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Monosynaptic Inputs to ErbB4 Expressing Inhibitory Neurons in Mouse Primary Somatosensory Cortex

Jiwon Choi and Edward M. Callaway¹

Systems Neurobiology Laboratories, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037

Abstract

Previous reports have described inputs to the somatosensory cortex (S1) in mouse or rat using retrograde or anterograde tracers. Such studies do not, however, reveal which particular cell types within the S1 cortex receive direct monosynaptic connections from these input sources. Here we describe the monosynaptic inputs to a subpopulation of mouse S1 inhibitory neurons that express ErbB4. We used a previously described “bridge protein”, composed of the ErbB4 ligand, neuregulin (NRG1), fused to the avian viral receptor TVB (TVB-NRG1), along with EnvB pseudotyped lentivirus (LV) and rabies virus (RV), to selectively co-infect ErbB4 expressing neurons (Choi et al., 2010). The RV had its glycoprotein gene deleted and replaced with mCherry, so that infected cells express mCherry and the virus cannot spread without provision of rabies glycoprotein (RG) by transcomplementation. The LV encoded and expressed RG to allow transcomplementation in co-infected neurons, so that the RV could spread transsynaptically and label their direct monosynaptic inputs. The RV could not spread beyond the direct inputs, due to the lack of RG in presynaptic cells. This method revealed long-range connections from thalamus, nucleus basalis, Raphe, and distant cortical areas, including ipsilateral motor, secondary somatosensory, retrosplenial, and perirhinal cortex and contralateral S1. In addition, local connections from ipsilateral pyramidal neurons within S1 were labeled. These input sources account for all of the known inputs to S1 described with standard tracers, suggesting that the subpopulation of ErbB4 positive inhibitory neurons infected using the TVB-NRG1 bridge protein receives inputs indiscriminately from S1 input sources.

Keywords

S1; Rabies Virus; Bridge Protein; Neuregulin; Transcomplementation

Introduction

Normal cortical function depends on the precise interplay of numerous inhibitory and excitatory cell types within the cortex, as well as connections within and between cortical and subcortical structures. A crucial step toward understanding the contributions of each of these brain structures and the particular cell types within those structures is to generate “wiring diagrams”, so that realistic models of circuit function can be generated and potential interactions inferred from connectivity can be functionally tested. Many studies have demonstrated that not all of the “potential connections” (Stepanyants and Chklovskii, 2005) suggested by the gross anatomical overlap of axons and dendrites are in fact realized (Brown and Hestrin, 2009). Instead, when neurons send axons to, and form terminal fields within

¹Correspondence to: Edward M. Callaway, Systems Neurobiology Laboratories, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, callaway@salk.edu, phone: 858-453-4100 x1158 FAX:858-455-7933.

particular cortical areas or layers, they often selectively synapse onto only a subset of the dendritic elements found within the terminal field. This has been documented most extensively for local cortical circuits, where functional connections to particular cell types can be readily investigated in living brain slices. For example, such studies have revealed that different types of inhibitory neurons in layer 2/3 of rodent primary visual cortex or S1 receive local connections from different cortical layers (Dantzker and Callaway, 2000; Xu and Callaway, 2009). This selectivity is observed for both excitatory and inhibitory inputs. More recently, the ability to express channelrhodopsin in the axons of neurons from a potential input source and then optically stimulate them selectively while recording from particular cell types in living brain slices (Cruikshank et al., 2010; Petreanu et al., 2007), has made it more tractable to investigate cell type specificity of distant inputs. These studies, along with older studies using more traditional methods have demonstrated that cell type selectivity is also common for inputs from distant sources, such as the thalamus (Beierlein et al., 2003; Cruikshank et al., 2010).

The development of novel rabies-based tracing tools (Wickersham et al., 2007b) has now made it possible to readily investigate the cell-type specificity of both long-distance and local connections to particular cortical areas in the intact brain (Haubensak et al., 2010; Marshel et al., 2010; Miyamichi et al., 2010; Rancz et al., 2011; Stepien et al., 2010; Wall et al., 2010; Yonehara et al., 2011). Previous studies have established rabies virus (RV) as a powerful tool for revealing neuronal connectivity, due to its ability to spread between neurons exclusively in the retrograde direction (Kelly and Strick, 2003; Ugolini, 1995). Furthermore, as first suggested by Ugolini (Ugolini, 1995) all available evidence indicates that the virus spreads only “transsynaptically”, exclusively between neurons with synaptic contacts (Ugolini, 1995; 2010; Wickersham et al., 2007b) (see further below). The development of glycoprotein (G)-deleted RVs (Etessami et al., 2000) allows the spread of the virus to be genetically controlled. Because G-deleted viruses are unable to generate new infectious particles in the absence of an alternative source of rabies G (RG) (Etessami et al., 2000; Larsen et al., 2007; Wickersham et al., 2007a), transcomplementation combined with selective infection can be used to label the direct inputs to specific cell types both in vitro (Wickersham et al., 2007b) and in vivo (Haubensak et al., 2010; Marshel et al., 2010; Miyamichi et al., 2010; Rancz et al., 2011; Stepien et al., 2010; Wall et al., 2010; Yonehara et al., 2011).

