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Genetic labeling of both the axons of transduced, glutamatergic neurons in rat postrhinal cortex and their postsynaptic neurons in other neocortical areas by Herpes Simplex Virus vectors that coexpress an axon-targeted β -galactosidase and wheat germ agglutinin from a vesicular glutamate transporter-1 promoter

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Abstract

Neuronal circuits comprise the foundation for neuronal physiology and synaptic plasticity, and thus for consequent behaviors and learning, but our knowledge of neocortical circuits is incomplete. Mapping neocortical circuits is a challenging problem because these circuits contain large numbers of neurons, a high density of synapses, and numerous classes and subclasses of neurons that form many different types of synapses. Expression of specific genetic tracers in small numbers of specific subclasses of neocortical neurons has potential to map neocortical circuits. Suitable genetic tracers have been established in neurons in subcortical areas, but application to neocortical circuits has been limited. Enabling this approach, Herpes Simplex Virus (HSV-1) plasmid (amplicon) vectors can transduce small numbers of neurons in a specific neocortical area. Further, expression of a particular genetic tracer can be restricted to specific subclasses of neurons; in particular, the vesicular glutamate transporter-1 (VGLUT1) promoter supports expression in VGLUT1-containing glutamatergic neurons in rat postrhinal (POR) cortex. Here, we show that expression of an axon-targeted β -galactosidase (β -gal) from such vectors supports mapping specific commissural and associative projections of the transduced neurons in POR cortex. Further, coexpression of wheat germ agglutinin (WGA) and an axon-targeted β -gal supports mapping both specific projections of the transduced neurons and identifying specific postsynaptic neurons for the transduced neurons. The neocortical circuit mapping capabilities developed here may support mapping specific neocortical circuits that have critical roles in cognitive learning.

Keywords

neocortical circuits; axon tracer; GAP-43; transneuronal tracer; wheat germ agglutinin; glutamatergic neuron-specific expression; vesicular glutamate transporter-1 promoter; herpes simplex virus vector

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1. Introduction

Neuronal circuits represent the physical foundation for neuronal physiology and synaptic plasticity, and thus for behaviors and learning (Dudai, 1989; Milner et al., 1998), but our knowledge of neocortical circuits is incomplete. Mapping neocortical circuits is a difficult problem because neocortex contains large numbers of neurons, a high density of synapses, and numerous classes and subclasses of neocortical neurons that form multiple different types of synapses (Alonso-Nanclares et al., 2008; Arlotta et al., 2005; Douglas and Martin, 2004; Markram et al., 2004; Peters and Jones, 1984; Sugino et al., 2006). Expression of specific genetic tracers in small numbers of specific subclasses of neurons has potential to map neocortical circuits. Enabling this approach, a virus vector can transduce small numbers of neurons in a specific neocortical area (Fraefel et al., 1996; Zhang et al., 2005). Further, expression of a particular genetic tracer can be restricted to specific subclasses of neurons by both targeting gene transfer to specific subclasses of neurons (Cao et al., 2010; Wang et al., 2005) and advantageous choice of a neuron class- or subclass-specific promoter to express the tracer (Jin et al., 1996; Kaplitt et al., 1994; Rasmussen et al., 2007; Song et al., 1997; Zhang et al., 2000; Zhang and Geller, 2010). In subcortical areas, suitable genetic tracers have been established for mapping the projections of neurons, and their synaptic targets (Braz et al., 2002; Braz and Basbaum, 2008; Dobi et al., 2010; El-Husseini Ael et al., 2001; Hanno et al., 2003; Kato et al., 2000; Liu et al., 1994; Livet et al., 2007; Ohmoto et al., 2008; Okada et al., 1999; Strittmatter et al., 1994; Yoshihara et al., 1999; Yoshihara, 2002; Zubair et al., 2002). However, application of these genetic tracers to neocortical circuits has been limited.

Genetic tracers have been developed that can label either the axons of transduced neurons or the postsynaptic neurons for the transduced neurons. The axon targeting domain in GAP-43 was localized to the 20 N-terminal amino acids (aa) of the protein (Liu et al., 1994). Fusing this axon targeting domain to a specific reporter, including *E. coli* β -galactosidase (β -gal) or a specific fluorescent protein, supports labeling the axons of the transduced neurons (El-Husseini Ael et al., 2001; Kato et al., 2000; Liu et al., 1994; Livet et al., 2007; Okada et al., 1999; Strittmatter et al., 1994; Zubair et al., 2002). Such genetic labeling of axons has supported mapping projections for specific classes of subcortical neurons, either in transgenic mice or after direct gene transfer into small groups of neurons using a virus vector (see references just cited). Of note, wheat germ agglutinin (WGA) protein is a well-known neuroanatomical tracer that has been extensively used to map the projections of specific neocortical neurons. More recently, the WGA gene has been used as a transneuronal tracer; recombinant WGA protein is secreted from the transduced neurons, and the secreted WGA protein is taken up by postsynaptic neurons, thereby labeling postsynaptic neurons for the transduced neurons (Braz et al., 2002; Braz and Basbaum, 2008; Dobi et al., 2010; Hanno et al., 2003; Ohmoto et al., 2008; Yoshihara et al., 1999; Yoshihara, 2002). WGA has been used as a transneuronal tracer to map specific subcortical circuits, either in transgenic mice or after direct gene transfer using a virus vector (see references just cited).

