

**Research Article: New Research / Development**

**Axonal localization of integrins in the CNS is neuronal type and age dependent**

Axonal localization of Integrins

Melissa R. Andrews<sup>1</sup>, Sara Soleman<sup>2</sup>, Menghon Cheah<sup>2</sup>, David A. Tumbarello<sup>3</sup>, Matthew R. J. Mason<sup>4</sup>, Elizabeth Moloney<sup>4</sup>, Joost Verhaagen<sup>4</sup>, Jean-Charles Bensadoun<sup>5</sup>, Bernard Schneider<sup>5</sup>, Patrick Aebischer<sup>5</sup> and James W. Fawcett<sup>2</sup>

<sup>1</sup>*School of Medicine, University of St Andrews, Medical & Biological Sciences Bldg, North Haugh, St Andrews, KY16 9TF, UK*

<sup>2</sup>*Department of Clinical Neurosciences, John van Geest Centre for Brain Repair, University of Cambridge, Robinson Way, Cambridge, CB2 0PY, UK*

<sup>3</sup>*Biological Sciences, University of Southampton, Highfield Campus, Life Sciences Building 85, Southampton, SO17 1BJ, UK*

<sup>4</sup>*Department of Regeneration of Sensorimotor Systems, Netherlands Institute for Neuroscience, Institute of the Royal Netherlands Academy of Arts and Science Amsterdam, Netherlands; Centre for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, Amsterdam, Netherlands.*

<sup>5</sup>*Neurodegenerative Disease Laboratory, Brain Mind Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland*

DOI: 10.1523/ENEURO.0029-16.2016

Received: 15 February 2016

Revised: 27 June 2016

Accepted: 29 June 2016

Published: 7 July 2016

**Author Contributions:** MRA and JWF designed Research; MRA, MC, SS, DAT performed Research; MM, EM, JV, JCB, BS, PA contributed unpublished reagents and tools; MRA and JWF Analyzed data; MRA and JWF wrote manuscript.

**Funding:** International Foundation for Research In Paraplegia: P117. American Association of Anatomists; Bryon Riesch Paralysis Foundation; Christopher and Dana Reeve Foundation; Plasticise European Network (7th Framework programme); NIHR Cambridge Biomedical Research Centre;

James Fawcett is a paid consultant for Acorda Therapeutics.

**Correspondence should be addressed to** either James W. Fawcett, Brain Repair Centre, Robinson Way, Cambridge CB2 0PY, UK. Email: [jf108@cam.ac.uk](mailto:jf108@cam.ac.uk), tel: +441223331160, fax: +441223331174 or Melissa R. Andrews, School of Medicine, University of St Andrews, Medical & Biological Sciences Bldg, North Haugh, St Andrews, KY16 9TF, UK. Email: [mra5@st-andrews.ac.uk](mailto:mra5@st-andrews.ac.uk), tel: +441334463558, fax: +441334463393

**Cite as:** eNeuro 2016; 10.1523/ENEURO.0029-16.2016

**Alerts:** Sign up at [eneuro.org/alerts](http://eneuro.org/alerts) to receive customized email alerts when the fully formatted version of this article is published.

Accepted manuscripts are peer-reviewed but have not been through the copyediting, formatting, or proofreading process.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

# Axonal localization of integrins in the CNS is neuronal type and age dependent

**Abbreviated title:** Axonal localization of Integrins

Melissa R. Andrews<sup>1\*</sup>, Sara Soleman<sup>2</sup>, Menghon Cheah<sup>2</sup>, David A. Tumbarello<sup>3</sup>, Matthew R. J. Mason<sup>4</sup>, Elizabeth Moloney<sup>4</sup>, Joost Verhaagen<sup>4</sup>, Jean-Charles Bensadoun<sup>5</sup>, Bernard Schneider<sup>5</sup>, Patrick Aebischer<sup>5</sup>, James W. Fawcett<sup>2\*</sup>

## Addresses:

1. School of Medicine, University of St Andrews, Medical & Biological Sciences Bldg, North Haugh, St Andrews, KY16 9TF, UK
2. John van Geest Centre for Brain Repair, Department of Clinical Neurosciences, University of Cambridge, Robinson Way, Cambridge, CB2 0PY, UK
3. Biological Sciences, Life Sciences Building 85, University of Southampton, Highfield Campus, Southampton, SO17 1BJ, UK
4. Department of Regeneration of Sensorimotor Systems, Netherlands Institute for Neuroscience, Institute of the Royal Netherlands Academy of Arts and Science Amsterdam, Netherlands; Centre for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, Amsterdam, Netherlands.
5. Neurodegenerative Disease Laboratory, Brain Mind Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

## Address for correspondence: (\*co-corresponding authors)

James W. Fawcett, Brain Repair Centre, Robinson Way, Cambridge CB2 0PY, UK.  
Email: [jf108@cam.ac.uk](mailto:jf108@cam.ac.uk), tel: +441223331160, fax: +441223331174

Melissa R. Andrews, School of Medicine, University of St Andrews, Medical & Biological Sciences Bldg, North Haugh, St Andrews, KY16 9TF, UK  
Email: [mra5@st-andrews.ac.uk](mailto:mra5@st-andrews.ac.uk), tel: +441334463558, fax: +441334463393

**Key words:** Adeno-associated virus, axon, axon initial segment, dorsal root ganglia, integrin, lentivirus, retinal ganglion cell, sensorimotor cortex

**Conflict of interest:** James Fawcett is a paid consultant for Acorda Therapeutics.

Number of pages	31
Number of illustrations	7

44	Words in Abstract	246
45	Words in Significance Statement	120
46	Words in Introduction	621
47	Words in Discussion	1474

48  
49

50 Author Contributions: MRA and JWF designed Research; MRA, MC, SS, DAT performed  
51 Research; MM, EM, JV, JCB, BS, PA contributed unpublished reagents and tools; MRA  
52 and JWF Analyzed data; MRA and JWF wrote manuscript.

53

54 Acknowledgements: This work was supported by the International Foundation for Research  
55 in Paraplegia (MRA), the Bryon Riesch Paralysis Foundation (MRA), the American  
56 Association of Anatomists (MRA), the Christopher and Dana Reeve Foundation (JWF), the  
57 Medical Research Council (JWF), the Plasticise European Network (seventh framework  
58 program) (JWF) and the NIHR Cambridge Biomedical Research Centre. We are grateful to  
59 Dr. Natalie Bull (Univ of Cambridge) for assistance with intravitreal injections.

60

61 Funding sources: This work was supported by the International Foundation for Research in  
62 Paraplegia (MRA), the Bryon Riesch Paralysis Foundation (MRA), the American Association  
63 of Anatomists (MRA), the Christopher and Dana Reeve Foundation (JWF), the Medical  
64 Research Council (JWF), the Plasticise European Network (seventh framework program)  
65 (JWF) and the NIHR Cambridge Biomedical Research Centre.

66

67 **Abstract**

68       The regenerative ability of CNS axons decreases with age however this ability  
69 remains largely intact in PNS axons throughout adulthood. These differences are likely to  
70 correspond with age-related silencing of proteins necessary for axon growth and elongation.  
71 In previous studies, it has been shown that reintroduction of the alpha9 integrin subunit  
72 (tenascin-C receptor,  $\alpha 9$ ) that is downregulated in adult CNS can improve neurite outgrowth  
73 and sensory axon regeneration after a dorsal rhizotomy or a dorsal column crush spinal cord  
74 lesion. In the current study, we demonstrate that virally-expressed integrins ( $\alpha 9$ ,  $\alpha 6$ , or  $\beta 1$   
75 integrin) in the adult rat sensorimotor cortex and adult red nucleus are excluded from axons  
76 following neuronal transduction. Attempts to stimulate transport by inclusion of a cervical  
77 spinal injury and thus an upregulation of extracellular matrix molecules at the lesion site, or  
78 co-transduction with its binding partner,  $\beta 1$  integrin, did not induce integrin localization  
79 within axons. In contrast, virally-expressed  $\alpha 9$  integrin in developing rat cortex (postnatal  
80 day 5 or 10) demonstrated clear localization of integrins in cortical axons revealed by the  
81 presence of integrin in the axons of the corpus callosum and internal capsule as well as in the  
82 neuronal cell body. Furthermore, examination of dorsal root ganglia neurons and retinal  
83 ganglion cells demonstrated integrin localization both within peripheral nerve as well as  
84 dorsal root axons and within optic nerve axons, respectively. Together, our results suggest a  
85 differential ability for *in vivo* axonal transport of transmembrane proteins dependent on  
86 neuronal age and subtype.

87

88 **Significance Statement**

89 Most CNS neurons have an intrinsically low ability to regenerate their axons. This study has  
90 asked whether the transport into axons of integrins, the receptors that mediate growth through

91 extracellular matrix, might reveal reasons for the poor regenerative ability of CNS axons.  
92 Tagged integrins were expressed in sensory, retinal ganglion cell, cortical and red nucleus  
93 neurons. The integrins were transported down the axons of sensory and retinal ganglion cell  
94 axons, but not down the axons of adult cortical or red nucleus neurons. However, during the  
95 postnatal period of corticospinal axon growth, cortical neurons admitted integrins into their  
96 axons. The findings suggest that exclusion of integrins and other receptors from CNS axons  
97 may be a cause for their poor regenerative ability.

98

99 **Introduction**

100           Deficiencies in the regeneration of adult central nervous system (CNS) axons are due  
 101 to several factors including inhibitory molecules in the lesion environment and a neuronal  
 102 regenerative response that diminishes with neuronal maturation. Previously, integrin biology  
 103 has been studied as a window into mechanisms of axon regeneration and their failure with  
 104 maturation (Andrews et al., 2009; Eva et al., 2010; Eva et al., 2012; Tan et al., 2011;  
 105 Franssen et al., 2015). Integrins are heterodimeric transmembrane proteins that bind  
 106 extracellular matrix (ECM) molecules to induce cell proliferation, survival, and promote  
 107 outgrowth (Hynes, 2002). These proteins are an integral part of CNS development, but many  
 108 become downregulated in neurons upon CNS maturation (Hammarberg et al., 2000; Andrews  
 109 et al., 2009). Consequently, when either integrins or their ligands are downregulated, axon  
 110 growth fails. Specifically, when adult dorsal root ganglia (DRG) neurons are grown on  
 111 marginal ECM substrates such as low levels of laminin and fibronectin, neurite outgrowth  
 112 requires forced expression of  $\alpha 1$  integrin and  $\alpha 5$  integrin, respectively (Condic, 2001). It is  
 113 therefore of interest that  $\alpha 9 \beta 1$ , the integrin that recognizes tenascin-C, the main extracellular  
 114 matrix glycoprotein of the adult CNS, is developmentally downregulated. Following CNS  
 115 injury, there is a substantial upregulation of tenascin-C without a concurrent upregulation of  
 116  $\alpha 9$  integrin (Zhang et al., 1997; Tang et al., 2003; Andrews et al., 2009). As a strategy to  
 117 induce axon regeneration in the CNS, this integrin has been re-expressed in the CNS. This  
 118 allows sensory axons to grow prolifically on tenascin-C *in vitro* but addition of inhibitory  
 119 substrates such as CSPGs and Nogo, as occurs *in vivo*, blocks this growth through  
 120 inactivation of integrins (Tan et al., 2011). Forced activation of integrins can allow axons to  
 121 overcome this inhibition (Hu et al. 2008; Tan et al. 2011). By increasing the pool of  $\alpha 9$   
 122 integrin in DRG neurons using adeno-associated virus (AAV), a modest increase in the  
 123 regenerative response at the site of injury after dorsal rhizotomy and after a dorsal column

124 spinal cord lesion has been demonstrated (Andrews et al., 2009). In addition, expression of  
125 the integrin activator kindlin-1 in DRG neurons only promotes limited regeneration in the  
126 spinal cord while co-expression of  $\alpha 9$  and kindlin promotes extensive regeneration and  
127 robust functional recovery (Tan et al., 2011; Cheah et al., *In Press*). An obvious next step is  
128 to use this combination to promote regeneration of intrinsic CNS axons. However, *in vitro*  
129 studies suggest that this strategy may fail because of the exclusion of integrins from CNS  
130 axons as they mature (Franssen et al., 2015).

