A conditional transgenic reporter of presynaptic terminals reveals novel features of the mouse corticospinal tract

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INTRODUCTION
Numerous murine models of neurodegenerative diseases exist and, in many of them, synaptic alterations predate the demise of the neuronal cell body and can be used as a predictor of disease onset and progression (reviewed in Conforti et al., 2004). In amyotrophic lateral sclerosis (ALS), synaptic alterations precede the demise of the neuronal cell, making synapses a useful vantage point from which to monitor the onset and progression of clinical signs and pathological changes. While murine models of ALS display many features in common with the clinical picture observed in patients, corticospinal tract (CST) involvement is usually less severe in mice than the picture observed in humans. In this paper we describe the characterization of a new conditional transgenic line obtained by targeted integration of a GFP-VAMP2 fusion gene into the Rosa26 locus, and devised to permit the detection of genetically defined presynaptic terminals in wild type mice and murine models of neural disorders. This reporter molecule is selectively enriched in presynaptic boutons, significantly reducing the background signal produced by fibers of passage. The specific features of this reporter line allow us to strongly support the view that murine CST terminals give rise to very few direct contacts with spinal motor neurons. Moreover, the evidence described here reveals the existence of previously uncharacterized, putative direct connections between CST presynaptic boutons and Renshaw neurons in the spinal cord. These results constitute a proof of concept for the potential application of this indicator line to morphological analyses of wild type and diseased synapses.

In many neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), synaptic alterations precede the demise of the neuronal cell, making synapses a useful vantage point from which to monitor the onset and progression of clinical signs and pathological changes. While murine models of ALS display many features in common with the clinical picture observed in patients, corticospinal tract (CST) involvement is usually less severe in mice than the picture observed in humans. In this paper we describe the characterization of a new conditional transgenic line obtained by targeted integration of a GFP-VAMP2 fusion gene into the Rosa26 locus, and devised to permit the selective characterization of a new conditional transgenic line obtained by targeted integration of a GFP-VAMP2 fusion gene into the Rosa26 locus, and devised to permit the detection of genetically defined presynaptic terminals in wild type mice and murine models of neural disorders. This reporter molecule is selectively enriched in presynaptic boutons, significantly reducing the background signal produced by fibers of passage. The specific features of this reporter line allow us to strongly support the view that murine CST terminals give rise to very few direct contacts with spinal motor neurons. Moreover, the evidence described here reveals the existence of previously uncharacterized, putative direct connections between CST presynaptic boutons and Renshaw neurons in the spinal cord. These results constitute a proof of concept for the potential application of this indicator line to morphological analyses of wild type and diseased synapses.

Keywords: amyotrophic lateral sclerosis, synapses, transgenic mice, GFP labeled, presynaptic terminals, reporter mouse, neurodegenerative, axons.
In addition, we validate our murine model to study the distribution and connections of corticospinal tract (CST) terminations in the spinal cord, an application relevant to the analysis of murine models of motor neuron diseases.

MATERIALS AND METHODS

GENERATION OF THE Rosa26EGFP−VAMP2 MOUSE STRAIN

The DNA fragment coding for EGF-PVAMP2 was first inserted into a plasmid for homologous recombination downstream of a flxed translation/transcription STOP cassette, using standard cloning techniques. From the 5′ to the 3′ the plasmid encompassed a 5′ homology arm for Rosa26, the CAG promoter/enhancer (CMV enhancer + β-actin promoter), the STOP cassette flanked by loxP sites, the EGF-PVAMP2 fusion protein, the bovine growth hormone polyadenylation site (bGH pa) and a 3′ homology arm for Rosa26 (Figure 1). A minigene for 5′Neo (neomycin resistance) (NeoR) was also inserted within the two loxP sequences, while a suicide gene for negative selection (diphtheria toxin gene) was introduced past the 3′ homology arm. The plasmid was electroporated into murine ES cells, which were cultured in the presence of neomycin. Surviving clones were genotyped by Southern blotting; briefly, after a complete digestion with EcoRV, genomic DNA from these clones was electrophoresed on agarose gel and blotted on a membrane, which was eventually hybridized with two distinct radioactive probes, annealing upstream or downstream of agouti progeny were characterized genetically for germline transmission.

Tissue Lysates and Western Blotting

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models of human CNS disorders, with a low axonal background. Tissue lysates were prepared to detect EGFP PVAMP2 fusion protein. Briefly, slides were treated with 0.3% H2O2 in TN buffer for 1 h, blocked/permeabilized for 1 h in TN buffer (0.1% Triton X-100 in PBS 1X), and transcardially perfused with 4% paraformaldehyde (PFA) in PBS 1X. Brains and spinal cords were post-fixed o/n at 4°C in PFA. Tissues were sunk at 4°C in 30% sucrose. Finally, they were embedded within OCT (BiOptica) and sectioned on a cryostate (16 μm for immunofluorescence and 20 μm for immunohistochemistry).

