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4 **Descending systems direct**

5 **development of key spinal motor**

6 **circuits**

7 Abbreviated title: Postnatal development of spinal motor circuits

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Abstract

29 The formation of mature spinal motor circuits is dependent on both activity
30 dependent and independent mechanisms during postnatal development.
31 During this time, reorganisation and refinement of spinal sensorimotor circuits
32 occurs as supraspinal projections are integrated. However, specific features of
33 postnatal spinal circuit development remain poorly understood. This study
34 provides the first detailed characterisation of rat spinal sensorimotor circuit
35 development in the presence and absence of descending systems. We show
36 that development of proprioceptive afferent (PA) input to motoneurons (MN)
37 and Renshaw cells (RC) is disrupted by thoracic spinal cord transection (TX) at
38 postnatal day 5 (PN5). PN5TX also lead to malformation of GABApre neuron
39 axo-axonic contacts on Ia afferents and the recurrent inhibitory circuit between
40 MN and RC. Using a novel *in situ* perfused preparation for studying motor
41 control, we show that malformation of these spinal circuits leads to
42 hyperexcitability of the monosynaptic reflex. Our results demonstrate that
43 removing descending input severely disrupts development of spinal circuits and
44 identifies key mechanisms contributing to motor dysfunction in conditions such
45 as cerebral palsy and spinal cord injury.

46 *Significance statement*

47 Acquisition of mature behaviour during postnatal development correlates with
48 arrival and maturation of supraspinal projections to the spinal cord. However,
49 we know little about the role descending systems play in maturation of spinal
50 circuits. Here, we characterise postnatal development of key spinal
51 microcircuits in the presence and absence of descending systems. We show

52 that formation of these circuits is abnormal following early (PN5) removal of
53 descending systems, inducing hyperexcitability of the monosynaptic reflex. The
54 study is a detailed characterisation of spinal circuit development elucidating
55 how these mechanisms contribute to motor dysfunction in conditions such as
56 cerebral palsy and spinal cord injury. Understanding these circuits is crucial to
57 develop new and improve current therapeutics in such conditions.

58 **Introduction**

59 In most mammals, mature over ground locomotion is acquired throughout
60 postnatal development, despite the early emergence of functioning brainstem
61 and spinal locomotor circuits (Cazalets *et al.*, 1990; Van Hartesveldt *et al.*,
62 1991; Ozaki *et al.*, 1996). In rats for example, quadrupedal locomotion is
63 achieved at postnatal day (PN) 10-12, with a striking transition to motor-
64 maturity occurring at PN14 (Altman & Sudarshan, 1975). By PN21, locomotion
65 is indistinguishable from that of adult animals. This protracted acquisition of
66 over ground locomotion is thought to reflect activity dependent mechanisms of
67 motor circuit development as the animal begins to interact with its environment.
68 For example, corticospinal tract (CST) terminations only reach the grey matter
69 of caudal spinal segments at PN9-10, with innervation patterns maturing by
70 PN14, the same time as locomotion (Donatelle, 1977). Brainstem structures
71 reach the lumbar spinal cord at or shortly after birth, but also undergo
72 significant development during the first 2 weeks (Bregman, 1987; Brocard *et*
73 *al.*, 1999; Vinay *et al.*, 2005)
74 Conversely, sensory systems make functional connections with their spinal
75 targets prenatally, with reorganisation and refinement taking place PN
76 (Fitzgerald, 2005; Chakrabarty & Martin, 2011a). Proprioceptive Ia afferents

77 (PA) form functional monosynaptic connections with homonymous and
78 synergistic motoneurons (MNs) at embryonic day 19 (E19) (Kudo & Yamada,
79 1985; Chen *et al.*, 2003). This sensory-motor connectivity exhibits stringent
80 specificity that is mainly dependent upon MN positional cues and cell surface
81 signalling (Mears & Frank, 1997; Sürmeli *et al.*, 2011; Fukuhara *et al.*, 2013).
82 Whilst features such as sensory-motor fidelity appear hardwired, postnatal
83 retraction of dense PA projections in the cervical cord is thought to depend on
84 late integration of descending systems (Gibson & Clowry, 1999; Chakrabarty &
85 Martin, 2011a). This profile has never been demonstrated in the lumbar spinal
86 cord however, despite obvious functional differences, especially in fine motor
87 control.

88 Functional outcomes following neonatal injuries to descending systems
89 underline the importance of supraspinal projections to spinal sensorimotor
90 circuit development. In cerebral palsy (CP), perinatal insults to the brain lead to
91 debilitating spasticity resulting from aberrant growth and reduced modulation of
92 sensory afferents (Dietz & Sinkjaer, 2007; Rosenbaum *et al.*, 2014).

93 Interestingly, sensory afferents also contribute to the remarkable recovery of
94 locomotion seen following neonatal spinal transection in rodents (Weber &
95 Stelzner, 1977; Commissiong & Toffano, 1989; Saunders *et al.*, 1998; Petruska
96 *et al.*, 2007; Tillakaratne *et al.*, 2010; Ichiyama *et al.*, 2011). With locomotor
97 training, prospects of functional recovery in neonatally transected animals far
98 outweigh those of adult transected animals, suggesting spinal circuits in the
99 **former** are better able to utilise sensory information in the absence of
100 supraspinal control. Indeed, although sensory afferents are important to
101 recovery, Bui *et al.* (2016) show that an interposed sensorimotor microcircuit
102 become indispensable following adult spinal TX. It is vital therefore, that we

103 further understand the development of spinal sensorimotor circuits in normal
104 and injured states.

105 Recently, important steps have been taken towards understanding
106 development of specific spinal circuits. Siembab *et al.* (2016) genetically
107 manipulated PA inputs in mice to demonstrate that maturation of motor axon
108 inputs to RC is dependent upon the strength of PA input during early PN
109 development. Additionally, Mendelsohn *et al.* (2015) showed that genetically
110 blocking neurotransmission between PA and MN during development leads to
111 increased incidence of PA projections to heteronymous (synergist) MNs.

112 In this study, we provide the first detailed characterisation of spinal
113 sensorimotor circuit development in the presence and absence of descending
114 systems. We show that normal development of PA input to all regions of the
115 spinal cord, including MN and RCs is disrupted following neonatal transection.
116 This leads to malformation of microcircuits involved in recurrent inhibition of
117 MNs and presynaptic inhibition of afferents. We used an in situ, perfused whole
118 rat preparation to study physiological development of spinal motor circuits and
119 show that a lack of descending input and resultant malformation of inhibitory
120 microcircuits resulted in hyperexcitability of the monosynaptic reflex (MSR).

121 **Materials and Methods**

122 Experiments and procedures were performed in a manner that conformed to
123 the UK Home Office guidelines regarding the use of animals. Approval was
124 granted by the local ethics committee (University of Leeds).