An important consideration in interpreting results following the spread of rabies virus between neurons, is the extent to which the virus is able to spread between neurons that share synaptic contacts (transsynaptic spread) versus spread to neurons that are in close proximity but do not actually make synaptic contacts. While it would be nearly impossible to demonstrate that rabies never spreads between neurons in the absence of synaptic contacts, there are many published studies showing that rabies did not spread to neurons that were in close proximity but not connected (Graf et al., 2002; Kelly and Strick, 2003; Lyon et al., 2010; Nassi and Callaway, 2006; Tang et al., 1999; Ugolini, 1995; 2010; Wall et al., 2010; Yonehara et al., 2011). And despite numerous studies using rabies virus, it has never been reported to spread between neurons that are known to not be synaptically connected. Here we discuss some of the most compelling examples in detail, while referring the reader to further examples highlighted in a review article that states: “Rabies virus is the only viral transneuronal tracer that is entirely specific, since it propagates exclusively between connected neurons, without inducing neuronal degeneration or spurious spread regardless of the dose and time post-inoculation” (Ugolini, 2010).

One of the first examples suggesting synapse specific spread of rabies was described by Ugolini in 1995 (Ugolini, 1995). Following direct rabies infection of hypoglossal motor neurons, rabies spreads transneuronal to numerous brainstem structures known to provide

their direct inputs (Ugolini, 1995). But rabies did not spread spuriously to the inferior olive, contrasting with results using alpha-herpesviruses under similar conditions (Babic et al., 1993; Ugolini et al., 1987). Furthermore, there was not transneuronal spread from brainstem neurons providing input to hypoglossal motor neurons, to motor neurons in the 7th and 10th brainstem motor nuclei whose axons cross those neurons' dendritic arbors without forming connections. Thus, the dendrites of rabies infected neurons were in close enough proximity to provide the potential for non-specific spread (as occurred with herpes), yet the spread remained transsynaptic.

Another example comes from the primate visual cortex, where neurons in layer 4C β make extremely dense projections to layer 3B (Yabuta and Callaway, 1998) but selectively connect to only "local" pyramids and not to "projecting" pyramids that have overlapping dendrites (Sawatari and Callaway, 2000). In contrast, neurons in layer 4C α connect directly to both types of layer 3B pyramid (Sawatari and Callaway, 2000). When rabies virus is injected into locations that receive input exclusively from layer 3B projecting pyramids there is extensive retrograde transneuronal spread to layer 4C α , but not to layer 4C β (Nassi and Callaway, 2006). Thus, the rabies virus spread only to cells that make synaptic connections and not to cells with axons in close proximity but not making synaptic connections. In view of these observations we refer to the spread of rabies as transsynaptic.

In order to investigate the sources of input to a subset of cortical inhibitory neurons that express the neuregulin (NRG) receptor ErbB4, we combined transsynaptic tracing with G-deleted rabies, with a transductional targeting method for directing viral infection to particular cell types (Choi et al., 2010) (see Figure 1). Using an EnvB pseudotyped, G-deleted RV, along with a bridge protein composed of the ErbB4 receptor ligand NRG1 fused to the EnvB receptor TVB (TVB-NRG1) (Snitkovsky and Young, 2002), the primary RV infection was targeted to ErbB4 inhibitory neurons in mouse S1 (Choi et al., 2010) (Figure 1A). The same TVB-NRG1 bridge protein was also used to selectively deliver an EnvB pseudotyped lentivirus (LV) (Choi et al., 2010) expressing RG, to a subset of the rabies infected neurons (Figure 1B). Coinfection allowed transcomplementation, spread of the rabies to the direct inputs to the targeted ErbB4 neurons, and labeling by expression of fluorescent reporter (mCh) from the rabies genome (Figure 1A). Importantly, the rabies could not spread beyond the direct monosynaptic inputs due to the absence of RG in presynaptic cells.

We were interested in studying the inputs to ErbB4 expressing cortical inhibitory neurons because signaling between NRG1 and ErbB4 is implicated in regulation of the strength of functional inhibition in the mature cortex and in the development of normal patterns of connectivity (Fazzari et al., 2010; Mei and Xiong, 2008; Woo et al., 2007). Furthermore, mutations in both ErbB4 and NRG are implicated in schizophrenia (Mei and Xiong, 2008). Understanding whether these neurons sample from a selected subset of cortical inputs could provide insight into their functional roles for cortical computation.