131       Clearly integrins can only stimulate axon regeneration if they are present in the axon  
132 at the site of damage. In the current study we have asked whether integrins are transported  
133 into sensory axons including dorsal root ganglia (DRGs) and retinal ganglia neurons (RGCs),  
134 and into several types of adult neurons including adult cortical neurons, rubrospinal neurons,  
135 and we also evaluated early postnatal cortical neurons during their growth phase. This study  
136 has been enabled by improvements in viral technology that have made it possible to produce  
137 AAV vectors that can accommodate epitope-tagged full length integrins. We show here that  
138 the presence in axons of virally-expressed integrins *in vivo* is dependent upon neuronal type  
139 as well as age. Integrins are restricted to the cell body and dendrites of adult cortical and  
140 rubrospinal neurons whereas during the developmental growth period, we observed integrins  
141 in the axons of early postnatal cortical neurons. However in adult RGC and DRG neurons,  
142 which have better regenerative ability especially following conditioning lesions, integrins  
143 were present along the length of the axons. These results suggest that in two important  
144 pathways involved in motor control known to be incapable of axonal repair, integrins become  
145 excluded from the axons as they mature.

## 146 **Materials and Methods**

### 147 **Integrin viral constructs:**

149 cDNAs encoding wild-type  $\alpha 9$  integrin were obtained from Dean Sheppard  
 150 (University of California San Francisco, USA) as previously described (Andrews et al.,  
 151 2009). cDNAs encoding wild-type  $\alpha 6$  and  $\beta 1$  integrins and eGFP-N1 vectors were obtained  
 152 from Charles ffrench-Constant (University of Edinburgh, UK) and cDNAs encoding  $\beta 1$   
 153 integrin-GFP were obtained from Martin Humphries (University of Manchester, UK).  
 154 pcDNA3.1/V5-His was purchased from Invitrogen. cDNA of  $\alpha 6$  and  $\alpha 9$  integrin was cloned  
 155 into eYFP-N1 using HindIII and KpnI restriction sites. V5 was PCR amplified adding on the  
 156 AgeI and NotI restriction sites before being cloned into the  $\alpha 9$ integrin-eYFP plasmid upon  
 157 removal of eYFP. Integrin lentiviral plasmids were constructed by PCR amplifying BclI and  
 158 SacII onto the  $\alpha 6$ integrin-eYFP,  $\alpha 9$ integrin-eYFP, and  $\beta 1$ integrin constructs, before cloning  
 159 into the LV-PGK vector (Bensadoun et al., 2003). Integrin adeno-associated viral plasmids  
 160 using a short CAG promoter or a CMV promoter were constructed by inserting the tagged  
 161 integrin constructs between the ITRs of AAV2 followed by a short poly-adenylation signal  
 162 (49bp).

Virus	Area injected	Serotype if AAV
LV-PGK- $\alpha 9$ integrin-eYFP	cortex	-
LV-PGK- $\alpha 6$ integrin-eYFP	cortex	-
LV-PGK-eGFP	cortex	-
LV-PGK- $\beta 1$ integrin	cortex	-
AAV-CMV- $\alpha 9$ integrin-eYFP	DRG	5
AAV-CAG- $\alpha 9$ integrin-V5	retina	2
AAV-CAG- $\alpha 9$ integrin-V5	cortex, DRG	5
AAV-CAG- $\beta 1$ integrin-GFP	cortex	5

163 **Table 1: List of viruses produced and used for procedures**

164 Generation of viruses:

165 *Lentiviruses:* The production, generation, and titration of lentiviral particles for LV-PGK- $\alpha 9$ -  
 166 eYFP, LV-PGK- $\alpha 6$ -eYFP, LV-PGK- $\beta 1$ integrin, and LV-PGK-eGFP was performed with a  
 167 four-plasmid system as previously described (Bensadoun et al. 2003).

168 *Adeno-associated viruses:* The production and titration of AAV serotype 2 and 5 vector

169 particles for AAV5-CAG- $\alpha$ 9integrin-V5, AAV5-CMV- $\alpha$ 9integrin-eYFP, AAV2-CAG-  
 170  $\alpha$ 9integrin-V5, and AAV5- $\beta$ 1integrin-eGFP was performed as previously described (Fagoe  
 171 et al., 2015). Briefly, a typical batch of AAV was produced in six 15 cm Petri dishes of  
 172 HEK293T cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%  
 173 fetal calf serum and 1% penicillin/streptomycin. Transfer and helper plasmids were mixed in  
 174 a ratio of 1:3 and co-transfected using polyethylenimine (PEI, linear MW 250,000;  
 175 Polysciences Inc., Warrington, PA, USA). Three days post-transfection cells were harvested  
 176 in Dulbecco's phosphate buffered saline (D-PBS, Gibco) and lysed by 3 freeze-thaw cycles.  
 177 Genomic DNA was digested by adding DNase I (Roche diagnostics, GmbH, Mannheim,  
 178 Germany) and the AAV vector particles were purified from the crude lysate by the iodixanol  
 179 gradient method (Hermens et al 1999; Zolotukhin et al 1999) and concentrated using an  
 180 Amicon 100kDa Ultra-15 device (Millipore). Concentrated AAV stocks were stored at -80°C  
 181 until use. Titers (genomic copies/ml) were determined by quantitative PCR of viral DNA  
 182 using primers against the CMV-enhancer sequence.

#### 183 Surgeries:

185 Experiments were conducted in accordance with the UK Animals (Scientific  
 186 Procedure) Act, 1986. Adult male Sprague Dawley rats (250-400grams) were used for all  
 187 cortical injections (adult and neonate), red nucleus injections, and intravitreal injections  
 188 whereas adult male Lewis **rats** (250-400grams) were used for all DRG injections (Charles  
 189 River Laboratories). Food and water were provided *ad libitum* and there was 12 hr light/dark  
 190 exposure. During surgery, adult rats were anesthetized in 1-2% isoflurane, in 2L/min  
 191 oxygen.

Area of Injection and Age	Virus	Number of animals
SMC – Neonate	LV-PGK- $\alpha$ 9integrin-eYFP	n=9

SMC – Neonate	LV-PGK-eGFP	n=10
SMC – Adult	LV-PGK- $\alpha$ 9integrin-eYFP	n=3
SMC – Adult	LV-PGK- $\alpha$ 6integrin-eYFP	n=3
SMC – Adult	LV-PGK-eGFP	n=5
SMC – Adult	LV-PGK- $\alpha$ 9-eYFP + LV-PGK- $\beta$ 1	n=9
SMC – Adult	AAV5-CAG- $\alpha$ 9integrin-V5	n=5
SMC – Adult	AAV5-CMV- $\beta$ 1integrin-GFP	n=6
SMC – Adult	AAV5-CAG- $\alpha$ 9-V5 + AAV5-CMV- $\beta$ 1-eGFP	n=10
SMC – Adult + SCI	LV-PGK- $\alpha$ 9integrin-eYFP	n=3
SMC – Adult + SCI	LV-PGK- $\alpha$ 6integrin-eYFP	n=6
SMC – Adult + SCI	LV-PGK-eGFP	n=4
Red Nucleus – Adult	LV-PGK- $\alpha$ 6integrin-eYFP	n=3
Red Nucleus – Adult	LV-PGK-eGFP	n=3
Red Nucleus – Adult + SCI	LV-PGK- $\alpha$ 9integrin-eYFP	n=3
Red Nucleus – Adult + SCI	LV-PGK- $\alpha$ 6integrin-eYFP	n=6
Red Nucleus – Adult + SCI	LV-PGK-eGFP	n=5
DRG – Adult	AAV5-CAG- $\alpha$ 9integrin-eYFP	n=8
DRG – Adult	AAV5-CAG- $\alpha$ 9integrin-V5	n=6
Retina	AAV2-CAG- $\alpha$ 9integrin-V5	n=10

Table 2: List of animal groups with viral type used

### Cortical and Red nucleus Injections - Adult

Adult cortical injection groups (LV-PGK- $\alpha$ 6integrin-eYFP, n=3; LV-PGK- $\alpha$ 9integrin-eYFP, n=3; LV-PGK-eGFP, n=5; AAV5-CAG- $\alpha$ 9integrin-V5, n=5; AAV-CAG- $\beta$ 1integrin-GFP, n=6) sustained a single injection of 1 $\mu$ l LV or AAV into the left forelimb sensorimotor cortex (SMC) at (AP 1.5mm, ML 1.5mm, DV -1.5mm) (Krajacic et al., 2009). Red nucleus injection groups (LV-PGK- $\alpha$ 6integrin-eYFP, n=3; LV-PGK- $\alpha$ 9integrin-eYFP, n=3; LV-PGK-eGFP, n=3) sustained a single injection of LV into the left red nucleus at (AP -5.9mm, ML 0.7mm, DV -7.0mm). Injections were performed using a custom made 30-gauge stainless steel needle attached to a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) driven by an infusion syringe pump (World Precision Instruments, FL, USA) at 0.2 $\mu$ l/min. One microliter was injected into the left SMC (LV or AAV) or into the left red nucleus (LV) over a five-minute period, followed by a three-minute period before needle withdrawal.

206 *Cortical injection – neonates*

207 Neonatal cortical injections (LV-PGK- $\alpha$ 9integrin-eYFP, n=9; LV-PGK-eGFP, n=10)  
 208 were conducted under hypothermic anesthesia with a single manual injection of 1ul of LV  
 209 into the developing left SMC at the following coordinates: ML 1.0mm, AP -0.5mm, DV -  
 210 0.7mm (Altman and Bayer, 1995), over the course of 1-2min, followed by a one-minute  
 211 period before needle withdrawal. Pups were returned to their mother immediately following  
 212 the procedure. Half of each group was taken for immunohistochemical analysis at 5 days  
 213 post-injection or 10 days post-injection.

214 *Dorsal root ganglia injection*

215 DRG injections were performed as described previously with some variations  
 216 (Andrews et al., 2009). Briefly, under anesthesia, a left hemilaminectomy was performed at  
 217 cervical levels C5-C6 (AAV5-CMV- $\alpha$ 9-eYFP, n=8) to unilaterally expose the C5-C6 DRGs  
 218 and dorsal roots or at lumbar levels L4-L5 (AAV5-CAG- $\alpha$ 9-V5, n=8) to unilaterally expose  
 219 the L4-L5 DRGs and dorsal roots. One microliter injections were performed as described for  
 220 the adult cortical injections except with a 33-gauge stainless steel needle attached to a  
 221 Hamilton syringe (Hamilton) driven by an infusion syringe pump at a rate of 0.1 $\mu$ l/min,  
 222 followed by a three-minute period before needle removal.

223 *Spinal cord lesions*

224 Cervical dorsal column crush lesions at C4-C5 were performed as previously  
 225 described (Andrews et al., 2009) concurrent with the cortical injection. Briefly, under  
 226 anesthesia (LV-PGK- $\alpha$ 6integrin-eYFP, n=6; LV-PGK- $\alpha$ 9integrin-eYFP, n=3; LV-PGK-  
 227 eGFP, n=4), a laminectomy was performed at cervical levels C4 and C5 to expose the dorsal  
 228 surface of the spinal cord. The dura was retracted and the dorsal columns, identified within

the immediate extents of the dorsal root entry zones on either side, were crushed bilaterally for 10 sec using finely-milled forceps at a depth of 2mm. With regard to cervical lateral hemisection lesions at C4-C5 (LV-PGK- $\alpha$ 6integrin-eYFP, n=6; LV-PGK- $\alpha$ 9integrin-eYFP, n=3; LV-PGK-eGFP, n=5), access to the spinal cord was performed as described above. Following dura retraction and identification of dorsal roots, a scalpel blade was inserted into the spinal cord rostral to the root entry to a depth of 2mm with the cut extending laterally to the edge of the cord.

#### *Intravitreal injections*

          Injections were performed as described previously (Bull et al., 2012). Briefly, under isoflurane anesthesia with local anesthetic applied topically to the cornea of the left eye, the left eyelid was gently retracted. Five microliters of AAV2-CAG- $\alpha$ 9integrin-V5 (n=10 animals) was injected into the vitreous of the left eye using a custom made 30-gauge stainless steel needle attached to a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland). Care was taken during the injection to ensure the lens was not damaged. The needle remained in position for 1 min following injection before needle withdrawal.