125 **Animals**

126 Wistar rats, both male and female were used for all experiments. The ages of
127 assessment for both intact and TX rats were PN10, PN14 and PN21. These
128 ages were used because they represent significant behavioural milestones
129 based on previous literature (Altman & Sudarshan, 1975; Westerga &
130 Gramsbergen, 1993a; Westerga & Gramsbergen, 1993b). For example, at
131 PN10 (immature time point) rats are capable of some quadrupedal weight
132 bearing but locomotor abilities are minimal. At PN14 (transition time point) there
133 is a striking transition to adult-like locomotion with some deficits in speed and
134 accuracy. At PN21 (mature time point) over ground locomotor kinematics are
135 indistinguishable from the adults.

136 **Decerebrate perfused rat preparation**

137 *Preparation Set up*

138 Wistar rats aged 10-21 days PN were weighed then anaesthetised with
139 Isoflurane (95%O₂, 5% CO₂ mix, 5% induction, 2% maintenance) until loss of
140 paw withdrawal. A midline laparotomy was performed and the stomach, spleen
141 and free intestine were ligated and removed. Once evisceration was complete,
142 the animal was transferred to an ice bath containing artificial cerebrospinal fluid
143 (ACSF composition listed below) bubbled with carbogen gas (95%O₂, 5% CO₂)
144 and a pre-collicular decerebration was performed and the skin removed. The
145 animal was then transferred to a cold dissecting dish (ACSF, < 3°C), where a
146 midline sternotomy permitted access to the chest cavity. Most of the lung
147 parenchyma and thymus were removed for easy access to the heart. An
148 incision was made into the apex of the heart for later insertion of the cannula (Ø
149 1.25 mm, DLR-4, Braintree Scientific, MA, USA) before another small incision
150 was made into the right atrium to allow draining of the perfusate. The animal

151 was then transferred to the recording chamber and rested (supine) on a custom
152 sylgard (Dow Corning) bed. The cannula was immediately inserted
153 transcardially into the ascending aorta (Figure 1A). The cannula was fixed in
154 place using a single suture (no 2, silk, MERSILK). Once the cannula was
155 secured the animal was carefully transferred to the prone position and the flow
156 rate was increased gradually over 1 minute until further increases in flow failed
157 to increase pressure (~40 mmHg). The pressure before and after turning the
158 animal were compared to ensure the cannula had not retracted from the aorta.
159 The oxygenated perfusate was pumped from a flask to the preparation via a
160 heat exchanger (32°C), bubble traps and a particle filter (25µm screen,
161 Millipore) (Figure 1B). The flow was generated by a pump (Gilson Minipuls 3)
162 with the volume maintained at 200 mL. After initiation of flow, the heart
163 resumed beating immediately and the preparation started to warm up.
164 Successful perfusion was indicated by liver blanching, filling of the scull cavity,
165 atrial distension and spontaneous motor activity. 100-200 pM vasopressin (Arg-
166 8-Vasopressin, AVP, AbcamBiochemicals) was added to the perfusate to
167 increase the systemic resistance and pressure to 50-80 mmHg which has been
168 deemed sufficient for adequate perfusion of the caudal spinal cord (Pickering &
169 Paton, 2006). Respiratory contractions were observed once the pressure
170 reached 40-50 mmHg, indicating brainstem viability (Figure 2A-C). Lumbar
171 spinal cord viability was initially observed by spontaneous rhythmic hind limb
172 movements and nociceptive reflexes in response to tail and hind limb pinches
173 (Figure 2D-E). Once stimulation and recording equipment were in place, robust
174 H reflex responses confirmed viability of the lumbar cord.

175 **Solutions**

176 Modified Ringers solution (ACSF) was made up of: (mM) NaCl (125); NaHCO₃
177 (25); KCL (3); CaCl₂ (2.5); MgSO₄ (1.25); KH₂PO₄ (1.25); D-Glucose (10). A PH
178 of 7.35-7.4 was attained once carbogenated. Polyethylene glycol (1.25%) was
179 added as an oncotic agent.

180 **H reflex**

181 *Isolation of nerve*

182 Once the cannula was secure and the animal was fixed in place using ear bars,
183 a small incision was made into the fascia separating the quadriceps and
184 hamstring muscles. Using blunt dissection, the muscles were reflected, the
185 sciatic nerve carefully freed from surrounding tissue (apart from its insertion
186 into gastrocnemius) and tibial nerve located and mounted on a bipolar hook
187 electrode (Figure 3D).

188 *Stimulation and Recording Methods*

189 The nerve was stimulated via hook electrodes with a monophasic square pulse
190 generated by an ISO-Flex isolated stimulator. Square pulses (0.3ms) were
191 generated every 3 seconds using the A.M.P.I Master-8 to trigger the stimulator,
192 allowing 5s recovery between pulses. Evoked intramuscular responses were
193 recorded using fine bipolar needle electrodes (SpesMedica) inserted into the
194 gastrocnemius muscle. For each animal, graded stimulation of the nerve
195 produced a recruitment curve which was used to determine Ia afferent and
196 motor axon thresholds (Figure 3A-C). H reflex threshold was determined as the
197 intensity at which the smallest visible response occurred at a frequency of 50%.
198 H reflexes on the ascending portion of the recruitment curve (small M wave,
199 large H wave) were deemed optimal for paired pulse analysis. At the end of the
200 experiment to further verify the H reflex, an axotomy was performed by

201 severing the nerve between the hook electrode and the exit of the mixed sciatic
202 nerve from the spinal cord (Figure 3D). This severed the sensory and motor
203 nerves to and from the spinal cord but retained the motor axons between the
204 hook and recording electrodes. Axotomy demonstrated that the H reflex was
205 abolished but a strong M wave was retained (Figure 3E).

206 Paired pulse stimulation was used to assess excitability of the central circuits
207 by evaluating the H reflex and its modulation. Dual pulses were administered to
208 the nerve at intervals ranging from 1-700ms and the level of depression was
209 calculated to produce a depression curve. At least 15 traces were recorded for
210 each interval tested and there was a minimum rest period of 1 minute between
211 trials. Control (single pulse) trials, also consisting of 15 traces, preceded each
212 test trial for direct comparison and normalisation.

213 **Data Analysis**

214 EMG signals were recorded using a unit gain amplifier headstage (Digitimer,
215 NL100) connected to a Neurolog amplifier (x 1000 amplification; Digitimer,NL
216 900D). Signals were bandpass filtered between 50Hz and 10000 Hz and
217 digitised using an analog to digital interface (1401micro, CED, Cambridge,UK)
218 and the data stored on a PC. The data was visualised using Spike2 software
219 (CED, Cambridge, UK) running on the PC, where the data was saved for offline
220 analysis. Raw traces were analysed using Signal 2.7 (CED, Cambridge, UK).
221 Signal 2.7 was used to generate the H reflex trace averages (n=15) at each
222 threshold/interval. Latencies were measured as peak to peak and amplitudes
223 as the absolute maximum of the averaged traces. H waves were always
224 normalised to either M max or the amplitude of the averaged control H wave
225 (15 averaged waves).