Of the three major, non-overlapping groups of mouse cortical inhibitory neurons, parvalbumin (PV)-, somatostatin (SOM)-, and vasoactive intestinal peptide (VIP)-expressing (Xu et al., 2010), ErbB4 is expressed most prominently in PV cells, but is also expressed in SOM and VIP cells (Choi et al., 2010; Fazzari et al., 2010). Studies and theories about the role of ErbB4 have therefore focused primarily on PV+, fast-spiking basket cells (Fischbach, 2007; Mei and Xiong, 2008). The relevance of ErbB4 expression in PV+ interneurons is strongly supported by the prominent effects seen when ErbB4 is selectively removed from these cells (Ting et al., 2010; Wen et al., 2010). Due to the selectivity of the TVB-NRG1 bridge protein viral targeting used, the studies described here

reveal inputs to PV and VIP expressing ErbB4 interneurons, but do not include SOM cells (Choi et al., 2010).

Previous studies have revealed several sources of input to mouse or rat primary somatosensory cortex. These include: cholinergic neurons in the nucleus basalis of Meynert (NBM) (Baskerville et al., 1993), serotonergic neurons in the Raphe (Lee et al., 2008), thalamic nuclei (Fabri and Burton, 1991b; Kuramoto et al., 2009; Pierret et al., 2000), contralateral S1 (Aronoff et al., 2010; Larsen et al., 2007), and several ipsilateral cortical areas (Aronoff et al., 2010; Fabri and Burton, 1991a). These known sources are therefore potential sources of synaptic input to ErbB4 expressing inhibitory neurons.

Materials and Methods

Preparation of EnvB pseudotyped rabies virus and lentivirus

Generation of G-deleted, mCherry expressing RV pseudotyped with EnvB (EnvB-mCh-RV) was described previously (Choi et al., 2010). Briefly, mCh-RV enveloped with its own native SAD-B19 rabies glycoprotein was used to infect a BHK cell line expressing EnvB. 5 hours later the cells were thoroughly washed with PBS (pH 7.4) and supplied with DMEM with 10% fetal bovine serum (FBS, HyClone) and antibiotics (Invitrogen). 5 days later, the media which contained virus was filtered through a 0.2µm filter unit (Chemicon), ultracentrifuged in SW 28 swing buckets for 2 hours at 19.4 K rpm. The pellet was dissolved in PBS, overlaid onto 20% sucrose, and ultracentrifuged in SW55 swing buckets for 2 hours at 21K rpm. The virus pellet was dissolved in Hank's balanced salt solution (HBSS), aliquotted, and stored at -80°C.

LV expressing GFP and the SAD-B19 rabies glycoprotein under the enhanced synapsin promoter (LV-eSyn-GFP-B19G) was produced by coupling GFP and B19G with a F2A element under the control of eSyn, cloned into p156RRLsinPPTCMV-GFP WPRE as a backbone, to produce p156RRLsinPPT eSyn-GFP-F2A-B19G WPRE. Enhanced synapsin promoter (eSyn) was constructed by PCR to amplify eSyn using the plasmid pBSII-SK-Pro-GFP-WPRE containing eSyn promoter (Hioki et al., 2007) to replace the CMV promoter in p156RRLsinPPTCMV-GFP WPRE with Cla I and BamHI. The stop codon of GFP in p156RRLsinPPTCMV-GFP WPRE was removed by replacement of PCR product encoding GFP without a stop codon by Bam HI and Bsr GI. B19G gene, which was produced by PCR, was inserted between two sites, Bsr GI and PmeI in p156RRLsinPPTCMV-GFP WPRE. In the resulting plasmid, the F2A element (Szymczak et al., 2004) (produced by PCR) was inserted between the Bsr GI and Bsu36I sites.

EnvB-LV-eSyn-GFP-B19G was produced by calcium phosphate transfection with 4 plasmids from HIV-1 lentivector production system (Wickersham et al., 2007a), 225µg of p156RRLsinPPT eSyn-GFP-F2A-B19G WPRE, 146 µg of pMDL.gp.RRE.Oded, 56µg of pRSV-Rev and 79µg of pCI-EnvB from 293T cells. 1 day after transfection, the cells were washed with PBS and supplied with DMEM supplemented with 10% FBS and antibiotics. 4 days posttransfection, ultracentrifugation of media and virus preparation were processed as described for RV, above.

Stereotaxic animal surgery, virus injection and perfusion

All procedures using live animals were conducted according to procedures approved by the Salk Institute Animal Care and Use Committee.