#### Immunohistochemistry:

          At the end of each experimental time point, animals were administered an overdose of sodium pentobarbital and transcardially perfused with PBS (pH 7.4), followed by 4% PFA (pH 7.4). Tissues (including brain, spinal cord, eyes, optic nerves, DRGs, dorsal roots and sciatic nerves depending on the paradigm) were removed and postfixed in 4% PFA and cryoprotected overnight in 30% sucrose in 0.1 M PB (pH 7.4).

#### *Brain, spinal cord, dorsal roots, sciatic nerves and ganglia*

          Brain tissue was sectioned coronally on a sliding microtome at a thickness of 40 $\mu$ m and stored in PBS with 0.02% sodium azide at 4°C until further processing and analysis.

253 Spinal cord tissue was embedded in O.C.T. (RALamb UK, Eastbourne, UK) and sectioned  
254 longitudinally (spinal cord) or transversely with spinal cord attached (for dorsal roots,  
255 ganglia, and sciatic nerves) on a cryostat at 14- $\mu$ m thickness, mounted on slides (Superfrost  
256 Plus; VWR International, Lutterworth, UK) and stored at -20°C until further processing and  
257 analysis.

258       Sections were washed with PBS and blocked in 10% NGS, 0.4% TX-100 in PBS.  
259 Primary antibodies, anti- $\beta$ 3 tubulin (1:400, mouse, Sigma), anti-GFP (1:500, rabbit,  
260 Molecular Probes), anti-V5 (1:250, mouse, Invitrogen), anti-GFAP (1:400, mouse, Sigma),  
261 anti-ankyrin G (1:100, mouse, Neuromabs), anti-ankyrin G (1:100, rabbit, Santa Cruz), anti-  
262 tenascin C (1:200, mouse, IBL America), anti-collagen IV (1:500, rabbit, Sigma), anti-  
263 fibronectin (1:100, rabbit, Sigma), and anti-laminin (1:500, rabbit, Sigma) were incubated  
264 overnight at 4°C. Sections were rinsed in triplicate in 0.1M PBS and incubated with  
265 secondary antibodies (1:500, goat anti-mouse or rabbit alexa fluor 568 or 488; Invitrogen),  
266 followed by a brief incubation with bisbenzimidazole (Sigma) nuclear stain. Slides were rinsed  
267 and coverslipped with Fluoromount. Certain antibodies (anti-tenascin C, anti-ankyrin G)  
268 required amplification using streptavidin. In these cases, pre-treatment of the tissue with  
269 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min was performed, along with incubation with biotinylated goat anti-  
270 rabbit or anti-mouse antibodies (1:500, Vector Labs) used in place of fluorescent secondary  
271 antibodies. Tissue was then incubated with streptavidin-conjugated to Alexa Fluor 568  
272 (1:250, Invitrogen).

#### 273 *Retinal whole mounts and optic nerve processing*

274       Animals were perfused as described above and the eyes, optic nerve and brains were  
275 removed and postfixed overnight in 4% paraformaldehyde at 4°C. For retinal whole mounts,  
276 retinas from both eyes were dissected and prepared as flattened whole mounts by making four

277 radial cuts (Bull et al., 2012). Retinal tissue was further post-fixed overnight as above.  
 278 Following PBS washes, tissue was incubated in blocking buffer (5% normal goat serum,  
 279 0.2% triton X-100 in PBS) prior to incubation overnight at 4°C in primary antibodies (rabbit  
 280 anti-tubulin, 1:1000, Covance; mouse anti-V5, 1:250, Invitrogen) diluted in blocking buffer.  
 281 Following further PBS washes, tissue was incubated overnight at 4°C in secondary antibodies  
 282 (goat anti-mouse 488 and goat anti-rabbit 568, 1:750, Invitrogen) diluted in blocking buffer.  
 283 Following the final PBS washes, retinal tissue was mounted on microscope slides and  
 284 coverslipped with Fluorosave. For optic nerve sections, optic nerves were cryoprotected in  
 285 30% sucrose, embedded in OCT embedding media, cryosectioned longitudinally at a  
 286 thickness of 14µm and mounted onto Superfrost Plus microscope slides. Optic nerve sections  
 287 were processed as described above for spinal cord cryosections using anti-V5 and anti-β3  
 288 tubulin antibodies.

#### 289 Microscopy and Analysis:

290 Fluorescence imaging was performed using a Leica DM6000 epifluorescent  
 291 microscope, a Leica TCS SP2 and a Leica SP8 confocal microscope (Leica, Wetzlar,  
 292 Germany). Images were captured using LAS AF Leica software, and processed with Adobe  
 293 Photoshop CS4.

#### 295 Results

##### 296 *Axons are transported into the axons of developing postnatal cortical neurons*

297 Integrins are vital for CNS development including neuronal migration and axonal  
 298 elongation (Graus-Porta et al., 2001; Denda and Reichardt, 2007), so it would be expected  
 299 that they would be transported to advancing axonal growth cones. We therefore examined  
 300 axonal localization of integrins during the immediate postnatal period when certain classes of

axons such as the corticospinal tract are still undergoing development and elongation.  
 Initially, we utilized many integrin antibodies, but were unable to find one that demonstrated  
 sufficiently good staining of endogenous integrins in tissue sections to show unequivocally  
 whether or not integrins are present in classes of CNS axons. We therefore transduced  
 neurons with tagged integrins that have been used previously in *in vitro* transport studies and  
 which traffic normally in cellular vesicles to and from the cell surface (Eva et al. 2010; Eva et  
 al. 2012; Franssen et al. 2015). Lentiviruses with their large cloning capacity (~8kB) were  
 utilized in this study since they allow for expression of a fluorescently tagged, full length  
 integrin and have been shown to successfully transduce cortical neurons *in vivo* achieving  
 maximum expression levels as early as 5-10 days (Hutson et al., 2012). In this group, we  
 injected a lentivirus encoding eYFP-tagged  $\alpha 9$  integrin under the control of a  
 phosphoglycerate kinase promoter (LV-PGK- $\alpha 9$ -eYFP) into the sensorimotor cortex of rat  
 pups on the day of birth (P0) and assessed axonal localization 5 or 10 days later (P5 and P10)  
**(Figure 1a)**. These early time points were selected based on minimal time required for *in*  
*vivo* lentiviral transduction and gene expression and the time window during which CST  
 axonal elongation is still ongoing. Results from this group demonstrated high levels of  
 neuronal transduction illustrated by co-labelling of NeuN and eYFP at the injection site, as  
 early as 5 days post-injection **(Figure 1b, c)**. Quite strikingly at P5, we also observed eYFP-  
 tagged  $\alpha 9$  integrin localized in axons within the corpus callosum **(Figure 1d)**, while at P10  
 we observed YFP-tagged  $\alpha 9$  integrin within axons of the internal capsule **(Figure 1e)**. In  
 both cases, the localization was characterized by a punctate and vesicular appearance of the  
 eYFP-tagged integrin **(Figure 1d, e)**.  
  
*Adult dorsal root ganglia neurons transport integrins into both peripheral and central*  
*axons*

326 We next investigated the presence of tagged  $\alpha 9$  integrin in the axons of adult dorsal  
 327 root ganglion (DRG) neurons. For these experiments we utilized AAV serotype 5 to achieve  
 328 optimal transduction of DRG neurons since lentivirus will not transduce DRG neurons *in vivo*  
 329 (Mason et al., 2010). Recently modified AAV plasmids (including plasmids with deleted  
 330 WPRE sequences and small polyA sequences) made it possible to clone full length  $\alpha 9$   
 331 integrin fused to either an eYFP or V5 epitope tag. Cervical or lumbar DRGs were injected  
 332 with either AAV5-CMV- $\alpha 9$ -eYFP or AAV5-CAG- $\alpha 9$ -V5, respectively, and analysed 3 or 6  
 333 weeks post-injection. We saw a high level of integrin within the cell bodies and in the axons  
 334 within the DRG (**Figure 2a-c**). After injection into the L4 and L5 lumbar DRGs,  
 335 examination of the sciatic nerve revealed clear localization of V5-tagged  $\alpha 9$  integrin within  
 336 peripheral axons (**Figure 2d-f**). Likewise, examination of the central branch of the DRG, the  
 337 dorsal root, revealed V5-tagged  $\alpha 9$  integrin localized within these axons extending towards  
 338 the spinal cord (**Figure 2g-i**). Within the dorsal root entry zone (DREZ), eYFP-tagged  $\alpha 9$   
 339 integrin could be observed having reached and entered the spinal cord extending along the  
 340 dorsal columns following injection into the C5 and C6 DRGs (**Figure 2j, k**).

#### 341 342 *Adult optic nerve axons contain integrins*

343 Retinal ganglion cells (RGCs) share some similarities with DRGs in their response to  
 344 injury and their regenerative ability, specifically with regard to the conditioning lesion  
 345 response, including the significantly enhanced growth response following a peripheral  
 346 preconditioning lesion (Leon et al., 2000). They also both respond to treatment with cyclic  
 347 AMP (cAMP) (Monsul et al., 2004). We therefore asked whether adult RGCs would transport  
 348 integrin into optic nerve axons similar to DRGs. In these experiments, intravitreal injections  
 349 in adult rat were performed unilaterally using a serotype 2 AAV (AAV2-CAG- $\alpha 9$ -V5),  
 350 which is the optimal AAV serotype for transducing RGCs (Hellström et al., 2009). Three

351 weeks post-injection we observed transduced cell bodies of retinal ganglia neurons along  
 352 with axonal fibers co-localizing  $\alpha 9$  integrin and  $\beta 3$  tubulin within axons extending towards  
 353 the optic disc as well as transport into the branching dendrites (**Figure 3a, c**). Within the  
 354 retina, we were able to see integrin transported into the axons of all the brightly transduced  
 355 RGCs. Further examination into the optic nerve three weeks post-injection revealed the  
 356 presence of  $\alpha 9$  integrin-V5 as punctate vesicles throughout the length of some axons (**Figure**  
 357 **3b**) including the presence of integrin in axons up to the chiasma resulting from this  
 358 relatively short-term (3 weeks) experiment.

359

360 *Virally-expressed integrin does not enter the axons of adult cortical neurons or adult red*  
 361 *nucleus neurons*

362 To further assess axonal localization of virally-expressed integrins in the CNS, we  
 363 initially utilized lentivirus for *in vivo* neuronal transduction of adult cortical neurons.  
 364 Unilateral injections into adult rat sensorimotor cortex resulted in a region of transduced  
 365 neurons with either LV-PGK- $\alpha 6$ -eYFP (**Figure 4c**) or LV-PGK- $\alpha 9$ -eYFP (**Figure 6f**). This  
 366 was comparable to the transduction efficiency found with injections of LV-PGK-eGFP  
 367 (**Figure 4a**). Upon examination of the cervical spinal cord, many GFP-filled corticospinal  
 368 axons were visible in the LV-eGFP group (**Figure 4b**). On the other hand, there was no  
 369 indication of eYFP-tagged integrin in corticospinal axons in the cervical spinal cord  
 370 expressing either  $\alpha 6$  (**Figure 4d**) or  $\alpha 9$  integrin (data not shown). Assessment of the  
 371 corticospinal tract more proximal to the injection site revealed no tagged integrin at any  
 372 point. At the injection site, eYFP-tagged integrin ( $\alpha 9$  integrin) was present in the cell body,  
 373 dendrites, and in some proximal axonal processes of the transduced cortical neurons (**Figure**  
 374 **5b-d**). In these cases,  $\alpha 9$  integrin-eYFP could be localized within apical dendrites extending  
 375 towards the outer layers of the cortex (**Figure 5c**), and in some cases the labelling could be

376 seen in dendritic spines (**Figure 5d**). We saw no integrin transport beyond the proximal  
377 processes of the neurons, including the subcortical white matter or anywhere further down the  
378 corticospinal tract.