226 **Surgical Procedures**

227 **Spinal cord transection**

228 In order to assess the development of spinal circuits in the absence of
229 descending input, neonatal (PN5) spinal cord transections were performed. To
230 correctly determine litter age with an accuracy of ± 3 hours, pregnant mothers
231 were checked 3 times per day from gestational day 19 (E19) until the litter was
232 born. Litters born overnight were not used as there could be error of up to 12
233 hours. The day of birth was called postnatal day 0 (PN0) and all transections
234 took place at PN5.

235 All the pups were separated from the mother immediately prior to surgery. Pups
236 were washed thoroughly with saline and then deeply anaesthetised (loss of
237 paw withdrawal) using isoflurane (4% induction, maintained at 1.5%). Upon
238 loss of withdrawal reflex, pups were subcutaneously injected with warm saline
239 (37°C, 200 μ l) and a midline, dorsal incision was made to the skin near the base
240 of the scapulae. The paravertebral muscles were retracted laterally to reveal
241 the mid-thoracic vertebral laminae. A mid thoracic (T8-10) laminectomy was
242 performed and the cord and meninges were completely severed using micro-
243 scissors. Completion of the transection was ensured by observing the base of
244 the vertebral canal and complete separation of the two distal stumps of the
245 spinal cord. Post mortem histological examination of the injury confirmed
246 completeness. A piece of Gelfoam® was then inserted into the cavity and the
247 wound was closed with silk sutures. Buprenorphine was administered at
248 0.1mg/kg before the pups regained consciousness to relieve pain. Pups were
249 placed in an incubator to recover fully before being returned to the mother.

250 **Cholera toxin β injections**

251 During the same surgery, cholera toxin β (7.5mg/ml) was pressure injected into
252 the gastrocnemius muscle using a glass micropipette attached to a Hamilton
253 syringe. A small incision was made over the medial gastrocnemius muscle, the
254 needle was inserted and 1 μ l was slowly injected. The needle was left in the
255 muscle for 5 minutes and retracted very slowly to prevent leakage.

256 **Immunohistochemistry**

257 **Tissue Processing**

258 Following H reflex recordings, preparations were moved to a fume hood
259 perfusion circuit and transcardially perfused with 4% paraformaldehyde in 0.1M
260 phosphate buffer. Vertebral columns containing the spinal cords of animals
261 were harvested and post fixed overnight (4% PFA). Spinal cords were
262 dissected out of the bony encasing and cryoprotected in sucrose (30% in PBS)
263 for at least 48 hours. Segments T13-S1 of the spinal cord were individually
264 separated and frozen in optimal cutting temperature compound (OCT, Leica). A
265 cryostat (1850, Leica Biosystems) was then used to cut transverse serial
266 sections (25 μ m) of L4-5 which were used for all analyses in this study.

267 All Immunohistochemical staining was conducted using the same principle
268 steps. Free floating sections were washed in phosphate buffered saline (PBS, 3
269 x 10 minutes) and then blocked in 3% normal donkey serum and phosphate
270 buffer (0.01M) with 0.2% Triton X-100 (PBST) for 1 hour before being
271 incubated overnight in primary antibodies (Table 1) for 24-48 hours. Sections
272 were then incubated in secondary antibodies which had been raised primarily in
273 donkey and against the immunoglobulin (IgG) in which the primary antibody
274 had been raised. The secondary antibodies were conjugated to Alexa 488 or
275 555 and biotinylated antibodies were revealed using avidin-Pacific Blue

276 (Invitrogen). All antibodies were diluted in blocking solution (same
277 concentrations as blocking step).

278 **Imaging and Analysis of IHC data**

279 **Confocal Imaging and quantification of boutons**

280 All images were taken using a confocal LSM 700 at 63x (oil immersion). The
281 same microscope was used for the entirety of any experiment. For most
282 analyses the pinhole was adjusted to 1 μ m optical slices and images were taken
283 as close to the centre of the cell as possible (nucleus visible). All motoneurons
284 on triplicate sections from L4-5 were imaged per animal. All stated 'n' relate to
285 animal numbers. Synaptic boutons were only included if they were in close
286 apposition to the neurone and the count was normalized to 100 μ m of the
287 neuron perimeter. Tile scans of whole sections were taken at 20x magnification
288 through 1 confocal plane and all sections were imaged using the same settings.
289 For quantification of lamina distribution of VGLUT1, 2 regions of interest (ROI)
290 were defined: dorsal (ROI^d) and intermediate (ROIⁱ). To account for changes in
291 size of the spinal cord sections with age, the ROIs were normalised to the size
292 of the section based on anatomical landmarks. The dorsal ROI box dimensions
293 were normalised by: height-25% of the length of a straight line from base of
294 dorsal column to cord dorsum, width-20% of horizontal line from base of dorsal
295 column laterally to termination of grey matter. The intermediate ROI was the
296 height of the central canal and extended from the lateral edge of the central
297 canal to the lateral termination of the grey matter. Image J particle analysis was
298 used to quantify the density of VGLUT1⁺ terminations in the defined regions.
299 First, images were converted to binary and a minimum bouton diameter of 2 μ m
300 was defined as the threshold (Ichiyama *et al.*, 2006). All measurements of

301 density were expressed as percentage coverage of the total area of the ROI
302 analysed. Motoneurons selected for analysis of synaptic coverage were chosen
303 based on size. All motoneurons were imaged, but only cells with a diameter
304 greater than 25 μ m were analysed as γ MNs tend to be under 20 μ m in diameter
305 (Ichiyama *et al.*, 2006).

306 **Z stacks and 3D reconstruction**

307 In some cases, it was necessary to use Z stack images in order to analyse cells
308 which received very few contacts, or if the contacts were mainly distributed on
309 dendrites (Mentis *et al.*, 2006). This protocol was also used for quantification of
310 axo-axonic presynaptic contacts because it is impossible to reliably identify the
311 centre of the VGLUT1⁺ terminal in the z plane. In these cases, 0.5 μ m steps
312 were used with a 63x lens through the entirety of the cell and its dendrites.
313 Images were reconstructed using Imaris 8.1 (Bitplane) and 3D rendered cells
314 were used to quantify surface area and verify terminal contact.

315 ***Analyses of VGLUT1⁺ contacts on RCs***

316 For at least 3 animals per group, RC from 3 free floating sections were
317 analysed. Total cell numbers per group were: Intact- PN10= 72, PN14= 59,
318 PN21= 56, PN5TX-PN10=74, PN14= 55, PN21= 62. The length of the
319 dendrites analysed was the first 50 μ m for every cell. 3D reconstructions were
320 quantified by normalising VGLUT1⁺ contacts to RC surface area (number of
321 VGLUT1 boutons/100 μ m²).

322 ***Analyses of P bouton contacts on VGLUT1⁺ PA***

323 3D reconstructions were quantified by normalising number of P boutons to
324 VGLUT1+ PA surface area (number of P boutons/ $10 \mu\text{m}^2$). P bouton contacts
325 were only quantified on VGLUT1+ PA that were closely apposed to ChAT+ MNs.
326 There were at least 3 animals per group, with 3 free floating sections analysed
327 per animal. The total number of VGLUT1+ PA on which P boutons were
328 quantified were: Intact- PN10= 572, PN14= 500, PN21= 474, PN5TX-PN10=
329 400, PN14= 483, PN21= 463.