Helper virus-free Herpes Simplex Virus (HSV-1) plasmid vectors (amplicons) (Fraefel et al., 1996; Geller and Breakefield, 1988) have attractive properties to support mapping neocortical circuits. These vectors efficiently transduce neurons. Further, they have a large capacity that can support coexpressing multiple neuroanatomical tracers from large neuron class- or subclass-specific promoters (51 kb and 149 kb vectors have been described (Wade-Martins et al., 2003; Wang et al., 2000)). Importantly, HSV-1 vectors containing specific cellular promoters support neuron-specific, or neuron class-specific expression, including catecholaminergic-, enkephalinergic-, glutamatergic-, or GABAergic-specific expression (Jin et al., 1996; Kaplitt et al., 1994; Rasmussen et al., 2007; Song et al., 1997; Zhang et al., 2000). Further, expression in specific subclasses of glutamatergic neurons has been obtained

by using the vesicular glutamate transporter-1 (VGLUT1) promoter. The three VGLUTs are expressed in distinct populations of neurons, and VGLUT1 is the major vesicular glutamate transporter in the neocortex, cerebellar cortex, hippocampus, and amygdala basolateral nuclei; specifically, most neocortical glutamatergic neurons contain VGLUT1, but some glutamatergic neurons, mostly in layer 4, contain VGLUT2 (review (Freneau et al., 2004)). Of note, a HSV-1 vector containing the VGLUT1 promoter supports expression in VGLUT1-containing glutamatergic neurons in rat postrhinal (POR) cortex (Rasmussen et al., 2007; Zhang and Geller, 2010).

In this study, we have developed HSV-1 vectors for mapping specific neocortical circuits by expressing specific neuronal tracers from the VGLUT1 promoter. Expression of an axon-targeted β -gal supports mapping specific commissural and associative projections of rat POR cortex neurons. Further, coexpression of WGA and the axon-targeted β -gal can map both specific projections of the transduced neurons and identify specific postsynaptic neurons for the transduced neurons.

2. Results

2.1. A HSV-1 vector expressing an axon-targeted β -gal from the VGLUT1 promoter labels commissural and associative projection axons of POR cortex neurons

Both of the HSV-1 vectors developed in this study contain our standard vector backbone (Fig. 1A (Song et al., 1997; Zhang et al., 2000)). These two vectors use the mouse VGLUT1 promoter (Fig. 1A; 7 kb promoter and 4.6 kb first intron (Rasmussen et al., 2007)) to express recombinant genes. After injection into POR cortex of an HSV-1 vector containing this promoter, >90 % of the expressing cells are VGLUT1-containing glutamatergic neurons (Rasmussen et al., 2007; Zhang and Geller, 2010). First, we developed a HSV-1 vector for mapping axon projections; we constructed a HSV-1 vector that uses the VGLUT1 promoter to express a chimeric protein that fused the 22 N-terminal aa of GAP-43, containing the axon targeting domain (Liu et al., 1994), to the N-terminus of β -gal (Fig. 1B; pVGLUT1gap-lac).

pVGLUT1gap-lac was packaged into HSV-1 particles using a helper-virus free packaging system (Fraefel et al., 1996; Sun et al., 1999). The numbers of infectious virus particles (IVP/ml) were quantified by titrating the vector stock on Baby Hamster Kidney (BHK) cells; at 1 day after transduction, positive cells were visualized using 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal) staining; this assay was previously used to titer pVGLUT1lac (Rasmussen et al., 2007; Zhang and Geller, 2010). Of note, most neuronal cell lines, such as PC12 cells, do not form a monolayer, and the titers obtained on BHK cells are higher than the titers obtained on specific neuronal cell lines (Yang et al., 2001; Zhang et al., 2000). Expression from the VGLUT1 promoter in fibroblast cells represents ectopic expression that declined rapidly at longer times after gene transfer (not shown). The titer of the pVGLUT1gap-lac vector stock was 1.0×10^6 infectious vector particles (IVP)/ml. We previously used a PCR assay to determine the titer of vector genomes (VG/ml) and the packaging efficiency (VG/ml / IVP/ml) for pVGLUT1lac, which were similar for pVGLUT1lac and a number of other vectors we have studied (Rasmussen et al., 2007); we did not repeat the VG/ml assay here.