379 To confirm that the lack of integrin transport into adult corticospinal tract (CST)  
380 axons was not due to experimental parameters of viral type (LV), viral promoter (PGK), or  
381 the large eYFP tag (722 base pairs) attached to the integrins, we repeated these experimental  
382 groups with consideration of these parameters. In these cases we used AAV with an  
383 alternative promoter, CAG, and the small V5 epitope tag (42 base pairs) fused to the integrin  
384 (AAV-CAG- $\alpha$ 9-V5). For these experiments, AAV5 was utilized as it has been shown to  
385 successfully transduce cortical neurons (Hutson et al., 2012) as well as other neuronal  
386 subtypes (Mason et al., 2010). Following adult cortical injections using AAV5-CAG- $\alpha$ 9-V5,  
387 a larger and more diffuse area of neuronal transduction at the injection site was observed  
388 compared to LV, however an absence of axonal localization of integrins beyond the proximal  
389 process of the axon remained (**Figure 5a**). In order to show that this selective transport  
390 pattern is not restricted to alpha integrins, we also injected AAV-CAG- $\beta$ 1-eGFP, an adeno-  
391 associated viral vector encoding tagged  $\beta$ 1 integrin and showed the same localization with  
392 transport into dendrites but no transport down axons (**Figure 5e-g**). Normal intracellular  
393 trafficking of integrins requires the presence of the  $\alpha\beta$  heterodimers in order to be correctly  
394 transported from the endoplasmic reticulum through the Golgi to the plasma membrane  
395 (Tiwari et al., 2011). It is currently unclear how much  $\beta$ 1 integrin, the binding partner of  $\alpha$ 9  
396 integrin and  $\alpha$ 6 integrin, is expressed in adult cortical neurons *in vivo* despite its presence  
397 being documented in hippocampal and cerebellar neurons (Pinkstaff et al., 1999), and in  
398 neurons of the red nucleus demonstrated by *in situ* hybridization (Plantman et al., 2005). To  
399 examine whether co-transduction of  $\alpha$ 9 integrin and  $\beta$ 1 integrin had an effect on axonal  
400 localization, we introduced both subunits by performing combined injections into adult rat

401 cortex. These were performed in two groups; 1) LV-PGK- $\alpha$ 9-eYFP and LV-PGK- $\beta$ 1  
 402 (untagged); and 2) AAV5-CAG- $\alpha$ 9-V5 and AAV5-CAG- $\beta$ 1-eGFP. We saw no change to the  
 403 overall localization of the eYFP- or V5-tagged  $\alpha$ 9 integrin, with integrins remaining in the  
 404 cell body and dendrites (data not shown). We conclude that, in agreement with our previous  
 405 observations *in vitro* (Franssen et al., 2015) integrins in mature cortical neurons are excluded  
 406 from axons, but transported into dendrites (**Figure 5a-e**).

407 Investigation within another motor tract in the adult, the rubrospinal tract (RST),  
 408 revealed a similar pattern. Following lentiviral injections into the red nucleus of adult rat  
 409 there were many neurons expressing high levels of GFP from LV-eGFP and LV-integrin ( $\alpha$ 6)  
 410 injections (**Figure 4e-h**). Examination of the rubrospinal tract in the cervical spinal cord  
 411 revealed GFP-filled axons in the LV-eGFP group (**Figure 4f**), while there was no indication  
 412 of any eYFP-tagged integrin localizing in rubrospinal axons (**Figure 4h**).

413 One of the main requirements for integrins to induce intracellular signaling and  
 414 downstream cellular processes such as neurite outgrowth is for ligand binding to occur with  
 415 extracellular matrix (ECM) molecules (Reichardt et al., 1989; Hynes, 2002). In the above  
 416 experimental groups, cortical injections were performed in uninjured naïve adult animals in  
 417 the absence of an injury-induced upregulation of ECM, thus, although tenascin-C is present  
 418 in the uninjured adult CNS, it could be suggested that transport into axons did not occur due  
 419 to a lack of an ECM-stimulus, or that integrin transport might only occur after an axotomy.  
 420 In order to address this issue, we performed cortical injections of LV-PGK- $\alpha$ 9-eYFP or LV-  
 421 PGK- $\alpha$ 6-eYFP along with a concurrent dorsal column crush spinal lesion at C4/C5 injuring  
 422 the corticospinal tract (CST). Alternatively, we also performed red nucleus injections of LV-  
 423 PGK- $\alpha$ 9-eYFP with a concurrent lateral overhemisection lesion at C4/C5 injuring the  
 424 rubrospinal tract (RST). Following spinal cord lesions there is a large increase in ECM  
 425 molecules including tenascin-C, the ligand for  $\alpha$ 9 $\beta$ 1 integrin (Andrews et al., 2009), and

426 other ECM molecules such as collagen, fibronectin, and laminin, a ligand for  $\alpha 6 \beta 1$  integrin  
 427 (**Figure 6a-d**). These cervical lesions did not result in the presence at the injury site of  $\alpha 6$   
 428 integrin or  $\alpha 9$  integrin in CST axons (**Figure 6e**) or of integrins in RST axons (data not  
 429 shown). Instead in these cases, we observed a similar localization of integrins within the cell  
 430 bodies and dendrites at the injection site (**Figure 6f**). To rule out distance as a factor  
 431 influencing integrin transport to the injury site, a further group with a nearby cortical stab  
 432 injury was included, but these cases also showed no axonal localization to the injury site (data  
 433 not shown).

434         There has been mounting evidence in the literature suggesting that the axon initial  
 435 segment (AIS) acts as a selective barrier, only permitting some classes of molecules to access  
 436 the axon (Song et al., 2009; Franssen et al., 2015). Although the role of the AIS in the  
 437 localization of sodium channels is established, there was also some evidence in our *in vitro*  
 438 study that the AIS might present a developmental barrier for molecules such as integrins  
 439 (Franssen et al., 2015; Normand and Rasband 2015). Immunohistochemical analysis using  
 440 antibodies against ankyrinG was performed on naïve early postnatal cortex (postnatal day 3)  
 441 and adult cortex. Results demonstrated that at both ages, ankyrinG-immunopositive  
 442 structures were present in the axons of the cortex (**Figure 7a-c**). Immunostaining for  
 443 ankyrinG and  $\alpha 9$  integrin-V5 in virally-injected adult cortex showed that in many neurons  
 444 integrins did not enter the initial segment, while in others there was some integrin in the  
 445 proximal-most part of the axon, colocalizing with ankyrin G (**Figure 7d-f**). In the cortex,  
 446 therefore, the AIS is present at a timepoint when integrins are localized within axons (early  
 447 postnatally) suggesting that ankyrin-G by itself is not the barrier to axonal transport of  
 448 integrins or equally that there is a developmental regulation of this barrier that occurs  
 449 between early postnatal development and adulthood.

450

## 451 Discussion

452       This study has focused on the axonal localization of virally-expressed integrin  
453 receptors *in vivo*. The work was stimulated by demonstrations that expression of appropriate  
454 integrins can drive a growth response in damaged neurites and axons (Condic et al., 1999;  
455 Condic, 2001; Andrews et al., 2009). In particular, it has been shown that expression of the  
456 integrin  $\alpha 9$ , the receptor for tenascin-C, can enhance the intrinsic regenerative response of  
457 sensory axons in the spinal cord. Transduction of DRGs with  $\alpha 9$  integrin, particularly if  
458 accompanied by the integrin activator kindlin-1, enables cut axons to regenerate through the  
459 inhibitory tenascin-rich extracellular milieu of the damaged spinal cord (Andrews et al.,  
460 2009, Tan et al., 2012, Cheah et al., *In Press*). However, a recent *in vitro* study on cortical  
461 neurons indicated that integrins (both endogenous and expressed) are excluded from their  
462 axons as they mature (Franssen et al., 2015), suggesting that overexpression of integrin by  
463 itself will not stimulate regeneration of the axons of cortical neurons. In these CNS neurons  
464 the mechanisms controlling transport into axons will also have to be addressed. In this study  
465 we have addressed the issue of integrin distribution *in vivo*. We have expressed a variety of  
466 integrins in sensory neurons and three CNS neuronal types to see whether or not they are  
467 excluded from axons. We used viral-mediated expression of tagged integrins for two reasons.  
468 This is an established regeneration-inducing strategy and because integrin antibodies stain  
469 tissue sections poorly, potentially due to low endogenous expression levels in the adult CNS.  
470 We show that axonal localization of virally-expressed integrins is highly dependent on  
471 neuronal type and age. Young cortical neurons during their growth phase as well as adult  
472 sensory and retinal ganglion cell neurons permit virally-expressed integrins into their axons,  
473 whereas axons of adult motor (cortical and red nucleus) neurons do not contain integrins.  
474 Rather in the latter cases, integrins remain within the somatodendritic compartment,  
475 sometimes entering the very proximal axons where they coincide with the ankyrinG-

476 immunopositive axon initial segment. Our finding in the rubrospinal tract is somewhat  
 477 surprising due to published data that demonstrates that RST axons have been shown to  
 478 regenerate for short distances into peripheral nerve grafts following certain treatments  
 479 including BDNF (Kobayashi et al., 1997). These findings of integrin exclusion from the  
 480 axon however, remain consistent regardless of viral type, viral promoter, integrin subunit,  
 481 fluorescent/epitope tag and axotomy. This confirms *in vivo* our previous *in vitro* findings that  
 482 integrins are transported into the axons of immature cortical neurons, but are progressively  
 483 excluded as the neurons mature (Franssen et al., 2015), that integrins are transported freely  
 484 into adult DRG axons (Eva et al., 2010; Eva et al., 2012), and that integrins can be detected in  
 485 adult retinal ganglion cell axons (Vecino et al., 2015).

486       The observation that integrin is transported into the axons of two populations of  
 487 sensory neurons, adult DRG and RGC neurons, is interesting because both have a relatively  
 488 high intrinsic regenerative ability. For example, following a (pre)conditioning lesion  
 489 consisting of a peripheral nerve cut or crush (Richardson and Issa; Neumann and Woolf,  
 490 1999) or lens injury (Leon et al., 2000), both neuronal types have been found to have  
 491 increased levels of GAP-43 (growth associated protein, 43kDa) and concurrently significant  
 492 axon regeneration. Likewise, following neuronal application of cAMP, both axonal  
 493 populations respond with increased growth after injury (Qiu et al., 2002; Monsul et al., 2004).  
 494 Both neuronal populations continue to express integrins at higher levels than cortical neurons  
 495 into adulthood (Vecino et al., 2015; Werner et al., 2000), so why do they not regenerate better  
 496 in the CNS environment when cut? We suggest two reasons: first they do not express  $\alpha 9$ , the  
 497 key integrin for interacting with the CNS extracellular matrix, and second any integrins  
 498 present on their axons are inactivated by CSPGs and by NogoA (Hu et al., 2008; Tan et al.,  
 499 2011). In support of these ideas, we have shown prolific regeneration of sensory axons in the  
 500 spinal cord by transduction with  $\alpha 9$  integrin and/or the integrin activator kindlin-1 (Andrews

et al. 2009, Tan et al. 2012, Cheah et al. *In Press*). In the combined study applying both  $\alpha 9$  integrin and kindlin-1, we have shown integrin transport into regenerating sensory axons in the dorsal column, and the fact that integrin-stimulated regenerating axons reached the medulla implies that active integrin was transported over this distance (Cheah et al., *In Press*).

Conversely, in our experiments with **neurons in two motor pathways**, the corticospinal and rubrospinal tracts, there was an obvious exclusion of integrins from axons. These pathways are well known to show only very low levels of regeneration following injury. Furthermore, neither a spinal cord injury-induced upregulation of ECM nor dual expression of both alpha and beta subunits were enough to stimulate transport in these axons. Likewise, in spinal cord lesions, transported proteins have been shown to accumulate at the terminals of cut axons (Ertürk et al., 2007), so had the integrins been axonally transported in adult CST axons post-injury, they should have accumulated and been present at the damaged axon ends even if less dense integrins within the length of the axons were below levels of detection. A correlation may exist between the endogenous regenerative capacity of axons and the ability of transmembrane receptors to localize/transport within axons. Moreover it is not only integrins that are excluded from these axons, but also TrkB and IGFR (Hollis et al. 2009a, Hollis et al. 2009b). CNS axons appear to become specialized for connectivity through selective transport of presynaptic molecules and exclusion of growth-related molecules.

