7000
LMO3	649,2401,6211	600,10,000

Figure 9 Verification of pLV by enzyme digestion. (A) Digestion of pLV-TRPS1-eGFP by NotI. (B) Digestion of pLV-ODZ3-eGFP by xbaI and EcoRV. (C) Digestion of plv-LMO3-eGFP by xbaI and EcoRV. The gel pictures have been altered to remove unnecessary information, the height of the bands relative to the DNA ladder has been maintained. (D) Comparison of expected and observed band size.

3.1.1.3 Verification of enhancers in pLV by sequencing

To verify the presence and proper amplification of the enhancer to the pLV plasmids we sequenced the enhancer regions. For this sequencing reaction we used the same primers as the ones originally used for the PCR amplification of the enhancers (Table 1). We used DNA dynamo to compare the expected sequence with the results we got from sequencing. For all plasmids, the entire reads (after trimming) matched the expected sequence (Table 2).

Table 2. Sequencing result of the primer used for initial PCR reaction.

	Start of read	End of read	Length of read	Mismatch	Enhancer start and end position (bp)
TRPS1(L)	6078	6692	614	no	4249-6710
TRPS1(R)	4896	4238	658	no	
ODZ3(L)	4265	5046	781	no	4286-6467
ODZ3(R)	6419	5803	616	no	
LMO3(L)	4743	5396	653	no	4225-5449
LMO3(R)	4925	4276	649	no	

The sequencing data shows that enhancers were cloned correctly. Stefan Blankvoort used the plasmids to make several different types of lentiviral vectors and inject these lentiviral vectors to adult mouse brains. This allowed us to compare enhancer driven expression of a transgene in transgenic mice with putatively enhancer driven expression after transfer with a viral vector.

3.2 Comparison of transgene expression between transgenic mice and virus injected mice

We compared the enhancer driven expression in transgenic mice and in mice injected with viral vectors using two different methods. The first one is anatomical distribution across the layers of the MEC and second is by colocalization with a molecular marker. We started by investigating the anatomical distribution of expression of enhancer driven tTA in transgenic mice. We already have a reasonably idea of the anatomical distribution in transgenic mice from previous in situ hybridization

experiments (Figure 2). To verify layer specific expression we did a stain that allows for the identification of individual layers in the MEC.

3.2.1 Anatomical distribution of transgene expression in transgenic mice

To find the exact anatomical location of the transgene expressing cells we need to combine two different types of information. Firstly the location of the transgene expression cells within the tissue and secondly the cytoarchitecture of the tissue to identify structures and the layers within the structures.

We bred enhancer-tTA lines with tetO-TVAG lines. TVAG mice are generated to allow for monosynaptic tracing (Wall et al., 2010) , and by detecting part of the transgenic construct (the peptide 2A) we can identify the transgene expressing cells. 2A is a viral peptide that results in equal expression of both TVA and the Rabies G transgenes (Weible et al., 2010). We detect the transgene using 2A staining for transgenic mice.The location of transgene is tracked by neurolucida software in fluorescent microscopy. To view the cytoarchitecture I have done a NeuN staining on adjacent sections.

To combine the two types of information (first the location of transgene and second the cytoarchitecture of tissue), we overlaid the vector files from neurolucida (with transgene count) (Figure 10 D) with scans of the NeuN stained tissue (Figure 10 C). The high resolution scanned pictures (Figure 10 A) is used for delineation of the layers of MEC (Figure 10 B)

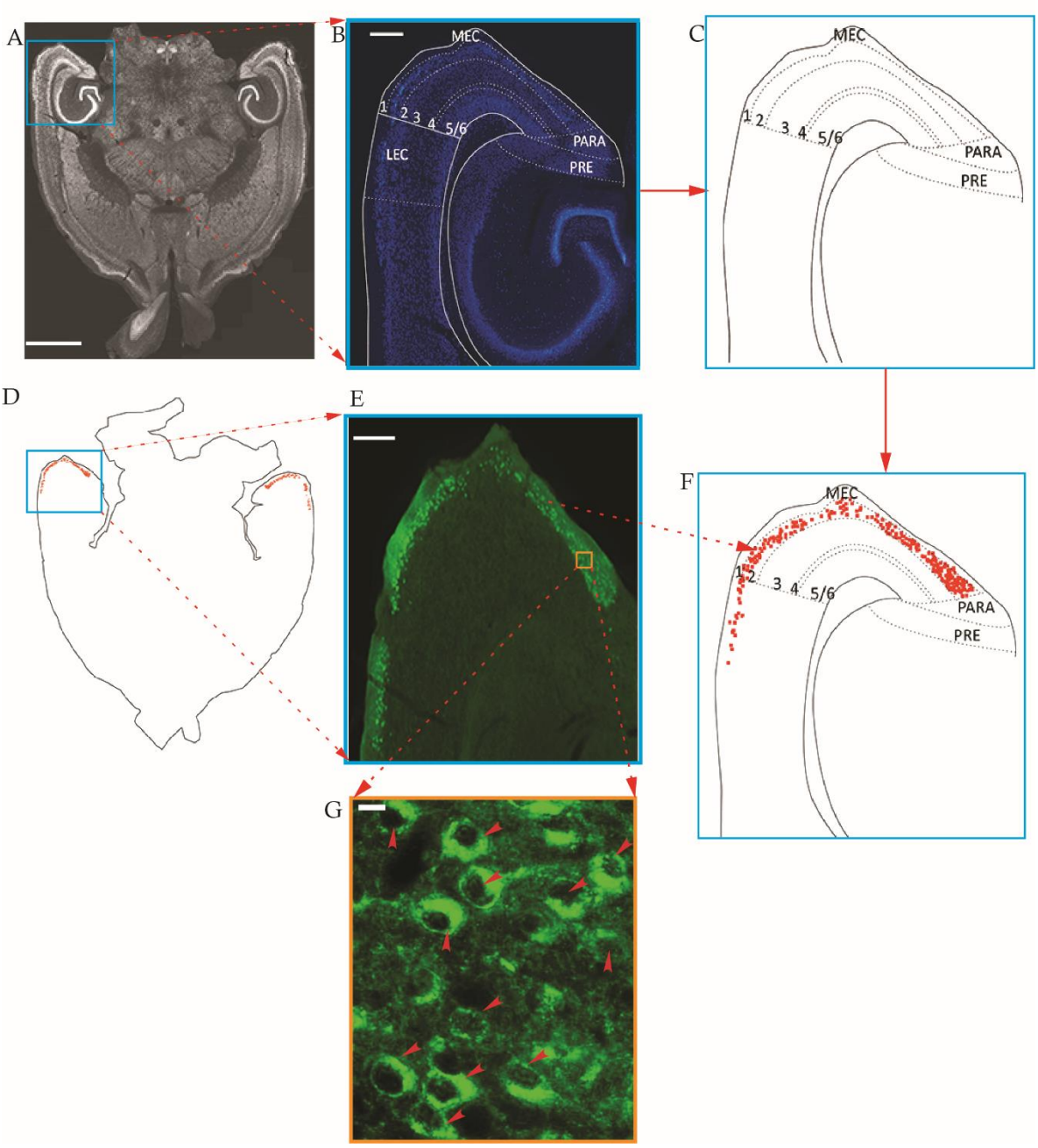


Figure 10 Method of creating integrated representations of cytoarchitecture and transgene expression. (A) NeuN staining of a full horizontal section (50µm) of a transgenic mouse. (B) MEC with delineation based on NeuN staining. (C) Outline based on figure B. (D) Vector file from Neuolucida exported to illustrator, orange mark represent the 2A counting as seen as green signal in figure E. (E) 2A staining showing transgene expression, MEC. (F) Overlaying of delineation of MEC (fig C) with vector file (fig D). (G) High powered magnification showing individual bodies. The scale bars in fig A and fig B are 2000µm and 250µm respectively.

In the Odz3-tTA x tetO-TVAG mice 54114 and 54337 I have counted both sides of 3 horizontally cut sections. The sections correspond to dorsal-ventral levels -2.94mm to -3.3mm from bregma. The expression of 2A is predominantly in the layer2 in the ODZ3 mouse line (Figure 11, 12, supplementary figure Appendix 1 figure 30, 31). There were few cells in parasubiculum that expressed 2A. The expression of 2A was continued in layer 2 up to LEC as well (Figure 11 C, D).

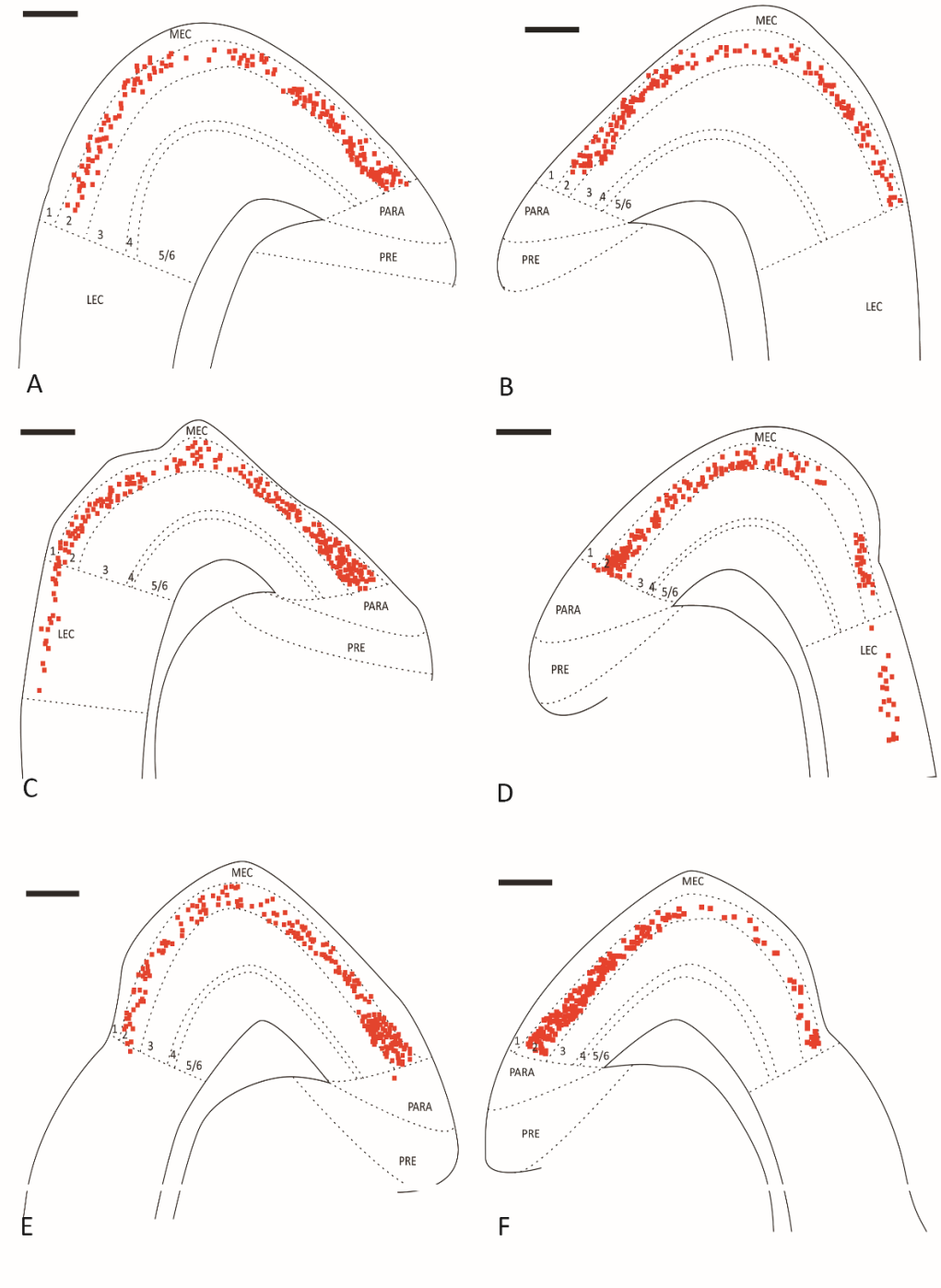


Figure 11 Enhancer driven expression of transgene in ODZ3 transgenic mouse 54114. The solid line indicates the outline of obvious borders whereas the dotted line indicates borders we based on cytoarchitecture, the orange boxes represent the 2A labelled cell bodies. A total of 3 sections on both sides of MEC were counted. (A) and (B) dorso ventral level 3.3mm ventral to bregma, (C) and (D) dorso ventral level 3.18mm ventral to bregma, (D) and (E) dorso ventral level 2.94mm ventral to bregma. Scale bars are 250 µm

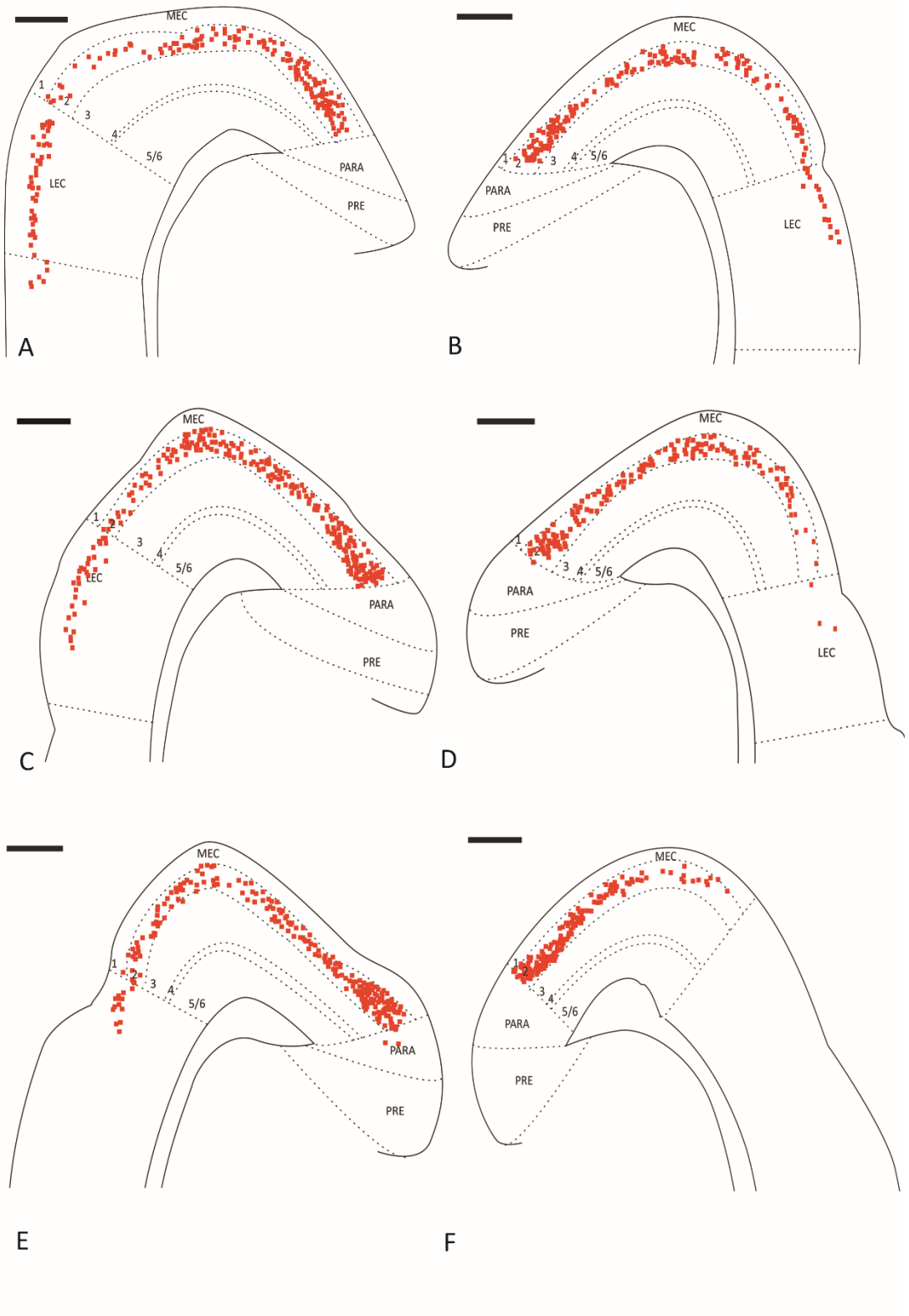


Figure 12.Enhancer driven expression of transgene in ODZ3 transgenic mice 54337. The solid line indicates the outline of obvious borders whereas the dotted line indicates borders we based on cytoarchitecture, the orange boxes represent the 2A labelled cell bodies. A total of 3 sections on both sides of MEC were counted. (A) and (B) dorso ventral level 3.3mm ventral to bregma ,(C) and (D) dorso ventral level 3.18mm ventral to bregma, (D) and (E) dorso ventral level 2.82mm ventral to bregma. Scale bar are 250 µm.

In the Trps1-tTA x tetO-TVAG mice 53900 and 53516, I have counted both sides of 3 horizontally cut sections for 53900 that correspond to dorsal ventral levels -2.94mm to -3.3mm from bregma and for 53516 I have counted 2 horizontally cut section corresponding to -2.94mm and -3.18mm from bregma. The expression of 2A is predominantly in layer 2 and layer 3 in the TRPS1 mouse line and in few sections in the parasubiculum (Figure 13, 14, supplementary figure, Appendix 1 figure 32, 33)).

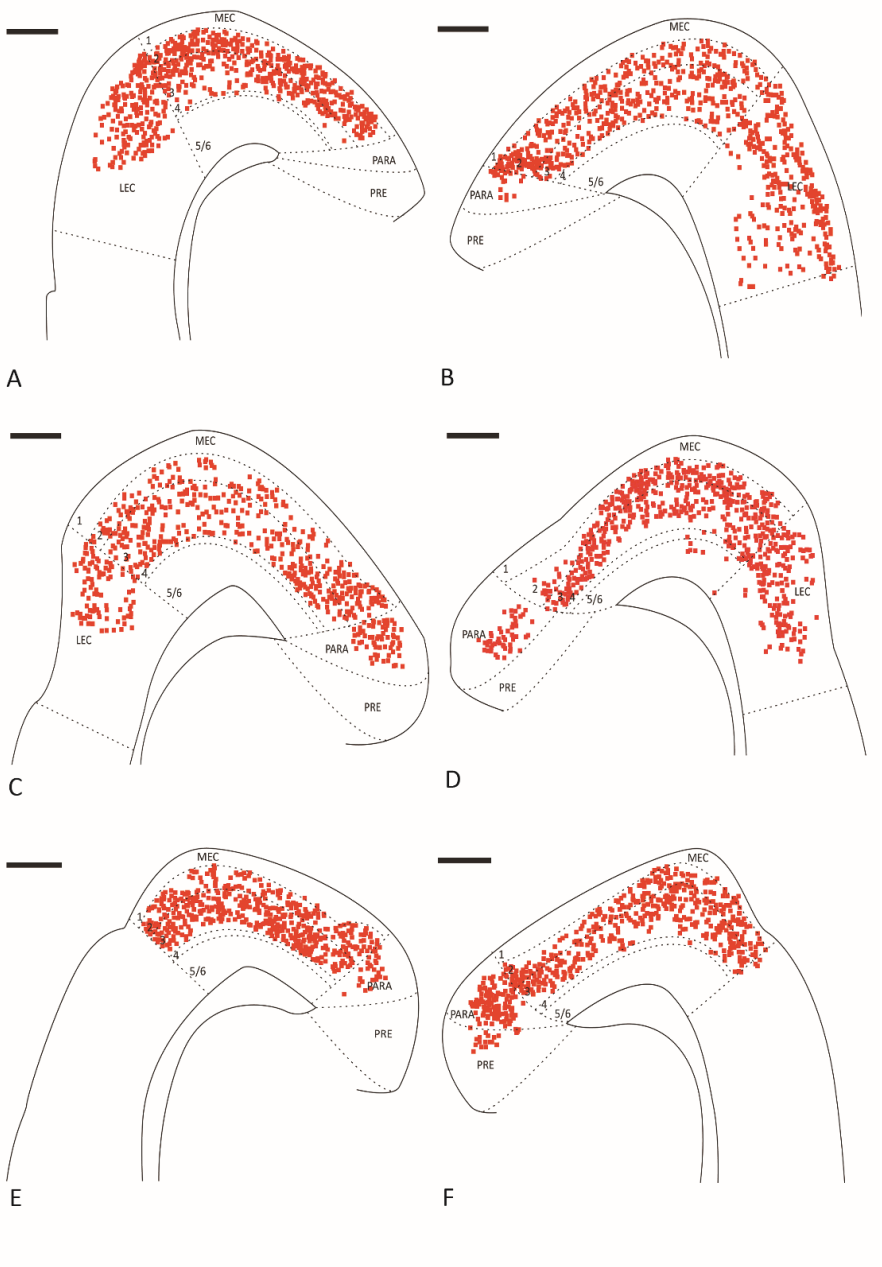


Figure 13 Enhancer driven expression of transgene in TRPS1 transgenic mice 53900. The solid line indicates the outline of obvious border, whereas the dotted line indicates border we based on cytoarchitecture, the orange boxes represent the 2A labelled cell bodies .A total of 3 sections on both side of MEC were counted. (A) and (B) dorso ventral level 3.3mm ventral to bregma, (C) and (D) dorso ventral level 3.18mm ventral to bregma, (E) and (F) dorso ventral level 2.94mm ventral to bregma .Scale bar are 250 µm.