Adult mice, C57BL/6, were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine (3.6mg/Kg and 0.36mg/Kg respectively). EnvB-LV-eSyn-GFP-B19G was mixed with the supernatant containing TVB-NRG1 (produced as described in (Choi et

al., 2010)) with 1:1 volume ratio between virus and supernatant and incubated 30mins at room temperature. This mixture was stereotactically injected to somatosensory cortex (coordinates: 0.8–1.5 mm posterior and 2–2.5mm lateral to bregma; 2 depths, 0.7, 0.4mm below the pia) using a glass pipette. 2–3 weeks after LV injection, EnvB-RV-mCh was mixed with the TVB-NRG1 supernatant with 1:10 volume ratio between virus and supernatant and injected to the same location where LV had been injected. Eight days after RV injection, mice were overdosed with Nembutal (100mg/Kg IP) and perfused with saline followed by 4% paraformaldehyde. Brains were removed from the skull and submerged in 30% sucrose in 4% paraformaldehyde for one day and then moved to 30% sucrose in PBS. The brain was sectioned coronally to a thickness of 40 μ m using a freezing microtome. A period of 8 days was chosen for survival after RV injections because we wanted to maximize the time available for the spread of rabies virus, but eliminate the possibility that labeled neurons would be killed by the rabies infection. We have previously found that rabies infected neurons remain healthy and viable for at least 10 days after infection (Wickersham et al., 2007a).

Immunohistochemistry against mCh, fluorescent immunohistochemistry and image Acquisition

To label mCh positive cells, brain sections were processed using rabbit antisera raised against DsRed (Clontech #632496) with 1:250 dilution as a primary antibody followed by Cy3 conjugated donkey anti rabbit secondary antibody (Jackson Immunoresearch Laboratories), and counterstained against DAPI as described previously (Wall et al., 2010). The specificity of the primary antibody for DsRed is shown by the lack of labeling in numerous brain sections that did not contain labeled neurons, as well as a lack of labeling in sections stained from animals that were not infected with mCh expressing rabies virus. Immunostained sections were mounted on slides, dehydrated, defatted, and coverslipped with Krysalon (EM Science). In some sections, mCh was observed from its native fluorescence instead of immunostaining, and these were mounted on glass slides and coverslipped with Vectashield (VectorLabs). In every mouse a complete set of all sections was examined, from the most anterior cortical areas extending posteriorly through the full extent of the midbrain to the junction with the pons. In cases where numbers of labeled neurons were small, every neuron was counted. When numbers of labeled neurons were not greater than about 20 in any given brain region, numbers of labeled neurons were not explicitly counted. Images were acquired with either an Olympus BX51 microscope and MicroFire digital camera (Optronics) or a Leica confocal microscope (Leica TCS SP2 AOBS). Contrast and brightness were adjusted linearly using Adobe Photoshop.

Results

Identification of monosynaptic inputs to ErbB4 inhibitory neurons in mouse S1 cortex

To identify the direct monosynaptic inputs to ErbB4 expressing inhibitory neurons in mouse S1 cortex, selective infection of ErbB4 interneurons was combined with transcomplementation of G-deleted RV. ErbB4 neurons were selectively infected with EnvB-pseudotyped viruses mixed with TVB-NRG1 bridge protein; the selectivity of this approach has been described previously for both EnvB-pseudotyped rabies and LV (Choi et al., 2010). In the absence of RG expression, the RG-deleted rabies is not able to produce infectious particles (Etessami et al., 2000), and remains confined to the initially infected ErbB4 interneurons (Choi et al., 2010). When neurons infected with RG-deleted rabies express RG *in trans*, transcomplementation allows RV to spread transsynaptically to directly presynaptic neurons (Wickersham et al., 2007b). We therefore first infected ErbB4 interneurons with an EnvB pseudotyped helper LV expressing RG (EnvB-LV-eSyn-GFP-B19G), mixed with TVB-NRG1, injected into S1 cortex. After waiting 2–3 weeks for RG

expression, a mixture of TVB-NRG1 and EnvB-pseudotyped, RG-deleted rabies expressing mCherry (EnvB-mCh-RV) was injected into the same cortical location. The RG expressed from the LV transcomplemented the RG-deleted rabies, allowing transsynaptic spread. The spread of RV to neurons presynaptic to the ErbB4 interneurons resulted in expression of mCh in numerous pyramidal neurons locally, near the S1 cortical injections (Figure 2). mCh expressing pyramidal neurons were also found in numerous distant structures known to contain neurons with projections to S1 (see further below). These results contrast sharply with results from injections of the same EnvB-mCh-RV and TVB-NRG1 mixture in the absence of prior injections of the helper LV; in such cases neuronal infection is confined to local interneurons, and pyramidal neurons expressing mCh are not detected (Choi et al., 2010).