During the period of axon growth, integrins are important for enabling growth cone advance (Denda and Reichardt, 2007). It is therefore not surprising that we found that integrins are transported into corticospinal axons during their growth phase. At an early postnatal age, injection of LV-PGK- $\alpha 9$ -eYFP in the sensorimotor cortex at postnatal day 0 resulted in  $\alpha 9$  integrin localization in a punctate pattern within axons of the corpus callosum

525 and the internal capsule at day 5 and 10 postnatal, respectively, during which time axons are  
526 still elongating. It is also within this early postnatal age group that CST axon regeneration is  
527 possible following injury (Bates and Stelzner, 1993). The developmental change from  
528 integrin transport to exclusion correlates with a general age-related reduction in axonal  
529 transport that occurs within both CNS and PNS axons, which can be rescued in the PNS with  
530 a conditioning lesion (Milde et al., 2015).

531 For sensory neurons, a previous conditioning crush of the peripheral nerve can  
532 increase the regenerative ability of the central branch of the axons and stimulate local  
533 regeneration in the spinal cord (Neumann and Woolf 1999). Retinal axons in the optic nerve  
534 show little regeneration unless a lens lesion or modification of signaling is applied (Leon et  
535 al. 2000, Sun et al. 2011). It will be interesting to see whether these various interventions  
536 alter integrin transport into axons. In the current work, lesion of the corticospinal tract made  
537 no difference to integrin transport into the axons. In the case of dorsal root ganglia neurons,  
538 we have obtained long-distance regeneration in the spinal cord of many axons with excellent  
539 sensory recovery through expression of  $\alpha 9$  integrin and the integrin activator kindlin-1 in  
540 sensory neurons without needing to make a conditioning lesion (Cheah et al. *In Press*).

541 Recent work has suggested mechanisms for axonal transport and selective exclusion  
542 of integrins. In sensory axons, integrins are transported in recycling vesicles marked by the  
543 GTPases Rab11 and Arf6 (Eva et al., 2010) and TrkB receptors in hippocampal neurons are  
544 associated with Rab11 (Huang et al., 2013). As cortical neurons mature, Arf6 transport of  
545 integrins becomes retrograde, acting to exclude integrins, driven by increases in the Arf6  
546 GEFs, Efa6 and ARNO. Additionally, there are many studies that demonstrate that the axon  
547 initial segment acts as a filter at the axon hillock between the cell body and axon, allowing  
548 only select proteins to access the axonal compartment (Song et al., 2009; Normand and

549 Rasband 2015; Eva et al., 2012; Petersen et al. 2014; Arnold 2009). However, our  
 550 observation of the presence of an ankyrin G-immunoreactive axon initial segment as early as  
 551 postnatal day 3 in cortical neurons *in vivo* suggests that the AIS may exist but has not  
 552 developed a full barrier function at an early age.

553       The findings in this study suggest differences in the transport of transmembrane  
 554 integrin receptors and their subsequent localization within axons, which is dependent on  
 555 neuronal subtype and age. In order for integrin receptors to induce a growth response, they  
 556 have to be able to interact with the ECM of the external lesion environment and must  
 557 therefore be transported to the tips of cut axons. In DRG and RGC axons this appears to be  
 558 the case, and if transport into other CNS axons could be enabled, there is a strong potential  
 559 for an enhanced regenerative response using integrin gene therapy.

560

## 561 **References**

- 562 Altman J, Bayer SA (1995) Atlas of prenatal rat development. Boca Raton, FL: CRC Press.
- 563 Andrews MR, Czvitkovich S, Dassie E, Vogelaar CF, Faissner A, Blits B, Gage FH, ffrench-  
 564 Constant C, Fawcett JW (2009) Alpha9 integrin promotes neurite outgrowth on  
 565 tenascin-C and enhances sensory axon regeneration. *J Neurosci* 29:5546–5557.
- 566 Bates CA, Stelzner DJ (1993) Extension and regeneration of corticospinal axons after early  
 567 spinal injury and the maintenance of corticospinal topography. *Exp Neurol* 123:106–  
 568 117.
- 569 Bensadoun JC, De Almeida LP, Fine EG, Tseng JL, Déglon N, Aebischer P (2003)  
 570 Comparative study of GDNF delivery systems for the CNS: Polymer rods, encapsulated  
 571 cells, and lentiviral vectors. *J Control Release* 87:107–115.

- 572 Bull ND, Chidlow G, Wood JPM, Martin KR, Casson RJ (2012) The mechanism of axonal  
573 degeneration after perikaryal excitotoxic injury to the retina. *Exp Neurol* 236:34–45.
- 574 Cheah M, Andrews MR, Chew DJ, Moloney E, Verhaagen J, Fassler R, Fawcett JW (*In*  
575 *Press*) Expression of an Activated Integrin Promotes Long-distance Sensory Axon  
576 Regeneration in the Spinal Cord. *J Neurosci. In Press*.
- 577 Condic ML (2001) Adult neuronal regeneration induced by transgenic integrin expression. *J*  
578 *Neurosci* 21:4782–4788.
- 579 Condic ML, Snow DM, Letourneau PC (1999) Embryonic neurons adapt to the inhibitory  
580 proteoglycan aggrecan by increasing integrin expression. *J Neurosci* 19:10036–10043.
- 581 Denda S, Reichardt LF (2007) Studies on Integrins in the Nervous System. *Methods Enzymol*  
582 426:203–221.
- 583 Ertürk A, Hellal F, Enes J, Bradke F (2007) Disorganized microtubules underlie the  
584 formation of retraction bulbs and the failure of axonal regeneration. *J Neurosci* 27:9169–  
585 9180.
- 586 Eva R, Crisp S, Marland JRK, Norman JC, Kanamarlapudi V, ffrench-Constant C, Fawcett  
587 JW (2012) ARF6 directs axon transport and traffic of integrins and regulates axon  
588 growth in adult DRG neurons. 32:10352–10364.
- 589 Eva R, Dasse E, Caswell PT, Dick G, ffrench-Constant C, Norman JC, Fawcett JW (2010)  
590 Rab11 and its effector Rab coupling protein contribute to the trafficking of beta 1  
591 integrins during axon growth in adult dorsal root ganglion neurons and PC12 cells. *J*  
592 *Neurosci* 30:11654–11669.
- 593 Fagoë ND, Attwell CL, Kouwenhoven D, Verhaagen J, Mason MRJ (2015) Overexpression  
594 of ATF3 or the combination of ATF3, c-Jun, STAT3 and Smad1 promotes regeneration  
595 of the central axon branch of sensory neurons but without synergistic effects. *Hum Mol*  
596 *Genet* 24:6788–6800.

- 597 Franssen EHP, Zhao R-RR-R, Koseki H, Kanamarlapudi V, Hoogenraad CC, Eva R, Fawcett  
598 JW (2015) Exclusion of Integrins from CNS Axons Is Regulated by Arf6 Activation and  
599 the AIS. *J Neurosci* 35:8359–8375.
- 600 Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, Orban P,  
601 Klein R, Schittny JC, Müller U (2001)  $\beta$ 1-Class Integrins Regulate the Development of  
602 Laminae and Folia in the Cerebral and Cerebellar Cortex. *Neuron* 31:367–379.
- 603 Hammarberg H, Wallquist W, Piehl F, Risling M, Cullheim S (2000) Regulation of Laminin-  
604 Associated Integrin Subunit mRNAs in Rat Spinal Motoneurons During Postnatal  
605 Development and After Axonal Injury. 304:294–304.
- 606 Hellström M, Ruitenber MJ, Pollett M a, Ehlert EME, Twisk J, Verhaagen J, Harvey a R  
607 (2009) Cellular tropism and transduction properties of seven adeno-associated viral  
608 vector serotypes in adult retina after intravitreal injection. *Gene Ther* 16:521–532.
- 609 Hermens WT, ter Brake O, Dijkhuizen PA, Sonnemans MA, Grimm D, Kleinschmidt JA VJ  
610 (1999) Purification of Recombinant Adeno-Associated Virus by Iodixanol Gradient  
611 Ultracentrifugation Allows Rapid and Reproducible Preparation of Vector Stocks for  
612 Gene Transfer in the Nervous System. *Hum Gene Ther* 10:1885–1991.
- 613 Hu F, Strittmatter SM (2008) The N-terminal domain of Nogo-A inhibits cell adhesion and  
614 axonal outgrowth by an integrin-specific mechanism. *J Neurosci* 28:1262–1269.
- 615 Huang S-H, Wang J, Sui W-H, Chen B, Zhang X-Y, Yan J, Geng Z, Chen Z-Y (2013)  
616 BDNF-dependent recycling facilitates TrkB translocation to postsynaptic density during  
617 LTP via a Rab11-dependent pathway. *J Neurosci* 33:9214–9230.
- 618 Hutson TH, Verhaagen J, Yáñez-Muñoz RJ, Moon LDF (2012) Corticospinal tract  
619 transduction: a comparison of seven adeno-associated viral vector serotypes and a non-  
620 integrating lentiviral vector. *Gene Ther* 19:49–60.
- 621 Hynes RO (2002) Integrins: Bidirectional, allosteric signaling machines. *Cell* 110:673–687.
- 622 Kobayashi NR, Fan DP, Giehl KM, Bedard a M, Wiegand SJ, Tetzlaff W (1997) BDNF and  
623 NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate

- 624 GAP-43 and  $\alpha$ -tubulin mRNA expression, and promote axonal regeneration. *J*  
625 *Neurosci* 17:9583–9595.
- 626 Leon S, Yin Y, Nguyen J, Irwin N, Benowitz LI (2000) Lens injury stimulates axon  
627 regeneration in the mature rat optic nerve. *J Neurosci* 20:4615–4626.
- 628 Lewis TL, Mao T, Svoboda K, Arnold DB (2009) Myosin-dependent targeting of  
629 transmembrane proteins to neuronal dendrites. *Nat Neurosci* 12:568–576.
- 630 Mason MRJ, Ehlert EME, Eggers R, Pool CW, Hermening S, Huseinovic A, Timmermans E,  
631 Blits B, Verhaagen J (2010) Comparison of AAV serotypes for gene delivery to dorsal  
632 root ganglion neurons. *Mol Ther* 18:715–724.
- 633 Milde S, Adalbert R, Elaman MH, Coleman MP (2015) Axonal transport declines with age in  
634 two distinct phases separated by a period of relative stability. *Neurobiol Aging* 36:971–  
635 981.
- 636 Monsul NT, Geisendorfer AR, Han PJ, Banik R, Pease ME, Skolasky RL, Hoffman PN  
637 (2004) Intraocular injection of dibutyryl cyclic AMP promotes axon regeneration in rat  
638 optic nerve. *Exp Neurol* 186:124–133.
- 639 Neumann S, Woolf CJ (1999) Regeneration of dorsal column fibers into and beyond the  
640 lesion site following adult spinal cord injury. *Neuron* 23:83–91.
- 641 Petersen JD, Kaech S, Banker G (2014) Selective Microtubule-Based Transport of Dendritic  
642 Membrane Proteins Arises in Concert with Axon Specification. *J Neurosci* 34:4135–  
643 4147.
- 644 Pinkstaff JK, Detterich J, Lynch G, Gall C (1999) Integrin subunit gene expression is  
645 regionally differentiated in adult brain. *J Neurosci* 19:1541–1556.
- 646 Plantman S, Novikova L, Novikov L, Hammarberg H, Wallquist W, Kellerth J-O, Cullheim S  
647 (2005) Integrin messenger RNAs in the red nucleus after axotomy and neurotrophic  
648 administration. *Neuroreport* 16:709–713.
- 649 Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, Filbin MT (2002) Spinal axon  
650 regeneration induced by elevation of cyclic AMP. *Neuron* 34:895–903.