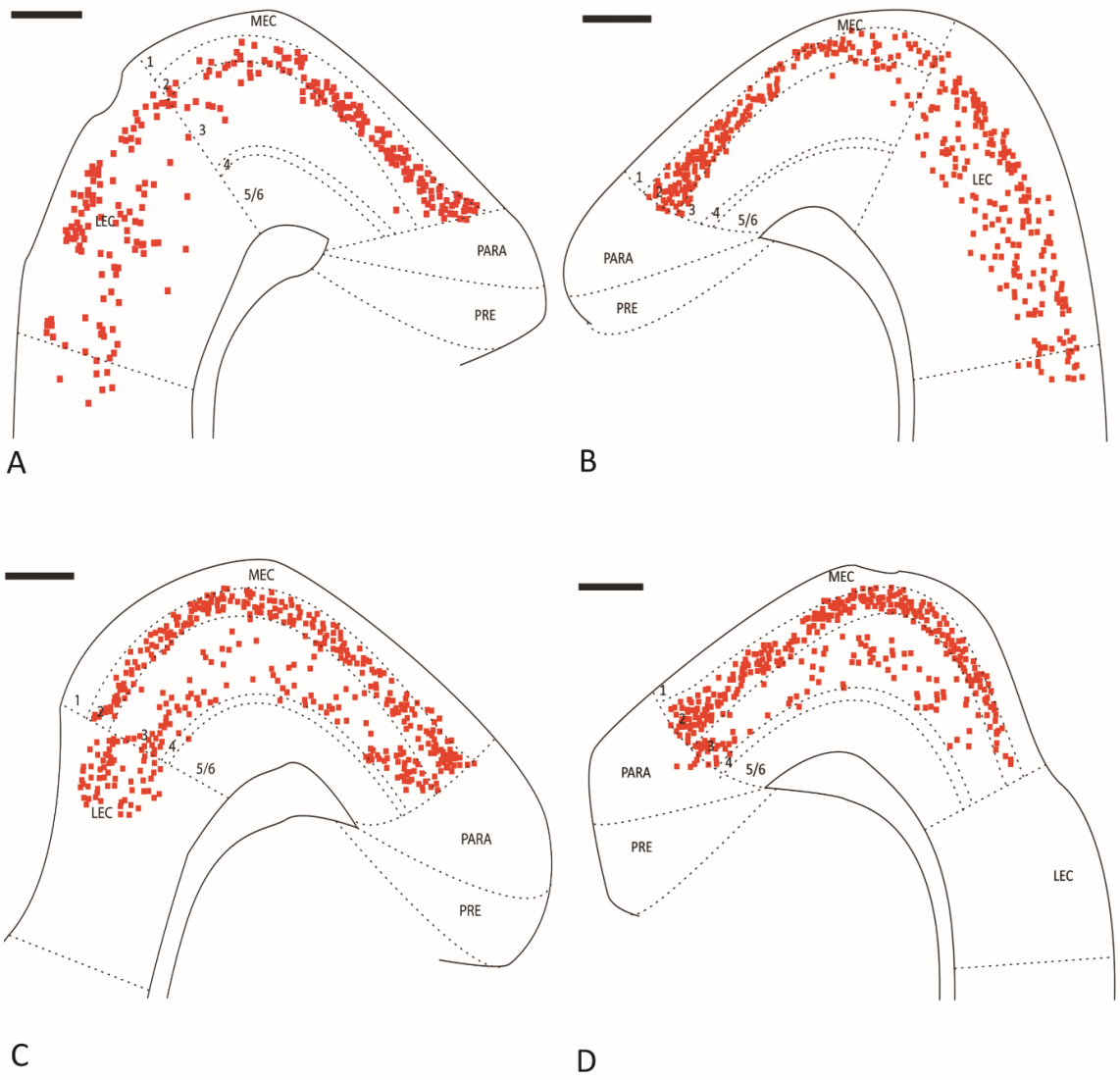


Figure 14 Enhancer driven expression of transgene in TRPS1 transgenic mice 53516. The solid line indicates the outline of obvious border whereas the dotted line indicates borders we based on cytoarchitecture, the orange boxes represent the 2A labelled cell bodies. A total of 2 sections on both sides of MEC were counted. (A) And (B) dorso ventral level 3.18 ventral to bregma, (C) and (D) dorso ventral level 2.94 ventral to bregma .Scale bar are 250µm.

3.2.1.1 Quantification of 2A expression in transgenic mice

To calculate the percentage distribution of 2A expression across different layers of MEC, the total of 2A expressing cells in each layer is represented as a percentage of the total number of 2A expressing cells in the MEC (Figure 15). In the ODZ3 line 99,45% (+/-0.12) of 2A positive cells were found in layer 2 and 0,55% (+/-0.11) of cells were found in layer1.

For TRPS1 transgenic lines the percentage distribution of 2A was 61,13% (+/-6,24) in layer 2 and 38,63% (+/-6,13) in layer 3 (Figure 15 percentages are noted as mean +/- SEM).

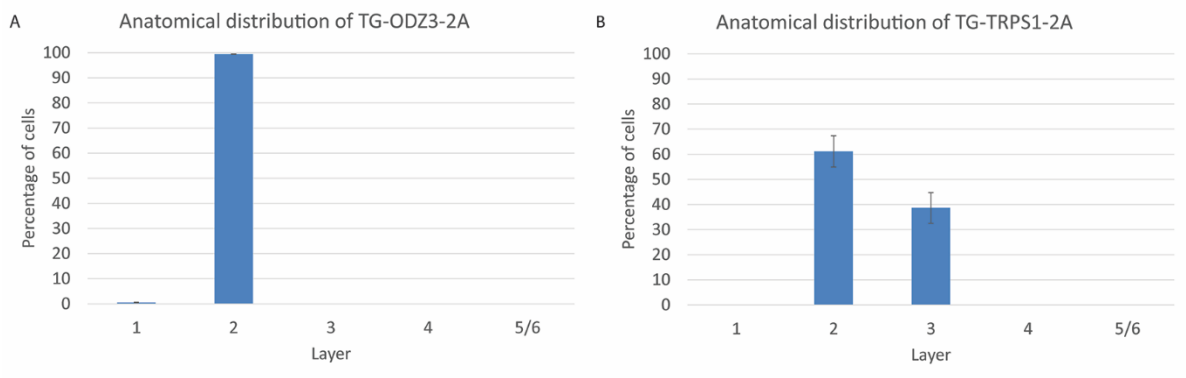


Figure 15 Percentage distribution of 2A in transgenic lines. (A) Percentage of 2A expression in MEC layer 2 in ODZ3 transgenic mice. (B) Percentage of 2A expression in MEC layer 2 and layer 3 in TRPS1 transgenic mice. Error bars give the SEM.

3.2.2 Anatomical distribution of transgenically labeled neurons after lentiviral vector injection

To compare expression of enhancer driven transgenes in transgenic mice with virus injected mice, we did stereotactical injections to the MEC of adult mice. We injected 11 mice with three different lentiviral vectors corresponding with the three different transgenic lines we described previously (ODZ3 (4 mice), TRPS1 (4 mice) and LMO3 (3 mice)). In LV injected mice eGFP expressing cells were counted. The sections were stained with a fluorescent Nissl to identify the cytoarchitectural borders and layers within MEC. Overlaying of the vector file resulting from the counting in Neurolucida with the scanned picture (of the fluorescent Nissl stain) was done in a similar fashion to the transgenic mice sections (Figure 10). For virus injected mice the expression of transgene (eGFP) is in both cytoplasm and nucleus so the expression is more like solid balls (Figure 16 C).

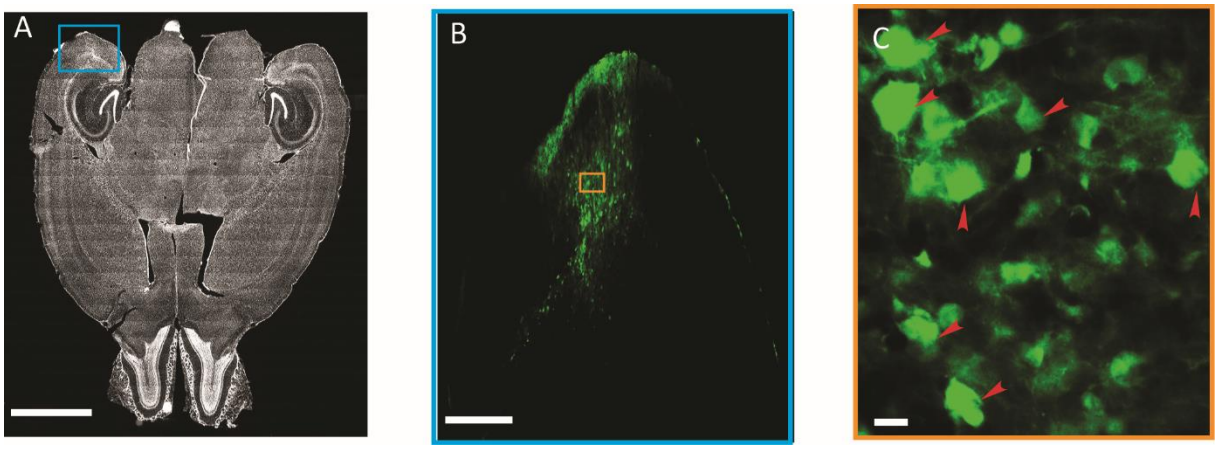


Figure 16 Expression of transgene in LV injected mice(A) Neurotrace staining of a full horizontal section (50µm) of a virus injected mice (B) eGFP expression showing transgene expression in MEC. (C) High powered magnification showing individual bodies. Scale bar are 2000,250 and 10 µm respectively.

To assay the eGFP, approximately 7 horizontal sections covering the entire dorsal-ventral range of the brain were mounted after fluorescent Nissl staining. Out of the 7 sections we selected one section to count based on the highest level of transgene expression. The mice were bilaterally injected with virus, in the cases where we found expression in both hemispheres, we counted both hemispheres. The total labelled eGFP cells is given in the table, not all labeled cells were found in the MEC (Table 3).

Table 3 Total virus injected mice with total transgene expression. Mouse number along with the associated gene and the total transgene expressed cell in Lentivirus injected mice here (L) and (R) represent the left and right MEC of same section.

Mouse Number	Gene Associated	Total eGFP labelled cells	Dominant eGFP expressed layer in MEC with percentage
53130	ODZ3	475	Layer 5/6 (46%)
53131	ODZ3	97(L),46(R)	Layer 3(34,45%)(L), layer 3(48,07%)(R)
53132	ODZ3	119	Layer 3(47,52%)
53133	ODZ3	121	Layer 3(71,87%)
52930	TRPS1	217	Layer 3(56,41%)
52931	TRPS1	347(L), 311(R)	Layer 3(45,23%)(L), layer 3(56,96%)(R)
53312	TRPS1	300	Layer 5/6(56,17%)
53313	TRPS1	375	Layer 5/6(43,63%)
52927	LMO3	439	Layer 5/6(77,70%)
52928	LMO3	189(L), 65(R)	Layer 5/6(55,26%)(L), layer 5/6(71,87%)(R)
52929	LMO3	270(L), 325(R)	Layer 3(64,10%)(L), layer 3(40,18%)(R)

The 4 mice injected with LV-Odz3-eGFP had variable numbers of eGFP labeled cells, with the lowest number of cells we counted in a single section being 46 and the highest 475 (Table 3). In all mice the eGFP labeled cells were spread over the layers. The largest fractions of eGFP labeled cells we found per layer was variable between mice. The maximum expression in mouse 53130 was in layer 6 (Figure 17-A) with transgene expression in presubiculum as well. In mouse 53131 the expression is mostly in layer 3 (Figure 17 B). In mouse 53132, the expression of transgene was spread on all layers of MEC (Figure 17 C, D) and in Mouse 53133 (Figure 17 E) the expression was mostly in layer 3. The original data is supplied in Appendix 1 (Figure 34).

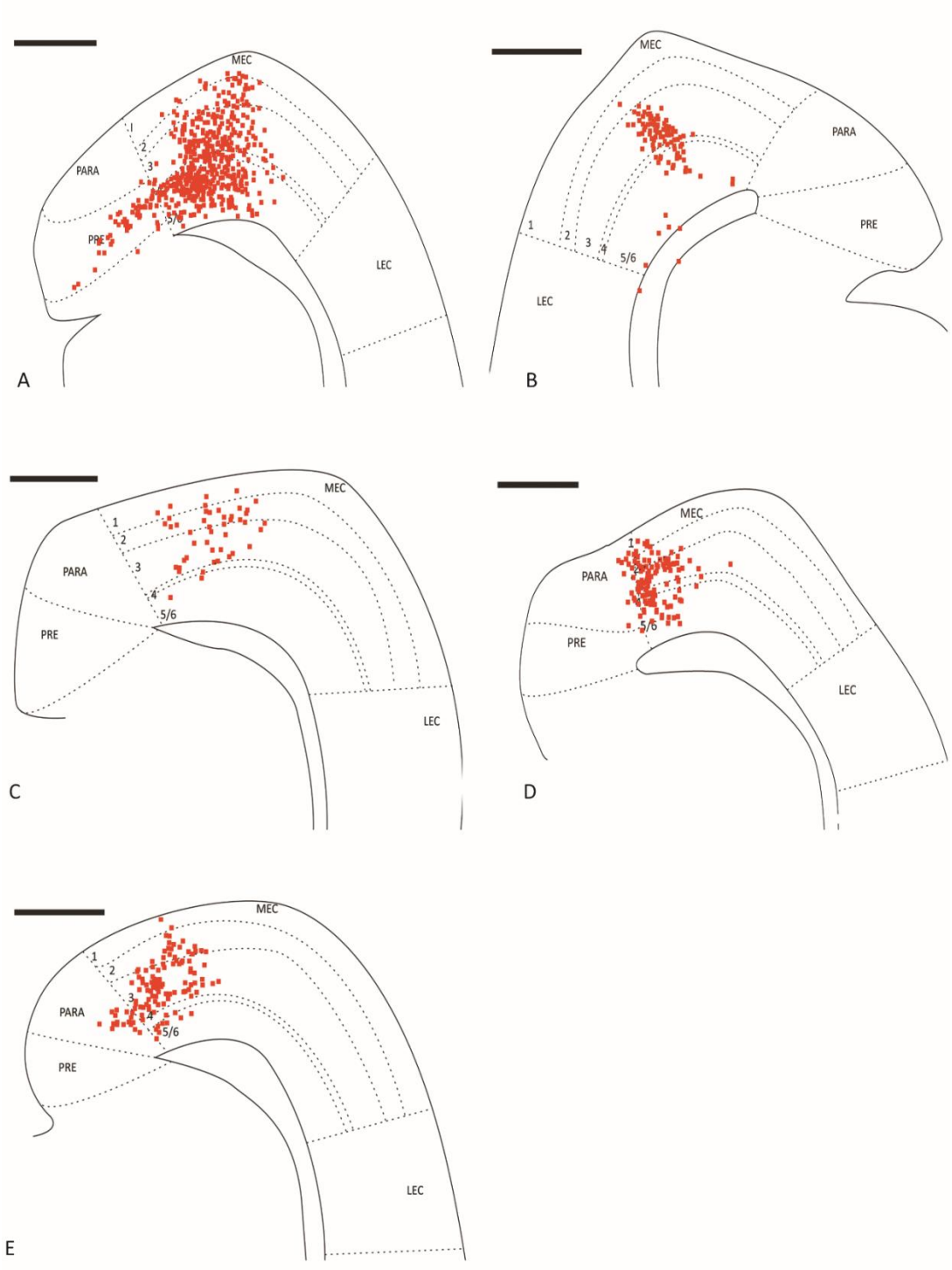


Figure 17 Enhancer driven expression of transgene in virus injection with ODZ3. The solid line indicates the outline of obvious borders, while the dotted line indicates borders we have based on cytoarchitecture, the orange boxes represent the eGFP labeled cell bodies. A total of 5 sections in 4 mice were counted (A) mouse 53130, (B) mouse 53131 left, (C) mouse 53131right, (D) mouse 53132, (E) mouse 53133. The black scale bars are 250 µm.

In all 4 mice injected with LV-TRPS1-eGFP the eGFP labelled cells were spread across layers. The largest fraction of eGFP labelled cells we found per layer was variable between mice, lowest being 217 and highest being 347. In mouse 52930 (Figure 18 A) the expression of transgene was high in

layer 3. In mouse 52931 (Figure 18 B, C) the transgene was expressed highly in layer 6 as well as Parasubiculum and presubiculum. In mouse 53312 (Figure18 D) the expression was mostly on layer 3 and the parasubiculum whereas in case 53313 (Figure 18 E) the expression was more in layer 3. The original data is supplied in Appendix 1 figure 35.

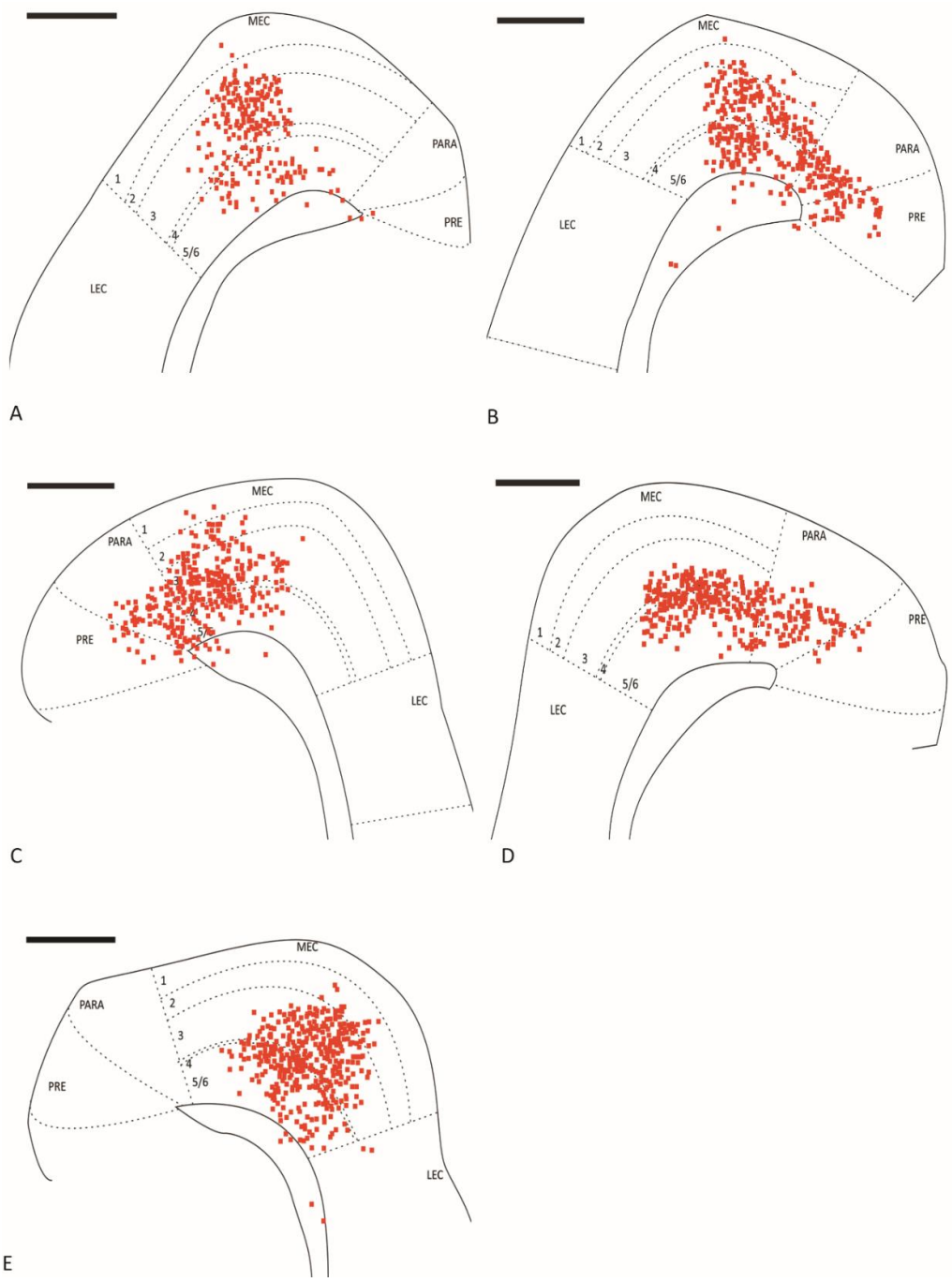


Figure 18 Enhancer driven expression of transgene in virus injection with TRPS1.The solid line showing the outline of obvious borders while the dotted line indicates borders we have based on cytoarchitecture, the orange boxes represent the eGFP labeled cell bodies. A total of 5 sections in 4 mice were counted.(A) mouse 52930, (B) mouse 52931 left, (C) mouse 52931 right,(D) represent case 53312,(E) mouse53313. The black scale are 250 µm. All sections correspond to a dorso-ventral level of 2.82mm ventral to bregma.