The gene transfer conditions used here were previously characterized using a HSV-1 vector containing a neuron-specific promoter, a modified neurofilament heavy gene promoter, that supports expression in both glutamatergic and GABAergic neurons in POR cortex (Zhang et al., 2000; Zhang et al., 2005). HSV-1 vectors can potentially transduce axon terminals and be retrogradely transported to neurons in distant areas that project to the injection site. Of note, we previously showed that following injection of a vector stock into POR cortex, ten

specific neocortical areas that receive large projections from POR cortex, including perirhinal cortex (Burwell and Amaral, 1998a; Burwell and Amaral, 1998b; Burwell, 2000; Burwell, 2001), each contained less than 1 % of the number of β -galIR cells as POR cortex (Zhang et al., 2005). Further, specific subcortical areas, including the hippocampus, amygdala, specific cholinergic basal forebrain areas, and specific catecholaminergic midbrain areas, lacked transduced cells (Zhang et al., 2005). Thus, following injection of pVGLUT1gap-lac into the POR cortex of one hemisphere, any β -gal-containing processes in distant neocortical areas are most likely the axons of the transduced neurons.

pVGLUT1gap-lac supported mapping both commissural and associative projections for POR cortex neurons. The vector stock was injected into the POR cortex in the left hemisphere of five rats. The rats were sacrificed four days later, and β -gal-immunoreactivity (IR) was visualized. Similar results were obtained from these five rats, and results from one representative rat are shown. Under low power, there were numerous β -gal-IR cells near the injection site (Fig. 2A). Under high power, near the injection site we observed both β -gal-IR large cell bodies and β -gal-IR axon-like processes (Fig. 2B). Using a pVGLUT1lac vector stock with a titer of 2×10^6 IVP/ml, we previously reported 544 ± 79 (mean $\pm$ s.e.m.) β -gal-IR cells per POR cortex (Zhang and Geller, 2010); we did not quantify the number of transduced cells in the present rats, as this pVGLUT1gap-lac stock had a modestly lower titer and the same gene transfer conditions were used. Neurons in POR cortex are known to send large projections to both the ipsilateral perirhinal cortex and the contralateral POR cortex (Burwell and Amaral, 1998a; Burwell and Amaral, 1998b; Burwell, 2000; Burwell, 2001). In the ipsilateral perirhinal cortex, under high power, we observed numerous β -gal-IR axons, but no β -gal-IR cell bodies (Fig. 2C). Similarly, in the contralateral, uninjected POR cortex, we observed numerous β -gal-IR axons, and again no β -gal-IR cell bodies (Fig. 2D). As a negative control for the β -gal-IR assay, in a rat that received pVGLUT1lac, a microscope field within the injected POR cortex, but distant from the injection site, lacked β -gal-IR neurons or processes (Fig. 2E). Counts yielded $1,296 \pm 175$ β -gal-IR axons in perirhinal cortex per hemisphere ($n=5$ rats), and 667 ± 77 β -gal-IR axons in the contralateral POR cortex per hemisphere ($n=5$ rats); in contrast, control rats contained <5 β -gal-IR axons in the contralateral POR cortex per hemisphere. We recognize that specific axons may bifurcate, and specific axons may be contained in more than one section: Thus, this quantification is useful for comparing the sizes of specific projections, but does not specify the number of neurons that project to a specific area.

2.2. Coexpression of WGA and an axon-targeted β -gal from the VGLUT1 promoter supports labeling both presynaptic axons and postsynaptic dendrites and cell bodies

WGA protein is a well-known neuroanatomical tracer, and more recently the gene has been used in transgenic mice and virus vectors as a transneuronal tracer (Braz et al., 2002; Braz and Basbaum, 2008; Dobi et al., 2010; Hanno et al., 2003; Ohmoto et al., 2008; Yoshihara et al., 1999; Yoshihara, 2002). We expected that expression of the WGA gene from a HSV-1 vector would label the transduced neurons, and WGA protein would be secreted and taken up by the postsynaptic neurons. Thus, we anticipated that WGA protein would label both the transduced neurons and specific postsynaptic neurons for the transduced neurons. Above, we established that the gap-lac construct labels presynaptic axons (Fig. 2). Thus, to distinguish between presynaptic axons and postsynaptic dendrites and cell bodies, we constructed a cassette that contains WGA, an internal ribosome entry site (ires), and gap-lac (Fig. 1B). We previously constructed cassettes that contain a specific neurotrophic factor gene, an ires, and the *Lac Z* gene (Sun et al., 2005). We used these constructs as a model to design a wga/ires/gap-lac cassette; we replaced the neurotrophic factor gene with the WGA gene, and we inserted sequences encoding the 22 N-terminal aa of GAP43 at the 5' end of the *Lac Z* gene. The wga/ires/gap-lac cassette was inserted into a HSV-1 vector containing the VGLUT1

promoter. pVGLUT1wga/ires/gap-lac was packaged into HSV-1 particles, and the titer of the vector stock was 1.5×10^6 IVP/ml.