- 651 Reichardt LF, Bixby JL, Hall DE, Ignatius MJ, Neugebauer KM, Tomaselli KJ (1989)  
652 Integrins and cell adhesion molecules: neuronal receptors that regulate axon growth on  
653 extracellular matrices and cell surfaces. *Dev Neurosci* 11:332–347.
- 654 Richardson PM, Issa VM Peripheral injury enhances central regeneration of primary sensory  
655 neurones. *Nature* 309:791–793.
- 656 Song AH, Wang D, Chen G, Li Y, Luo J, Duan S, Poo MM (2009) A Selective Filter for  
657 Cytoplasmic Transport at the Axon Initial Segment. *Cell* 136:1148–1160.
- 658 Sun, F., K.K. Park, S. Belin, D. Wang, T. Lu, G. Chen, K. Zhang, C. Yeung, G. Feng, B.A.  
659 Yankner, and Z. He. 2011. Sustained axon regeneration induced by co-deletion of PTEN  
660 and SOCS3. *Nature*. 480:372-375.
- 661 Tan CL, Andrews MR, Kwok JCF, Heintz TGP, Gumy LF, Fässler R, Fawcett JW (2012)  
662 Kindlin-1 enhances axon growth on inhibitory chondroitin sulfate proteoglycans and  
663 promotes sensory axon regeneration. *J Neurosci* 32:7325–7335.
- 664 Tan CL, Kwok JCF, Patani R, Ffrench-Constant C, Chandran S, Fawcett JW (2011) Integrin  
665 Activation Promotes Axon Growth on Inhibitory Chondroitin Sulfate Proteoglycans by  
666 Enhancing Integrin Signaling. *J Neurosci* 31:6289–6295.
- 667 Tang X, Davies JE, Davies SJ a (2003) Changes in Distribution , Cell Associations , and  
668 Protein Expression Levels of NG2 , V2 , and Tenascin-C During Acute to Chronic  
669 Maturation of Spinal Cord Scar Tissue. *J Neurosci Res* 444:427–444.
- 670 Tiwari S, Askari J a, Humphries MJ, Bulleid NJ (2011) Divalent cations regulate the folding  
671 and activation status of integrins during their intracellular trafficking. *J Cell Sci*  
672 124:1672–1680.
- 673 Vecino E, Heller JP, Veiga-Crespo P, Martin KR, Fawcett JW (2015) Influence of  
674 extracellular matrix components on the expression of integrins and regeneration of adult  
675 retinal ganglion cells. *PLoS One* 10:e0125250.
- 676 Werner A, Willem M, Jones LL, Kreutzberg GW, Mayer U (2000) Impaired Axonal  
677 Regeneration in  $\alpha 7$  Integrin-Deficient Mice. *20:1822–1830*.

678 Zhang Y, Winterbottom JK, Schachner M, Lieberman a. R, Andersen PN (1997) Tenascin-C  
679 expression and axonal sprouting following injury to the spinal dorsal columns in the  
680 adult rat. J Neurosci Res 49:433–450.

681 Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C,  
682 Samulski RJ, Muzyczka N (1999) Recombinant adeno-associated virus purification  
683 using novel methods improves infectious titer and yield. Gene Ther 6:973–985.

684

685

686

687 **Figure legends:**

688 **Figure 1: eYFP-tagged  $\alpha 9$  integrin is transported into early postnatal cortical axons**

689 Schematic of experimental design indicating the ages at which the lentivirus was injected and  
690 the time points of perfusion and tissue analysis, P = postnatal day (**A**). Fluorescent images of  
691 cortical injection site five days following LV-PGK-  $\alpha 9$ integrin-eYFP injection showing  
692 eYFP-labelled cortical neurons (green) (**B**) co-labelled with NeuN (red) and bisbenzamide  
693 nuclear label (blue) (**C**). Fluorescent images of eYFP-labelled  $\alpha 9$ integrin within axons of the  
694 corpus callosum (**D**) or internal capsule (**E**), five or ten days following cortical injection,  
695 respectively. Coronal brain illustrations (left) indicate approximate area of injection site (**of**  
696 **boxed area for B, C**) and area of image of corpus callosum (**in D**) and (right) indicate  
697 approximate area of image of internal capsule (**in E**) (**F**). Scale bars = 100 $\mu$ m (**B, C**); 50 $\mu$ m  
698 (**D, E**).

699

700 **Figure 2: V5 and eYFP-tagged  $\alpha 9$  integrin expressed in DRG neurons is transported to**  
701 **the central and peripheral branches of DRG axons**

702 DRG neurons express  $\alpha 9$  integrin-V5 (green in all panels) four weeks following injection of  
703 AAV5-CAG- $\alpha 9$ integrin-V5 into the L4 and L5 DRGs (**A**) including within the proximal  
704 neuronal processes (**arrows in C**), shown co-labelled with  $\beta 3$  tubulin (red) (**B**). Confocal (**D**)  
705 and epifluorescent (**E**) images show V5-labelled  $\alpha 9$  integrin within axons in the sciatic nerve,  
706 four weeks following DRG injection, co-labelled with anti- $\beta 3$  tubulin (red) (**F**). Confocal  
707 (**G**) and epifluorescent (**H**) images show V5-labelled  $\alpha 9$  integrin within axons in the dorsal  
708 root, four weeks following DRG injection, co-labelled with anti- $\beta 3$  tubulin (red) (**I**).  
709 Epifluorescent image of eYFP-labelled  $\alpha 9$  integrin (green in J and K) in the axons of the  
710 dorsal root entry zone leading into the dorsal column, six weeks following DRG injection of  
711 AAV-CMV- $\alpha 9$ integrin-eYFP into the C5 and C6 DRGs (**J**), and in axons in the dorsal

columns (**arrows in K**) observed in sagittal section at level C2 (**K**). Scale bars = 200 $\mu$ m (**A**, **B**, **J**, **K**); 100 $\mu$ m (**C**, **E**, **F**, **H**, **I**); 20 $\mu$ m (**D**, **G**).

714

**Figure 3:  $\alpha$ 9integrin-V5 expressed in adult RGCs is transported into optic nerve axons**

Confocal images of flat mount retina show RGCs immunolabelled with anti-V5 (green) and co-labelled with anti- $\beta$ 3 tubulin (red) three weeks after intravitreal injection of AAV2-CAG- $\alpha$ 9integrin-V5 (**A**, **C**). **A\*** shows a high magnification image (**from A**) of integrin-containing axons in a fascicle (arrows) travelling towards the optic nerve. Epifluorescent images in **B** of optic nerve indicate V5-labelled  $\alpha$ 9integrin within axon fibers of the optic nerve three weeks following AAV injection. Arrows in **C** indicate V5-labelled axons following along the course of  $\beta$ 3 tubulin axons. Scale bars = 20 $\mu$ m (**A**, **C**); 50 $\mu$ m (**B**).

723

**Figure 4:  $\alpha$ 6integrin expressed in adult cortical or rubrospinal neurons is not transported down CST or RST axons.**

Adult motor cortex three weeks following injection of LV-PGK-eGFP (**A**) or LV-PGK- $\alpha$ 6integrin-eYFP (**C**). In cervical spinal cord, axons are filled with eGFP in the CST following LV-PGK-eGFP cortical injection (**B**), but no integrins are observed after LV-PGK- $\alpha$ 6integrin-eYFP injection (**D**). Insert in **C** (**C\***) shows high magnification view of LV- $\alpha$ 6 integrin transduced cortical neurons. Adult red nucleus three weeks following injection of LV-PGK-eGFP (**E**) or LV-PGK- $\alpha$ 6integrin-eYFP (**G**). Within the cervical spinal cord, only in the LV-PGK-eGFP injected groups are RST fibers found labelled with GFP (**F**) and not following LV-PGK- $\alpha$ 6integrin-eYFP injection (**H**). Scale bars = 500 $\mu$ m (**A**, **C**, **E**, **G**); 100 $\mu$ m (**B**, **D**); 200 $\mu$ m (**F**, **H**).

735

**Figure 5:  $\alpha$ 9integrin- and  $\beta$ 1integrin-transduced adult cortical neurons express integrins in their dendrites but not in their axons.**

$\alpha$ 9- and  $\beta$ 1-transduced neurons (**A-E**) show prominent apical and basal dendrites (white arrows), but integrins have not entered the axon beyond the very proximal processes (**A**, **E**, **F**). **B** shows a region of cortex above the injection site demonstrating  $\alpha$ 9-transduced neurons with YFP-immunopositive integrin within apical dendrites. **C** demonstrates detail of YFP-immunopositive  $\alpha$ 9integrin dendritic arbors branching (white arrows) near the surface of the cortex. **D** shows a confocal image of YFP-immunopositive  $\alpha$ 9integrin within a dendrite with prominent dendritic spines (white arrows). **F** and **G** show the base of the cortex and the underlying white matter four weeks following AAV5- $\beta$ 1-GFP cortical injections demonstrating transduction of neurons throughout a wide area of cortex. Most of the white matter is devoid of tagged integrin, but a few fine processes of neurons very close to the white matter can be seen, demonstrating that labelled processes in white matter can be seen if

749 present. **G** is a composite showing subcortical white matter from the midline (left of picture)  
 750 to lateral cortex. Although there are many integrin-transduced neurons in the overlying  
 751 cortex, no integrin-containing axons are observed in the white matter of the corpus callosum.  
 752 Scale bars = 50µm (**A, C, E, F**); 100µm (**B, G**); 10µm (**D**).

753

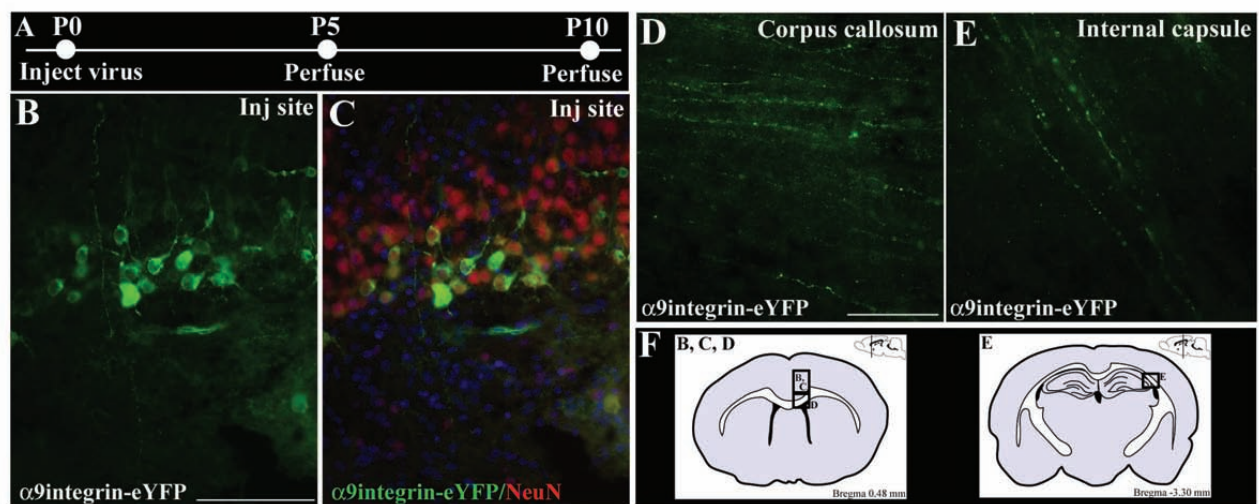
754 **Figure 6: CNS injury induces upregulation of ECM expression but does not induce**  
 755 **integrin localization in adult CST axons**

756 Cervical dorsal column crush lesion leads to upregulation of ECM molecules such as  
 757 collagen (**A**), fibronectin (**B**), laminin (**C**), and tenascin-C (**D**). Dashed lines in **A-E** indicate  
 758 approximate borders of lesion site. Following injections of LV- $\alpha 9$ integrin-eYFP (**E'**) into  
 759 adult sensorimotor cortex with concurrent cervical spinal cord crush lesion did not induce  
 760 CST axonal localization three or six weeks following injury and injection (**E**). High  
 761 magnification image (**F**) demonstrates perinuclear appearance of neuronally-expressed  $\alpha 9$   
 762 integrin (arrowheads) also localized within dendrites (arrows). Scale bars = 100µm (**in A for**  
 763 **A-C, D, E, F**); 200µm (**F'**).