The 3 mice injected with LV-LMO3-eGFP had variable number of eGFP labelled cells, with the lowest number of cells we counted in single section being 65 and the highest 439. In all mice the eGFP labelled cells were spread over the layers. The largest fraction of eGFP labelled cells we found per layer was variable between mice. In mouse 52927 (Figure19 A) the expression of transgene was mostly in layer 5/6 with some expression in the para and pre-subiculum. In mouse 52928 (Figure19 B) the expression of transgene was mostly in the Para- and pre-subiculum. In mouse 52929 (Figure 19 D, E) the expression of transgene was mostly on layer 3 with some expression in the para-subiculum. The original data is supplied in Appendix 1 figure 36.

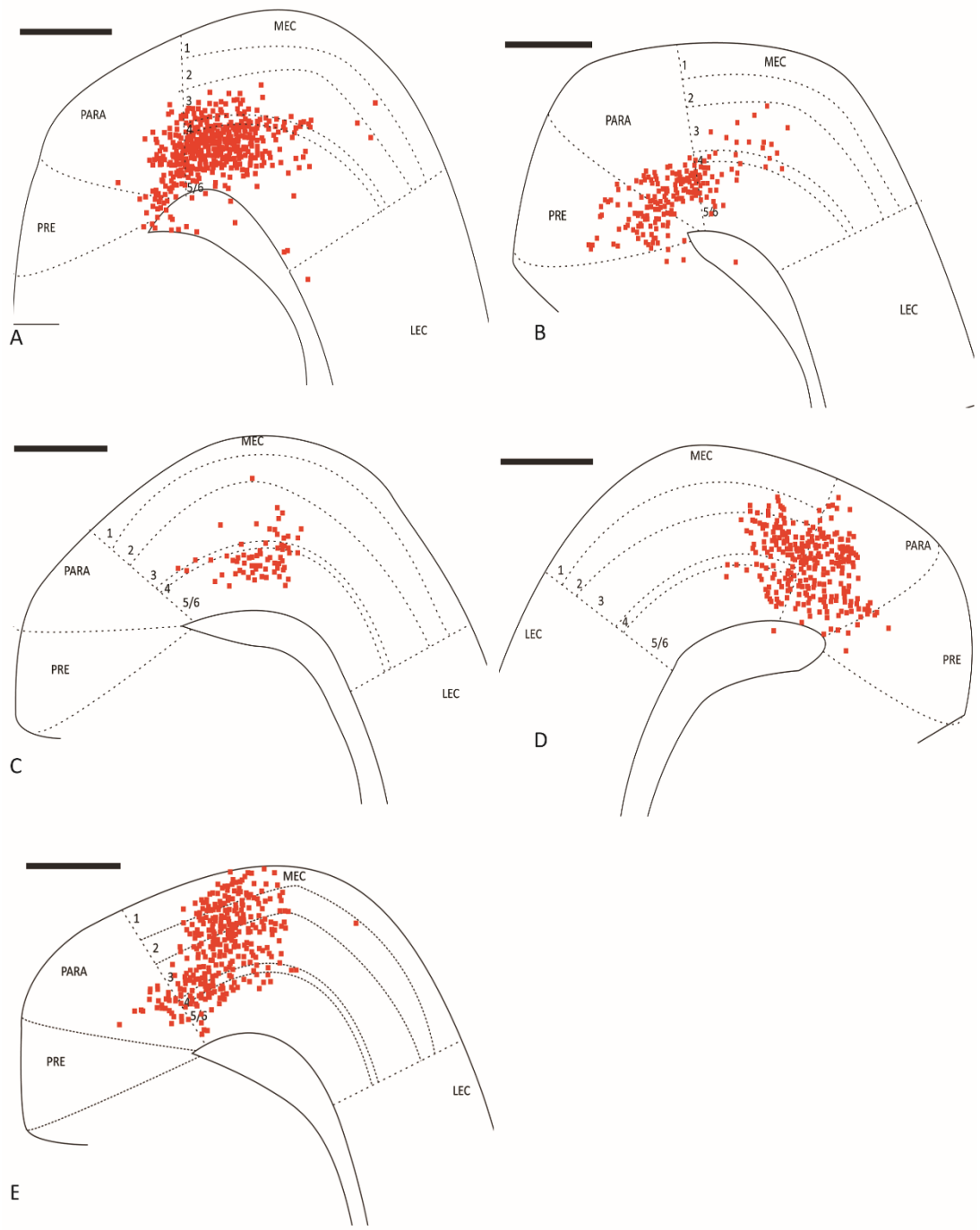


Figure 19 Enhancer driven expression of transgene in virus injection with LMO3.The solid line indicates the outline of obvious borders, while the dotted line indicates borders we have based on cytoarchitecture, the orange boxes represent the eGFP labelled cell bodies. A total of 5 sections in 4 mice were counted. (A) mouse 52927,(B) mouse 52928 left,(C) mouse 52928 right,(D) mouse 52929 left,(E) 52929 right. The black scale are 250 µm. All sections correspond to a dorso-ventral level of 2.82 mm ventral to bregma.

3.2.2.1 Summary of anatomical distribution after injection with lentiviral vectors

The average percentage of eGFP across the different layers of MEC can be calculated from the count of eGFP labelled cells. The percentage distribution of eGFP labeled cells in LV-ODZ3 injected mice was 3,33% (+/-1,18) ,12,42% (+/-6,27), 55,22% (+/-9,50) ,6,85% (+1,76/-1,76), 22,18 (+/-8,44)% in layer 1,

2, 3 ,4 and 5/6 respectively (Figure 20 A). The percentage distribution of transgene in LV-TRPS1 injected mice was 0%, 5,95% (+/-2,11), 46,52% (+/-4,85), 8,56% (+/-1,55), 38,70% (+/-5,56) in layer 1, 2, 3 ,4 and 5/6 respectively (Figure 20 B). The percentage distribution of transgene in LV-LMO3 injected mice was 1,58% (+/-1,58) , 7,48% (+/-5,46), 34,23% (+/-8,71), 8,04% (+/-1,80) ,48,62% (+/-12,75) in layer 1,2,3,,4,,5/6 respectively (Figure 20 C). In ODZ3 injected mice the expression of transgene was highest in layer 3 compared to other layers. In TRPS1 injected mice the expression of transgene was highest in layers 3 and 5/6. In LMO3 injected mice the expression of transgene was more significant in layer 5/6. (Figure 20, percentages are noted as mean +/- SEM).

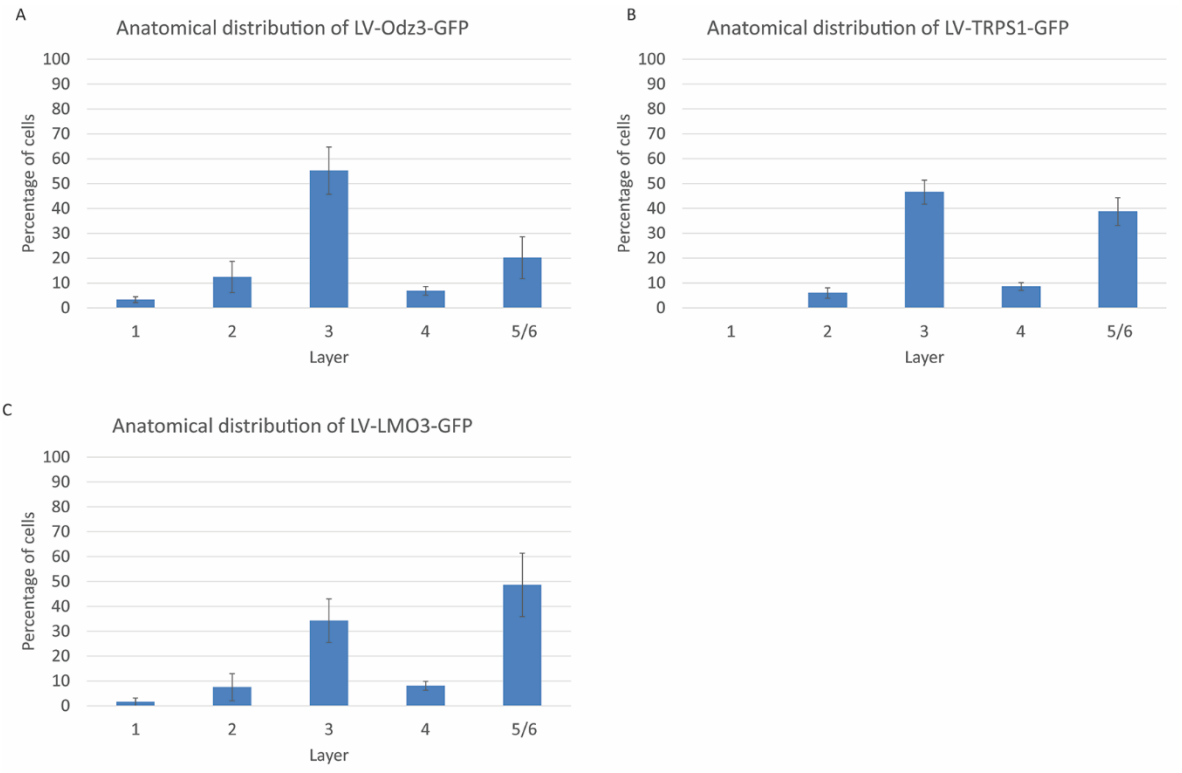


Figure 20 Percentage distribution of eGFP labeled cells across different layers in MEC of virus injected mice. (A) eGFP labeled cells in LV-odz3-eGFP injected mice, (B) eGFP labeled cells in LV-trps1-eGFP injected mice (C) eGFP labeled cells in LV-lmo3-eGFP injected mice. The error bars represent the standard error of mean.

3.3 Summary of anatomical distribution of transgene expression

In general in the virus injected mice, the expression of the transgene was not as confined to particular layers as in transgenic mice (Figure 21).

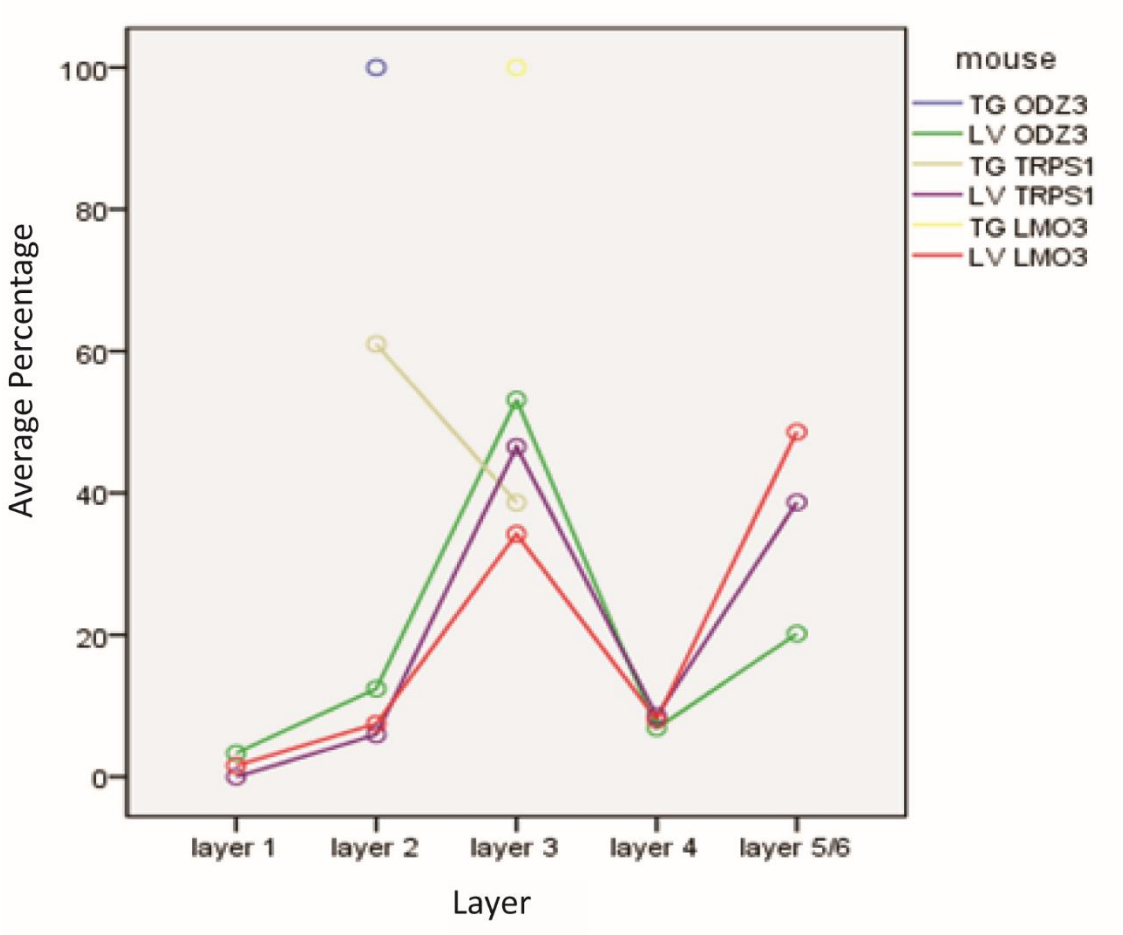


Figure 21 Comparison of transgene expression in transgenic mice and virus injected mice. The blue dot represent the TG ODZ3, blue line represent the TG TRPS1 and yellow dot represent TG LMO3, the green line represent the LV ODZ3,the purple line represent the LV TRPS1 and the red line represent the LV LMO3 transgene expression.

3.4 Comparison between transgenic mice and virus injected mice based on a molecular marker (calbindin)

In addition to anatomical location, the specific cell types may be identified by molecular markers. A common marker for specific cell types in the MEC is the calcium binding protein calbindin. We compared transgene expression in cell types marked by calbindin between transgenic mice and virus injected mice.

3.4.1 Distribution of calbindin positive neurons and transgenes in transgenic mice

We did immunohistochemistry against calbindin and 2A on sections from ODZ3 and TRPS1 transgenic lines to specify further which cell types express the transgenes. Since anatomical location of the cells is less important here, we did not include a NeuN stain to delineate layers and all delineations are based on 4', 6-Diamidino-2-Phenylindole (DAPI) staining. The sections were stained against calbindin

and 2A (Figure 22 B) and counterstained with DAPI (Figure 22 A). Both calbindin positive and 2A cells were marked (Figure 22 C). Special attention was payed to colocalization between 2A and calbindin.

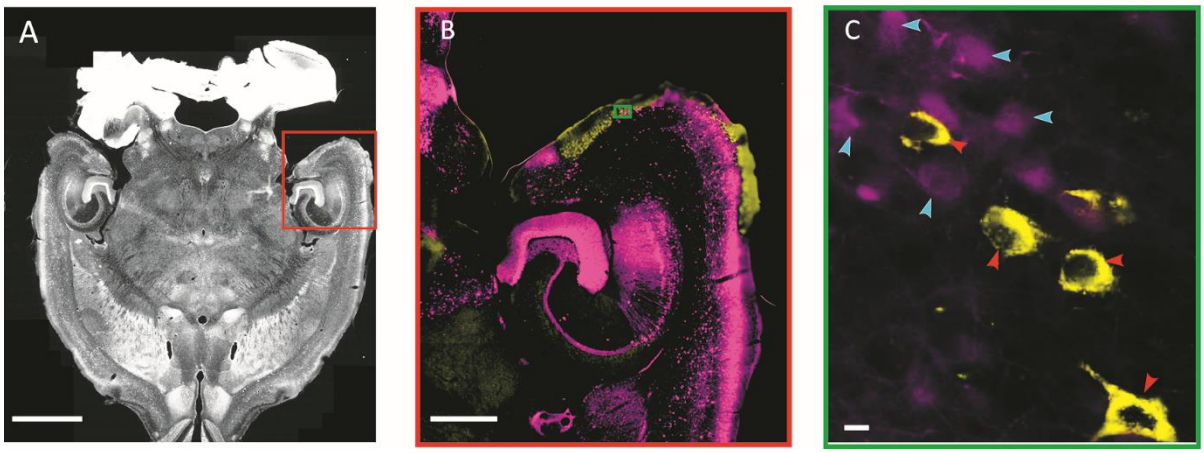


Figure 22 Immunohistochemistry against Calbindin and 2A in transgenic mice(A) section of mouse 54114 with 2A and calbindin staining,(B).Zoomed version of MEC in which there is expression of transgene and calbindin,(C) Highly magnified view of individual cells, cells expressing transgene 2A in yellow, calbindin positive cells in pink. Scale bar are 2000, 250 and 10 µm

3.4.1.1 Calbindin expression in transgene expressing cells of Odz3 transgenic mouse lines

Unpublished data on the Odz3 line has shown that none of the 2A+ cells colocalized with a marker for inhibitory neurons, GAD67. This means that we can assume that the transgene expressing cells in MEC of these transgenic mice are excitatory. Layer II of the MEC contains calbindin positive excitatory cells. To specify the type of excitatory cells, I stained and counted both sides of 5 sections of 2 mice from the Odz3 transgenic mouse line. In MEC, transgene was expressed in layer 2, whereas the calbindin were located in layer 2 and 3 and in the pre and para subiculum (Figure 23, 24). In the combined sections of both mice I have counted 1304 calbindin positive cells and 1257 2A labeled cells in the MEC. Of all these cells only one cell showed colocalization between 2A and calbindin (Figure 23 D). This means 0,55% (+/-0,1) of the transgene expressing cells are calbindin positive.

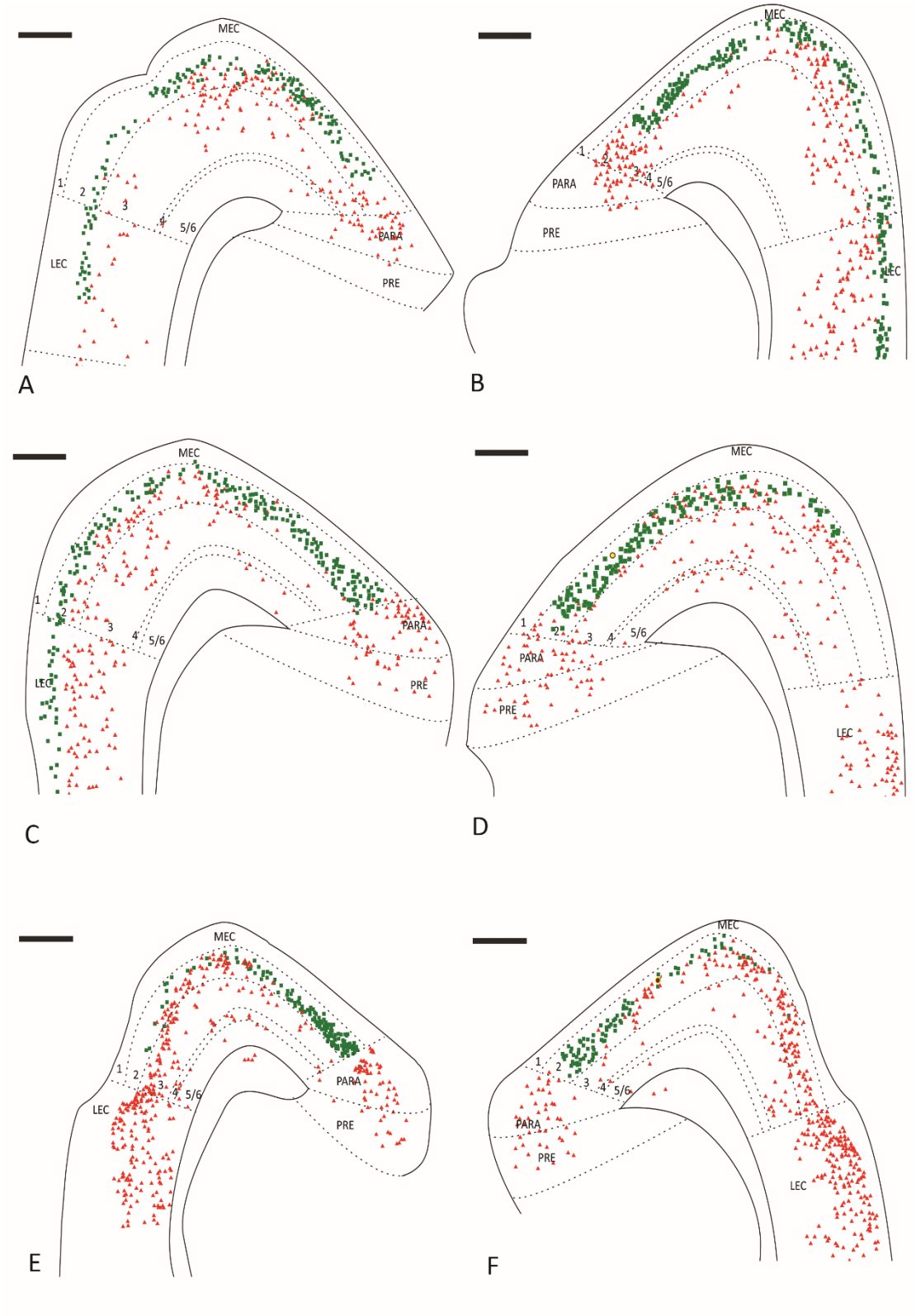


Figure 23 Expression of transgene and calbindin in transgenic line odz3.Outlines of horizontal sections of the MEC of mouse 54114. Red triangles represent calbindin positive cells whereas green rectangles represent 2A expressing cells. (A) and (B) dorso ventral level 3.3mm ventral to bregma (C) and (D) dorso ventral level 3.18mm ventral to bregma (D) and (E) dorso ventral level 2.94mm ventral to bregma scale bars are 250µm.