Tissue Processing for Immunodetection

Adult mice were anesthetized with Avertin (0.2 ml/10 g body weight), and transcardially perfused with 4% paraformaldehyde (PFA) in PBS 1X. Brains and spinal cords were post-fixed o/n at 4°C in PFA. Tissues were sunk at 4°C in 30% sucrose. Finally, they were embedded within OCT (BiOptica) and sectioned on a cryostate (16 μm for immunofluorescence and 20 μm for immunohistochemistry).

ImmunoFluorescence

Slides were washed in PBS and blocked/permeabilized in blocking solution (10% Goat Serum, 0.3% Triton X-100 in PBS 1X) for 1 h, then incubated overnight at 4°C with the proper primary antibody: rabbit α-GFP (Invitrogen) 1:500, mouse α-NeuN (Chemicon) 1:300, mouse α-CaBP (Swant) 1:1000 in blocking solution. After several washes, sections were treated for 2 h with secondary antibodies (Goat α-rabbit Molecular Probes Alexa Fluor 488, Goat α-mouse Alexa Fluor 546) 1:1000 in blocking solution. Nuclei were counterstained with Hoechst 1:5000 in PBS for 5 min. To perform immunofluorescence on Rosa26EGFP−VAMP2 slides, we used a Tyramide Signal Amplification (TSA) Kit (Perkin Elmer) to detect EGFP-PVAMP2 fusion protein. Briefly, slides were treated with 0.3% H2O2 in TN buffer for 1 h, blocked/permeabilized for 1 h in TN buffer (0.1% Triton X-100 in TN buffer) then incubated overnight at 4°C with the rabbit α-GFP primary antibody 1:4000 in TNB. Subsequently, slides were washed in TTN buffer (0.1% Tween20 in TN buffer), incubated for 1 h with a biotin-conjugated goat α-rabbit secondary antibody (VectorStain) 1:200 in TNB, washed again, treated for 30 min with HRP-streptavidin (supplied with the kit; 1:150 in TNB) and processed with the tyramide solution for 10 min. Other markers on the same sections were revealed using protocols for ordinary immunohistochemistry, as explained above. We used mouse α-synaptotagmin1 (1:500, Synaptic Systems), mouse α-CalB (1:300 (Swant) and goat α-Choline Acetyltransferase (ChAT) 1:50 (Choline O-acetyltransferase, Millipore) to label presynaptic terminals, Renshaw cells, and motor neurons, respectively. Rabbit α-VGlut2 (1:1000 (Vesicular Glutamate Transporter 2, Synaptic Systems) was also used to label cerebellar mossy fibers terminals. After several washes, sections were treated for 2 h with fluoresceinated secondary antibodies 1:1000 in blocking solution. Nuclei were counterstained with Hoechst 1:5000 in PBS for 5 min.

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FIGURE 1 | Generation of the Rosa26 EGFP-VAMP2 line. (A) Schematic representation of the wt Rosa26 locus, recombinant (rec.) construct and rec. locus obtained by homologous recombination within the 5′ and 3′ homology arms. See Section “Results” for a description of the rec. construct. CAG, chicken β-actin promoter and CMV enhancer; bGH, bovine growth hormone. (B) Southern analysis of ES cell clones using the 5′ probe sketched in [(A)]. Lanes marked by asterisks (*) correspond to recombinant clones (note 9.8 kb band). (C) Western blot immunostained with an anti-GFP Ab. Adult forebrain (Br) and cerebellar (Cbl) lysates were gel-separated as indicated. Lanes 1–4 contain lysates from Rosa26EGFP−/−VAMP2+/+ mice, revealing a 49 kDa band corresponding to the size of the EGFP-VAMP2 fusion protein; lane 5 contains lysates from Rosa26YFP+/+ mice (27 kDa). Lanes 1 and 4 are from Syn1-Cre negative mice; Lanes 2, 3, and 5 are from Syn1-Cre positive mice.

IMMUNOPEROXIDASE STAINING – DAB AMPLIFICATION

Emx1-Cre+ Rosa26EGFP−/−VAMP2+/+ mouse brain sections were treated with 0.3% H2O2 in PBS for 30 min, permeabilized in P-solution (1.72 M sucrose, 50 mM NaCl, 3 mM MgCl2, 20 mM HEPES, 0.3% Triton X-100) for 10 min, blocked 1 h in a goat serum-based blocking solution then incubated overnight at 4°C with the rabbit α-GFP primary antibody 1:500 in blocking solution. Subsequently, slides were treated with a biotin-conjugated goat α-rabbit secondary antibody 1:200 in blocking solution for 2 h. Several washes followed, then a streptavidin-HRP containing solution (ABC solution, Vectastain) for 30 min. The chromogenic solution contained 0.3 mg/ml diaminobenzidine (DAB, Sigma), 0.03% H2O2 in PBS. The reaction was blocked with 0.001% NaN3 in PBS. Nuclei were counterstained as described above.