We injected pVGLUT1wga/ires/gap-lac into POR cortex in the left hemisphere of three rats, and sacrificed the rats 8 days later. Results from the three rats were similar, and we show results from one rat. First, we verified expression of gap-lac: We observed β -gal-IR cells and processes proximal to the injection site and β -gal-IR axons in the contralateral, uninjected POR cortex (not shown); these results were similar to those obtained using pVGLUT1gap-lac (Fig. 2). Next, we showed that WGA labels transduced neurons and processes proximal to the injection site. We detected WGA using an anti-WGA antibody, and identified neurons in the same sections by costaining for a neuronal marker, NeuN. We observed WGA-IR positive cells that also contained NeuN-IR proximal to the injection site (Fig. 3A-C); this field also contains WGA-IR processes in cross section and untransduced NeuN-IR neurons that lacked WGA-IR. Slightly further from the injection site, we observed WGA-IR processes in both cross section and oblique views (Fig. 3D-F). As a negative control, omission of the primary antibodies resulted in no IR (Fig. 3G-I).

In the uninjected, contralateral POR cortex, we observed different types of WGA labeling in different layers of POR cortex. A lower layer of POR cortex, above the white matter, contains numerous WGA-IR processes, but no WGA-IR cell bodies (Fig. 4A-C), although many NeuN-IR neurons were observed. Specific WGA-IR processes could be axons from the transduced neurons or dendrites from the postsynaptic neurons that form synapses with the transduced neurons. A middle layer of POR cortex contains WGA-IR cell bodies that contain NeuN-IR (Fig. 4D-F); these neurons are likely postsynaptic neurons that form synapses with the transduced neurons. These cell bodies contain relatively uniform WGA-IR throughout the cell body (Fig. 4D-F, arrows). We also observed punctate WGA-IR in cell bodies that could represent either WGA-IR in a cell body or WGA-IR in presynaptic axons that are proximal to the cell body. This field also contains WGA-IR processes; again, specific WGA-IR processes could be axons from the transduced neurons or dendrites from the postsynaptic neurons that form synapses with the transduced neurons. An upper layer of POR cortex, near the surface of neocortex, contains a WGA-IR neuron (Fig. 4G-I), and a limited number of WGA-IR processes. Interestingly, in some sections, we observed a narrow, columnar-like region of WGA-IR processes (Fig. 4J-L).

To distinguish between WGA-IR in presynaptic axons or postsynaptic dendrites, in the uninjected contralateral POR cortex, we detected WGA-IR and costained the same sections for a dendrite marker, MAP2-IR (Huber and Matus, 1984). In specific sections, we observed WGA-IR and MAP2-IR costained cell bodies (Fig. 5A-C); these cells are likely postsynaptic neurons that form synapses with transduced neurons and are actively synthesizing MAP2 in the cell body. This section also contains some WGA-IR processes that lack MAP-IR that are likely axons of transduced neurons, and some MAP2-IR dendrites that lack WGA-IR which represent dendrites of neurons that do not form synapses with the transduced neurons. In another section, we observed numerous WGA-IR processes in cross section that contain MAP2-IR (Fig. 5D-F); these processes are likely the dendrites of postsynaptic neurons that form synapses with transduced neurons. Again, this section also contains some WGA-IR processes that lack MAP-IR, and these are likely axons of transduced neurons. In a third section, we observed numerous WGA-IR processes in oblique or longitudinal views that contained or lacked MAP2-IR (Fig. 5G-I); these are likely the dendrites of postsynaptic neurons that form synapses with transduced neurons or the axons of transduced neurons, respectively.

3. Discussion

Because HSV-1 vectors can transduce limited numbers of neurons in a chosen neocortical area, they have potential for elucidating neocortical circuits. In this report, we establish HSV-1 vectors for both mapping the projections of transduced neocortical neurons and for identifying their postsynaptic neurons. The vectors used a VGLUT1 promoter that supports expression selectively in VGLUT1-containing glutamatergic neurons (Rasmussen et al., 2007; Zhang and Geller, 2010). We fused the axon-targeting domain of GAP43 to β -gal and showed that this protein labels the axons of transduced neurons; this fusion protein identifies both commissural and associative projections for POR cortex neurons. Further, we showed that expression of WGA labels the dendrites and cell bodies of the postsynaptic neurons for the transduced neurons.