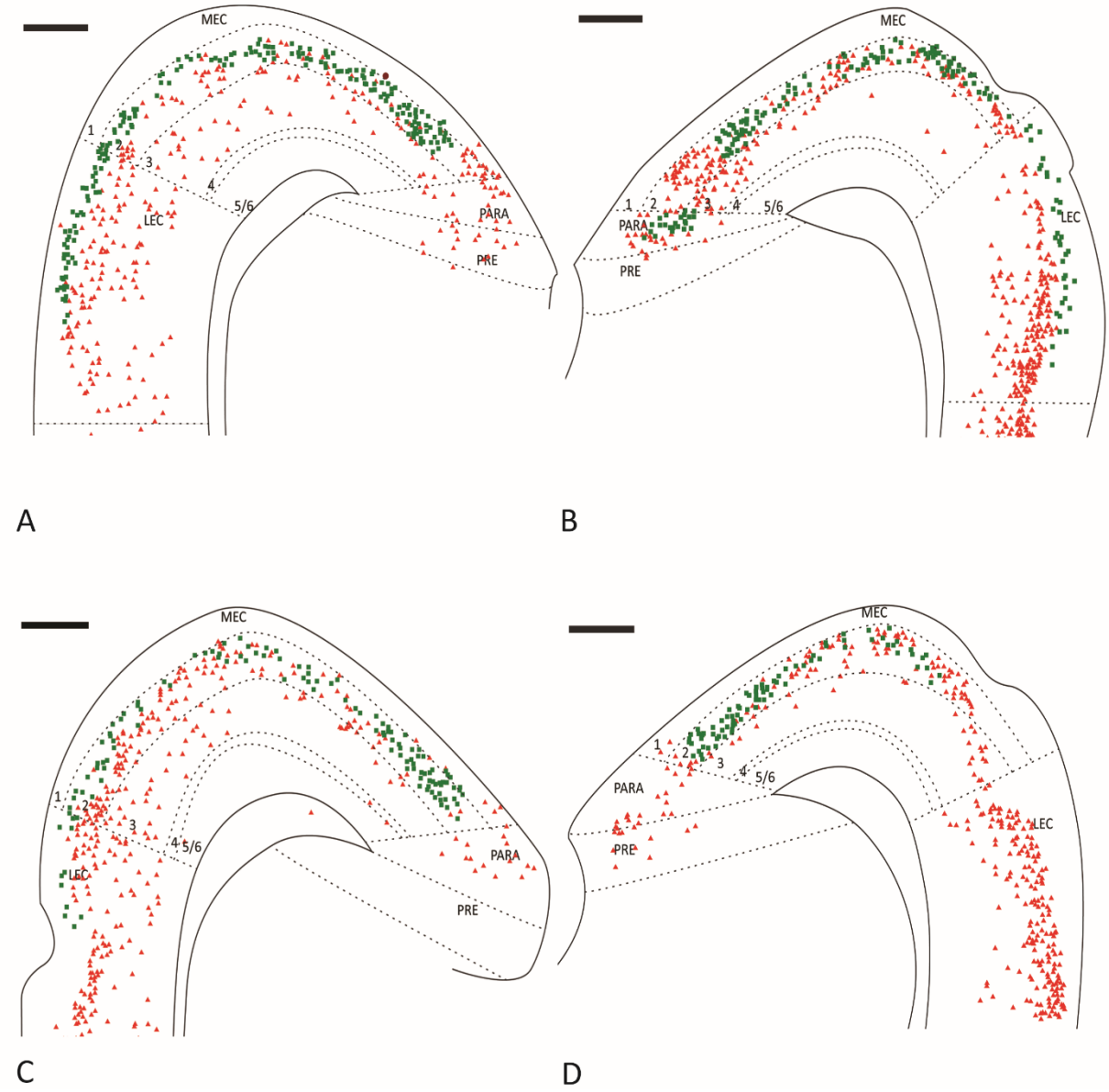


Figure 24 Expression of transgene and calbindin in transgenic lines ODZ3. Oultines of horizontal sections of the MEC of mouse 54337. Red triangles represent calbindin positive cells whereas green rectangles represent 2A expressing cells. (A) and (B) dorso ventral level 3.3 ventral to bregma,(C) and (D) dorsoventral level 3.18 ventral to bregma. Scale bars are 250µm.

3.4.1.2 Calbindin expression in transgene expressing cells of Trps1 transgenic mouse lines

In TRPS1 transgenic lines the expression of transgene 2A was in both layer 2 and layer 3. In ventral section (bregma -3.3) (Figure 25 A, B) calbindin was present mainly in between layer 2 and layer 3 but in case of dorsal section (bregma -2.82) (Figure 25 E, F) the calbindin was found mostly in layer 2. There was no colocalization between 1642 calbindin positive cells and 3985 2A expressed cells in both mice 53900 and 53516 (Figure 25, 26). No colocalization means 0% of the transgene expressing cells in the TRPS1 line express calbindin.

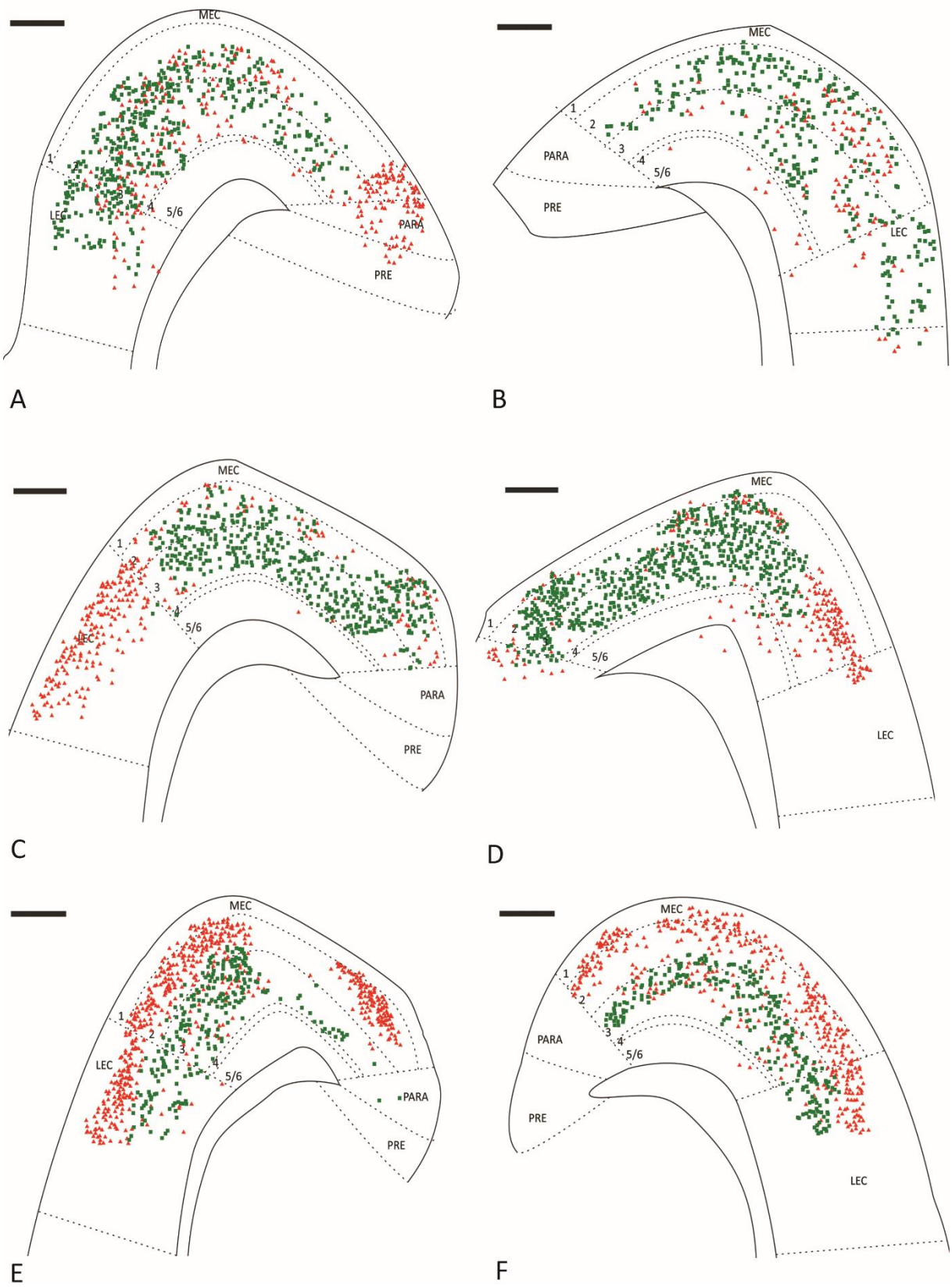


Figure 25 Expression of transgene and calbindin in transgenic line TRPS1Outlines of horizontal sections of the MEC of mouse 53900. Red triangles represent calbindin positive cells whereas green triangles represent 2A expressing cells. (A) and (B) dorso ventral level 3.3mm ventral to bregma, (C) and (D) dorso ventral level 3.18mm ventral to bregma , (E) and (F) dorso ventral level 2.94mm ventral to bregma. Scale bars are 250µm.

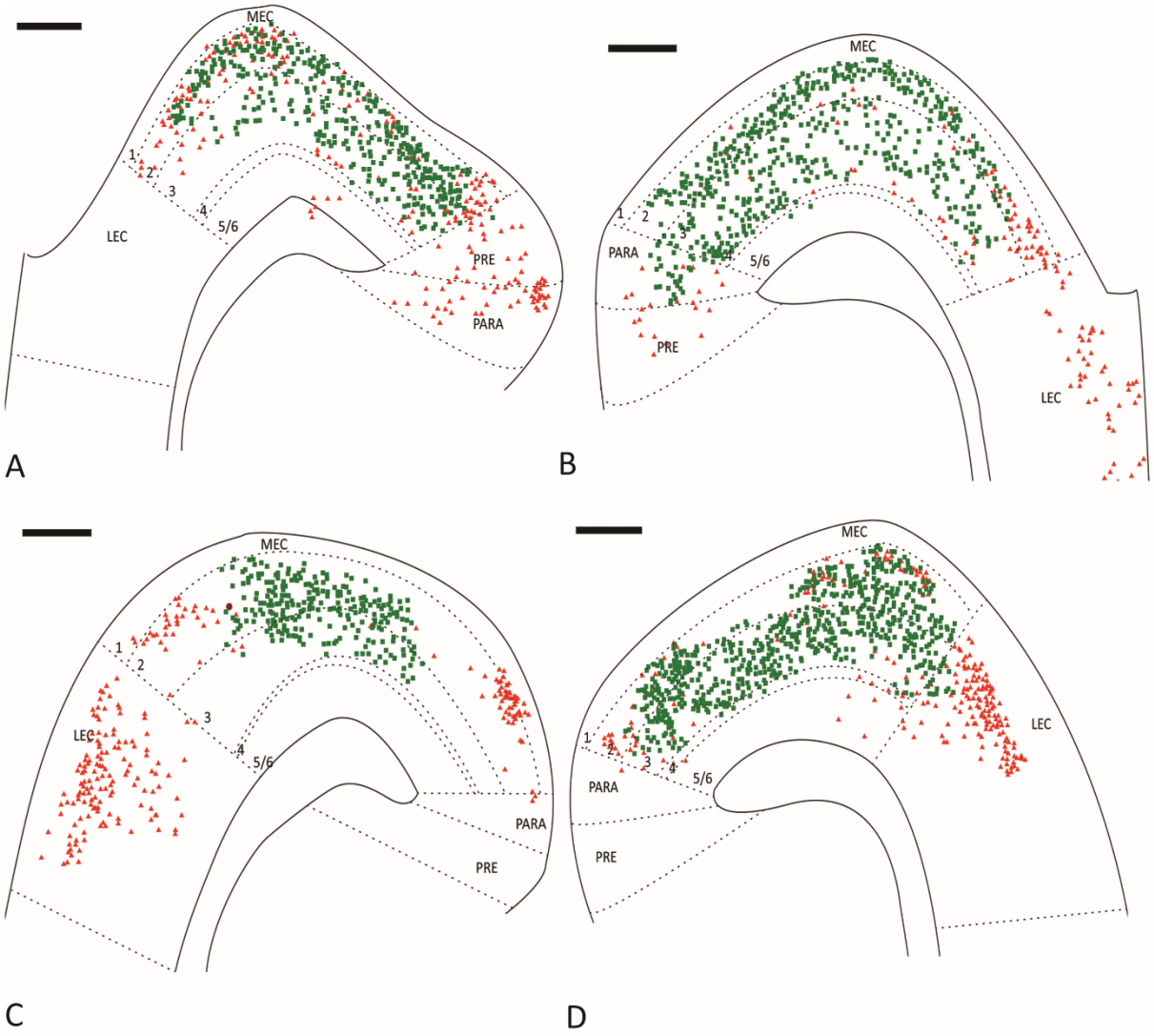


Figure 26 Expression of transgene and calbindin in transgenic lines TRPS1. Outlines of horizontal sections of the MEC of mouse 53516. Red triangles represents calbindin positive cells whereas green rectangles represent 2A expressing cells. (A) and (B) dorso ventral level 3.18mm ventral to bregma, (C) and (D) dorso ventral level 2.82mm ventral to bregma. Scale bars are 250µm

3.4.2 Distribution of calbindin positive neurons and transgenes in virus injected mice

Immunohistochemistry against calbindin was done on the sections of mice injected with LV-ODZ3-eGFP and LV-TRPS1-eGFP to specify the cell types in which the transgene is expressed.

The data was collected in the same way data was collected from sections of transgenic mice. The sections were stained against calbindin (Figure 23 B) and counterstained with DAPI (Figure 27 A). In the MEC eGFP expressing cells and calbindin expressing cells were marked (Figure 27 C). Special attention was payed to colocalization between eGFP and calbindin.

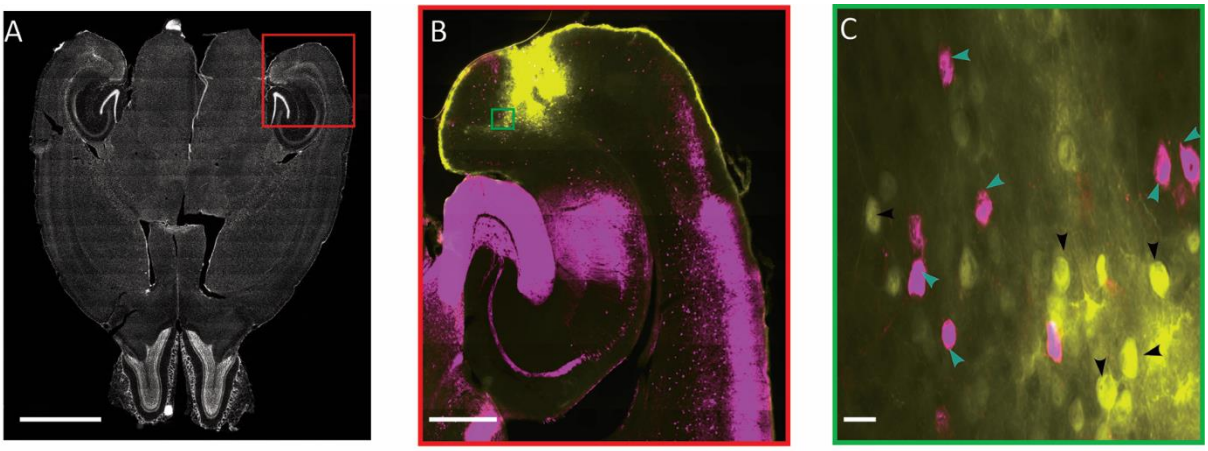


Figure 27 Calbindin and eGFP expression in Lentivirus injected mice. (A) A full horizontal section of mouse 52927 with DAPI staining. (B) MEC in which there is expression of eGFP (in yellow) and calbindin (in pink). (C) Highly magnified view of individual cells. Scale bars are 500, 200 and 10 µm respectively.

3.4.2.1 Distribution of calbindin positive neurons and transgenes in virus injected mice (ODZ3)

In 3 sections from 3 mice, we counted 3 sections for calbindin positive cells and eGFP labeled cells in the MEC (Figure 28).Since the injection was made in MEC, the transgene (eGFP) expression was in this area (MEC). In mouse 53130 calbindin was spread in all layers in MEC whereas transgene (GFP) was expressed in layer 3 and layer 5/6. In mouse 53132, few calbindin positive cells were observed in MEC, whereas in mouse 53133 calbindin positive cells were dominant in presubiculum and parasubiculum and in LEC. But there was no colocalization between calbindin and eGFP expressing neurons. eGFP expression was confined to MEC, but very few calbindin positive cells were present in MEC. There was no overlapping zone in transgene (eGFP) expression and calbindin positive cells (Figure 28 B).