330 **1.1 Statistics**

331 All statistical tests were performed using IBM SPSS 22 statistics package.
332 Tests for normal distribution of data were performed before comparisons were
333 made. In cases where the data were normally distributed, univariate analysis of
334 variance tests were performed with Bonferroni post hoc for pairwise
335 comparisons. For data that were not normally distributed, Kruskal-Wallis tests
336 were used with Bonferroni corrections performed on multiple comparisons
337 produced by Dunns tests. For comparing Hmax/Mmax or threshold values
338 between intact and PN5TX at PN14 only, Mann-Whitney U tests were
339 performed.

340 **Results**

341 To create a longitudinal profile of sensorimotor circuit development, we
342 targeted ages PN10, 14 and 21. At PN10 motor control in rats is immature.
343 PN14 marks a dramatic transition to near mature motor control and at PN21 the
344 motor system is considered largely indistinguishable from adult rats.

345 **Normal development of proprioceptive afferents in the presence** 346 **and absence of descending systems**

347 In the cervical spinal cord, proprioceptive afferents (PA) and descending
348 systems develop in an activity-dependent, competitive manner. The cervical
349 cord is functionally distinct from the lumbar cord of the rat in that the forelimbs
350 are used for skilled tasks (grasping and manipulation) as well as locomotion.
351 Reaching and grasping involves fine control of small muscles in the forepaws
352 whereas the lumbar spinal circuitry is less associated with such fine control.
353 This led us to investigate whether PA in the lumbar cord develop differently
354 from the cervical cord and if their developmental profile was dependent on the
355 presence of descending systems.

356 **Normal PN development of PA innervation of the lumbar cord.**

357 Using an antibody against vesicular glutamate transporter 1 (VGLUT1) to
358 identify PA, density of terminals was assessed in dorsal and intermediate
359 regions of interest in the spinal cord, as well as directly onto MNs. Results
360 showed reduced VGLUT1⁺ terminal density in dorsal, intermediate and ventral
361 regions (MNs) of the spinal cord between PN10 and 14, however in most cases
362 there was little change between PN14 and 21. In the dorsal horn there was a
363 significant effect of development on PA termination densities ($p=0.008$, Figure
364 4H). Densities were significantly greater in PN10 animals ($63.64 \pm 1.46\%$, $n=3$)
365 compared to PN14 ($39.11 \pm 3.76\%$, $p=0.012$, $n=4$,) and PN21 ($42.31 \pm 5.27\%$,
366 $p=0.023$, $n=5$, Figure 4H) but there was no difference between PN14 and 21
367 ($p=.830$), suggesting development of these inputs ceases at this stage. In the
368 intermediate region, there was also a significant effect of age on VGLUT1⁺
369 terminal density ($p=0.020$, Figure 4I). Similar to the dorsal horn, there was a
370 reduction in VGLUT1⁺ terminal density between PN10 ($14.31 \pm 3.32\%$) and
371 PN14 ($3.78 \pm 0.70\%$, $p=0.029$) and PN21 (5.59 ± 0.65 , $p=0.049$) but there was

372 no difference between PN14 and PN21 ($p=0.803$). We appreciate that in the
373 dorsal and intermediate grey, VGLUT1⁺ boutons are also sourced from the CST
374 and low threshold mechanosensory fibres, which both undergo significant
375 postnatal changes (Varoqui *et al.*, 2002; Todd *et al.*, 2003; Fitzgerald, 2005;
376 Martin, 2005; Du Beau *et al.*, 2012). However, in contrast to both proprioceptive
377 and cutaneous afferents, CST axons proliferate in the lumbar grey between
378 PN10 and 14, indicating that reduced VGLUT1⁺ bouton density in dorsal and
379 intermediate regions is solely due to afferent retraction, and most likely
380 underestimated (Donatelle, 1977; Joosten *et al.*, 1989).

381 In the ventral horn, there was a significant effect of age ($p=0.005$) on VGLUT1⁺
382 terminal density in close apposition with MNs (Figure 4J). At PN10 (4.51 ± 0.24
383 boutons/100 μ m soma perimeter, $n=3$, Figure 4E) the density of VGLUT1⁺
384 boutons on MNs were greater than at PN14 (3.46 ± 0.26 , $p=0.031$, $n=3$, Figure
385 4F) and PN21 (3.1 ± 0.26 , $p=0.006$, $n=3$, Figure 4G), however there was no
386 difference between PN14 and 21 ($p=0.632$).

387 **Neonatal transection prevents retraction of proprioceptive afferents in the** 388 **lumbar spinal cord.**

389 Following PN5TX, retraction of VGLUT1⁺ terminals observed during normal
390 development was completely abolished. In dorsal and intermediate regions,
391 CST axons and cutaneous afferents are also VGLUT1⁺ (Alvarez *et al.*, 2004; Du
392 Beau *et al.*, 2012). Considering that CST is absent following TX and the only
393 other sources of this VGLUT1 are proprioceptive and cutaneous afferents, we
394 can conclude that developmental retraction of afferents is abolished in the
395 absence of descending systems. In the dorsal region, there was no difference
396 between intact and TX groups at PN10 (Intact= 63.64 ± 1.46 , TX= 67.33 ± 6.63 ,

397 $p=.512$, $n=3$, Figure 4B & B') however at both PN14 (Intact= 39.10 ± 3.75 , TX=
 398 51.23 ± 2.10 , $p=0.030$, $n=3$, Figure 4C & C') and PN21 (Intact= 42.32 ± 5.27 ,
 399 TX= 60.88 ± 1.46 , $p=0.030$, $n=3$, Figure 4D & D') transected groups had
 400 greater densities of VGLUT1⁺ puncta (Figure 4H). For intermediate regions
 401 (Figure 4I), again the normal developmental trend was abolished for VGLUT1⁺
 402 boutons ($p=0.356$). There was no difference between groups at PN10 either
 403 (intact= 14.31 ± 3.32 , TX= 7.88 ± 5.02 , $p=0.645$). At PN14 there was also no
 404 difference between intact and TX groups (intact= 3.78 ± 0.70 , TX= 9.67 ± 1.26 ,
 405 $p=0.060$). At PN21 neonatal TX resulted in a greater density of VGLUT1 puncta
 406 (intact= 5.59 ± 0.65 , TX= 14.33 ± 0.34 , $p= 0.010$).

407 On MNs, significant retraction of PA boutons during normal development was
 408 abolished by PN5TX ($p=.514$, Figure 4E-G' & J). At PN10 VGLUT1⁺ puncta
 409 densities were not different between groups (Intact= 4.508 ± 0.25 , TX= $4.53 \pm$
 410 0.44 , $p= .966$, $n=3$, Figure 4E-E'), however TX groups had greater densities at
 411 both PN14 (Intact= 3.46 ± 0.28 , TX= 4.99 ± 0.44 , $p= 0.009$, $n=3$, Figure 4F-F')
 412 and PN21 (Intact= 3.14 ± 0.28 , TX= 4.18 ± 0.36 , $p= 0.034$, $n=3$, Figure 4G-G').