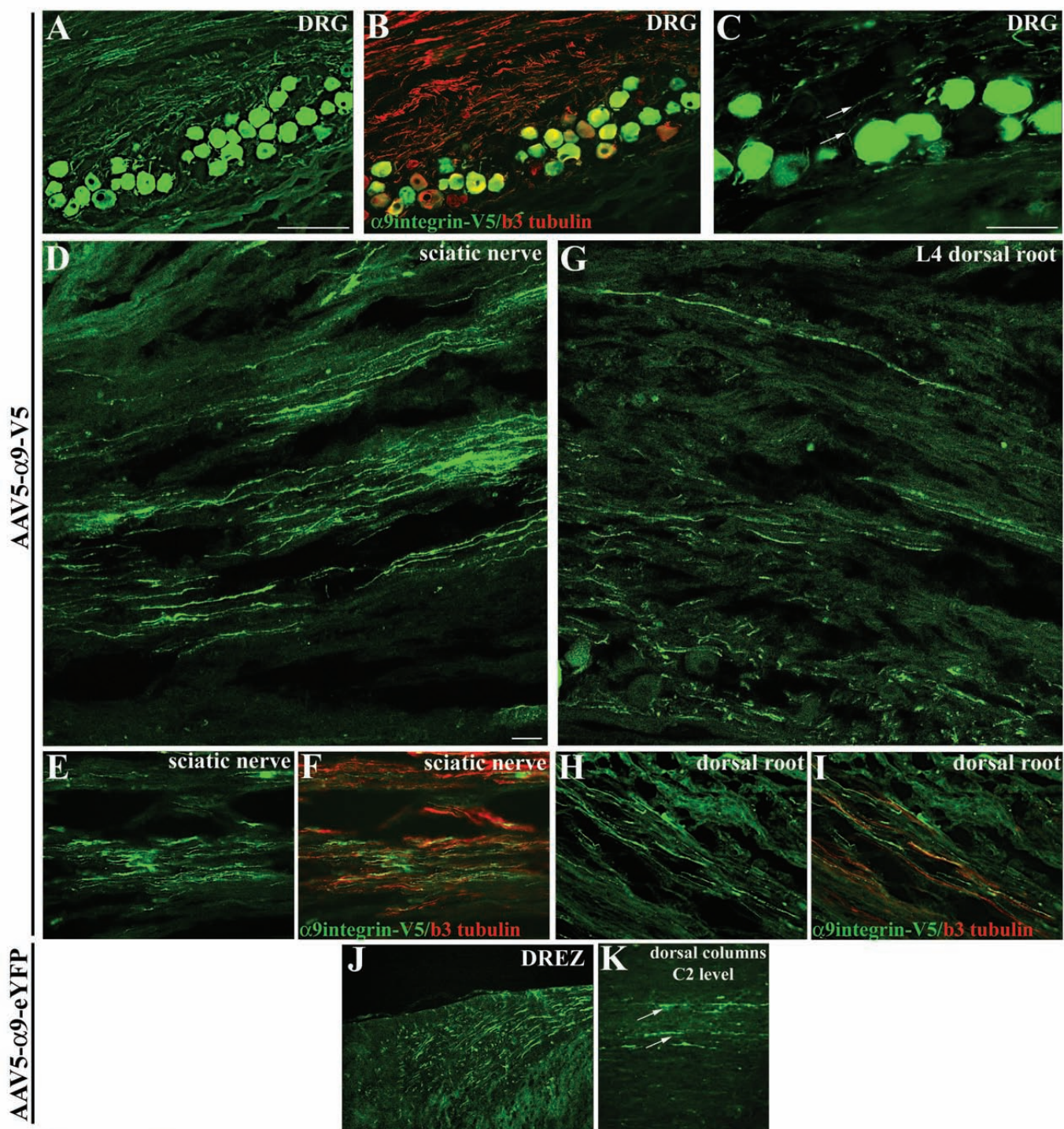
764

765 **Figure 7: Ankyrin G is expressed in both early postnatal (P3) and adult cortical**  
 766 **neurons, with integrin localization apparent in the axon initial segment in adult**  
 767 **injection sites.**

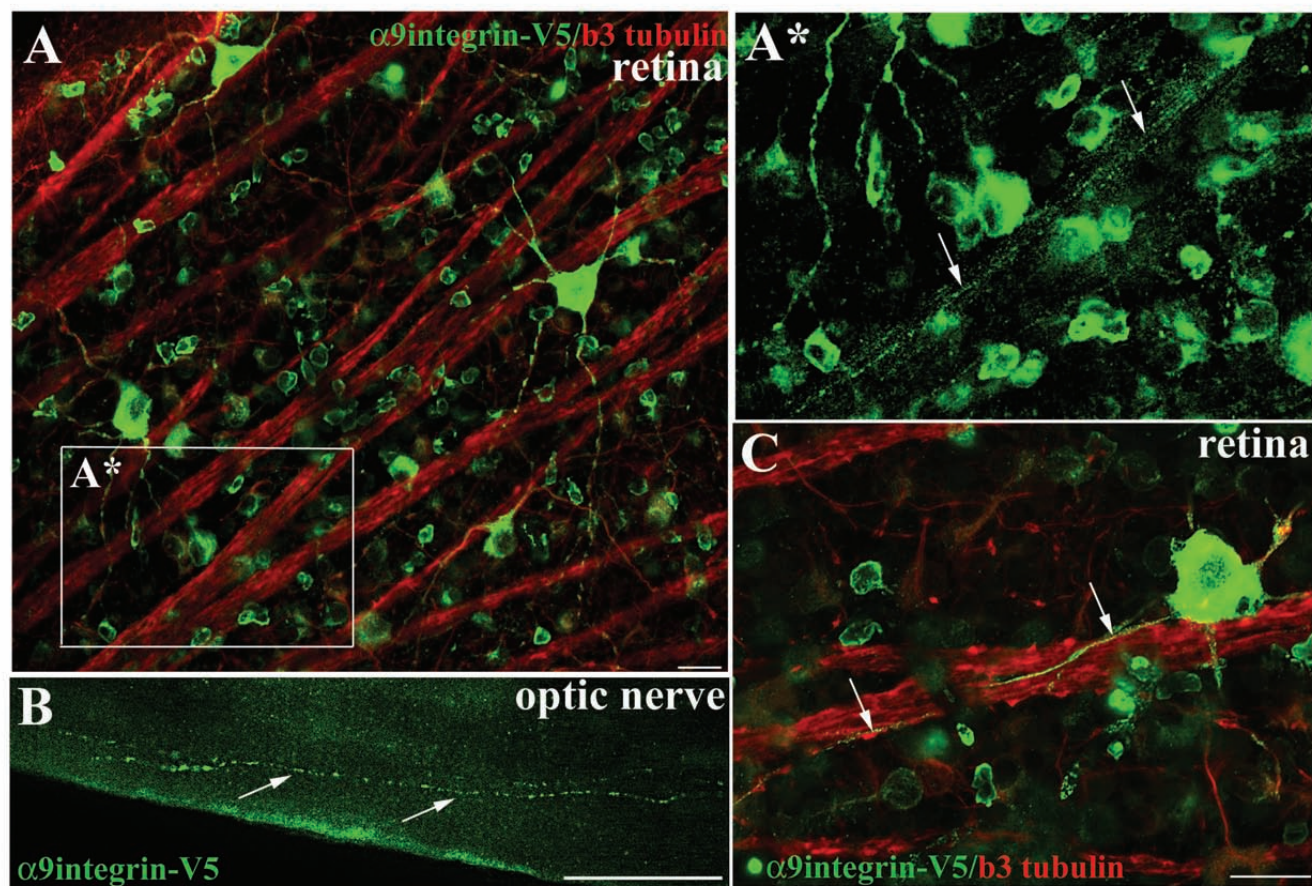
768 Epifluorescent images of anti-ankyrin G immunolabelled cortex of adult (**A**) or postnatal day  
 769 3 rat (**B, C**). Insert in B is shown in higher magnification in C. **D-F** show confocal images  
 770 near an adult cortical injection site (AAV5-CAG- $\alpha 9$ -v5) with V5-immunopositive  $\alpha 9$   
 771 integrin within neurons (**D**), co-labelled with anti-ankyrin G (**E**), indicating that in some  
 772 cases there was colocalization of virally-expressed integrin with the ankyrin G-  
 773 immunopositive axon initial segment. Scale bars = 100µm (**A, B**); 50µm (**C**); 10µm (**D-F**).