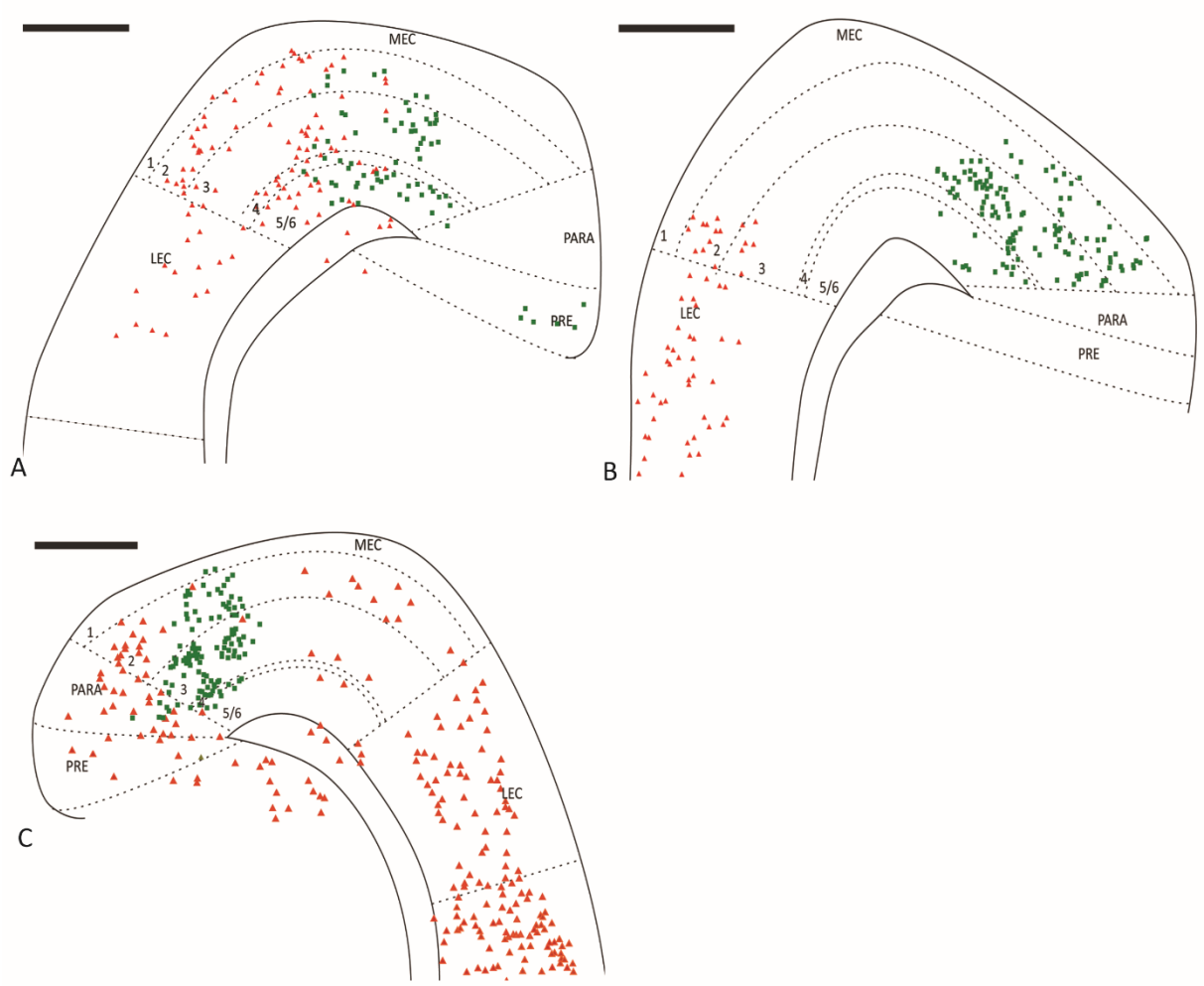


Figure 28 Expression of transgene and calbindin in LV-ODZ3-eGFP injected mice. Outlines of horizontal sections of MEC of (A) mouse 53130, (B) mouse 53132, (C) mouse 53133.The red triangles represent calbindin positive cells and the green rectangles represent transgene expressed cells. The black scale bar are 250µm. All sections correspond to dorso ventral level of 2.94mm ventral to bregma.

3.4.2.2 Distribution of calbindin positive neurons and transgenes in virus injected mice (TRPS1)

In 5 sections from 4 mice, we counted 5 sections for calbindin positive cells and eGFP labeled cells in the MEC (Figure 29). We did not find any colocalization between the eGFP and calbindin positive cells in all the virus injected mice with TRPS1. Note that in mice 52930, 52931 and 53312 (Figure 29 A, C), few calbindin positive neurons were found in MEC region. Whereas in mouse 52931 (Figure 29 B) calbindin positive neurons were quite significant in layer 2 of MEC but not around the eGFP labeled cells which were mostly present in layer3, 4, 5/6. In mouse 53313 (Figure 29 E) few calbindin positive cells were found in layer 2. Meaning generally there was little overlap between the spread of eGFP positive cells and the regions where we found calbindin positive cells.

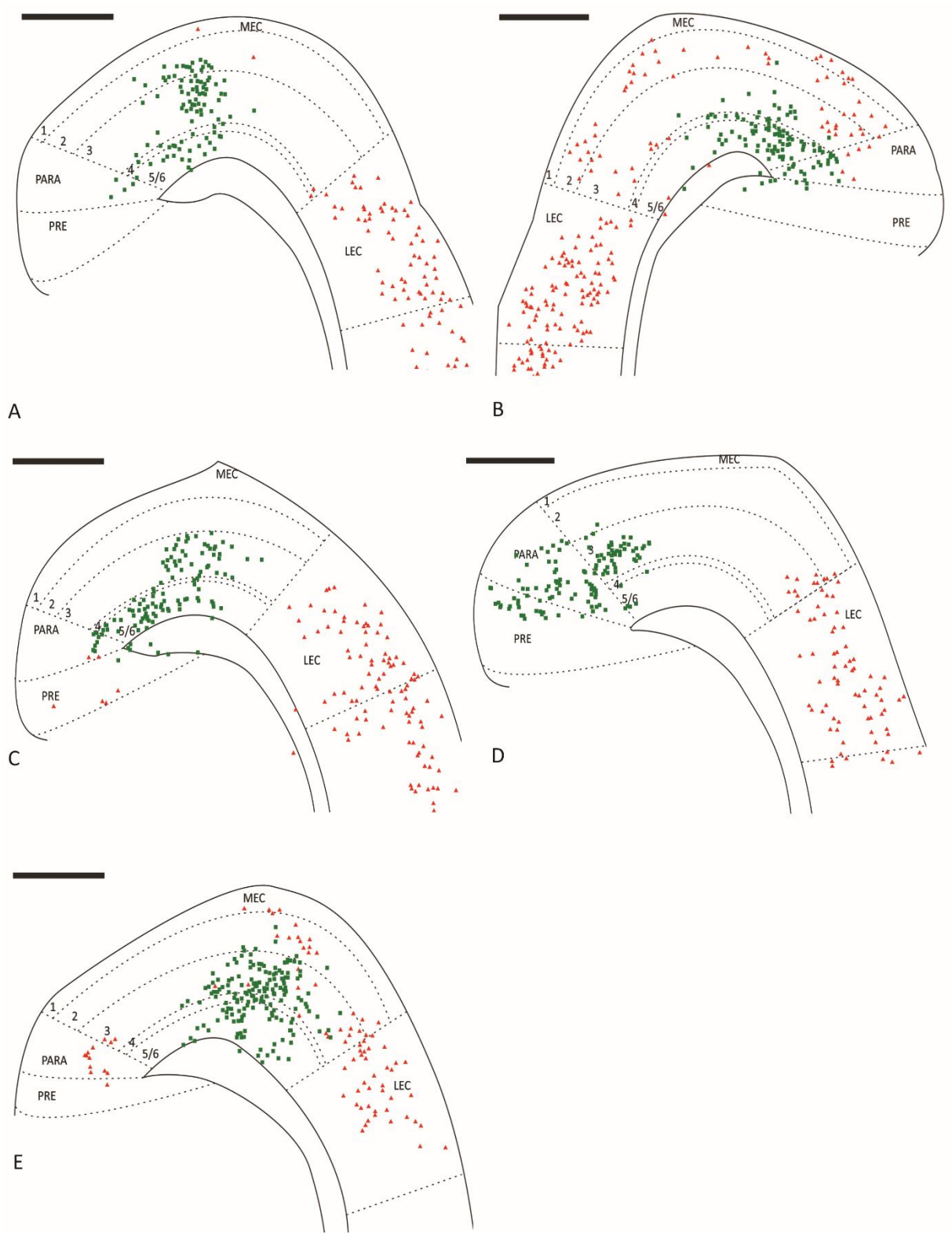


Figure 29 Expression of transgene and calbindin in LV-TRPS1-eGFP injected mice. Outlines of horizontal sections of (A) mouse 52930, (B) mouse 52931 left, (C) mouse 52931 right, (D) mouse 53312, (E) mouse 53313. The red triangles represent calbindin positive cells whereas the green rectangles represent the transgene expressed cells.The black scale bars are 250µm. All sections correspond to dorso ventral level of 2.94mm ventral to bregma.

3.4.3 Comparing transgenic mice and virus injected mice based on molecular marker

In neither transgenic mice nor in virus injected mice there was colocalization between calbindin and transgene expressing cells (except one cell in ODZ3 mouse (Figure 23 D)). Note that the pattern of distribution of calbindin positive cells in transgenic lines and virus injected mice was quite different. Lentivirus injected mice had fewer calbindin positive cells in MEC compared to transgenic mice in MEC.

4 Discussion

4.1 Summary of main research

This work was done to investigate if expression of enhancer driven transgenes introduced through pronuclear injection is similar to the expression of transgenes introduced through lentivirus into the adult mouse brain. The transgene expression was first analyzed on the basis of their expression in different layers of MEC and secondly on the basis of colocalization of calbindin and transgene. Our results show that the expression of transgene in virus injected mice was not as layer specific as the expression in transgenic mice. The transgenes in lentivirus injected mice were expressed in all layers of the MEC. The cell specificity was determined by analyzing the colocalization of calbindin and transgene expressed cells. There was no colocalization between calbindin and transgene in both virus injected and transgenic mice (except one in transgenic ODZ3 mouse). But the pattern of calbinidin staining in the transgenic mice and virus injected mice was different.

4.2 Integration of enhancers into pLV plasmids

Enhancers can drive specific transgene expression in transgenic mouse lines (Figure 2). To test if this enhancer driven specificity is maintained after lentiviral based transfer of transgenes, we first cloned the enhancer into plasmids that allow the production of lentiviral vectors. The PCR product of enhancers ODZ3, TRPS1 and LMO3 were of the expected size (Figure 7). The enzyme digestion of the pLV plasmid shows that the pattern of band size were as expected for ODZ3 and TRPS1 but not for LMO3. This may be explained by the fact that the XbaI enzyme used for digestion of LMO3 pLV is prone to Dam methylation. The GATC site may have been methylated giving different product sizes than expected. We observed bands at about 600 and 10,000. If the XbaI site at position 7196 was methylated and remained uncut this is the expected pattern (Figure 8 C).

The sequencing result for ODZ3, TRPS1 and LMO3 verified that the enhancer is present in the pLV plasmid. By the fact that we get reads for all 6 primers in the corresponding plasmids, we are certain that the enhancer sequence is integrated in the plasmid. All plasmids had a 100% match between the expected sequence and the reads we got back from the sequencing. Previous research has shown that single nucleotide mutations in enhancers can have a substantial effect on expression level of the

gene they control (Okhovat et al., 2015) . Since we had a 100% match, this is not a factor we need to consider.

4.3 Anatomical spread of transgene expression

4.3.1 Transgene expression in transgenic mice

The distribution of 2A expressing cells was 99,45% in layer 2 in ODZ3 transgenic mice (Figure 15). The 0,55% expression in layer 1 might be the result of using the adjacent sections for cytoarcitecture. The percentage of expressed 2A was 61% and 38% in layer 2 and layer 3 respectively in TRPS1 transgenic lines (Figure 17). Unpublished data on ODZ3 transgenic lines shows that there was no colocalization between GAD67 and 2A expressed cells suggesting that the transgene in both ODZ3 and TRPS1 lines are excitatory cells.

In mice 53900 and 53516 TRPS1 transgenic mice the percentage of transgene expressed cells in layer 2 and layer 3 is different (Figure 13 and Figure 14). As the sections varied in dorsoventral axis so the difference in gene expression might have varied in these two mice or it could be the result of differential staining between sections. The phenomenon of genetic drift might have occurred. The initial phenotype that is identified in earlier generation of transgenic mice can change with time. As these two mice though develop from same enhancer might have undergone different phenotypic selection overtime. To overcome the genetic drift the breeders should be refreshed from time to time after 5 generation (Jankowsky et al., 2001).

4.3.2 Transgene expression in lentivirus injected mice

None of lentivirus injections show any obvious layer specificity (Figure 20). In all of the lentivirus injected mice the spread of transgene is across several layers (Figure 17, 18, 19). In ODZ3 and TRPS1 the expression of transgene was highest in layer 3 whereas for LMO3 the transgene was highest in layer 5/6.

The expression of transgene within same virus vector was different as the transgene expressed cells range in number from 46 to 475 (in case of ODZ3), 217 to 347 (in case of TRPS1) and 65 to 439 (in case of LMO3). The percentage of transgene within the MEC layer varies in all virus injected mice the dominant being either layer 3 or layer 5/6. The pattern of spread of virus in ODZ3 enhancer looks

similar in 3 sections out of 5. In mice 52931 and 53312 the viral spread is observed in the parasubiculum (figure 18 B, D). For LMO3 the spread of transgene is different in each section. So we can say that the transgene expression is different in virus vectors with the same enhancer. This implies that the enhancer driven transgene after transfer by lentiviral vector does not have anatomical specificity. This also suggests expression of the transgene after transfer by lentiviral vectors does not have cell type specificity. This result may be biased as only one section from each mice's brain is counted.

4.4 Distribution and colocalization of calbindin and transgene in transgenic mice and lentivirus injected mice

Expression of certain molecular markers can be used for the classification and identification of neuronal subtypes (Xu et al., 2010). We used the molecular marker calbindin, which is readily detected by immunohistochemistry, to specify the cell type of the transgene expressed

4.4.1 Distribution of calbindin in transgenic mice

Unpublished data on ODZ3 line has shown that none of 2A positive cells colocalized with a marker specific to inhibitory neurons, GAD67. So we expect the 2A transgene is expressed in excitatory cells. The cells in MEC specially layer 2 can be distinguished based on differences in the immunoreactivity to calbindin (Fujimaru and Kosaka, 1996) and reelin (Chin et al., 2007) (Varga et al.).To know the subtypes of excitatory cells we are using a molecular marker calbindin.

The distribution of calbindin positive cells I found was different from the results by Kitamura et al. where they form the island like patches of cells. The cells in the island are calbindin positive whereas cells out of the islands are calbindin negative (Kitamura et al., 2014). Calbindin positive cells show a different pattern in case of TRPS1 (Figure 25 E, F and figure 26 C, D) because of different dorsoventral organization of the sections and these sections are the dorsal sections. Only one cell that showed colocalization between calbindin and transgene was observed in transgenic mice ODZ3 (Figure19 B) but no other colcalization observed in both ODZ3 and TRPS1 transgenic mice. Calbindin negative cells in layer 2 are usually stellate cells (Kitamura et al., 2014; Ray et al., 2014; Tang et al., 2014), so we expect the transgene is expressed mostly in stellate cells in case of ODZ3 transgenic mice.

4.4.2 Calbindin in lentivirus injected mice

There was no colocalization between calbindin and transgene in lentivirus injected mice, which was similar to transgenic mice. It was likely that the transgene expression in transgenic mice were calbindin negative (means no colocalization) as they were cell specific. However the unexpected staining in case of virus injected mice cast doubt about the cell specificty of transgene expressed cells. Fewer calbindin positive cells were observed in the virus injected mice compared to the transgenic mice. The zone of transgene expression and calbindin positive cells were quite distinct in case of TRPS1. The periphery of transgene expressed cells in case of TRPS1 shows almost no calbindin expression. It gives the impression that the integration of provirus may have alter the expression of cellular gene. Alternatively, this could be the effect of non-uniform staining during the experiment. Finally, it is possible that the supernatant during the viral production might contain contaminants affecting the tissue the concentrated virus is injected to.

4.5 Comparing the transgene expression and colocalization in transgenic and lentivirus injected mice

The expression of transgene in case of transgenic mice is limited to specific layers of MEC (layer 2 for ODZ3 mice and layer 2 and layer 3 for TRPS1 transgenic mice) and there was almost no colocalization with calbindin. This suggests that transgene expression in transgenic mice is cell type specific. Virus injected mice have expression almost in every layer of MEC which suggests that the transgene expression was neither layer nor cell type specific. The transgene expression in virus injected mice in TRPS1 was high in layer 3 and in layer 5/6, whereas transgenic lines exhibit the expression of transgene in layer 2 and 3. For ODZ3 lentivirus injected mice the expression was high in layer 3 whereas in transgenic lines the expression of transgene was dominant in layer 2. So this observation led to conclusion that there is significant difference in transgene expression in transgenic and lentivirus injected mice.

A near complete lack of co-localization between the transgene and calbindin was observed in both transgenic lines and virus injected mice. This could be used as an argument that transgene expressing cells were both calbindin negative. However we cannot say about the type of cells in which the transgene is expressed in lentivirus injected mice as it neither showed layer specificty nor cell type specificty. But in case of transgenic lines like ODZ3 we can say about the nature of cells in which transgene is expressed. The transgenes were expressed in layer 2 and as they do not colocalize with

GAD67 (from unpublished data), we are sure that the transgene were expressed in excitatory cells. The lack of colocalization with calbindin further supports the fact that the transgene was expressed in excitatory stellate cells.

The lack of overlapping zone between transgene and calbindin especially in case of TRPS1 lentivirus injected mice gives enough explanation for lack of colocalization between this two (Figure 29). Though in ODZ3 lentivirus injected mice the transgene and calbindin both have random distribution in MEC (Figure 28 A and C) but we didn't observe colocalization between calbindin and transgene.

4.6 Potential mechanism for unspecific expression in lentivirus injected mice

There could be several reason for the differences in the transgene expression in transgenic mice and lentivirus injected mice. The transgene expression by Lentivirus is regulated by factors like viral titer, envelope protein, enhancer and promoter of provirus. The spread of virus is difficult to control, potentially giving under or over expression in the region right around the injection site. The expression of transgene is highly titer regulated (Matrai et al., 2010), though the same titer has been used in injecting all the mice, the transgene expression in these mice varied. We know that lentivirus system is based on reverse transcribing of viral RNA into dsDNA and insertion of this dsDNA into host DNA usually by non-homologous recombination which is usually a random process (Ramezani and Hawley, 2002). This random insertion might result in expression of non-targeted gene. Hence the insertional effect might have influence the layer specificity of enhancers.

The unspecific expression of virally transferred transgenes we see may occur because of two reasons, firstly because of integration of the vector to transcriptionally active sites (Schroder et al., 2002) and secondly because of residual promoter activity of the 5'LTR (Naldini et al., 1996). Either one of these mechanisms or a combination of the two can be the reason of the unspecific expression of transgene.

Study on the site for integration for HIV-1 virus suggest that they are inclined to integrate in the transcriptionally active sites (Ciuffi, 2008; Schroder et al., 2002; Wang et al., 2007). As the lentivirus integrates in the gene rich transcriptionally active region the expression due to insertional effects is expected (Schroder et al., 2002) and in our case observed. The promoter trapping of HIV-1 reveals that the integration at distal site from promoter region result in abnormal transcription of transgene. (De Palma et al., 2005).

The destination plasmid we use for making lentivirus consists of 5'LTR which is usually a chimera of CMV or RSV promoter along with the LTR repeats from HIV-1 virus. In our case, it is a chimera of CMV

and a HIV 5'LTR. The CMV promoter is used in the production phase of lentivirus, where the lentivirus is produced from co-transfection of 3 helper plasmid and transfer plasmid into 293T cells. In this phase CMV promoter helps in the growth of lentivirus. During harvesting process of lentivirus, the virus is linearized, during which the CMV promoter region is cleaved but the 5'LTR repeat is still there. So the residual activity of 5'LTR could have been activated after integration in host (mouse) gene. As 5'LTR act as promoter (Klaver and Berkhout, 1994) it might influence the host gene as well which might result in different expression of transgene. Lentiviral vectors with flexed payload (such as (pLV-flexChR2-eGFP-WPRE) (Boyden et al., 2005)) do not show any expression in wild type brains showing that the transcription in antisense direction of WPRE sequence do not show leaky expression. But the presence of 5'LTR activity might result in leaky expression. The leaky expression might have result in unspecific transgene expression in every layers. Thus the specificity of enhancer might have been overshadowed by 5'LTR promoter activity. This explanation is also supported by study of lentivirus transgene delivery in neuron, where the strong promoter like CaMKII was used to drive the transgene expression in neurons but the expression of transgene was conferred by the RSV promoter (Holehonnur et al., 2015).

4.7 Improvements in Lentivirus to improve cell type specificity

Viruses do not render cell type specific transgene expression on their own. They are modified using envelope protein, specific promoter to achieve required specificity. Enveloped viral vectors have been used to express transgenes in specific neurons (Cronin et al., 2005). Later the neuron type specific promoter like CaMKII were used to restrict expression to excitatory glutamergic neurons (Dittgen et al., 2004). But the specificty of these neurons was not absolute. In our case we use the enhancer to drive the cell type specifity and observe that the expression of transgene was not cell type specific.

If we assume that the combination of 5'LTR promoter activity and integration in transcriptionally active site is the cause of a lack of specificity in our case then we can isolate the effect of 5'LTR by coding the construct in antisense with WPRE sequence. This will nullify the effect of 5'LTR and will allow the enhancer based cell specificty in the target region, as enhancers have high potential to drive cell type specific genetic labeling (Gray et al., 2015).

5 Conclusion

The main aim of this thesis was to compare enhancer driven expression in lentivirus injected mice and transgenic mice. The anatomical study of transgene suggest that the transgene expression in lentivirus injected mice were neither layer specific nor cell specific in comparison to transgenic mice with same enhancer which showed higher specificity. Thus the lentivirus along with enhancer is not sufficient enough to drive the cell specific transgene expression. Further improvements in making lentivirus is needed to ensure the specificity required to use lentivirus in studying the brain circuit and manipulating them with accuracy.