NISSL STAINING

Slides were washed in PBS, dehydrated with a rising ethanol scale (50% for 3 min, 70% for 3 min, 95% for 3 min, pure EtOH for 1 min), submerged in an ethanol/chloroform (1:1) solution for 20 min, rehydrated with a descending ethanol scale (95% for 5 min, 70% for 10 min, 50% for 10 min, pure ddH2O for 5 min), stained with cresyl violet acetate for 15 min, post-fixed with 4% cold PFA for 15 min, dehydrated again and treated with a de-differentiation solution (two drops of glacial acetic acid in 100 mL of 95% EtOH). Finally, they were rinsed in xylene and mounted with a xylene mounting gel.

IMAGE ACQUISITION AND PROCESSING

Slides were examined on a Leica Confocal (20x-40x-63x) and a Zeiss Axioplan 2 (5x, 10x, 20x) epifluorescence microscope. In some cases, images were further magnified digitally. Minor adjustments in term of contrast or brightness were made with Adobe Photoshop CS4 version 11.0.2. This program was also used for merging and collages. Spinal cord stacks were processed using the ImageJ software (NIH).

RESULTS

GENERATION OF Rosa26EGFP−/−VAMP2 KNOCK-IN MICE

We have developed a new Cre-inducible presynaptic reporter consisting of the presynaptic protein VAMP2 fused N-terminally to EGFP.

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The goal of this project was to produce an indicator/reporter line expressing a fusion protein targeted to the secretory vesicle wall, and to achieve a moderate expression level so as to avoid overexpression artifacts that are common with chimeric proteins, often due to defective subcellular trafficking of unfolded proteins.

The chimeric molecule was constructed as follows: starting from a 2 kb rat VAMP2 cDNA (Ellerbeck et al., 1989), the CDS was modified in order to generate an N-term fusion to EGFP and a C-term fusion to a spacer peptide derived from CDS was modified in order to generate an N-term fusion to MYC tag. The resulting doubly fused CDS was contained within a 2.7 kb fragment comprising 1.5 Kb of 3'UTR source but not at the termination of this tract, we utilized the Cre-dependent fluorescent reporter of presynaptic terminals

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ANATOMICAL CHARACTERIZATION OF CORTICOSPINAL TRACT TERMINATIONS IN Emx1-Cre+/− Rosa26YFP−/−; Rosa26VAMP2−/−; Syn1-Cre−/−; VAMP2+/− line (Zhu et al., 2001). In these mice, Cre recombinase is expressed only in a subset of synapsin 1+ neurons, possibly due to a positional effect or to the short stretch of promoter used to generate this transgene (Hoose et al., 1993). Syn1-Cre+/− mice were mated with Rosa26VAMP2+ animals (Srinivas et al., 2001) in order to label Cre positive cells. Many basal pontine neurons (Neun+ cells) were positive for YFP (Figures A1A,B). In the cerebellar cortex, the reporter decorated basket cells, stellate cells, and some calbinbin (CalB+) + PCs (Figure A1C); conversely, CGs were uniformly negative. Even if scattered large YFP+ cells in the granule cell layer (GL) were detected, they were invariably negative for NeuN (Figure A1D), which exclusively decorates GCs in the murine cerebellar cortex (Weyer and Schilling, 2003). Thus, they probably represent internal granule layer (IGL) GABA interneurons (likely Golgi cells).

Syn1-Cre−/− mice were then mated with Rosa26VAMP2+; YFP+/+ mice to analyze the spatial distribution of the reporter in sagittal cerebellar sections. We observed a clear signal in the GC layer of lateral hemispheres; this signal did not colocalize with the GC marker NeuN (Figure 2E, lobule IX). Interestingly, in the anterior lobe, which does not receive projections from the pons, only a weak, sparse signal was visible (Figures 2C,D, lobules I and II). Finally, adult cerebellar sections were stained for EGFP and for the vesicular glutamate transporter VGlut2. In postnatal and adult cerebellum, VGlut2 decorates mossy fiber terminals, climbing fiber terminals and a subgroup of unipolar brush cell terminals; as expected, EGFP-VAMP2 and VGlut2 colocalized tightly with presynaptic terminals located in the GC layer (Figures 3A–F). Higher magnifications of a single synaptic structure also confirmed the “rosette-like” shape and the expected size (arrowhead in Figure 3H). Note that the axonal stem and its collaterals (diameter ~1 μm, Wu et al., 1999) were also weakly labeled (arrow in Figure 3H). These results clearly indicate that the reporter is correctly produced and transported from pontine cell somata to their presynaptic terminal in the cerebellum of Syn1-Cre+/− Rosa26VAMP2−/− mice.