Although successful transcomplementation indicated co-infection with the helper LV and the EnvB-mCh-RV, as well as expression of RG from the LV genome, GFP expressing neurons expected following infection with EnvB-LV-eSyn-GFP-B19G were not observed. The cause of this failure is not known, however previous studies have also reported similar findings in which fluorescent proteins linked to other genes by 2A elements do not fluoresce (Hasegawa et al., 2007; Lengler et al., 2005; Wall et al., 2010).

The following descriptions summarize findings from viral injections made into S1 cortex of five mice (Table 1). In every mouse a complete set of all sections was examined, from the most anterior cortical areas extending posteriorly through the full extent of the midbrain to the junction with the pons. In cases where numbers of labeled neurons were small, every neuron was counted. When numbers of labeled neurons were greater than about 20 in any given brain region, numbers of labeled neurons were not explicitly counted.

Corticocortical inputs to ErbB4 neurons

Local label in ipsilateral S1—mCh expression in pyramidal neurons, indicative of rabies spread from ErbB4 neurons in S1 to their directly presynaptic inputs, was observed throughout the cortical layers of ipsilateral S1, with the exception of layer 1, which lacks pyramidal neurons (Figure 2). Pyramidal neurons were observed more frequently in L2/3 and L5 than in L4 or L6 (Figure 2) and local pyramidal neurons were abundant in all 5 animals (Table 1).

Label in distant cortical regions—In addition to local excitatory input to ErbB4 neurons, mCh label was found in pyramidal neurons located in all of the cortical regions known to project axons to rodent S1 cortex (Table 1). Pyramidal neurons were labeled in the secondary somatosensory cortex (S2) of all 5 animals (Table 1). In S2, pyramidal neurons were located lateral to the S1 virus injection location, separated by a clear gap (~2mm) containing no intervening labeled neurons (Figure 3). The S2 pyramidal neurons were observed in L2/3 and L5 (Figure 3). Four of 4 animals also had mCh labeled neurons in M1 motor cortex (Table 1, motor cortex was damaged in one animal preventing analysis). M1 pyramidal neurons were labeled primarily in layer 5 (e.g. Figure 4) but there were also smaller numbers of M1 pyramidal neurons observed in layers 2 and 6. Labeled pyramidal neurons were also detected in the retrosplenial cortex of all 5 animals and these were located in deep cortical layers (below layer 4)(Figure 5). Overall, the relative numbers of labeled neurons in each cortical area were similar for S2, M1 and RS (Table 1).

Contralateral S1 and the perirhinal cortex contained smaller numbers of labeled pyramidal neurons and these were not present in every animal (Table 1). In contralateral S1, pyramidal neurons were observed in 3 of 5 animals and these neurons were located in L2/3, (Figure 6A), L5 (Figure 6B) and L6. In the perirhinal cortex, only one mCh labeled neuron was found in L5 of just one of the 5 animals (Table 1, Figure 7).

Thalamic inputs to ErbB4 neurons in S1

Although the most prominent thalamic nuclei projecting to S1 cortex are VP and Po (Alloway et al., 2004; Fabri and Burton, 1991b; Koralek et al., 1988; Pierret et al., 2000) VA and VL, which are slightly anterior to VP and Po, have also been shown to project not only motor cortex, but also to S1 (Kuramoto et al., 2009; Wang and Kurata, 1998). mCh labeled neurons were found in all of these thalamic nuclei (VP, Po, and VA-VL complex, Table 1). The largest numbers of labeled neurons were invariably found in VP (5 of 5 animals, Table 1). Smaller numbers of labeled neurons were present in Po (3 of 5 animals, Table 1, Figure 8). Following virus injections at the 3 most anterior-medial sites injected in the 5 animals, mCh expressing neurons were also found in the VA-VL complex (Table 1). Although the VA-VL complex is typically considered a motor nucleus, it has previously been shown to provide input to S1 cortex (Kuramoto et al., 2009).

Nucleus Basalis of Meynert and Raphe inputs to ErbB4 neurons in S1

Small numbers of neurons providing monosynaptic input to ErbB4 inhibitory neurons in S1 were observed in the NBM, at the junction between globus pallidus and internal capsule (Figure 9A). These were present in four of five animals (Table 1). A single mCh labeled neuron was observed in the Raphe (Figure 9B) for two of 5 animals (Table 1). The two neurons were found in a similar location in each of the two animals, at the lateral side of the median Raphe (paramedian Raphe).