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APPENDIX 1 SUPPLEMENTARY FIGURES

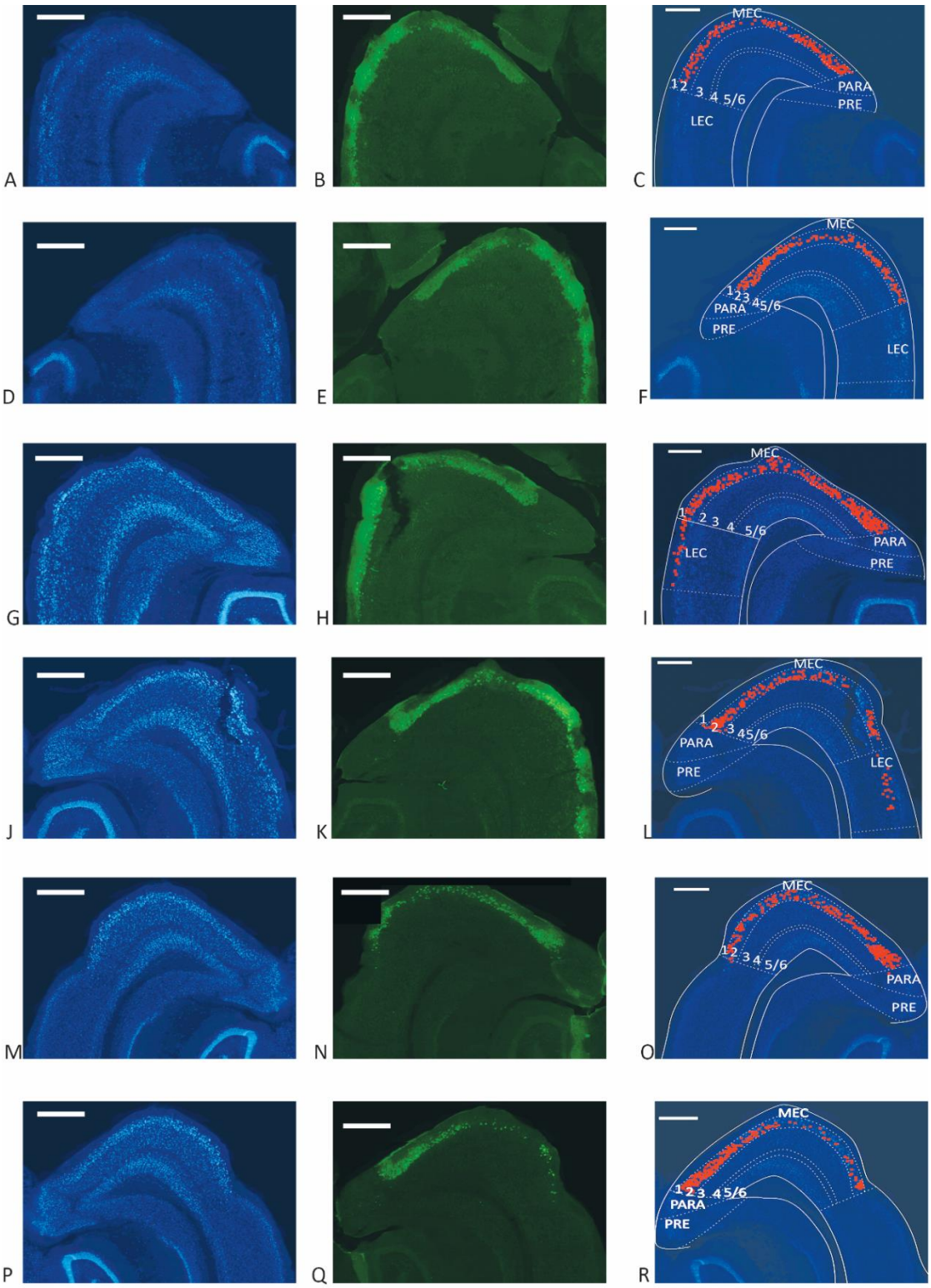

Figure 30 Enhancer driven expression of transgene in ODZ3 (mouse 54114). Left panel (NeuN staining), central panel (2A expression), right panel (overlap of left and central panel, the orange boxes represent the 2A labeled cell bodies. Fig A, B, C, D, E, and F belongs to dorso ventral level 3.3mm ventral to bregma. Fig G,H,I,J,K,L belongs to dorso ventral level 3.18mm ventral to bregma, whereas fig M,N,O,P,Q,R belongs to dorso ventral level 2.94mm ventral to bregma. White scale bar are 250µm.

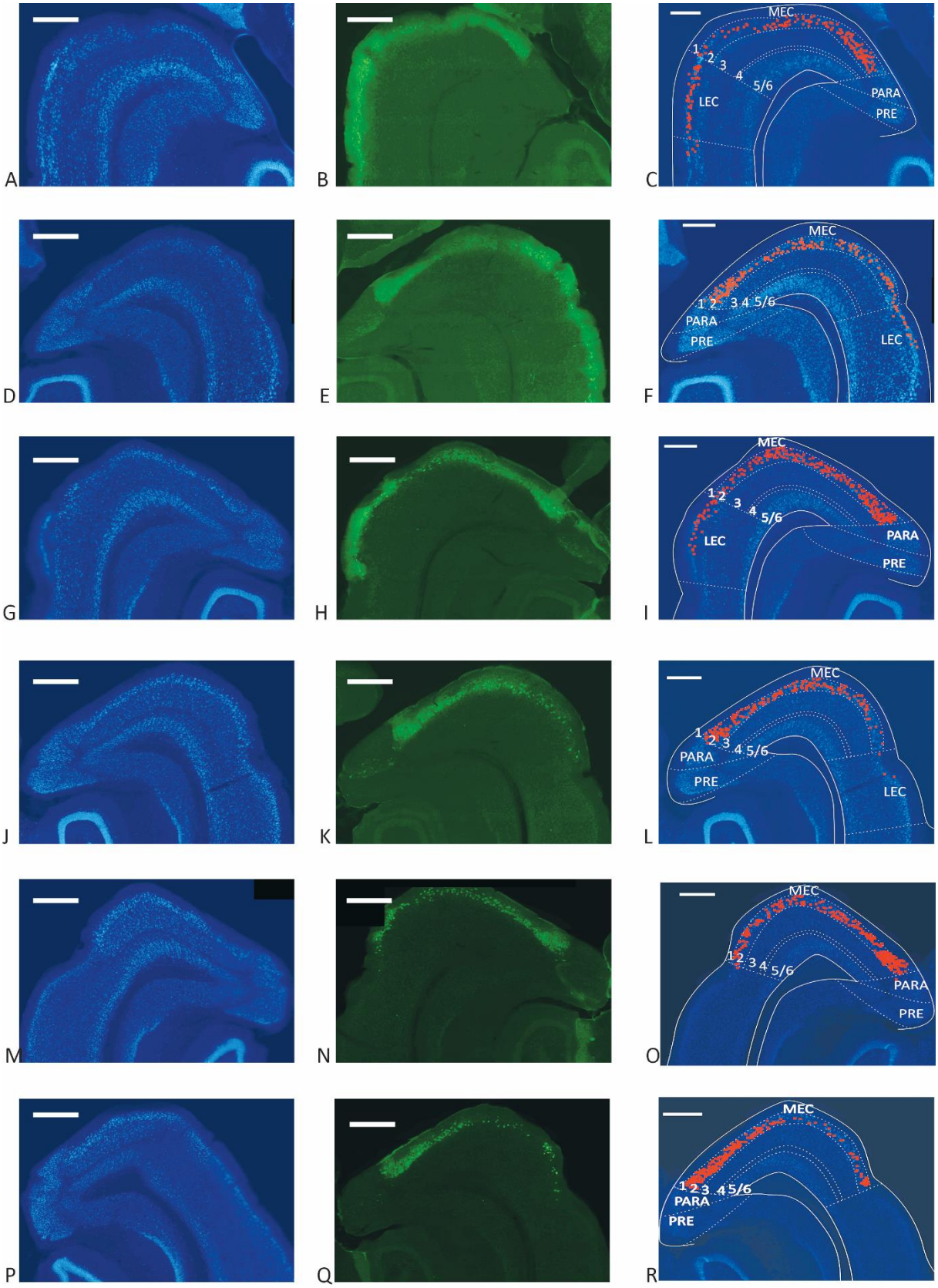

Figure 31 Enhancer driven expression of transgene in ODZ3 (mouse 54337). Left panel (NeuN staining), central panel (2A expression), right panel (overlap of left and central panel, the orange box represent the 2A labelled cell bodies. Fig A, B, C, D, E, and F belongs to dorso ventral level 3.3mm ventral to bregma. Fig G,H,I,J,K,L belongs to dorso ventral level 3.18mm ventral to bregma whereas fig M,N,O,P,Q,R belongs to dorso ventral level 2.94mm ventral to bregma.White scale bar are 250µm.

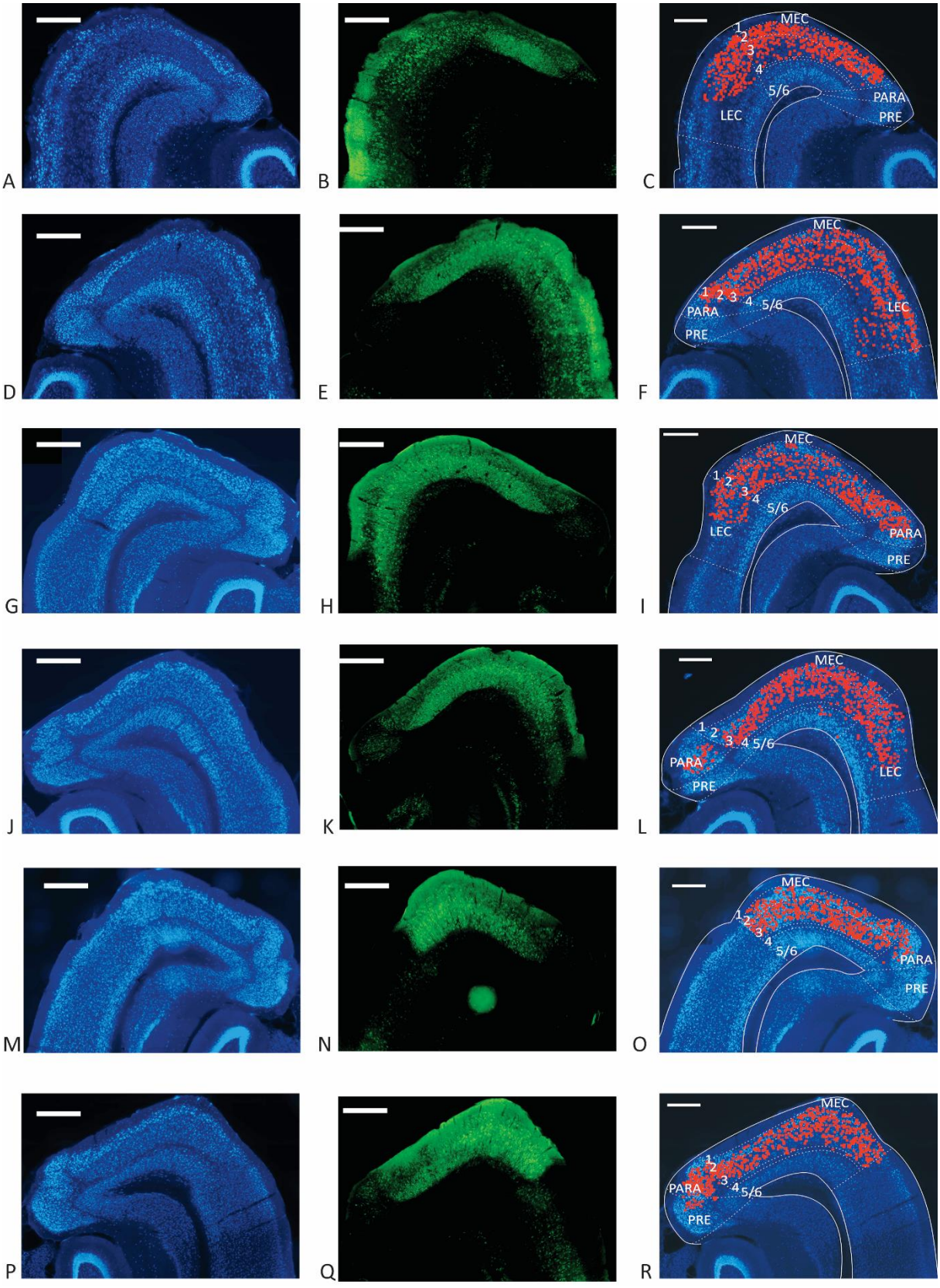

Figure 32 Enhancer driven expression of transgene in TRPS1 (mouse 53900). Left panel(NeuN staining),central panel(2A expression),right panel(overlap of left and central panel, the orange boxes represent the 2A labeled cell bodies .Fig A,B,C,D,E,F belongs to dorso ventral level 3.3 ventral to bregma. Fig G,H,I,J,K,L belongs to dorso ventral level 3.18mm ventral to bregma, whereas fig M,N,O,P,Q,R belongs to dorso ventral level 2.9mm ventral to bregma .White scale bar are 250µm.

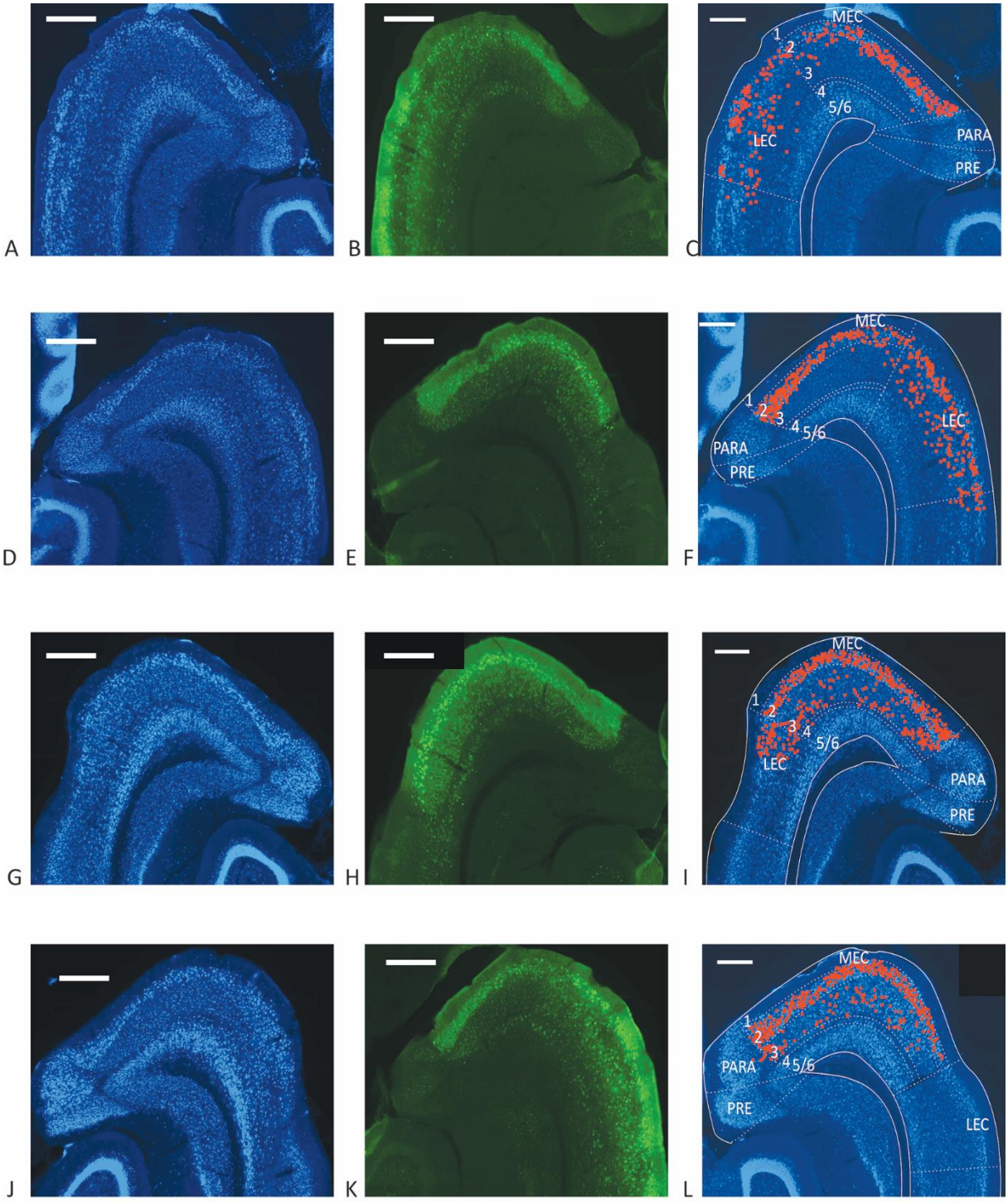

Figure 33 Enhancer driven expression of transgene in TRPS1 (mouse 53516). Left panel (NeuN staining), central panel (2A expression), right panel (overlap of left and central panel, the orange boxes represent the 2A labeled cell bodies. Fig A, B, C, D, E, and F belongs to dorso ventral level 3.18mm ventral to bregma. Fig G, H, I, J, K, L belongs to dorso ventral level 2.94mm ventral to bregma. White scale bar are 250µm.

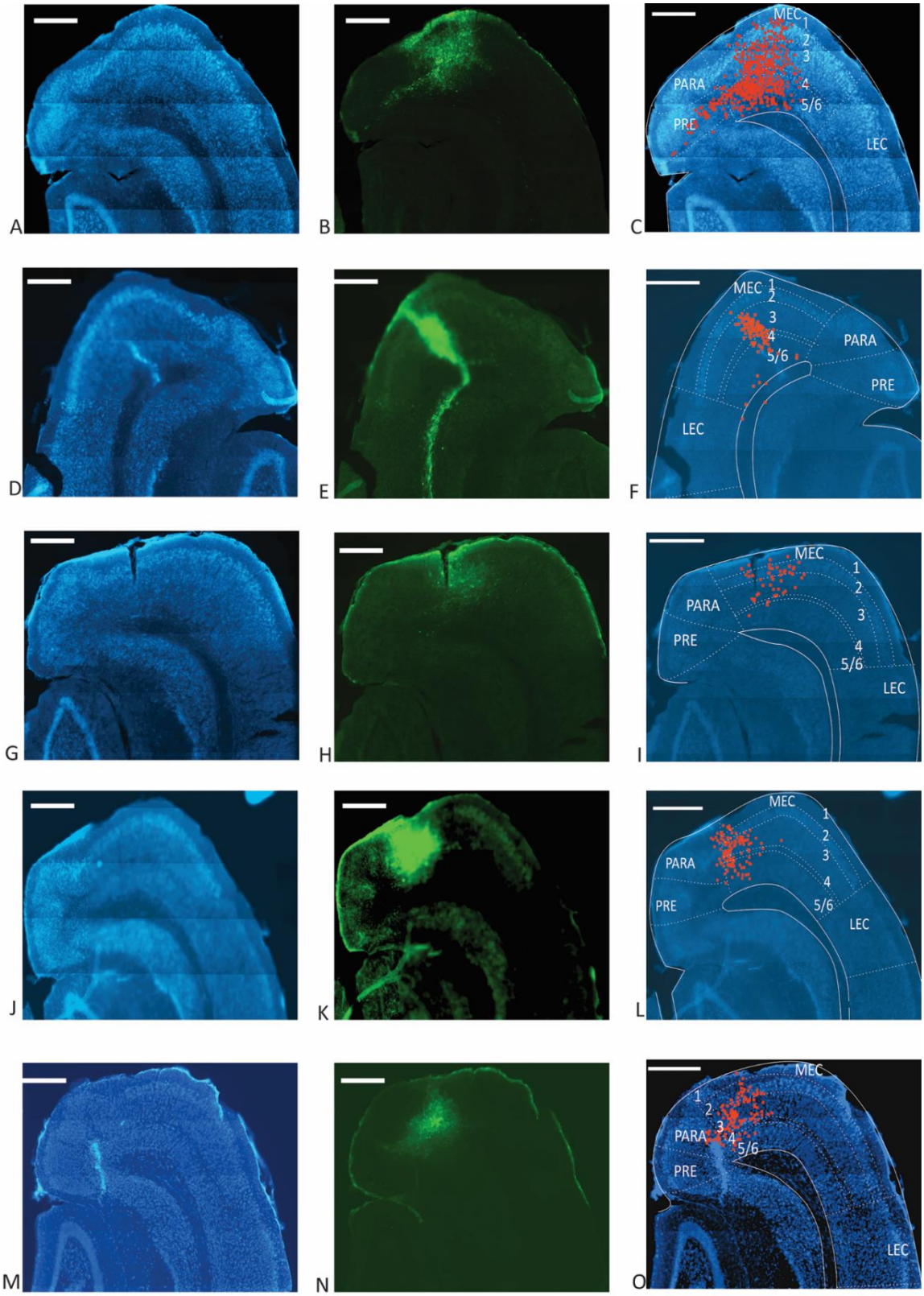

Figure 34 Enhancer driven expression of transgene in virus injection with ODZ3. Left panel (Nissil staining), central panel (eGFP expression), right panel (overlap of left and central panel, the orange boxes represent the eGFP labelled cell bodies. Fig A,B,C represent the mouse 53130 Fig D,E,F represent mouse 53131 left, fig G,H,I represent case 53131 right. Fig J.K.L represent mouse 53132. Fig M,N,O represent mouse 53133.The white scale bars are 250 µm. All sections correspond to a dorso ventral level of 2.82mm ventral to bregma.