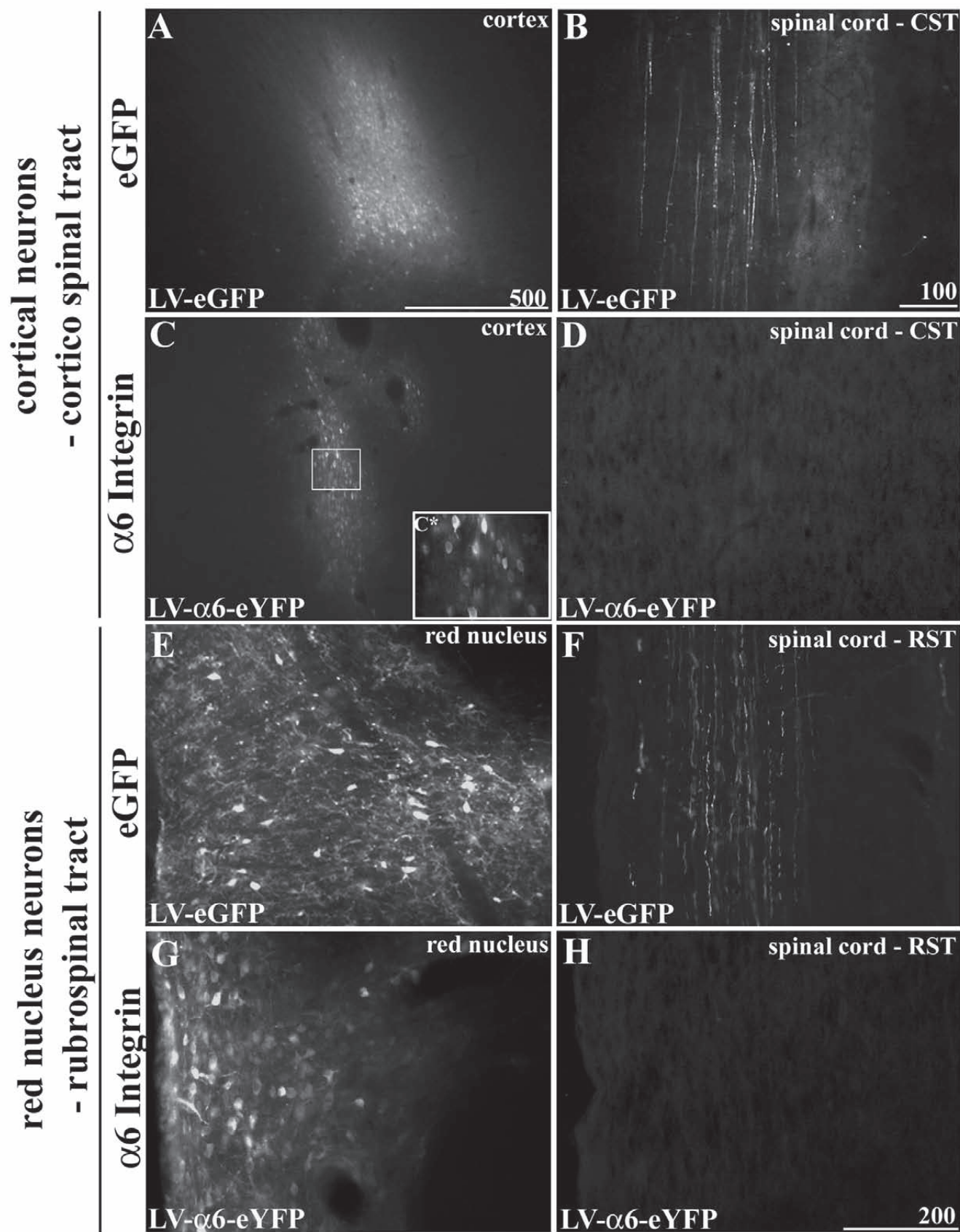
**Figure1**



**Figure 2**



**Figure 3**



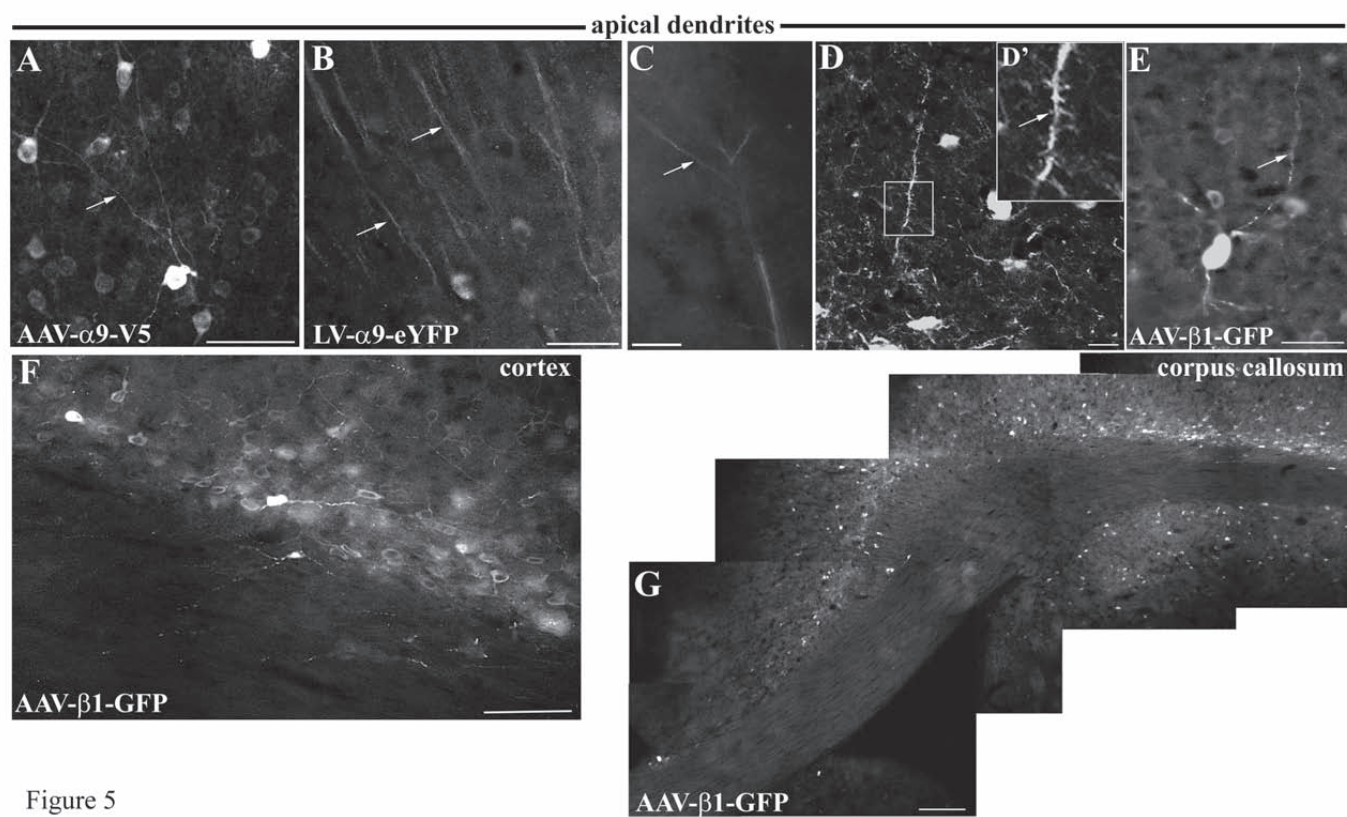
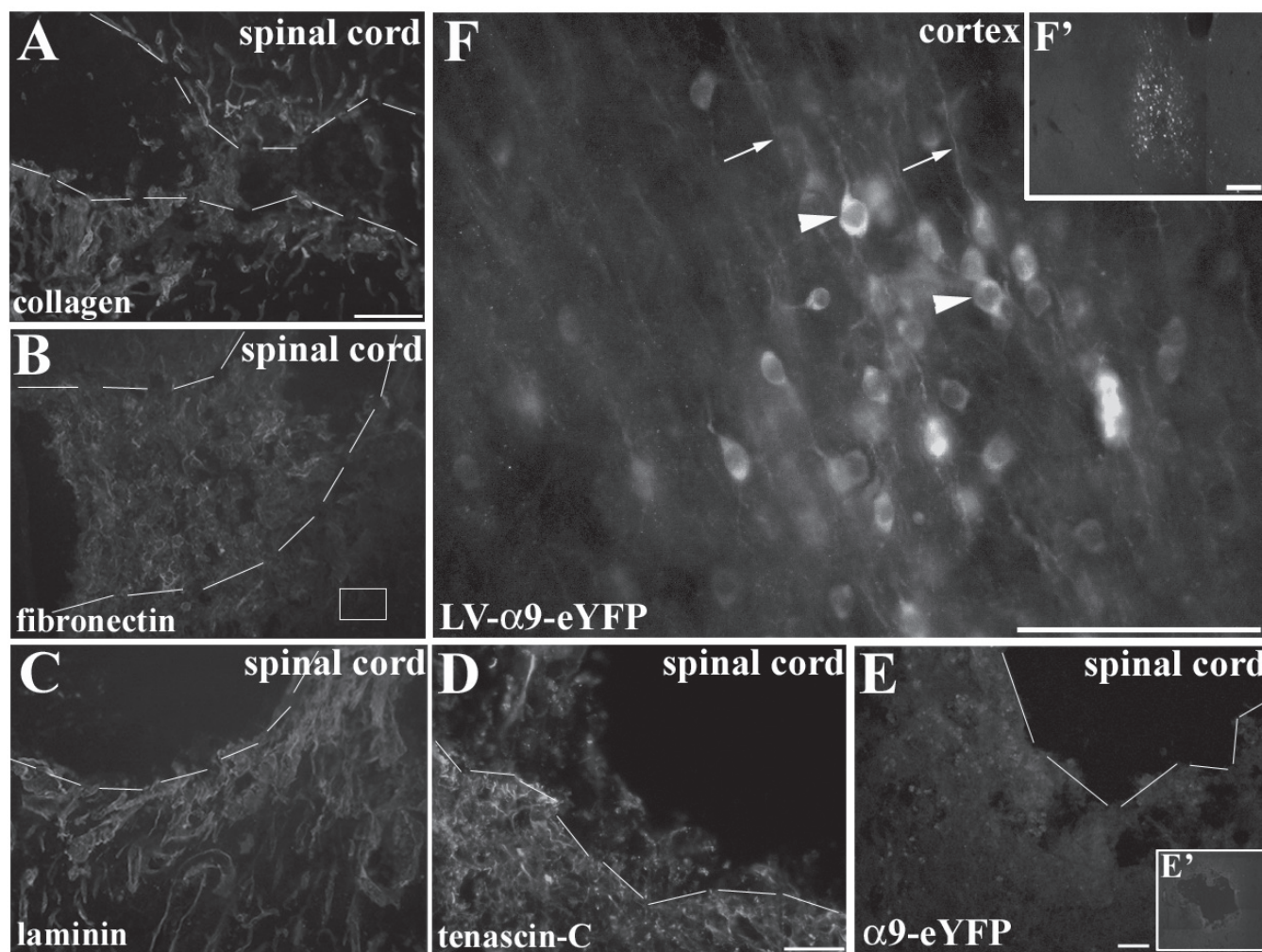
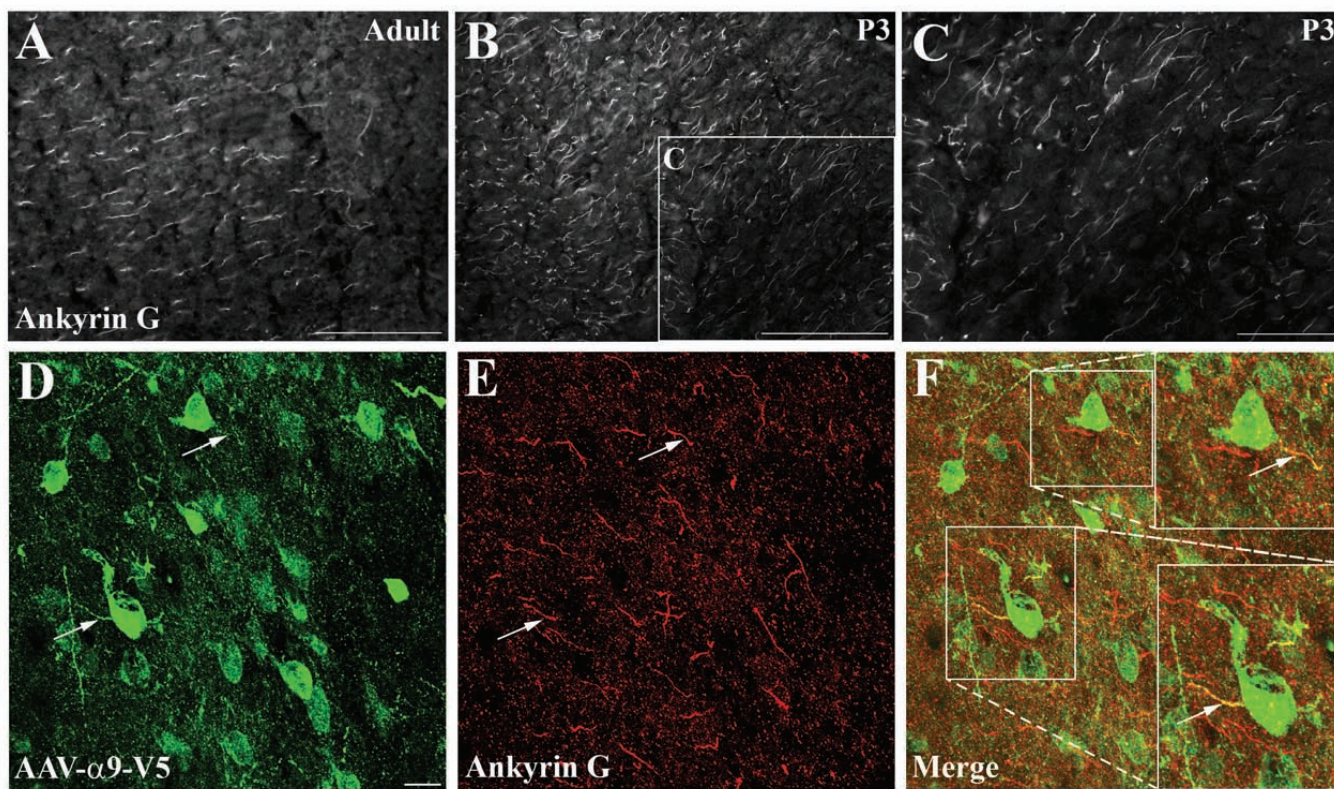


Figure 5



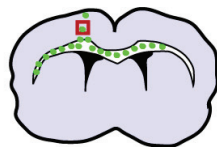
**Figure 6**



**Figure 7**

## AXONAL LOCALIZATION OF INTEGRINS

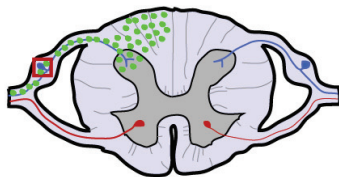
**Early postnatal ( $\leq P10$ )  
cortical neurons,  
corticospinal tract**



**Adult RGC,  
optic nerve**



**Adult DRG,  
dorsal root,  
dorsal column**



Dendrites

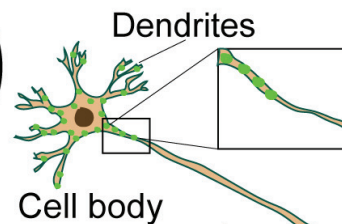
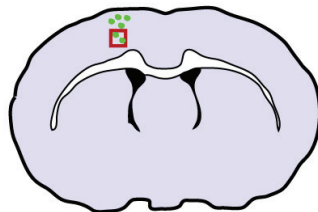
Cell body

Axon

Terminal

**Permissive  
Transport**

**Adult cortical  
neurons,  
corticospinal tract**

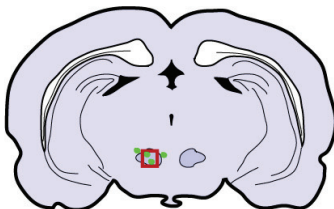


Cell body

Axon

Terminal

**Adult red  
nucleus neurons,  
rubrospinal tract**



**Restricted  
Transport**

□ Injection site

● Virally-expressed integrin