413 **Descending systems are necessary for retraction of PA from** 414 **Renshaw cells**

415 A lack of normal retraction of afferents from dorsal and intermediate zones as
 416 well as MNs suggests that interneuron populations may also be affected by
 417 PN5TX. In order to test this, we assessed VGLUT1⁺ terminal density on
 418 Renshaw cells, which reportedly originate exclusively from PA afferents (Mentis
 419 *et al.*, 2006).

420 **PA terminals are retracted from RCs during PN development**

421 Antibodies against Calbindin D-28k revealed Renshaw cells and extensive
422 portions of their proximal dendrites in the ventral horn of the lumbar spinal cord
423 (Figure 5A-A’). Because PA terminals on RCs are sparse, and mainly
424 distributed on their dendritic processes, 3D reconstructions of Calbindin⁺ RCs
425 and PA boutons were used to quantify terminals contacting the soma and
426 dendrites (Figure 5A-B’). During normal development, there was a significant
427 retraction of PA terminals from RCs ($p= 0.014$). This was true for the soma ($p=$
428 0.004 , Figure 5F), dendrites ($p= 0.003$) and cell overall ($p= 0.05$). For the whole
429 cell (soma and dendrites combined), there was a reduction in PA terminations
430 on RCs between PN10 (1.21 ± 0.06 per $100\mu\text{m}^2$, $n=3$) and PN14 (0.68 ± 0.15 ,
431 $n=3$, $p= 0.011$) and PN10 and PN21 (0.63 ± 0.11 , $n=3$, $p= 0.008$), however this
432 reduction in PA afferent input plateaus at PN14 as there was no significant
433 difference between PN14 and 21 ($p= 0.76$). On the soma, there was a
434 significant difference between PN10 (0.72 ± 0.02) and PN14 (0.52 ± 0.09 , $p=$
435 0.043) and PN21 (0.27 ± 0.02 , $p= 0.001$). Additionally, there was a decrease
436 between PN14 and 21 ($p= 0.022$). For the dendrites, we saw a significant
437 reduction in PA contacts between PN10 (1.92 ± 0.08) and PN14 ($1.17 \pm .14$, $p=$
438 0.002) and PN21 (1.15 ± 0.06 , $p= 0.002$), however there was no difference
439 between PN14 and 21 ($p= 0.94$).

440 **Disrupted development of PA boutons on RCs following neonatal** 441 **transection**

442 In the PN5TX group, there was no effect of age on PN development of PA
443 terminals contacting RCs ($p= 0.144$), suggesting development was significantly
444 disrupted. The effect of age was abolished on the soma, and there were no
445 significant differences between intact and TX groups at any of the ages

446 compared (PN10 intact= 0.716 ± 0.02 per $100\mu\text{m}^2$, TX= 0.66 ± 0.12 , n= 3, p=
447 0.62, PN14 intact= 0.52 ± 0.09 , TX= 0.76 ± 0.06 , n= 3, p= 0.057 and PN21
448 intact= 0.27 ± 0.02 , TX= 0.43 ± 0.11 , p=0.213, n=3, Figure 5F). On the proximal
449 dendrites of RCs, again PN5TX acted to disrupt retraction of PA. When each
450 time point was considered, intact and TX groups were not statistically different
451 at PN10 (intact= 1.92 ± 0.08 per $100\mu\text{m}^2$, TX= 1.85 ± 0.27 , p= 0.768, Figure
452 5G) and PN21 (intact= 1.15 ± 0.07 , TX= 1.23 ± 0.27 , p= 0.774) but the TX
453 group had greater PA puncta density at PN14 (intact= 1.17 ± 0.14 , TX= $1.80 \pm$
454 0.08 , p= 0.027, Figure 5D-D', G). When values for dendrites and soma were
455 combined to evaluate the whole cell, again development was shown to be
456 significantly disrupted (p= 0.063). There was no significant difference between
457 intact and TX groups at PN10 (intact= 1.21 ± 0.07 per $100\mu\text{m}^2$, TX= $1.07 \pm$
458 0.10 , p=.454) or PN21 (intact= 0.63 ± 0.10 , TX= 0.92 ± 0.05 , p=.454) but at
459 PN14 the transection group had a greater density of PA boutons apposing
460 Renshaw cells (intact= 0.67 ± 0.15 , TX= 1.36 ± 0.16 , p= 0.002). Therefore, we
461 show that PN5TX disrupts development of PA inputs to RCs due to significantly
462 greater density at PN14.

463 **Neonatal transection disrupts the normal formation of motor axon**
464 **collaterals on Renshaw cells.**

465 Motor axon collaterals provide excitatory drive to adult RCs in a recurrent
466 inhibition circuit exhibiting remarkable efficacy (Moore *et al.*, 2015). Because of
467 the lack of PA retraction following PN5TX shown above, we wanted to assess if
468 this had any bearing on the development of motor axon inputs to RCs. This
469 was done by quantifying VACHT⁺ boutons on RCs (Figure 6A-E) throughout
470 development in the presence and absence of descending systems.

471 Intact animals did not exhibit a significant effect of age on RC VACHT⁺
472 terminals, suggesting that these remained relatively stable throughout
473 development (Figure 6E). In the PN5TX group, there was a significant effect of
474 age ($p= 0.005$) suggesting that PN5TX significantly altered the developmental
475 profile. Comparisons of values for the two groups at each age revealed no
476 significant differences at PN10 (Intact= 3.86 ± 0.64 per 100 μm soma perimeter,
477 $n=3$ TX= 3.94 ± 0.33 , $n=3$) or 14 (Intact= 4.50 ± 0.82 , $n=3$, TX= 4.45 ± 0.12 ,
478 $n=3$, $p= 0.954$) however there were significantly fewer VACHT⁺ boutons on RCs
479 from PN5TX spinal cords at PN21 (intact= 4.77 ± 0.38 , $n=3$, TX= 2.78 ± 0.18 ,
480 $n=3$, $p= 0.013$).

481 In summary, it is likely that the lack of PA retraction following PN5TX results in
482 a subsequent decrease in motor axon synapses (Figure 6F-F').

483 **Postnatal development of GABApre projections to PA terminals in** 484 **the presence and absence of descending systems.**

485 Proprioceptive afferent activity is heavily modulated by axo-axonic contacts (P
486 boutons) from GABApre neurons, via presynaptic inhibition (PAD) (Hughes *et*
487 *al.*, 2005; Rudomin, 2009). It has recently been suggested that differentiation of
488 GABApre projections depend upon availability of target PA terminal and local
489 glutamate signalling (Betley *et al.*, 2009; Mende *et al.*, 2016). The extreme
490 developmental retraction of PA and lack thereof following PN5TX provided a
491 unique assay to characterise the developmental profile of GABApre projections
492 and their dependence on both sensory and descending inputs. GAD65⁺
493 boutons contacting PA terminals, which directly apposed MNs were quantified
494 using 3D IMARIS reconstructions. All PA terminals contacting MNs were
495 analysed (Figure 7A-B'').