IN Syn1-Cre+/− Rosa26VAMP2−/−; Rosa26YFP−/−; Rosa26EGFP−/−; Syn1-Cre−/−; VAMP2+/− mice, the recombinase is expressed exclusively in glutamatergic neurons and glial cells of the cerebral hemispheres (Gorski et al., 2002), at the origin of the CPT and CST tracks, but not in their target territories, i.e., the brainstem and spinal cord. First, we reproduced published results by breeding Emx1-Cre into the Rosa26YFP+/− background, to confirm Cre localization in the deep layers of the dorsal pallium, and particularly in the primary motor cortex (M1). As expected, we found a large percentage of YFP+ neurons (Figure A3, YFP+; NeuN+ cells).

TERRITORIES POSITIVE FOR EGFP-VAMP2 IN Emx1-Cre+/− Rosa26VAMP2−/−; Rosa26YFP−/−; Rosa26EGFP−/−; Syn1-Cre−/− mice were also analyzed by immunohistochemistry on brainstem and spinal cord sections. At the origin of the pyramidal tract, in the cerebral hemispheres, EGFP decorates cell-poor, fiber rich regions (Figure 2A). In the medulla oblongata (Figure 2B, inset magnified in C), where all cells are...
Cell bodies, stains axons weakly, and decorates axon terminals effectively synapse on mouse motor neurons, suggesting more strongly, as expected of a properly sorted synaptic vesicle marker.

**MOST EGFP-VAMP2+ CST TERMINALS DECORATE DORSAL AND INTERMEDIATE LAMINAE IN THE SPINAL CORD GRA Hammus**

Next, we focused on the analysis of CST terminals. In the spinal cord of *Emx1-Cre*+/+ *Rosa26EGFP+/+* mice, immunoreactivity for the fusion protein spanned cervical (Figure 5A) through sacral (Figure 5B) segments. More precisely, we found strongly labeled axons primarily in the funiculi of the dorsal column, where mouse CST axons descend after decussating in the medulla (Steward et al., 2004; Barreyn et al., 2005 and others). Obviously, the more caudal the segment, the less signal we detected in the white matter, due to the progressive depletion of corticospinal axons. However, the most remarkable feature was the punctate pattern clearly visible in the gray matter, matching the distribution of corticospinal synaptic terminals. To further characterize where corticospinal axons terminate, we analyzed the cytoarchitecture of a lumbar section immediately adjacent to the one stained by immunofluorescence, and based our analysis on segment-specific Rexed lamination (Figure 5C). The strongest signal was confined to the dorsal horn and to the zona intermedia, mainly in laminae III–VI, with only sparse immunoreactive puncta in laminae VII–IX. These data are in close agreement with published results obtained using anterograde tracing (Kuypers and Martin, 1982; Holstege, 1996; Steward et al., 2004). Again, almost all EGFP+ puncta residing in the gray matter colocalized with syt-1 (Figures 5D–F).

**EVIDENCE SUGGESTING THE EXISTENCE OF LOW-FREQUENCY MONOSYNAPTIC CORTICAL CONNECTIONS ON MOTOR NEURONS AND ON RENSHAW CELLS IN MICE**

Next, we searched for evidence of direct connections between corticospinal fibers and spinal motor neurons of the ventral horns. We performed double immunofluorescence on cervical spinal cord sections of *Emx1-Cre*+/+ *Rosa26EGFP+/+* (*VAMP−/−*) mice immunostaining sections for EGFP and ChAT, a well-established marker of cholinergic motor neuron of lamina IX. Only infrequent, scattered EGFP+/+ puncta were observed on or flanking ChAT immunoreactive motor neurons, and they localized mainly to proximal dendrites (Figure 6).

In addition, transverse spinal sections from the same Tg line were immunostained for Calbindin-D28k (CaBP), a marker of Renshaw cells, which are inhibitory interneurons residing in lamina VII of the spinal cord (Carr et al., 1998; Alvarez and Fyffe, 2007). The neuronal identity of Renshaw cells was established also based on morphology, position and size (Figure 7). Termination-like EGFP+ puncta were observed on Renshaw cell somata, or adjacent to presumptive dendrites, suggesting the previously unreported existence of likely monosynaptic connections between these glycinergic neurons and glutamatergic CST presynaptic boutons (Figure 7). In keeping with this observation, Renshaw cells express abundant AMPA receptors (Glur2 and Glur4), suggesting that they receive glutamatergic presynaptic terminals (Alvarez and Fyffe, 2007).