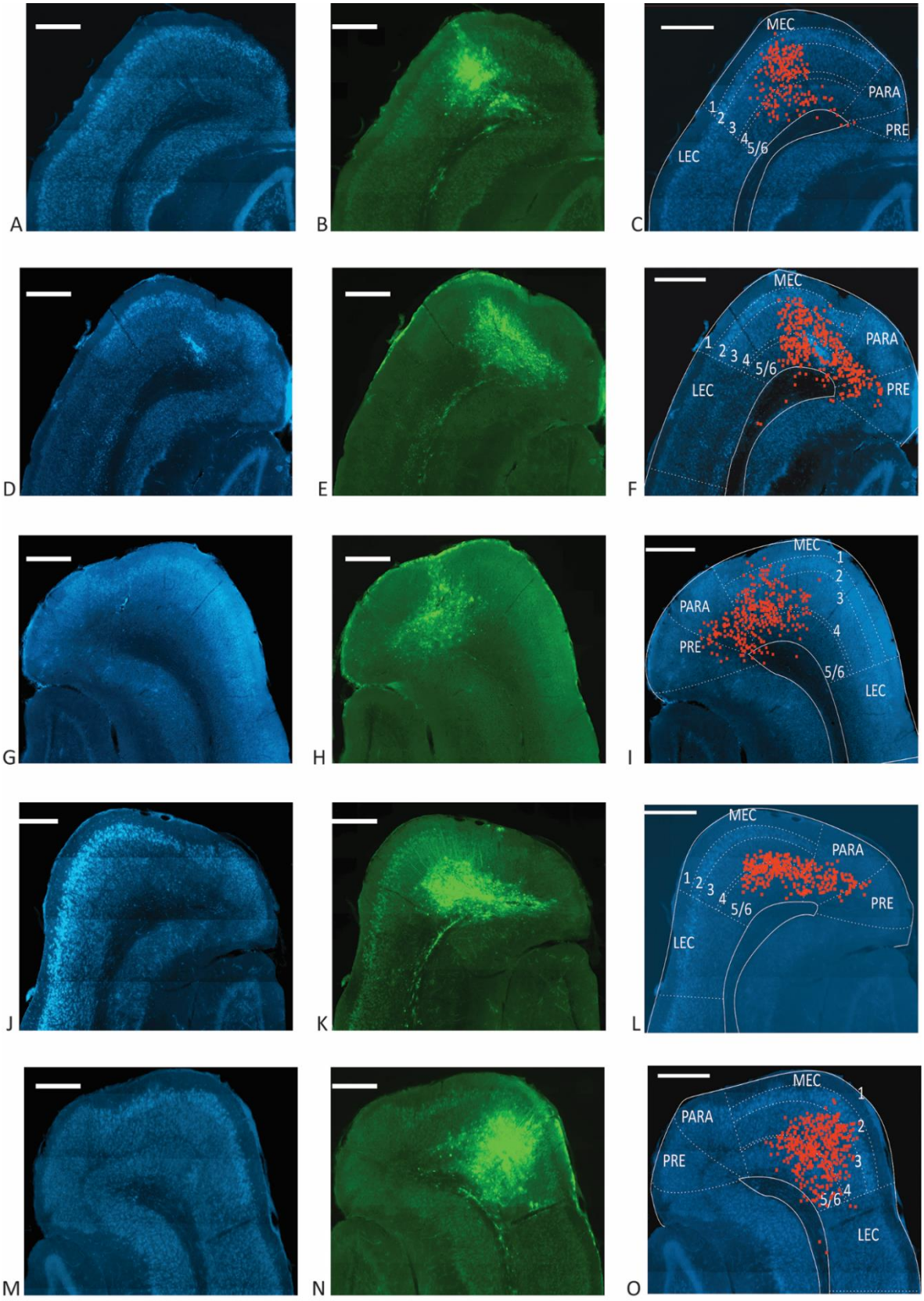

Figure 35 Enhancer driven expression of transgene in virus injection with TRPS1. Left panel (Nissl staining), central panel (eGFP expression), right panel (overlap of left and central panel, the orange boxes represent eGFP labeled cell bodies. Fig A, B, C represent the mouse 52930. Fig D, E, F represent mouse 52931 left. Fig G, H, I represent mouse 52931right.Fig J, K, L represent mouse 53312.Fig M, N, O represent mouse 53313. The white scale bars are 250 µm. All sections correspond to a dorso ventral level of 2.82mm ventral to bregma.

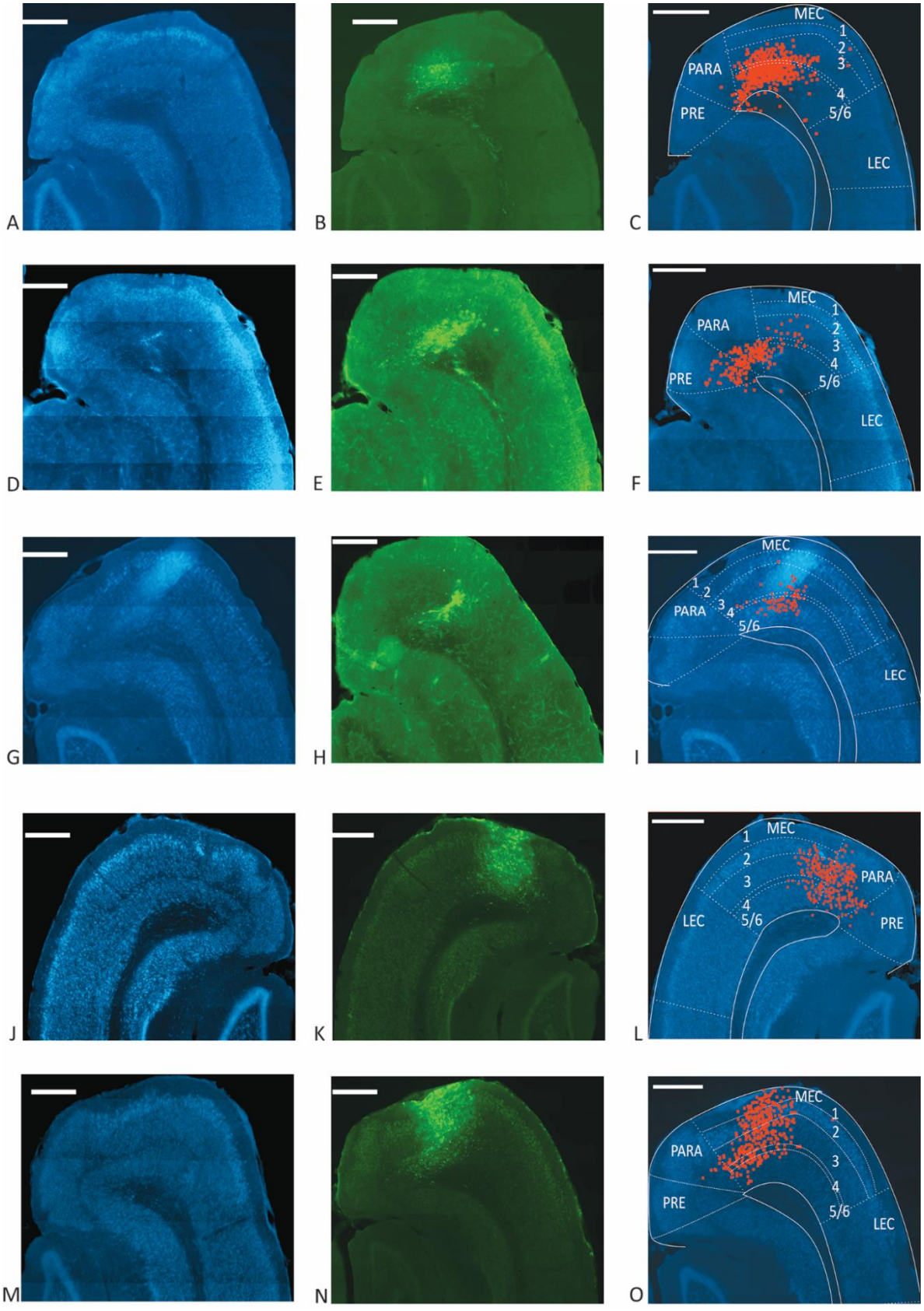

Figure 36 Enhancer driven expression of transgene in virus injection with LMO3. Left panel (Nissl staining), central panel (eGFP expression), right panel (overlap of left and central panel, the orange boxes represent the eGFP labeled cell bodies. Fig A, B, C represent the mouse 52927. Fig D, E, F represent mouse 52928 left .Fig G, H, I represent mouse 52928right. Fig J, K, L represent mouse 52929. Fig M, N, O represent mouse 52929. The white scale bars are 250 µm. All sections correspond to a dorso ventral level of 2.82mm ventral to bregma.

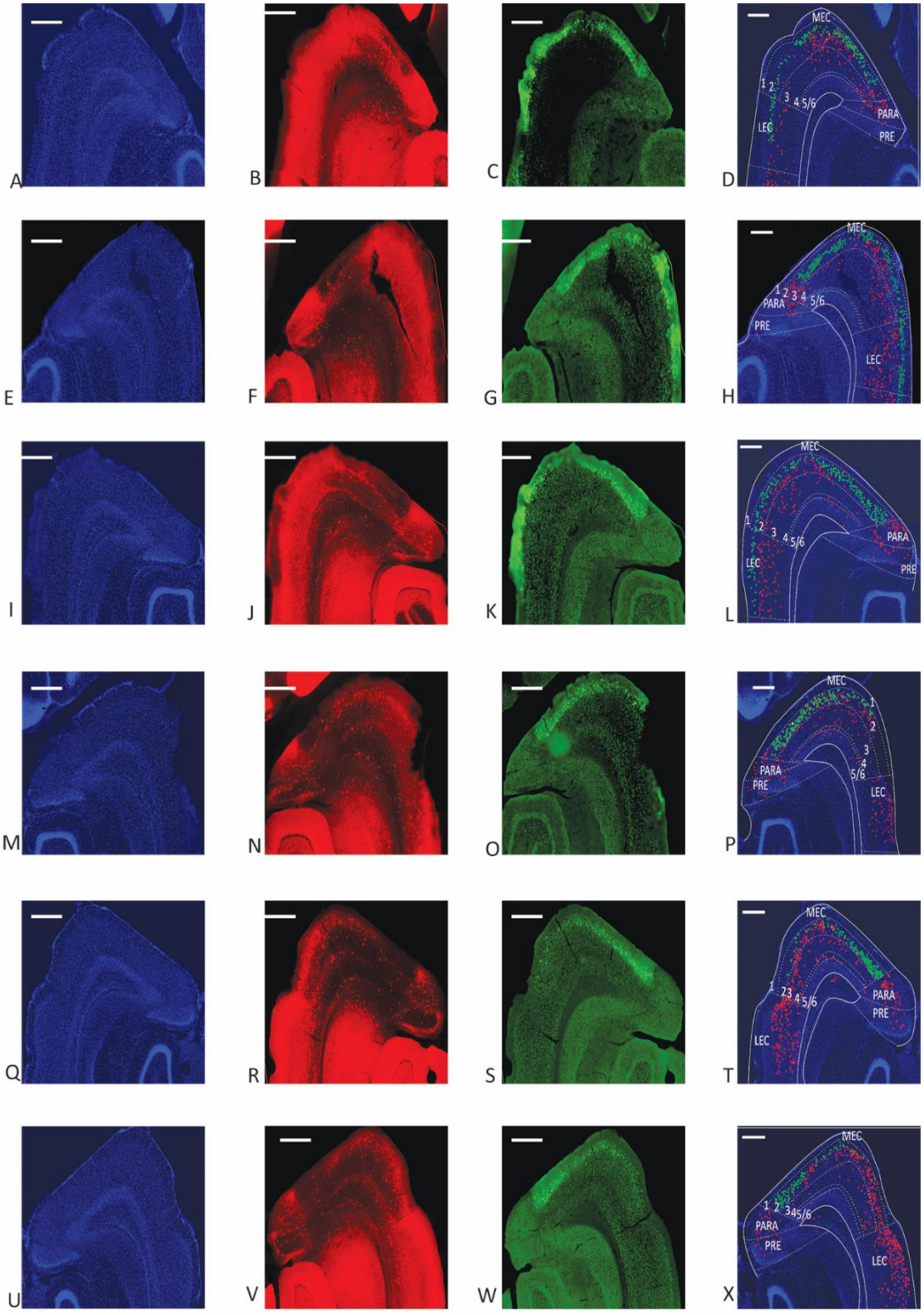

Figure 37 Expression of transgene and calbindin in transgenic ODZ3 (54114) ,the left panel represent the DAPI staining, the first column from left shows calbindin+ cells, the second column from left shows transgene 2A+ cells, the right panel is the combined of first three columns showing calbindin and 2A. Red triangle represents calbindin labeled cells whereas green rectangle represents 2A labeled cell bodies. Fig A, B, C, D, E, F, G, H belongs to dorso ventral level 3.3mm ventral to bregma. whereas fig I,J,K,L,M,N,O,P belongs to dorso ventral level 3.18 ventral to bregma and fig Q,R,S,T,U,V,W,X belongs to dorso ventral 2.94mm ventral to bregma. The white scale bar are 250µm.

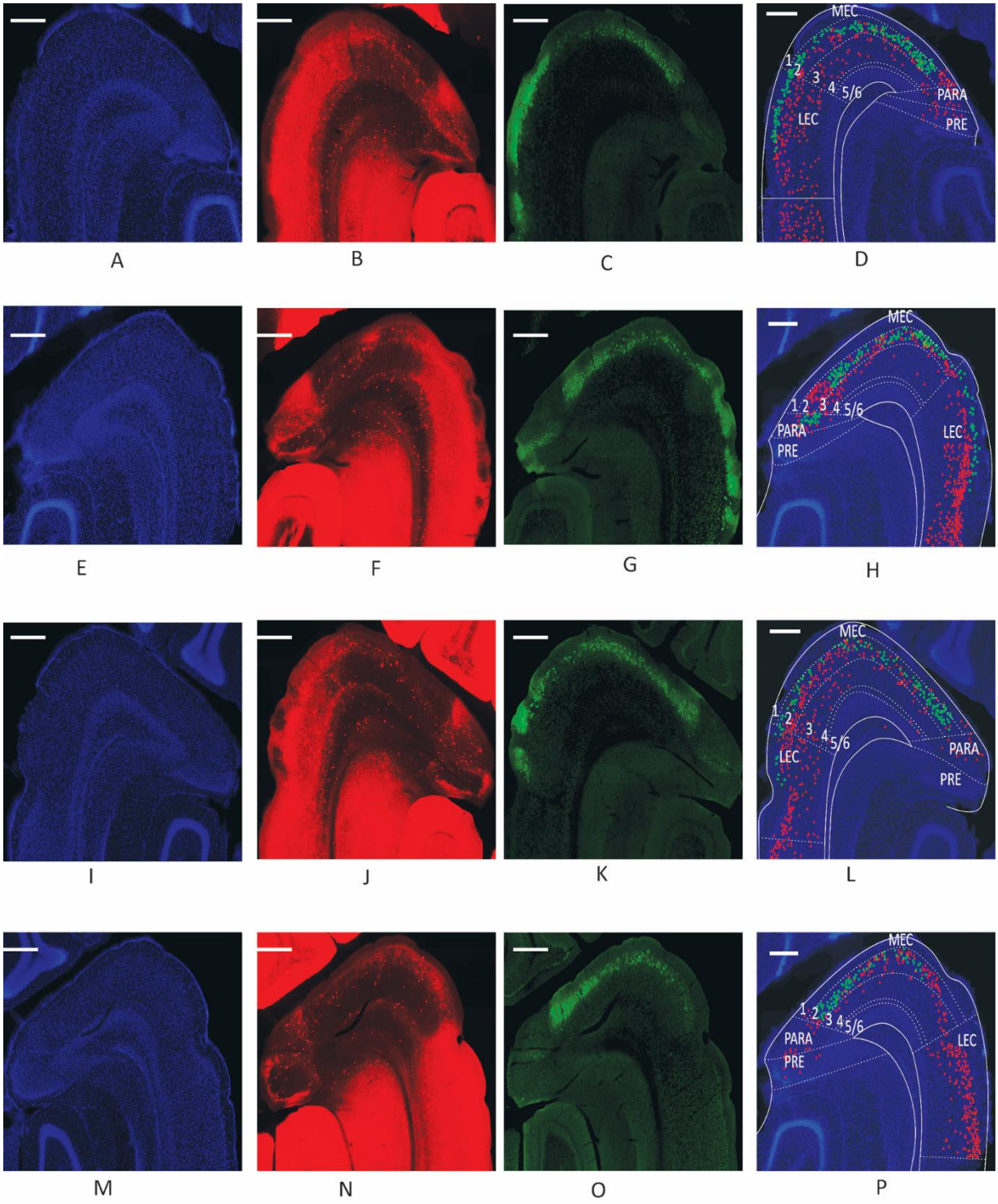

Figure 38 Expression of transgene and calbindin in transgenic line ODZ3 (mouse 54337), the left panel represent the DAPI staining, the first column from left shows calbindin+ cells, the second column from left shows transgene 2A+ cells, the right panel is the combined of first three columns showing calbindin and 2A.Red triangle represents calbindin labeled cells whereas green rectangle represents 2A labeled cell bodies. Fig A,B,C,D,E,F,G,H belongs to dorso ventral level 3.3mm ventral to bregma, whereas fig I,J,K,L,M,N,O,P belongs to dorso ventral level 3.18mm ventral to bregma. The whit scale bar are 250µm.

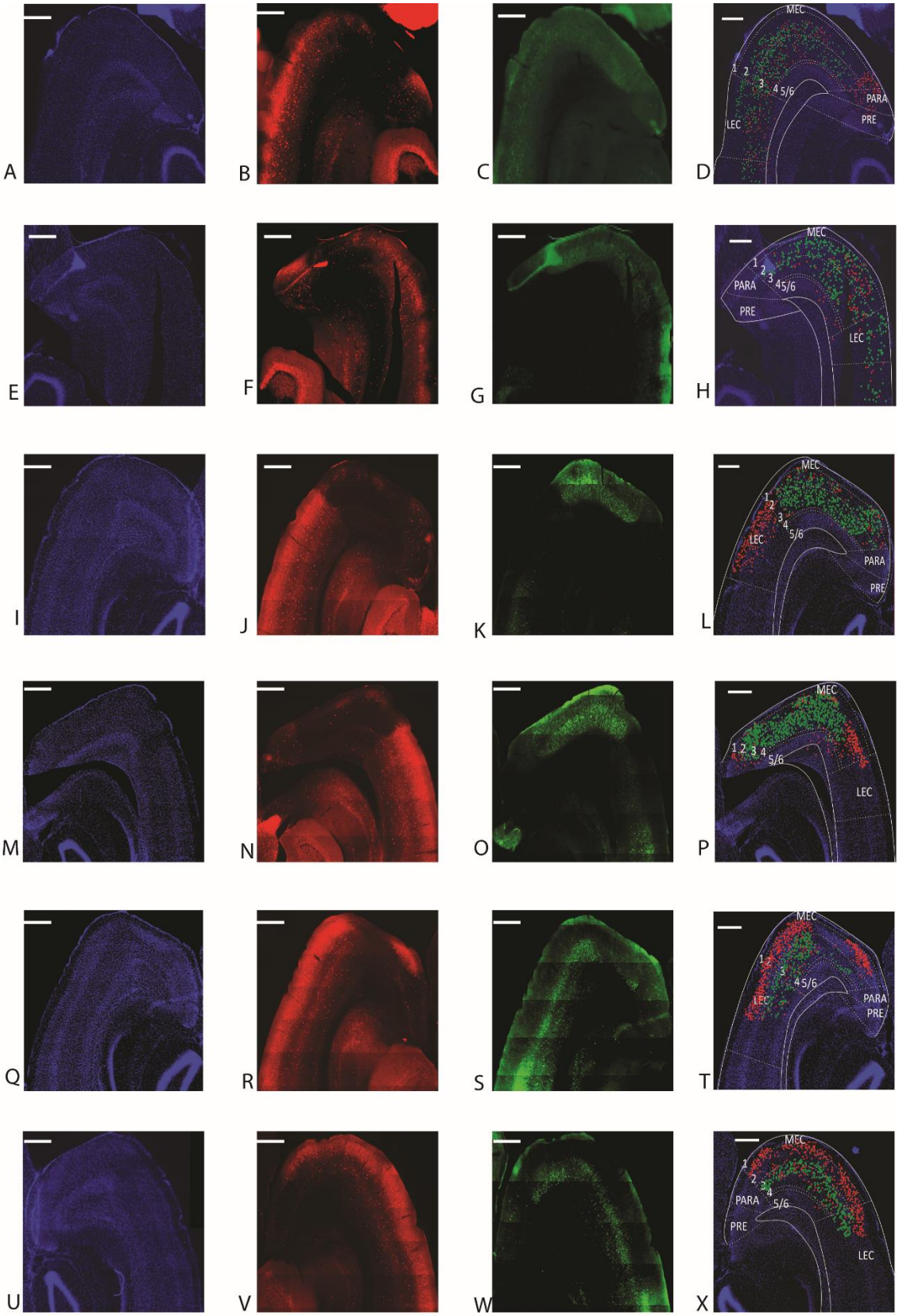

Figure 39 Expression of transgene and calbindin in transgenic line TRPS1 (mouse 53900), the left panel represent the DAPI staining, the first column from left shows calbindin+ cells, the second column from left shows transgene 2A+ cells, the right panel is the combined of first three columns showing calbindin and 2A expression. Red triangle represents calbindin labeled cell bodies whereas green rectangle represents 2A labeled cell bodies. Fig A,B,C,D,E,F,G,H belongs to dorso ventral level 3.3mm ventral to bregma, whereas fig I,J,K,L,M,N,O,P belongs to dorso ventral level 3.18 ventral to bregma and fig Q,R,S,T,U,V,W,X belongs to dorso ventral level 2.82mm ventral to bregma. The white scale bar are 250µm.