We labeled the axons of the transduced neurons by using an axon-targeted β -gal (gap-lac). The gene transfer conditions that were used support expression in ~500 neurons in POR cortex (Zhang and Geller, 2010), with minimal transduction of neurons in either other neocortical areas or subcortical areas that project to POR cortex (Zhang et al., 2005). Proximal to the injection site, we observed labeling of numerous cell bodies, and some processes. These labeled processes could represent axon collaterals of the transduced neurons or dendrites of these neurons that were labeled by passive diffusion of gap-lac. In both the contralateral, uninjected POR cortex, and in the ipsilateral perirhinal cortex, areas known to receive large projections from POR cortex (Burwell and Amaral, 1998a; Burwell and Amaral, 1998b; Burwell, 2000; Burwell, 2001), we observed numerous labeled axons, but no labeled cell bodies. Counts showed ~1,300 or ~670 β -gal-IR axons in perirhinal cortex or the contralateral POR cortex, respectively, per hemisphere. Specific axons may bifurcate, and specific axons may be contained in more than one section; this quantification estimates relative projection sizes to specific areas, but does not specify the number of neurons responsible for the projection. Nonetheless, given the significant numbers of labeled axons in perirhinal cortex and the contralateral POR cortex, and that ~500 neurons were transduced, it appears that many of the transduced neurons send axons to each of these areas, consistent with the large projections previously reported (Burwell and Amaral, 1998a; Burwell and Amaral, 1998b; Burwell, 2000; Burwell, 2001). Importantly, the large numbers of axons that were detected demonstrates high-level expression of gap-lac that should support specific neuroanatomical studies.

We expressed WGA to label the dendrites and cell bodies of postsynaptic neurons for the transduced neurons. After injection of the vector into the POR cortex of one hemisphere, specific neuronal cell bodies in the contralateral POR cortex contained WGA; these cell bodies were predominately located in the middle and upper neocortical layers. These cells were identified as neurons using a neuronal marker; analogously, the specific class of neuron could be identified using neuron class-specific markers. We observed numerous WGA-containing processes, and costaining for a dendritic marker, MAP2, showed that specific WGA-containing processes were postsynaptic dendrites, and other specific processes were likely the axons of the transduced neurons. Because the vector coexpressed both WGA and gap-lac, the axons of transduced neurons can be selectively visualized using β -gal-IR. Of note, progressively fewer WGA-containing processes were observed in the lower, middle, and upper layers of POR cortex, consistent with the axons of transduced neurons entering the contralateral POR cortex from the white matter and ascending to their targets. Interestingly, specific sections contained WGA positive processes in narrow columnar-like clusters, suggesting that one or a few transduced neurons send their axons to localized targets in the contralateral POR cortex. Analogous to the results with gap-lac, the significant numbers of postsynaptic dendrites and cell bodies that were visualized demonstrate high-level expression of WGA that should support specific neuroanatomical

studies. In summary, the WGA gene has been used in transgenic mice and virus vectors as a transneuronal tracer for specific neurons in subcortical areas (Braz et al., 2002; Braz and Basbaum, 2008; Dobi et al., 2010; Hanno et al., 2003; Ohmoto et al., 2008; Yoshihara et al., 1999; Yoshihara, 2002), the WGA protein is a commonly used for mapping neocortical connections, and we have now shown that the WGA gene can be used in combination with gap-lac, in a HSV-1 vector, to map neocortical connections.

The specificity of gene transfer and expression from HSV-1 vectors could be combined with the neuronal circuit tracing cassettes developed here to map both the axons and postsynaptic targets of specific subclasses of neocortical neurons. Neuron class- or subclass-specific expression has been obtained using both targeted gene transfer and specific promoters. Targeted gene transfer has been achieved to neurons containing specific neurotrophic receptors or a specific subunit of the NMDA receptor (Cao et al., 2010; Wang et al., 2005). Complementarily, promoters that support enkephalinergic-, catecholaminergic-, glutamatergic-, or GABAergic-specific expression have been reported (Jin et al., 1996; Kaplitt et al., 1994; Rasmussen et al., 2007; Song et al., 1997; Sun et al., 2004; Zhang et al., 2000). Further, the VGLUT1 promoter supports expression selectively in VGLUT1-containing glutamatergic neurons (Rasmussen et al., 2007; Zhang and Geller, 2010). Moreover, to potentially obtain greater specificity in the subclasses of glutamatergic neurons expressing these neuronal circuit tracing cassettes, the VGLUT1 promoter was recently subdivided into an upstream promoter and first intron fragments that support expression in specific subclasses of glutamatergic neurons (Zhang et al., 2010b). Further, additional specificity of expression could be obtained by combining targeted gene transfer to specific classes of neurons with a neuron class- or subclass-specific promoter. Thus, the neuronal circuit tracing cassettes developed here could be selectively expressed in specific subclasses of neocortical glutamatergic neurons to map the circuits containing those neurons.