From this analysis, we conclude that very few corticospinal terminals effectively synapse on mouse motor neurons, suggesting
FIGURE 3 | EGFP decorates presynaptic terminals in the internal granule layer of Syn1-Cre Rosa26EGFP-VAMP2/+ mice. Sagittal sections of an adult cerebellum, immunostained for EGFP (B,E,H) and counterstained for the glutamatergic axon terminal VGluT2 (A,D,G), which, in the IGL, labels mossy fiber presynaptic terminals. Overlay (ovl) in (C,F,I). In high magnifications (G–I) note EGFP+ terminal (arrowhead in H) and faintly labeled distal axon (arrow in H). Scale bar: (A–C) 75 μm; (D–F) 25 μm; (G–I) 10 μm.

that the CST plays a minor role in the control of fine movements in this species. Intriguingly, we also provide preliminary evidence of a possible direct connection between Emx1+ corticospinal neurons and Renshaw cells in lamina VII, which could have implications relevant to the study of normal and diseased spinal cord circuits.

DISCUSSION

THE ROSA26EGFP-VAMP2 LINE PERMITS SELECTIVE VISUALIZATION OF PRESUMPTIVE AXON TERMINALS

In the present paper we describe a new Rosa26 knock-in line carrying an EGFP-based Cre-inducible presynaptic reporter. We analyzed the pattern of EGFP-immunoreactivity in three different circuits (pontocerebellar, CPT and CST) and demonstrated in all cases a highly selective localization of the reporter molecule in the putative presynaptic terminal, while axons were only detectable in the context of densely fasciculated white matter tracts such as the dorsal funiculus. While EGFP-VAMP2 did not decorate cell bodies of Cre-positive cells in the brain cortex (Figure A2A) and labeled axons weakly (Figures A2B,C), it was spatially restricted and significantly enriched in axonal terminals (Figures 2 and 3); moreover, it colocalized with different presynaptic markers and labeled likely presynaptic puncta with the expected size and shape (Figures 3,4 and 5). Notably, all
FIGURE 4 | EGFP decorates presynaptic terminals in the pons of Emx1-Cre Rosa26EGFP-VAMP2/+ mice. Sagittal sections of the pontine nuclei, immunostained for EGFP (B) and counterstained for the synaptic vesicle protein synaptotagmin1 (Syt1) (A). Overlay in (C). (D–F) are magnifications of the corresponding insets in (C). Double-positive puncta (yellow, ovl) represent corticopontine axon terminals. Scale bar: (A–C) 75 μm; (D–F) 15 μm.

FIGURE 5 | EGFP decorates presynaptic terminals in the spinal cord of Emx1-Cre Rosa26 EGFP-VAMP2/+ mice and colocalizes with synaptotagmin 1 positive puncta. Transverse sections of the spinal cord at cervical (A), sacral (B), and lumbar (C) levels. In (A), strong signal is seen in the dorsal white matter (arrows; see text for discussion). In (C), a Nissl-stained lumbar cord hemisection is juxtaposed to its adjacent section, stained for EGFP. Stippled lines delimit Rexed laminae (I–IX). Note that the majority of corticospinal terminals are located in dorsal and intermediate laminae, while few occupy lamina IX, containing the bodies of spinal motoneurons. (D–F) Transverse sections of the spinal cord gray matter at the lumbar level. EGFP signal from corticospinal axon terminals in (B) colocalizes with synaptotagmin 1 (Syt1) in (A). Overlay (ovl) in (C) (arrows). Scale bar for (D–F) 25 μm.
**FIGURE 6** | Infrequent EGFP+ puncta in the proximity of lamina IX motor neuron somata. Transverse sections of the spinal cord gray matter at the cervical level, lamina IX. (A,D) Choline acetyltransferase (ChAT) immunostaining. (B,E) EGFP immunostaining; (C,F) overlay. Very few corticospinal terminations are found on motor neuron cell bodies, while sparse EGFP+ puncta are detected on ChAT+ presumptive dendrites (arrows). Note larger size of some EGFP+ dots likely due to tyramide mediated signal amplification. Scale bar: (A–C) 75 μm; (D–F) 30 μm.

**FIGURE 7** | EGFP+ presynaptic terminals are detectable on calbindin-IR neurons in lamina VII. Transverse sections of the spinal cord gray matter at the cervical level, lamina VII. EGFP+ axon terminals (arrows in B,E) are detected on the soma and dendrites of calbindin (CaBP) immunoreactive cell bodies (A,D) likely corresponding to Renshaw neurons. Overlay in (C,F). This finding suggests the previously unreported existence of CS afferents on these interneurons. Scale bar: (A–C) 75 μm; (D–F) 45 μm.
the targets of these tracts were globally Cre negative (with the exception of the few Syt-Cre+ GABAergic interneurons in the GL of the cerebellar cortex, (Figure A1). Significantly, reporter targeting was highly efficient even in very long range axonal tracts, such as the CST (Figures 5–7), whose length in the mouse exceeds the diameter of pyramidal neuron bodies by three orders of magnitude.