496 **GABApre projections (P boutons) proliferate as PA terminals are**
 497 **retracted in normal development.**

498 We found that while PA were retracted with normal development, the density of
 499 P boutons on PA terminals significantly increased with age ($p = 0.0001$, Figure
 500 7I). Interestingly, this increase occurred between PN10 (0.45 ± 0.05 , $n=3$, Fig
 501 7C-C'') and PN14 (1.04 ± 0.03 , $n=3$, $p = 0.0001$, Figure 7D-D'') with it reaching
 502 its maximal limits between PN14 and PN21 (1.04 ± 0.03 , $n=3$, $p=0.997$, Fig 7E-
 503 E''), when the PA retraction is maximal. PN10 and 21 were also significantly
 504 different from each other ($p= 0.0001$). At PN10, 41.61 % (± 2.65) of Ia boutons
 505 directly apposing MNs were devoid of P boutons; this was reduced to 11.72%
 506 (± 1.91) at PN14 ($p= 0.0001$) and 6.37% (± 0.14) at PN21 ($p= 0.0001$, Figure
 507 7K). There was a concomitant increase ($p<0.0001$) in the quantity of GABApre
 508 clusters on PA terminals containing 3 or more P boutons (3+ clusters, Figure
 509 7J) between PN10 (7.86 ± 0.89) and PN14 (38.84 ± 3.99 , $p= 0.0001$) and PN21
 510 (44.47 ± 3.44 , $p= 0.0001$) but again there was no change between PN14 and
 511 21 ($p= 0.06$). If PA terminal surface area was substantially increased with age,
 512 it could be argued that younger animals have less available space for P
 513 boutons to contact and this could be responsible for reduced 3+ clusters at
 514 PN10. Our analysis showed that there was no change in Ia afferent terminal
 515 surface area between PN10 (intact- $21.77 \pm 0.89 \mu\text{m}$, TX- $20.68 \pm 1.54 \mu\text{m}$) and
 516 PN14 (intact- $22.80 \pm 0.66 \mu\text{m}$, TX- $22.81 \pm 1.04 \mu\text{m}$) however, there was an
 517 increase between PN10 and PN21 (intact $26.54 \pm 0.57 \mu\text{m}$, $p= 0.006$, TX- 24.90
 518 $\pm 0.85 \mu\text{m}$, $p=0.03$). There was no difference between intact and TX groups at
 519 any age.

520 **Neonatal transection significantly attenuates proliferation of**
 521 **GABApre projections to PA terminals**

522 The results above show that during normal development, GABApre projections
523 proliferate as Ia afferents are retracted. Because PA terminal density is
524 increased following PN5TX, we assessed the impact this had on P bouton
525 density.

526 We found no difference in quantity of P boutons closely apposing PA terminals
527 between intact and PN5TX animals at PN10 (intact= 0.45 ± 0.048 , TX= $0.36 \pm$
528 0.032 , $n=3$, $p= 0.255$, Figure 7C-F") just as we saw no difference in PA density
529 at this stage. In contrast, there were significantly fewer P boutons in the TX
530 group at PN14 (intact= 1.04 ± 0.03 , TX= 0.56 ± 0.02 , $n=3$, $p= 0.001$, Figure 7D-
531 G") and PN21 (intact= 1.04 ± 0.03 , TX= 0.75 ± 0.12 , $n=3$, $p= 0.004$, Figure 7E-
532 H"). This was due to a greater proportion of PA terminals devoid of P boutons
533 at PN14 (intact= $11.79 \pm 1.95\%$, TX= $33.36 \pm 1.42\%$, $p= 0.002$) and PN21
534 (intact= $6.37 \pm 0.14\%$, TX= $23.61 \pm 6.91 \%$, $p= 0.008$). Similarly, PA terminals
535 apposed by clusters of >3 P boutons were also reduced at these ages (PN14-
536 intact= $38.84 \pm 3.99\%$, TX= $18.10 \pm 1.03\%$, $p= 0.001$, PN21- intact= $44.47 \pm$
537 3.45% , TX= $27.61 \pm 4.22\%$, $p= 0.001$). It is important to note however, that
538 despite this attenuation there was still a significant increase in P bouton density
539 in the PN5 TX group between PN10 and 21 (PN10- 0.36 ± 0.03 , PN21- $0.75 \pm$
540 0.12 , $p= 0.001$).

541 In summary, our analyses show that as PA terminals are retracted during
542 normal development, GABApre projections proliferate. This profile is severely,
543 but not completely attenuated by PN5TX (Figure 8).

544 **Hyper-excitability of the monosynaptic reflex following neonatal** 545 **transection.**

546 Our anatomical data shows reduced retraction of afferents, reduced modulatory
547 input from GABApre neurons and reduced motor axon input to RCs, collectively

548 suggesting that development in the absence of descending systems induces
549 hyper-excitability of the lumbar sensorimotor circuitry. We tested this
550 hypothesis by assessing H reflexes in a perfused whole rat preparation which is
551 viable throughout PN development (first described by Pickering and Paton
552 (2006)). The development of this preparation was vital to the success of these
553 experiments. The decerebration was necessary to negate the variable effects
554 of anaesthetics, such as ketamine on the developing CNS. Additionally, the low
555 blood volume of neonates means haemorrhaging upon severance of major
556 vessels (as occurs with decerebration) leads rapidly to hypovolaemic death.
557 Our perfused preparation allowed full control of the systemic volume, pressure
558 and oxygenation levels and as a result, greater success rates without the need
559 for anaesthesia.

560 Interestingly, we were not able to evoke H reflexes at PN10. H reflexes were
561 also absent at this age in ketamine anaesthetised rats (data not shown). H
562 reflex amplitudes, thresholds and interactions did not vary greatly between
563 PN14 and 21 in intact or TX groups, so these data were pooled. Therefore, H
564 reflexes were assessed between intact and PN5TX rats aged PN14-21.
565 Thresholding revealed that the excitability of the monosynaptic reflex was
566 increased in PN5TX animals due to lower thresholds for evoking H reflex
567 responses (intact = 37.3 ± 6.6 mA, n=6, TX= 23.9 ± 3.2 mA, p= 0.050, n=7,
568 Figure 9A). Similarly, PN5TX resulted in a significantly greater Hmax/Mmax
569 ratio (intact= 0.25 ± 0.04 mV, n=5, TX= 0.39 ± 3.21 mV, n=4, p= 0.046, Figure
570 9B). There were no significant differences between groups in H reflex latency.
571 For assessing homonymous paired pulse inhibition between groups we used 7
572 inter-pulse intervals from 700-1ms. The evoked H reflex response was
573 extremely low at intervals between 10 and 1 ms, presumably due to axonal

574 collisions. There were significant differences, however, at intervals 700 to 50
575 ms and an overall effect of PN5TX on paired pulse depression. At each time
576 interval, PN5TX animals displayed significantly attenuated PPD, resulting in
577 test responses with greater amplitudes represented as a percentage of the
578 control (single pulse) when compared to intact animals (**700ms**-intact= $53.24 \pm$
579 2.07% , $n=4$, TX= $73.47 \pm 7.12\%$, $n=3$, $p= 0.026$, **200ms**- intact= $46.80 \pm 2.32\%$,
580 $n=6$, TX= $74.62 \pm 13.33\%$, $n=4$, $p= 0.001$, **50ms**- intact= $29.64 \pm 4.14\%$, $n=6$,
581 TX= 46.81 ± 4.14 , $n=4$, $p= 0.040$, Figure 9C-D').