Discussion

We have demonstrated the long distance and local inputs to a subpopulation of inhibitory neurons in mouse S1 cortex, using selective viral targeting methods based on the TVB-NRG1 bridge protein (Choi et al., 2010) along with transcomplementation of G-deleted RV (Wickersham et al., 2007b). Previously, TVB-NRG1 was shown to direct infection with EnvB pseudotyped RV and LV to ErbB4 positive inhibitory neurons (Choi et al., 2010). Here, this selective infection method was used to co-infect ErbB4 cortical neurons with both G-deleted rabies and with LV expressing RG; co-infection allowed transcomplementation and labeling of the direct monosynaptic inputs to the ErbB4 neurons.

The results presented here demonstrate both the sources of input to ErbB4 neurons and the utility of this approach. Although previous studies have used cell-type specific infection and transcomplementation of G-deleted rabies to describe input to specific cell types (Haubensak et al., 2010; Wall et al., 2010), those studies relied on cell type specific gene expression in transgenic mice. The methods used here do not, however, rely on transgenic animals and should also be applicable to other species where use of transgenic animals is less practical. It is also likely that these methods can be used to target other cell types using different bridge proteins.

It is likely that the majority of primary neurons coinfecting with rabies and LV injected into S1 cortex in our experiments were VIP and/or CR expressing ErbB4 interneurons with bipolar morphologies, but injections might have also involved small numbers of PV expressing ErbB4 neurons (Choi et al., 2010). Because the LV used did not unambiguously label neurons, it is not possible to determine in these experiments whether the labeling patterns observed reflect contributions from a mixture of these populations, nor what are their relative proportions. Since different cell types within these populations might receive inputs of different strength from the input sources that were labeled, these labeling patterns should be interpreted conservatively.

Previous studies have used standard anterograde and retrograde tracers to investigate the sources of input to S1 cortex, but such studies cannot reveal the sources of input to specific

cell types. Our results demonstrate input to ErbB4 expressing S1 cortical neurons from all of these sources, but there are differences in the relative numbers of neurons that are labeled in each structure. The most abundant transynaptic labeling is observed in local S1 pyramidal neurons near the injection site. These neurons are located throughout the cortical layers (except layer 1), consistent with previous functional characterization of local inputs to VIP and/or CR expressing bipolar inhibitory neurons in mouse S1 cortex (Xu and Callaway, 2009). More moderate levels of labeling were observed in several cortical areas and in the thalamus. Moderate levels of labeling were observed consistently in ipsilateral S2, motor and retrosplenial cortex, while weaker labeling was observed in contralateral cortex of just 3 of 5 animals. Label in ipsilateral perirhinal cortex was observed as just one labeled neuron in just one animal.

Thalamic label was most extensive and consistent in VP. This is not surprising since VP provides the main lemniscal input to S1 (Aronoff et al., 2010; Fabri and Burton, 1991b; Pierret et al., 2000). In contrast, paralemniscal input from Po was less consistent and several-fold fewer cells were labeled. Interestingly, functional studies of local connections to bipolar interneurons, similar in morphology and immunolabeling profile to those labeled with RV via the TVB-NRG1 bridge protein (Choi et al., 2010), showed that they receive very strong input from the lemniscal recipient layer 4, and weak input from the paralemniscal recipient layer 5A (Xu and Callaway, 2009). This contrasted with other inhibitory cell types, including PV expressing inhibitory neurons, that received either more balanced input from layer 4 and 5A, or which received input predominantly from layer 5A (e.g. neurogliaform and chandelier cells (Xu and Callaway, 2009)). Thus, our observations are consistent with the possibility that the direct thalamic connections to different cortical inhibitory cell types are biased toward lemniscal versus paralemniscal pathways in a way that reinforces the bias of local inputs from layer 4 versus layer 5A.

Thalamic label was observed not only in VP and Po, but also in the VA-VL complex in 3 of 5 animals. These 3 cases correspond to the most anterior and medial S1 virus injection sites and are likely to include shoulder and neck representations, which are located closer to M1. Anterograde tracer injections and single afferent reconstructions following tracer injections into the VA-VL complex have shown that the inputs to M1 often extend posteriorly into S1 (Kuramoto et al., 2009). The absence of rabies label in VA-VL following more posterior injections suggests that the VA-VL afferents may limit their projections to the more anterior sites.