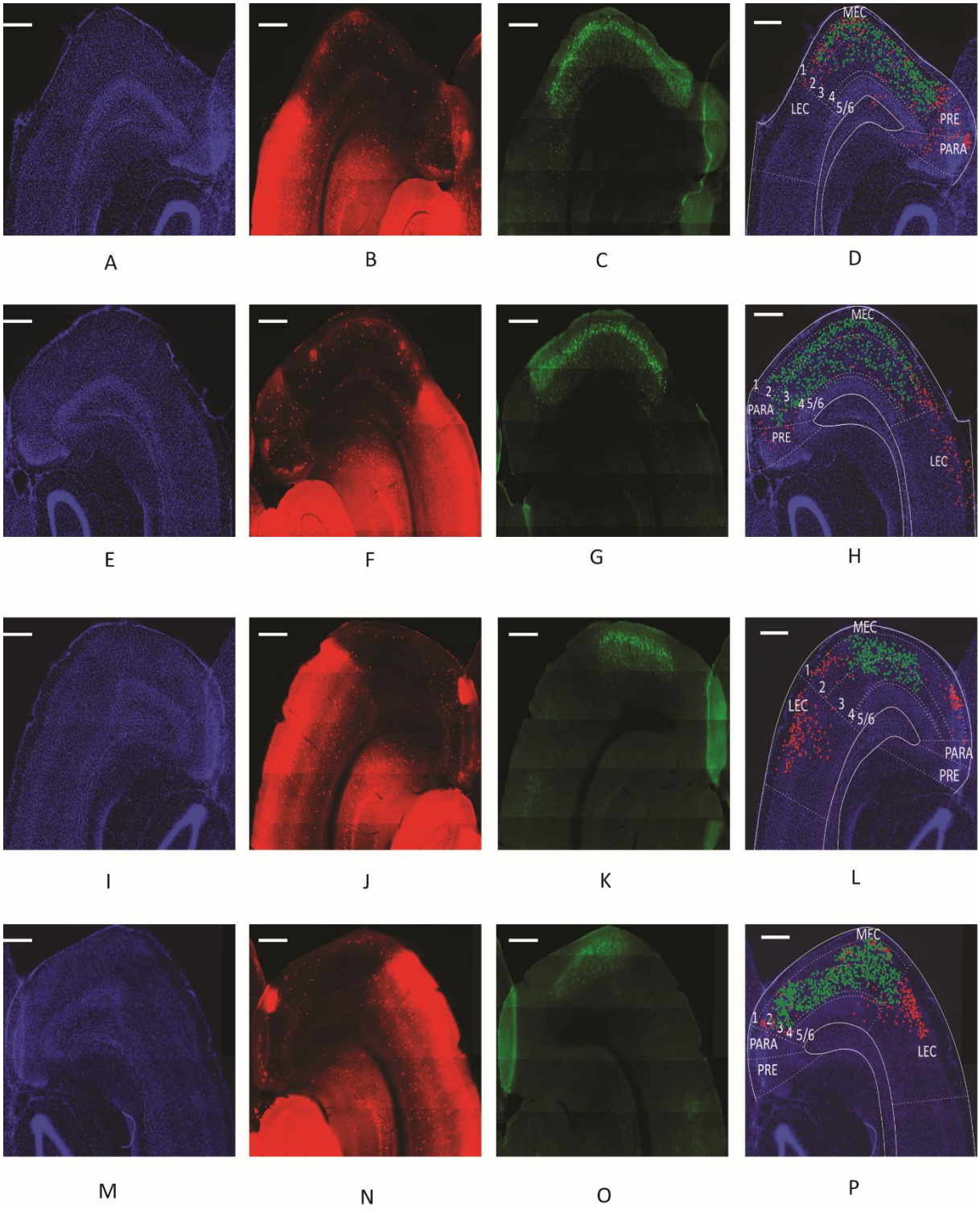

Figure 40 Expression of transgene and calbindin in transgenic line TRPS1 (mouse 53516), the left panel represent the DAPI staining, the first column from left shows calbindin+ cells, the second column from left shows transgene 2A+ cells, the right panel is the combined of first three columns showing calbindin and 2A expression. Red triangle represents calbindin labeled cell bodies whereas green rectangle represents 2A labeled cell bodies. ,fig A,B,C,D,E,F,G,H belongs to dorso ventral level 3.18mm ventral to bregma whereas fig I,J,K,L,M,N,O,P belongs to dorso ventral level 2.82mm ventral to bregma. The white scale bar are 250µm.

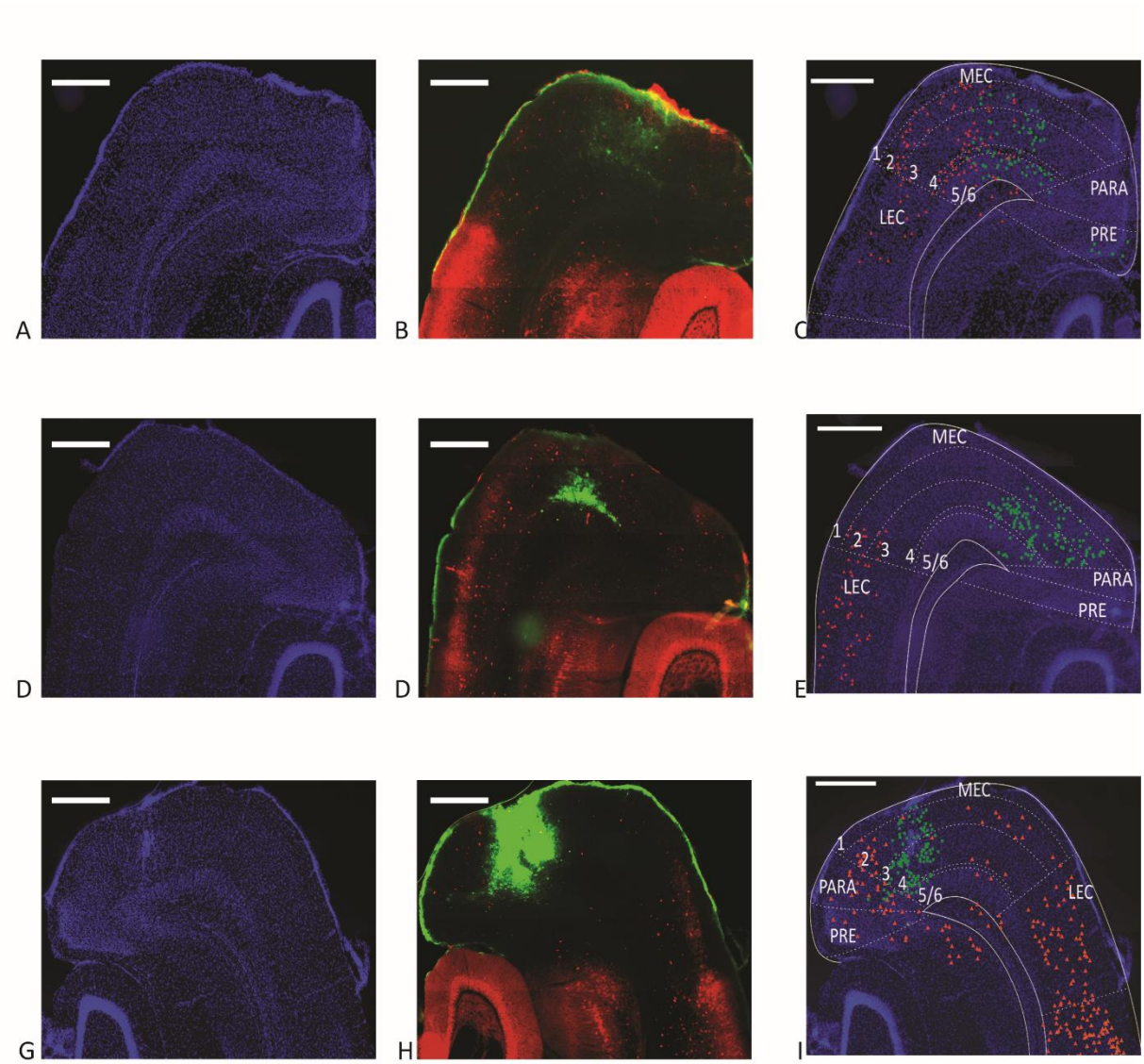

Figure 41 Expression of transgene and calbindin in LV-ODZ3-eGFP injected mice. The left panel represent the DAPI staining ,the central panel represent the calbindin+ and GFP+ cells, the right panel represent the combined form of left and central section showing calbindin and GFP (red triangle represents calbindin labeled cell bodies whereas green rectangle eGFP labeled cell bodies. Fig A,B,C mouse 53130, fig D,E,F mouse 53132 whereas fig G,H,I mouse 53133. The white scale bar are 250µm.All sections correspond to dorso ventral level 2.94mm ventral to bregma.

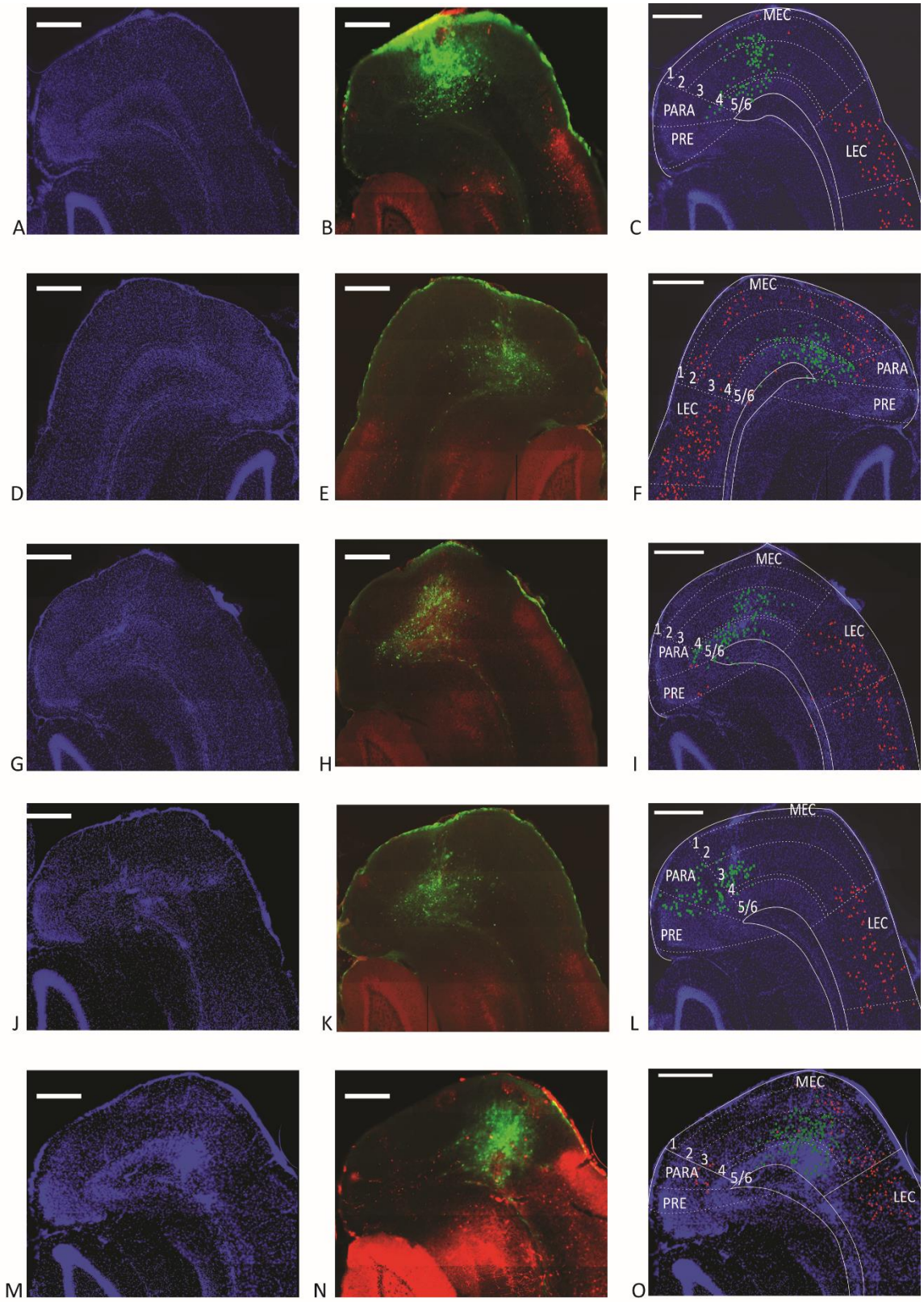

Figure 42 Expression of transgene and calbindin in LV- TRPS1-eGFP injected mice. The left panel represent the DAPI staining ,the central panel represent the calbindin+ and GFP+ cells, the right panel represent the combined form of left and central section showing calbindin and eGFP labeled cells (red triangle represents calbindin labeled cell bodies whereas green rectangle represents eGFP labeled cell bodies. Fig A,B,C mouse 52930, fig D,E,F mouse 52931 whereas fig G,H,I represent mouse 52931, fig J,K,L mouse 53312, fig M,N,O mouse 53313.The white scale bar are 250µm.All sections correspond to dorso ventral level 2.94mm ventral to bregma.

APPENDIX 2 PROTOCOL

Protocol for fluorescent Nissl staining

1. Optional: Incubate the sections in 1ml Scale A2 solution for 24 hours at 4**°**C in Eppendorf tube (4 sections in 1ml for better result).
2. Take 12 well plate. Incubate the sections in 0.2% Triton x-100 PBS for 10 minutes at room temperature to permeabilize the tissue. All incubation and washing should be done on a shaker to ensure even exposure of tissue.
3. Wash the sections 2 times 5 minutes in PBS at room temperature.
4. Dilute the Neurotrace stain in PBS. Dilution ratio of 1:1000 have been used successfully. Incubate the section in diluted Nissl stain for overnight at 4°C. The Neurotrace we used here is Nissl stain 435/455 blue (life technology, N-21479).
5. Incubate in 0.2% Triton X-100 for 10 minutes at room temperature.
6. Wash 2 times 5 minutes in PBS at room temperature.
7. Mount the sections onto glass slides with aqueous mounting medium.
8. Allow mounting medium to harden.
   (PBS used in all the steps is 1X)

Protocol for mouse anti-calbindin

1. Wash the sections 2 times 15 minutes in PBS at room temperature.
2. Permeabillize 10 minutes in PBS+1%TritonX.
3. Preincubate in PBS+1%TritonX+5%NDS (Donkey Serum) for 1 hour.
4. Incubate with primary antibody 1:5000 mouse anti-C, diluted in PB+1%Tritonx+5%NDS for 48 hours.
5. Wash 6 times 10 minutes in 1X PBS.
6. Incubate with secondary antibody 1:250 in PB+1%TritonX for 6 hours. Use Donkey anti-mouse CY3.
7. Wash once with 1:10,000 diluted DAPI in PBS.
8. Wash 6 times 10 minutes in PBS.

9. Mount in Tris-HCl.
10. Coverslip with PVA/2.5%DABCO.

Protocol for mouse anti-calbindin+2A

1. Wash the sections 2 times 15 minutes in PBS at room temperature.
2. Permeabillize 10 minutes in PBS+1%TritonX.
3. Preincubate in PBS+1%TritonX+5%NDS (Donkey Serum) for 1 hour.
4. Incubate with primary antibody 1:5000 mouse anti-CB+1:2500 rabbit anti-2A, diluted in PB+1%Tritonx+5%NDS for 48 hours.
5. Wash 6 times 10 minutes in 1X PBS.
6. Incubate with secondary antibody 1:250 in PB+1%TritonX for 6 hours. Use Donkey anti-mouse Cy3 and Donkey anti-rabbit 488.
7. Wash once with 1:10,000 diluted DAPI in PBS.
8. Wash 6 times 10 minutes in PBS.
9. Mount in Tris-HCl.
10. Coverslip with PVA/2.5%DABCO.

Protocol for NeuN and 2A staining

1. Wash the sections 2 times 15 minutes in PBS at room temperature.
2. Permeabillize 10 minutes in PBS+1%TritonX.
3. Preincubate in PBS+1%TritonX+5%NDS (Donkey Serum) for 1 hour.
4. Incubate with 1:500 NeuN, primary antibody 1:2500 rabbit anti-2A, diluted in PB+1%Tritonx+5%NDS for 48 hours.
5. Wash 6 times 10 minutes in 1X PBS.
6. Incubate with secondary antibody 1:250 in PB+1%TritonX for 6 hours. Use Donkey anti-mouse Cy3 and Donkey anti-rabbit 488.
7. Wash 6 times 10 minutes in PBS.
8. Mount in Tris-HCl.
9. Coverslip with PVA/2.5%DABCO.

APPENDIX 3 CHEMICAL, SOLUTION AND ANTOBODIES

RECEIPE, SOLUTIONS AND CHEMICALS

Agarose gel

-Add 1gm of agarose in 200ml TE buffer.

-Add (10µl/1ooml) of cybersafe in agarose gel before casting on tray.

LB broth (35g/l)

-Add 7 gm LB in 200ml water.

-Autoclave LB broth for half an hour at 121°C

Kanamycin stock (50mg/ml)

-dissolve 0,5g in 10ml ddH2O.

-Filter through a 0, 22µm filter.

-Aliquot and store at -20°C

Ampicillin stock (100mg/ml)

-Dissolve 1 gm in 10ml ddH2O.

-Filter through a 0, 22µm filter.

-Aliquot and store at -20°C

1%PBS (Phosphate Buffer Saline), pH 7, 4

-From 10X stock solution, add 10ml in 100ml distilled water.

PBS+ (1% PBS+ 1% TritonX 100)

-Add 5ml stock from20% TritonX in 100ml PBS.

PBS++ (1% PBS+ 1% TritonX 100+5% NDS) (Normal Donkey Serum)

-50 ml PB+ + 2,5ml NDS

0.2% TritonX from 10% Tritonx

-Add 1ml stock from 10% TritonX in 50ml PBS.

1% TritonX

-Add 5ml stock from20% TritonX in 100ml PBS.

Tissue Culture Solution

-Glycerin 250ml

-Ethylene glycol 300ml

-0.1M PB 500ml

Dissolve all above ingredients in 1 litre at 4°C.

Primary and secondary antibodies

Primary antibody	Dilution	Secondary antibody	Dilution	Supplier
Mouse anti calbindin	1: 5000	Donkey anti mouse Cy3	1: 250	Invitrogen
Rabbit anti-2A	1: 2500	Donkey anti rabbit 488	1: 250	Invitrogen
NeuN	1: 500	Donkey anti mouse 488	1: 250	Invitrogen