582 **Discussion**

583 This study demonstrates that neonatal transection precludes normal
584 development of spinal sensorimotor circuits (Figure 10). We show postnatal
585 retraction of VGLUT1⁺ boutons from dorsal, intermediate, and ventral regions of
586 the lumbar cord, which is completely absent following TX. Importantly, TX also
587 induced malformation of key modulatory microcircuits responsible for
588 presynaptic inhibition of afferents and recurrent inhibition, leading to
589 hyperexcitability of the H reflex in maturity. Therefore, we not only characterise
590 important features of spinal circuit development, but also highlight mechanisms
591 contributing to motor dysfunction in conditions such as cerebral palsy and
592 spinal cord injury.

593 **Developmental retraction of afferents from the lumbar spinal cord is** 594 **dependent upon the presence of descending systems.**

595 Postnatal development of PA in the cervical spinal cord has been well studied
596 in the rat and cat, but the functionally different lumbar segments have never
597 been assessed. Gibson and Clowry (1999) showed retraction of PA from the
598 ventral horn during development of the rat cervical spinal cord, while

599 Chakrabarty and Martin (2011a) identified refinement of PA in dorsal and
600 intermediate regions of the cat cervical spinal cord. We show that early
601 postnatally, when locomotion is immature, PA input to lumbar MNs is dense but
602 subsequently retracted as locomotion becomes mature (PN14-21). At the same
603 time points, retraction of VGLUT1⁺ puncta was also seen in dorsal and
604 intermediate regions as well as on RCs. It is important to note, however, that
605 Siembab *et al.* (2016) showed that not all interneuron populations are subject to
606 PA retraction. In addition, development of cutaneous fibres in dorsal and
607 intermediate regions (Fitzgerald, 2005) may have contributed to VGLUT1
608 retraction.

609 Our results showing retraction of PA from RC is consistent with previous work
610 demonstrating that PA innervate and activate RCs early postnatally, but inputs
611 in adulthood are greatly reduced and not capable of activation (Renshaw, 1946;
612 Eccles *et al.*, 1957; Mentis *et al.*, 2006).

613 The mechanisms responsible for afferent pruning during development of the
614 cervical spinal cord are not fully understood, but thought to be related to
615 competition with descending systems (Gibson *et al.*, 2000; Clowry *et al.*, 2004;
616 Martin, 2005; Clowry, 2007; Chakrabarty & Martin, 2011b). An important
617 consideration here is that cervical motor circuits control muscles responsible for
618 reaching and grasping, which require fine control of forelimb muscles and are
619 therefore critically dependent upon interactions between descending and
620 sensory systems (Alstermark & Isa, 2012). The lumbar spinal cord is less
621 associated with fine motor control and therefore may not develop in the same
622 manner. Despite these functional differences between cervical and lumbar
623 cords, we found that lumbar PA development is affected similarly to what has
624 previously been described for cervical PA after altering descending inputs.

625

626 ***Neonatal transection prevents postnatal retraction of proprioceptive***
627 ***afferents.***

628 In contrast to normal development, we show that in PN5TX rats, PA fail to
629 retract. The physiological manifestation of this anatomical finding was reduced
630 H reflex thresholds compared to intact counterparts.

631 It is appreciated that afferent sprouting in mature spinal cords can be induced
632 by lesions to descending tracts or supraspinal regions (Liu & Chambers, 1958;
633 Nelson & Mendell, 1979; Krenz & Weaver, 1998; Tan *et al.*, 2012). However,
634 the mechanisms leading to increased afferent input following neonatal lesions
635 may be different. Adult injuries are sustained when lumbar spinal circuits are
636 fully mature, having been organised under the influence of both descending
637 and sensory input. When the same injury is sustained neonatally, locomotor
638 circuits are immature and subsequently develop in the presence of sensory
639 input alone. Considering that we show PA retraction during normal
640 development, it is likely that following PN5TX afferents fail to retract rather than
641 sprout. This is consistent with the work of Levinsson *et al.* (1999) who found
642 that neonatal transection in rats results in immature nociceptive reflexes in
643 adulthood. In this regard, it is reasonable to suggest that descending input to
644 the lumbar cord is necessary for normal development of sensorimotor systems.
645 Indeed, the CST does not innervate the lumbar cord until PN8-9 meaning it has
646 no influence on spinal circuit development in PN5TX rats (Donatelle, 1977).
647 Furthermore, brainstem projections are present in the lumbar cord early PN,
648 but do not mature until the end of the 2nd postnatal week and thus also have
649 little influence on spinal circuit development in PN5TX rats (Bregman, 1987;
650 Vinay *et al.*, 2005). It could be argued that brainstem systems have greater

651 influence on developmental input of PA to ventral motor circuits, as CST lacks
652 direct functional connections here (Alstermark *et al.*, 2004). Indeed, Basaldella
653 *et al.* (2015) recently showed that genetically reducing vestibular sensation
654 using Nox3 mutant mice lead to greater densities of VGLUT1⁺ boutons on MNs.
655 Alternatively, a lack of retraction may have resulted from homeostatic
656 mechanisms serving to maintain functional levels of excitability following
657 PN5TX. In support of this hypothesis, Mendelsohn *et al.* (2015) showed that
658 significantly reducing sensory-motor synaptic transmission, without affecting
659 input density, resulted in sprouting of afferent collaterals onto synergistic,
660 heteronymous motoneurons. This, combined with other recent literature
661 suggests that development of PA projection densities is mediated by a
662 combination of activity, positional cues and recognition signals (Sürmeli *et al.*,
663 2011; Bikoff *et al.*, 2016). However, further work is needed to understand these
664 mechanisms in the context of spasticity in conditions such as CP and SCI.