In addition to these strong connections, labeled neurons were found in both the NBM, and the paramedian Raphe. NBM is known to provide cholinergic input to the S1 cortex, but also contains GABAergic neurons. Because we did not stain against choline acetyltransferase, it is uncertain whether the labeled neurons in NBM are cholinergic. However, since 90% of neurons projecting from NB to S1 in rat are cholinergic (Baskerville et al., 1993), it is likely that they include cholinergic projection neurons. The observation of two labeled neurons in the Raphe for two of 5 mice suggests that serotonergic neurons are also likely to be presynaptic to ErbB4 neurons in S1 (Lee et al., 2008). Nevertheless, due to the inability to definitively identify the numbers and identities of postsynaptic neurons in S1, it is not possible to understand the reason that small numbers of neurons were labeled in these brainstem structures, as well as some other areas. For example, one possibility is that the number of inputs to each S1 cell is in fact small and perhaps even some of these cells receive no input. An alternative, and perhaps more likely possibility, however, is that the population of ErbB4 expressing neurons is diverse and involvement of some types of cells was variable. For example, PV-expressing ErbB4 neurons, which are infected less frequently with the TVB-NRG1 bridge protein, might be the source of labeling that is weak or variable.

In the experiments described here, inputs to different subsets of ErbB4 inhibitory neurons were not unambiguously identified. In the future, more advanced combinatorial genetic methods and transgenic mouse lines might make it possible to separately identify the inputs to each of the subtypes of ErbB4 expressing interneurons and to determine whether ErbB4 cells receive inputs from different sources than their ErbB4 negative PV, VIP, and SOM expressing counterparts. For example, crossing an ErbB4 Cre line (Madisen et al., 2010) with an SOM Flp line or a PV Flp line and then expressing TVA and RG only in cells that express both Cre and Flp (e.g. (Wall et al., 2010)) could allow the inputs to either the SOM or PV subpopulation of ErbB4 cells to be identified.

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Figure 1.

Monosynaptic retrograde tracing strategy to identify direct presynaptic inputs to ErbB4 expressing inhibitory neurons.

A. ErbB4 expressing interneurons are selectively targeted for coinfection with both an EnvB pseudotyped, rabies glycoprotein (RG)-deleted rabies virus expressing mCherry (EnvB-mCh-RV) and an EnvB pseudotyped lentivirus expressing RG (EnvB-RG-LV). Selective infection with these EnvB pseudotyped viruses is mediated by the TVB-NRG1 bridge protein. In the absence of RG expression the RG-deleted rabies virus would not be able to spread, but transcomplementation due to expression of RG from the EnvB-RG-LV allows transsynaptic spread of rabies, from coinfecting neurons to their direct presynaptic inputs. Because RG is not expressed in the presynaptic neurons, RV does not spread beyond direct

inputs to the ErbB4 inhibitory neurons. mCh expression from the rabies genome therefore labels ErbB4 interneurons at the site of viral injections, as well as their direct presynaptic inputs across the whole brain.

B. Genomic structure of EnvB-RG-LV and EnvB-mCh-RV. In the lentiviral genome, coding sequences for GFP and RG are fused with a foot-and-mouth disease virus 2A element (F2A) controlled under the enhanced synapsin promoter (eSyn). GFP is grey to indicate that the GFP intended to express from this construct was not seen (see text). In the rabies genome, the native RG was deleted and replaced with mCh. N, nucleoprotein; P, phosphorylated protein; M, matrix protein; L, large RNA-dependent RNA polymerase.

Figure 2.

Cortical neurons expressing mCherry in S1 cortex.

The S1 cortex was injected first with a mixture of EnvB-LV-eSyn-GFP-B19G and TVB-NRG1 followed by EnvB-RV-mCh and TVB-NRG1. This resulted in co-infection of ErbB4 cortical neurons and transynaptic spread of the mCherry (mCh) expressing RV to their directly presynaptic neurons.

A. Overview of mCh expressing neurons near the injection site in S1. Pial surface is at the top. Layers are indicated as shown in the DAPI-stained image of the same section shown in panel **B**. Arrows, 1 and 2, indicate labeled pyramidal neurons that are illustrated at higher magnification in panels **C** and **D**, respectively. **E.** mCh expressing neurons in another section through S1 cortex located 300um away from the section shown in **A**. Arrows indicate labeled pyramidal neurons. Scale bars in **A** and **B** are 300μm. Scale bars in **C**, **D**, and **E** are 150μm.

Figure 3.

Cortical neurons expressing mCherry in S1 and S2 cortex.

A. Low power view illustrating the location of mCh-expressing neurons in S1 and S2 merged with a DAPI counterstained image of the same section. Arrows indicate neurons located in S2. **B.** Higher power view of the region outlined by the square in the square in panel A. Arrows indicate three projection neurons, two in layer 3 and one in layer 5, labeled as a result of the transynaptic spread of mCherry expressing rabies from the injection site in S1 cortex. Scale bars: A 500 μ m, B 100 μ m.

Figure 4.

Cortical neuron expressing mCherry in layer 5 of M1 motor cortex.