**EVIDENCE OF CORTICOSPINAL TERMINATIONS ON SPINAL MOTORNEURONS**

The Rosa26<sup>VAMP2</sup> line was used in this work to tackle a controversial issue: whether or not CS terminals establish direct connections with spinal motor neurons in the mouse (Figure 6). To date, neuroanatomists disagree in regard to the contribution of the corticospinal pathway on motor function in species other than primates. The CST is a composite, species-specific pathway; it has several functions and originates from a variety of cortical areas, including classical cortical motor areas (as the primary motor cortex, the premotor cortex, the supplementary motor area), the anterior cingulate cortex (Dum and Strick, 1996) and even sensorimotor areas, as the somatosensory cortex, the parietal operculum and the posterior parietal cortex (Kuypers and Martin, 1982). Fibers coming from each area will eventually synapse in the spinal cord in a discrete fashion: sensorimotor inputs preferentially terminate in the dorsal horn, while motor fibers ultimately synapse in the zona intermedia and, to a lesser extent, in the ventral horn (Dum and Strick, 1996; Lemon and Griffiths, 2005; Lemon, 2008). Ninety percent of the murine pyramidal tract is made up of sensorimotor projections, which terminate in lamina II–V (Steward et al., 2004; Barye et al., 2005; Lemon and Griffiths, 2005; Lemon, 2008); accordingly, our results show an accumulation of CST terminations in the spinal dorsal horn (Figure 5C).

Early observations made in rats by neuroanatomical tracing using low resolution first generation methods (Casale et al., 1988) have led neuroanatomists to the conclusion that CS terminals are fairly abundant in laminae III – VI, and sparse in lamina II, while no direct monosynaptic connections were reported in lamina IX. Likewise, attempts to elicit sustained excitation post-synaptic potentials (EPSP) in motor neurons through electrical stimulation of the CST have been unsuccessful (Aberdam et al., 2004). Other neuroanatomical studies have also led to the conclusion that the CST only plays a minor role in the initiation of limb movement (Lemon, 2008). At odds with these conclusions, corticospinal terminations have been detected by other authors on rat (Barye et al., 2002), mouse (Barye et al., 2005) and hamster (Kuang and Kalil, 1990) motor neurons. Contradictory results have emerged from electron microscopy studies as well (Carsi et al., 1996; Yang and Lemon, 2003). Our genetic evidence adds to the results of previous investigations, hopefully contributing to the establishment of a consensus in regard to this highly debated topic.

Our evidence speaks for the existence of only a small number of direct corticospinal connections on the surface of spinal motor neurons. In comparison to previously used genetic reporters, the Rosa26<sup>VAMP2</sup> line was used in this work to tackle a controversial issue: whether or not CS terminals establish direct connections with spinal motor neurons in the mouse (Figure 6). To date, neuroanatomists disagree in regard to the contribution of the corticospinal pathway on motor function in species other than primates. The CST is a composite, species-specific pathway; it has several functions and originates from a variety of cortical areas, including classical cortical motor areas (as the primary motor cortex, the premotor cortex, the supplementary motor area), the anterior cingulate cortex (Dum and Strick, 1996) and even sensorimotor areas, as the somatosensory cortex, the parietal operculum and the posterior parietal cortex (Kuypers and Martin, 1982). Fibers coming from each area will eventually synapse in the spinal cord in a discrete fashion: sensorimotor inputs preferentially terminate in the dorsal horn, while motor fibers ultimately synapse in the zona intermedia and, to a lesser extent, in the ventral horn (Dum and Strick, 1996; Lemon and Griffiths, 2005; Lemon, 2008); accordingly, our results show an accumulation of CST terminations in the spinal dorsal horn (Figure 5C).

Taken together, our results are consistent with the notion that while scattered monosynaptic connections exist in mice, most functional motor tasks are controlled through polysynaptic pathways in this species. It is now widely accepted that synaptic contacts and alterations may influence at least in part the progression of neurodegenerative diseases (reviewed in Conforti et al., 2007). ALS primarily involves the upstream and downstream halves of a monosynaptic circuit, including the pyramidal neuron of cortical layer 5 and the spinal motor neuron. If such a circuit is indeed less crucial in mice than in humans, then the results stemming from murine models of ALS should be interpreted cautiously, especially as regards CST involvement in this disease.

**EVIDENCE FOR THE EXISTENCE OF LIKELY DIRECT CONNECTIONS BETWEEN THE PYRAMIDAL TRACT AND RENSHAW CELLS**

The Rosa26<sup>VAMP2</sup> mouse strain has also made it possible to visualize putative direct connections from the cortex to Renshaw cells (Figure 7), glycineric neurons that receive antidromic signals from spinal motor neurons. To the best of our knowledge, this represents an entirely novel finding. Intriguingly, Emx1 is expressed by glutamatergic neurons, suggesting that a subset of CS fibers carries excitatory stimuli to Renshaw cells. It is well established that Renshaw cell axons make local arborizations but also extend to other ipsilateral spinal segments (Alvarez and Pynte, 2007). Thus, through a direct corticospinal connection with Renshaw cells, a pool of spinal motor neurons may be subjected to an inhibitory, inter-segmental control. If this were the case, it may have important implications for motor control in physiology and disease. Further studies are required to gauge the pathophysiological relevance of these observations.