Lastly, these neuronal circuit tracing cassettes may support mapping neocortical circuits that have an essential role in cognitive learning or specific diseases. HSV-1 vectors expressing a constitutively active protein kinase C (PKC) have been used to target some of the essential information for performing specific visual discriminations to an identified circuit in POR cortex (Zhang et al., 2005; Zhang et al., 2010a). Further, delivery of this PKC into small numbers of hippocampal neurons can correct deficits in spatial learning in aged rats (Zhang et al., 2009). Thus, inserting a specific neuronal circuit tracing cassette into a vector expressing this PKC may enable mapping specific forebrain circuits that encode some of the essential information for performing an advanced cognitive task.

4. Materials and Methods

4.1. Materials

Dulbecco's modified minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and OptiMEM were obtained from Invitrogen. G418 was from RPI. X-gal was from Sigma. Mouse anti-*E. coli* β -gal was obtained from Sigma, goat anti-WGA was from Vector Labs, mouse anti-NeuN was obtained from Millipore, and mouse anti-MAP2 was obtained from Sigma. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG), FITC-conjugated rabbit anti-goat IgG, and Texas red-conjugated horse anti-mouse IgG were from Vector Labs.

4.2. Vectors

The two vectors in this study used our standard vector backbone (Fig. 1A; (Song et al., 1997; Zhang et al., 2000)) and contain the mouse VGLUT1 promoter and first intron (7 kb and 4.6 kb fragments, respectively) (Rasmussen et al., 2007). The two cassettes in the

vectors are diagrammed in Fig. 1B; construction of these cassettes and insertion into HSV-1 vectors is detailed next.

To construct pVGLUT1gap-lac, pVGLUT1lac (Rasmussen et al., 2007) was digested with Kpn I, treated with calf intestinal phosphatase, and the large fragment containing the vector backbone, Lac Z gene, and 5' part of the VGLUT1 promoter was isolated. Two complementary oligonucleotides were inserted that encode Kpn I, Asc I, Hind III, a translation start sequence close to the Kozak consensus, the 22 N-terminal aa of mouse GAP-43 (MLCCMRRTKQVEKNDEDQKIEQ GenBank: AAH80758.1 (Liu et al., 1994)), and destroys the Kpn I site in the Lac Z gene by a G to A mutation (the Lac Z gene in our HSV-1 vectors is derived from pCH110 GenBank: U13845.1 (Geller and Breakefield, 1988; Hall et al., 1983)). The sequence of the sense strand oligonucleotide is 5' CTTGGTAGGCGCGCAAGCTTACCATGCTGTGCTGTATGAGAAGAACCAACAG GTTGAAGAAGA ATGATGAGGACCAAAAGATTGAACAACACTAGTAC 3'. The resulting vector was designated pVGLUT1-partial-promoter-gap-lac. pVGLUT1-partial-promoter-gap-lac was digested with Kpn I and Asc I, and the large fragment was isolated; pVGLUT1lac was digested with the same enzymes, and the 8.8 kb fragment containing the 3' part of the VGLUT1 promoter and the first intron was isolated; and these two fragment were ligated together to yield pVGLUT1gap-lac.

To construct a wga/ires/gap-lac cassette, a 1,041 bp fragment was synthesized (DNA synthesis by Genescript Inc.). This fragment encodes Asc I, Hind III, a translation start sequence close to the Kozak consensus, the WGA isolectin D gene (GenBank: M25537.1 (Smith and Raikhel, 1989)), an ires with an internal 51 bp deletion (omitting a poly C tract that would have been problematic to synthesize; ires from pINS-TH-NFHbdfn/ires/lac (Sun et al., 2005)), 81 bp representing the 5' untranslated region from pVGLUT1lac ((Rasmussen et al., 2007); the Lac Z gene in our HSV-1 vectors is derived from pCH110 GenBank: U13845.1 (Geller and Breakefield, 1988; Hall et al., 1983)), sequences encoding the 22 N-terminal aa of GAP-43 (above), and the 3' end contained a G to C mutation that changed the Kpn I site in the Lac Z gene to a BsiW I site. This fragment was inserted into pUC57 at the EcoR V site (pUC57wga/iresΔ/gap). To reconstitute the complete ires, pUC57wga/iresΔ/gap was digested with Bgl II and Dra III, and the large fragment was isolated (removing 54 bp). pcDNAires/lac (Sun et al., 2005) was digested with the same enzymes, and a 407 bp fragment containing internal ires sequences was isolated. These two fragments were ligated together to yield pUC57wga/ires/gap (Sup. Fig. 1 contains the complete wga/ires/gap DNA sequence).