A. Low power image showing a single mCherry labeled pyramidal neuron, also illustrated at higher power in panel C. **B.** DAPI counterstained image of the same section illustrated in A. Pial surface is toward the top for all images. Arrowheads in panels A and B indicate the border between S1 and M1 cortex. Dotted vertical lines indicate the midline between the two cortical hemispheres. The mCh labeled neuron is located in layer 5 of the M1 cortex and indicates transynaptic spread of RV to M1. Scale bars: A, B 500 μ m; inset to A 100 μ m.

Figure 5.

Cortical neurons expressing mCherry in S1 and retrosplenial cortex.

A. Low power view illustrating overall location of mCh expressing neurons. Arrows indicate neurons located in the retrosplenial cortex (RS). Many labeled neurons are also seen near the virus injection site in S1. Dotted vertical line indicates the midline between the two cortical hemispheres. **B.** Higher power view of the neurons indicated by arrows in A. Scale bars: A 500 μ m, B 100 μ m.

Figure 6. Cortical neurons expressing mCherry in contralateral S1 cortex. **A.** Callosally-projecting mCh labeled neuron located in layer 2/3 of S1, contralateral to viral injections. **B.** Callosally-projecting mCh labeled neuron located in layer 5 of S1, contralateral to viral injections. Scale bars: 250 μ m.

Figure 7.

Cortical neuron expressing mCherry in perirhinal cortex.

A. mCherry expressing neuron indicated by arrow in the perirhinal cortex (PRh). Inset shows a higher power view of the same neuron. **B.** DAPI counterstained image of the same section as A. Arrowhead in A and B indicates the rhinal fissure. Ect – ectrorhinal cortex. Pir – piriform cortex. Scale bars: A and B 500 μ m, inset to A 100 μ m.

Figure 8. Thalamic neurons expressing mCherry. **A,B.** Red fluorescent and DAPI images, respectively, illustrating the overall locations of mCh expressing thalamic neurons resulting from transynaptic spread of RV injected into S1 cortex. Labeled neurons indicated by arrows are located in both VP (ventral posterior nucleus) and Po (posterior nucleus). **C,D.** Additional red fluorescence and DAPI images showing another rabielabeled mCh expressing neuron in Po. **E.** Higher power view of the neuron indicated by arrow in C. Scale bars: in A-D 500 μ m, E 100 μ m.

Figure 9.

S1 projecting, mCherry expressing neurons in nucleus basalis of Meynert and in the Raphe. **A.** Low power view of a section containing the nucleus basalis of Meynert (NBM) including an mCh expressing neuron in NBM (arrow). The red fluorescence image is merged with the DAPI counterstained image to show NBM in the context of neighboring structures including the thalamic nucleus VP and the internal capsule (IC). The same mCh expressing neuron is shown at higher power in the inset. **B.** Low power view of a section containing the dorsal Raphe (DR) and showing an mCh expressing neuron in paramedian Raphe (located in the lateral part of median Raphe [MnR], indicated by arrow). The red fluorescence image is merged with the DAPI counterstained image to show the Raphe in the context of neighboring structures. The same mCh expressing neuron is shown at higher power in the inset. Scale bars: A, B 500 μ m, insets 100 μ m.

Table 1
Numbers of labeled cells in locations providing input to ErbB4 interneurons in S1 cortex.

Animal ID	Injection site (AP, ML, DV) (in mm)	Pyramids in S1	S2	M1	RS ctx	PRh ctx	CL S1	PMnR	NB	VP	Po	VA-VL
1	-1.2,2.0,0.7-0.3	>20	2	4	1	0	4	1	1	15	1	4
2	-1.0,2.3,0.7-0.4	>20	5	4	3	0	0	0	4	2	2	1
3	-0.8,2.0,0.7-0.4	>20	9	5	2	0	2	1	3	11	0	3
4	-1.5,2.5,0.7-0.4	>20	7	5	8	1	0	0	2	8	0	0
5	-1.3,2.5, 0.7-0.4	>20	3	na	7	0	2	0	0	7	4	0

Data are arranged according to the identifying number for each animal. The coordinates of each injection site relative to bregma are indicated. Remaining columns indicate numbers of mCh labeled neurons in each structure.

Abbreviations: S1 – primary somatosensory cortex; S2 – secondary somatosensory cortex; M1 – primary motor cortex; RS ctx – retrosplenial cortex; PRh ctx – perirhinal cortex; CL ctx – contralateral S1 cortex; PMnR-Paramedian Raphe; NB – nucleus basalis of Meynert; VP – ventral posterior thalamic nucleus; Po – posterior thalamic nucleus; VA-VL – ventral anterior/ventral lateral thalamic complex. na – no data were available for this structure in this animal due to inadvertent tissue damage.