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**REFERENCES**

Aberdam, B., Ogawa, J., and Lac, T. (2004). Lack of monosynaptic corticospinal neuronal EPSPs in mice: a new method to study indirect corticospinal connections in the adult spinal cord. J. Neurophysiol. 91, 1822–1830. doi: 10.1152/jn.00820.2003

Alvarez, F. J., and Fyffe, R. E. (2007). The continuing case for the Renshaw cell. Front. Neuroanat. 1, 1–15. doi: 10.3389/neuro.13.001.2007

Bareyre, F. M., Haudenschild, B., and Schwab, M. E. (2002). Long-lasting sprouting of corticospinal axons in spinal cord injury. Nat. Med. 22, 7097–7110. doi: 10.1038/nm1279

Bareyre, F. M., Korschentner, M., Mangel, T., and Samson, J. R. (2005). Transgenic labeling of the corticospinal tract for monitoring axonal responses to spinal cord injury. Nat. Med. 11, 1375–1380. doi: 10.1038/nm1279

Braida, L. D., Leinwand, T. R., Likelihood, S., Lisch, E., Monroe, I. A., Kjode, J. O., et al. (2005). Database and tools for analysis of topographic organization and map transformations in major projection systems of the brain. Neuroimage 26, 681–695. doi: 10.1016/j.neuroimage.2003.06.036
Braun, M. J., Rico, B., and Raab-Berio, A. I. (2002). Transneuronal tracing of diverse CNS circuits by Cre-mediated induction of wheat germ agglutinin in transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 99, 15148–15153. doi: 10.1073/pnas.222540999

Carr, P. A., Abraham, F. J., Lemus, E. A., and Prifti, B. E. (1998). Calbindin D28k expression in immunohistochemically identified Ramon cells. Neuroreport 9, 2057–2061. doi: 10.1097/00001756-199808030-00045

Casale, E. J., Light, A. R., and Rustioni, A. (1988). Direct projection of the corticospinal tract to the superficial laminae of the spinal cord in the rat. J. Comp. Neurol. 278, 275–286. doi: 10.1002/cne.902780210

Cost forti, L., Adhab, R., and Coleman, M. P. (2007). Neuronal death: where does the end begin? Trends Neurosci. 30, 159–166. doi: 10.1016/j.tins.2007.02.004

Cuthbert, M. H., Grobas, A. A., and Volkeren, P. F. (1996). Direct cortico-motorneuronal synaptic contacts are present in the adult rat spinal cervical cord and are first established at postnatal day 7. Neurons. Lett. 205, 123–126. doi: 10.1016/0304-3908(96)01276-X

Dudgeon, R. K., and Lemus, K. B. (2011). Neurotransmitter nuclear and cytoplasmic inositol differentially regulate alternative splicing and neuron-specific decay of Bdnf. PLoS ONE 6:e23185. doi: 10.1371/journal.pone.0023185

Dunn, R. E., and Strick, P. L. (1996). Sponal cord terminations of the medial wall motor area in macaque monkeys. J. Neurosci. 16, 6513–6525

Efronin, I. A., Tramblé, W. S., and Schneider, B. H. (1989). Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. J. Biol. Chem. 264, 11061–11064

Fischer, L. R., Culver, D. G., Tennant, P., Davis, A. A., Wang, M., Castellano-Sanchez, C. J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L., and Jones, K. R. (2002). Patterning and immunodetection. Curr. Protoc. Mol. Biol. Chapter 10. Unit 10. 8. doi: 10.1002/0471654024.mbb100846

Geroldi, C., Talley, T., Qin, M., Paletto, L., Babitt, A. J., and Jones, K. R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. J. Neurosci. 22, 6109–6124. doi: 10.1523/JNEUROSCI.22-19-061092002

Getz, E., Hao, J. C., Bennett, M. K., and Kelly, R. B. (1995). A targeting signal in VAMP regulating transport to synaptic vesicles. Cell 81, 583–593.

Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L., and Jones, K. R. (2002). Patterning and immunodetection. Curr. Protoc. Mol. Biol. Chapter 10. Unit 10. 8. doi: 10.1002/0471654024.mbb100846

Kuang, R. Z., and Kalil, K. (1990). Branching patterns of corticospinal axon arbors in the rodent. J. Comp. Neurol. 290, 1–9. doi: 10.1002/cne.902900101

Lemon, R. N. (2008). Descending pathways in motor control. Annu. Rev. Neurosci. 31, 50–72. doi: 10.1146/annurev.neuro.31.060607.093943

Leto, K., Carletti, B., Williams, I. M., Magrassi, L., and Ross, F. (2006). Different types of cortical GABAergic interneurons originate from a common pool of multipotent progenitor cells. J. Neurosci. 26, 11662–11664. doi: 10.1523/JNEUROSCI.1006-06.2006

Lo, L., and Andersen, P. J. (2011). A Cre-dependent, anterograde transneuronal viral tracer for mapping output pathways of genetically marked neurons. Neuron 72, 926–939. doi: 10.1016/j.neuron.2011.12.002

Mao, X., Fujimura, T., Chapdelaine, A., Yang, H., and Orkin, S. H. (2001). Activation of EGF expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. Blood 97, 52–58. doi: 10.1182/blood.V97.1.52

Rutten, A., Vrooman, C. L., Medina, L., Jiao, Y., Del Maç, N., and Honig, M. G. (2000). Pathway tracing using histone-derived alanine amino. J. Neurosci. Methods 103, 23–37. doi: 10.1016/S0165-0270(00)00293-9

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71. doi: 10.1038/9707

Steenstra, S., Varambula, T., Lin, C. S., William, C. M., Tanabe, J., Yoshii, T. M., et al. (2001). Cre reporter strains produced by targeted insertion of EFFP and ECFP into the ROSA26 locus. J. Exp. Biol. 1–4. doi: 10.1104/10.1117/2115-1-4

Stevens, O., Zhang, H., Hu, C., Anderson, K., and Tosner-Larson, M. (2004). The dorsolateral corticospinal tract in mice: an alternative route for corticospinal input to caudal segments following dural column lesions. J. Comp. Neurol. 472, 411–417. doi: 10.1002/jnr.20090

Weyer, A., and Schilling, K. (2003). Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. J. Neurosci. 23, 480–489. doi: 10.1523/JNEUROSCI.0765-00

Wu, H. S., Suguira, I., and Minoda, Y. (1999). Projection patterns of single motor neurons originating from the lateral vestibular nucleus in the rat cerebellar cortex and nuclei. J. Comp. Neurol. 411, 97–118. doi: 10.1002/(SICI)1096-9861(19990816)411:1<97::AID-CNE8>3.0.CO;2-O

Yang, H. W., and Lemon, R. N. (2003). An electron microscopic examination of the corticospinal projection to the cervical spinal cord in the rat: lack of evidence for cortico-motorneuronal synapses. Exp. Brain Res. 149, 489–496. doi: 10.1007/s00221-003-1195-9

Zaherovsky, J., Winterfeld, F., and Lacinova, I. (2006). Neuroanatomical Tracing 3 Singapore: Springer. doi: 10.1007/3-87294-292-9

Zhou, Y., Rimmer, M. I., Glick, P., Su, Z., Charney, P., Rushing, E. J., et al. (2001). Ablation of NSF function in neurons induces abnormal development of central cortical and reactive glial in the brain. Genes Dev. 15, 858–876. doi: 10.1101/gad.862101

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APPENDIX

**FIGURE A1 |** Syn1-Cre is expressed in pontine nuclei but not in cerebellar granule cells. (A) In Syn1-Cre Rosa26-YFP mice, YFP immunoreactivity is detected in pontine nuclei (inset magnification in B). In the cerebellum, YFP immunoreactivity (arrows in C) is detected in calbindin (+) Purkinje cells (arrows in D). Large YFP (+) cell bodies in the granule cell layer (arrow in D) are NeuN negative, likely representing Golgi interneurons. CaBP, calbindin; ovl, overlay; ML, molecular layer; PL, Purkinje cell layer; GL, granule cell layer. Scale bar: (A) 150 μm; (B) 70 μm; (C, D) 75 μm.
FIGURE A2 | At low resolution, EGFP-VAMP2 (Mausoloe) signal is detectable in fiber-rich, cell-body-poor territories. (A) Left: LacZ staining of a frontal adult Emx1-Cre Rosa26LacZ-/+ mouse forebrain hemisection reveals location of cell bodies (reproduced with permission from Gorski et al., 2002); right: similar section from an Emx1-Cre Rosa26EGFP-VAMP2-/+ brain immunostained for EGFP reveals complementary distribution of the reporter, which is enriched in cell-poor regions. (B) Likewise, in a medulla oblongata hemisection, EGFP immunoreactivity is concentrated at the level of the pyramids (inset magnification in (C)).

FIGURE A3 | Abundant YFP+ cells in the Emx1-Cre Rosa26YFP-/+ motor cortex. Sagittal section of the Emx1-Cre Rosa26YFP-/+ motor cortex immunostained for YFP and the neuronal marker NeuN, which labels all cortical neurons. Note the presence of NeuN- YFP+ cells, likely corresponding to glia.