To construct pVGLUT1wga/ires/gap-lac, pVGLUT1lac was digested with Asc I and EcoR V, and a 19.6 kb fragment containing the vector backbone, VGLUT1 promoter, and 3' part of the Lac Z gene was isolated. pUC57wga/ires/gap was digested with Asc I and BsiW I, and the 1.4 kb fragment containing wga/ires/gap was isolated. pHSVlac (Geller and Breakefield, 1988) was digested with Acc65 I (produces the same overhang as BsiW I) and EcoR V, and a 1.2 kb fragment containing part of the Lac Z gene was isolated. These three fragments were ligated together to yield pVGLUT1wga/ires/gap-lac.

4.3. Cells and vector packaging

BHK21 and 2-2 cells were maintained in DMEM supplemented with 10 % FBS, 4 mM glutamine and penicillin/streptomycin. They were grown in an incubator at 37 °C, 5 % CO₂, and 100 % humidity. 2-2 cells were used for HSV-1 vector packaging; G418 (0.5 mg/ml), present during the growth of 2-2 cells, was removed before plating cells for packaging; 2-2 cells contain the HSV-1 immediate early 2 (IE 2) gene and were maintained under previously characterized selective conditions (Smith et al., 1992). Late-log phase, confluent cultures of BHK21 cells were used for titering the resulting vector stocks.

Vectors were packaged into HSV-1 particles using a modified form of the helper-virus free packaging protocol described previously (Fraefel et al., 1996; Sun et al., 1999). Purified vectors were titered on BHK cells, by counting X-gal positive cells at 24 hrs after transduction; control, mock-transduced cultures lacked X-gal positive cells. The titers of pVGLUT1gap-lac and pVGLUT1wga/ires/gap-lac are in the results section. We previously quantified the titer of vector genomes (VG/ml) and the packaging efficiency (VG/ml / IVP/ml) for pVGLUT1lac using a PCR assay, and the VG/ml titer for pVGLUT1lac was similar to a number of other vectors we have studied (Rasmussen et al., 2007). We did not repeat the VG/ml assay here because the two vectors examined in this report are similar to pVGLUT1lac. Wild-type HSV-1 was not detected (<10 plaque forming units/ml) in either of the vector stocks studied here.

4.4. Stereotactic injections of vectors into rat POR cortex

The VA Boston Healthcare System IACUC approved all the animal procedures. Adult male Long-Evans rats (150-200 g) were anesthetized by ip injection of a Ketamine (20 mg/ml) Xylazine (2 mg/ml) mixture with a final dose of 60 mg/kg and 6 mg/kg, respectively. Additional anesthesia was administered as needed. Each rat received a single injection of a specific vector into the left POR cortex: The injection coordinates were anterior-posterior (AP) -8.0, medial-lateral (ML) -6.0, dorsalventral (DV) -5.2 (Paxinos and Watson, 1986). AP is measured relative to bregma, ML is relative to the sagittal suture, and DV is relative to the bregma-lambda plane. A micropump (model 100, KD Scientific) was used for the injections; 3 µl of vector stock was injected over 5 minutes, and after 5 additional minutes, the needle was slowly retracted.

4.5. Immunohistochemistry

Brains were perfused as described (Zhang et al., 2000), and 25 µm coronal sections were prepared using a freezing microtome. Immunohistochemistry was performed on free-floating sections as described (Zhang et al., 2000). β-gal-IR was detected using mouse anti-β-gal (1:300 dilution), WGAIIR was detected using goat anti-WGA (1:100 dilution), neuronal cell bodies were identified using mouse anti-NeuN (1:1,000 dilution), and dendrites were identified using mouse anti-MAP2 (1:300 dilution). This anti-MAP2 antibody is a dendrite-specific marker (Huber and Matus, 1984). Primary antibodies were visualized with FITC-conjugated goat anti-mouse IgG or with FITC-conjugated rabbit anti-goat IgG and Texas red-conjugated horse anti-mouse IgG (1:200 dilutions).

4.6. Axon counts

Using pVGLUT1gap-lac, the numbers of axons in the contralateral POR cortex and perirhinal cortex were counted in digital images taken at 60X magnification. In the fields that were examined, all the β-gal-IR axons were scored. All counts were done at least two separate times, and results differed by <10 %.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Schematic diagrams of the HSV-1 vector backbone and the two neuronal circuit tracing cassettes. (A) Schematic diagram of the HSV-1 vector backbone (Song et al., 1997; Zhang et al., 2000). The HSV-1 ori_s and **a** sequence (contains the DNA cleavage/packaging site) support replication and packaging of a vector into HSV-1 particles, respectively. The tri-A cassette contains three SV40 early region polyadenylation sites that are placed to block any transcription from the HSV-1 ori_s fragment (contains the HSV-1 IE 4/5 promoter). The VGLUT1 promoter fragment contains a 7 kb promoter fragment, which contains the transcription start site, and a 4.6 fragment that contains the first intron (Rasmussen et al., 2007). The expression cassette is followed by the α-globin second intron and the SV40 early region polyadenylation site. The *E. coli* col E1 origin of DNA replication and amp^r gene support propagation of the vector in *E. coli*. (B) Schematic diagrams of the two neuronal circuit tracing cassettes. The gap-lac cassette encodes the 22 N-terminal aa of GAP-43, which contains the axon targeting sequence (Liu et al., 1994), fused to the N-terminus of β-gal. The wga/ires/gap-lac cassette contains the WGA gene, an ires, and gap-lac.

Fig. 2.

After injection of pVGLUT1gap-lac into POR cortex, β -gal is observed in cell bodies and processes proximal to the injection site, and in axons in the ipsilateral perirhinal cortex and contralateral POR cortex. The rats were sacrificed at 4 days after gene transfer, and β -gal-IR was detected using an anti- β -gal antibody. (A and B) Low and high power views show β -gal-IR cell bodies and processes proximal to the injection site in POR cortex. Arrows, β -gal-IR cell bodies; arrowheads, β -gal-IR processes. (C and D) High power views show β -gal-IR axons in the ipsilateral perirhinal cortex (C) and in the contralateral POR cortex (D). (E) A high power view of the POR cortex that received the vector injection, but distant from the injection site, lacks β -gal-IR cells or processes. Scale bars: 50 μ m (A), and 100 μ m (B -E).

Fig. 3.

After injection of pVGLUT1wga/ires/gap-lac into POR cortex, WGA is observed in neuronal cell bodies and processes proximal to the injection site. The rats were sacrificed at 8 days after gene transfer. WGA-IR was detected using anti-WGA, and neurons were identified by costaining using anti-NeuN. (A-C) WGA-containing neurons, and processes in cross section, proximal to the injection site; (A) WGA-IR, (B) NeuN-IR, or (C) merged. Long arrows with narrow arrowheads, costained cells; arrowheads, WGA-IR only; short arrows with wide arrowheads, NeuN-IR only neurons. (D-F) WGA-containing processes proximal to the injection site; (D) WGA-IR, (E) NeuN-IR, or (F) merged. (G-I) Omission of the primary antibodies resulted in no IR; (G) fluorescein filter, (H) Texas red filter, or (I) merged. Scale bar: 50 μ m.

Fig. 4.

After injection of pVGLUT1wga/ires/gap-lac into POR cortex, the contralateral POR cortex contains WGA in neuronal cell bodies and processes. The rats were sacrificed at 8 days after gene transfer. WGA-IR was detected using anti-WGA, and neurons were identified by costaining using anti-NeuN. (A-C) A lower layer of POR cortex contains numerous WGA positive processes; (A) WGA-IR, (B) NeuN-IR, or (C) merged. Arrowheads, WGA-IR only; short arrows with wide arrowheads, NeuN-IR only neurons. (D-F) A middle layer of POR cortex contains WGA positive neurons and processes; (D) WGA-IR, (E) NeuN-IR, or (F) merged. Long arrows with narrow arrowheads, costained cells. (G-I) An upper layer of POR cortex contains a WGA positive neuron; (G) WGA-IR, (H) NeuN-IR, or (I) merged. (J-L) Numerous WGA positive processes in a narrow band in a lower layer of POR cortex; (J) WGA-IR, (K) NeuN-IR, or (L) merged. Scale bar: 50 μ m.

Fig. 5.

After injection of pVGLUT1wga/ires/gap-lac into POR cortex, the contralateral POR cortex contains WGA in presynaptic axons, and in postsynaptic dendrites and neuronal cell bodies. The rats were sacrificed at 8 days after gene transfer. WGA-IR was detected, and postsynaptic dendrites and neuronal cell bodies were identified by costaining using anti-MAP2 (Huber and Matus, 1984). (A-C) WGA positive presynaptic axons and postsynaptic neuronal cell bodies; (A) WGA-IR, (B) MAP2-IR, or (C) merged. Long arrows with narrow arrowheads, contained postsynaptic neuronal cell bodies; arrowheads, WGA-IR only presynaptic axons; short arrows with wide arrowheads, MAP2-IR only neurons. (D-F) WGA positive presynaptic axons and post-synaptic dendrites in cross section; (D) WGA-IR, (E) MAP2-IR, or (F) merged. Long arrows with narrow arrowheads, contained post-synaptic dendrites; arrowheads, WGA-IR only presynaptic axons; short arrows with wide arrowheads, MAP2-IR only dendrites. (G-I) Longitudinal and oblique views of WGA positive presynaptic axons and post-synaptic dendrites; (G) WGA-IR, (H) MAP2-IR, or (I) merged. Scale bar: 50 μ m.