665 ***Neonatal transection disrupts formation of Renshaw cell - motoneuron***
666 ***circuit***

667 Renshaw cells powerfully modulate motor output by forming a remarkably
668 efficient recurrent inhibitory circuit with MNs (Bhumbra *et al.*, 2014; Moore *et*
669 *al.*, 2015). During normal development, both motor axon and PA synapses
670 proliferate on RCs up to PN15, after which PA terminals retract (Mentis *et al.*,
671 2006a). Siembab *et al* 2016 recently showed that genetically up or down
672 scaling proprioceptive inputs to RCs significantly regulated the development of
673 motor axon input density. In our study, PA input to RCs was increased as a
674 result of PN5TX. In agreement with Siembab *et al* (2016), between PN14 and
675 21 motor axon synapses on RCs were severely reduced following TX,
676 suggesting that similar mechanisms are involved. The fact that there was no

677 difference in PA input between intact and PN5TX animals at PN21 suggests
678 that retraction from RCs was restored. Therefore, considering the loss of motor
679 axon input between PN14-21, RCs experience a significant developmental loss
680 of excitatory input following PN5TX, which could be an important determinant of
681 spasticity in our model and CP.

682 **Neonatal transection severely disrupts development of GABApre**
683 **neuron projections.**

684 Accurate control of movement depends on the gating and directing of sensory
685 information in the spinal cord. This control is mediated by GABApre
686 interneurons exerting presynaptic inhibition via axo-axonic projections (P
687 boutons) to sensory terminals (Frank & Fuortes, 1957; Eccles *et al.*, 1961;
688 Hughes *et al.*, 2005; Rudomin, 2009). Neonatal animals often display poorly
689 directed, exaggerated responses to sensory stimuli (Weed, 1917; Stelzner,
690 1971) which are attenuated with PN development, suggesting refinement of
691 afferent projections and/or their modulation. While PN afferent retraction has
692 been demonstrated, development of axo-axonic GABApre contacts on sensory
693 terminals is not understood. In adult cats, Pierce and Mendell (1993) showed
694 that 86% of Ia terminals have P boutons, but it is not known when this profile is
695 established. Betley *et al.* (2009) demonstrate that GABApre projections express
696 stringent specificity for sensory terminals, shunning MNs even when PA
697 terminations were genetically reduced using Er81^{-/-} mutant mice. Further, the
698 lack of available targets resulted in significant retraction of GABApre
699 projections from the ventral horn. Our data shows co-development of PA
700 terminals and P boutons, with both reaching a plateau with motor maturity
701 (PN14-21). However, P bouton density significantly increased as PA terminals
702 were retracted, showing an inverse rather than direct relationship (Fig 7).

703 Between PN10 (41.61%) and 21 (6.37%) there was a 35.24% decrease in PA
704 terminals lacking P boutons, indicating that the increase is due to greater
705 GABApre projections to PA, rather than a redistribution of P boutons from
706 retracted PA terminals. This suggests that regulation of GABApre projections
707 cannot be governed solely by local spinal mechanisms. Indeed, adult sacral
708 spinal cord TX also results in P bouton retraction, even though PA terminal
709 density remains unchanged (Kapitza *et al.*, 2012).

710 Following PN5TX in our study, P boutons apposing PA terminals were severely
711 reduced, but these axo-axonic contacts still proliferated with development.

712 Interestingly, Mende et al (2016) showed that the efficacy of presynaptic
713 inhibition can be regulated by local glutamate and BDNF signalling between
714 sensory terminals and P boutons. Reducing VGLUT1 availability lead to
715 reduced presynaptic inhibition via downregulation of GAD65/67 in GABApre
716 boutons. Given the high PA input and reduced paired pulse depression seen in
717 our study, similar mechanisms may be responsible for the developmental
718 increase in P bouton density despite PN5TX.

719 Our findings contribute detail and depth towards comprehension of postnatal
720 sensorimotor circuit formation. Although the core locomotor circuitry is
721 functional prenatally, acquisition of mature organisation and therefore
722 behaviour, depends on postnatal integration of descending systems. By
723 removing descending input early postnatally, development of spinal
724 sensorimotor circuits is severely disrupted leading to hyperreflexia. We also
725 identify several features of the sensorimotor circuitry which contribute directly to
726 hyperreflexia. Similar mechanisms have previously been shown to contribute to
727 spasticity in adult injuries, however direct comparisons with neonatal
728 transections will be needed to identify differences and similarities. It is likely

729 that after neonatal injuries, the same circuits contributing to spasticity may also
 730 contribute to enhanced functional recovery, thus it is crucial that we target
 731 these circuits in order to better understand and treat perinatal and adult lesions
 732 to descending systems.

733 **Author contributions**

734 R.M.I and S.C devised the project and designed experiments. C.C.S designed
 735 and performed experiments, including all data collection and analysis. In situ
 736 preparation was learned initially at the lab of J.F.R.P, who also provided advice
 737 as C.C.S further developed it for this project. C.C.S prepared the manuscript
 738 with input from R.M.I, S.C and J.F.R.P.

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1027 **Figure Legends**
1028
- 1029 **Table 1. Antibodies, sources and concentrations.**
1030
- 1031 **Figure 1. Preparation set up.** (A) Schematic of animal set up with cannulation, recording and
1032 stimulation sites shown. Animal is in prone position. (B) Components of the perfusion circuit
1033 and flow of ACSF. Red line represents flow from reservoir to preparation and the blue line
1034 represents the return flow. 2 pumps are shown for clarity, all tubing actually inserts into a single
1035 pump.
1036
- 1037 **Figure 2. Establishing preparation viability.** (A) Vasopressin increases systemic pressure
1038 leading to higher frequency of respiratory contractions. Red box shows respiratory contractions
1039 at low, non-viable pressure, green box indicates high, viable pressure. (B) Shows weak, low
1040 frequency respiratory contractions at low pressure. (C) Respiratory frequency markedly
1041 increases with increased pressure indicating brainstem viability. (D-E) Motor (EMG) outputs
1042 from the left gastrocnemius in response to toe pinch at viable and non-viable systemic
1043 pressures.
1044
- 1045 **Figure 3. Stimulation and recording set up.** (A-B) shows typical H reflex recruitment curves,
1046 demonstrating classical H and M wave responses to increased stimulation strength. (C) Typical
1047 H reflex responses to graded stimulation. (D) Experimental setup showing stimulation,
1048 recording and axotomy sites. (E) H reflex was confirmed by severing the sciatic nerve at the
1049 site marked in (D), resulting in loss of H wave but unaffected M wave.
1050
- 1051 **Figure 4. Postnatal development of VGLUT1⁺ terminations in the lumbar spinal cord.** (A)
1052 Typical VGLUT1⁺ staining with dorsal, intermediate and ventral regions of interest marked. (A')
1053 Images were converted to binary for assessment of bouton density in dorsal and intermediate
1054 regions of interest. (B-D') Representative heat maps of VGLUT1⁺ puncta densities at PN10-21
1055 in intact and PN5TX rats. Thresholding is based on maximum and minimum densities. (E-G')
1056 Representative images of MNs contacted by PA boutons throughout development in intact and
1057 PN5TX rats. (H-J) Quantification of boutons in dorsal (H) and intermediate (I) regions of interest
1058 as well as on MNs (J). Scale bars: A=200µm, E=10 µm.
1059

1060 **Figure 5. Postnatal development of PA boutons on Renshaw cells.** (A-B'') 3D
 1061 reconstruction of Renshaw cell and PA contacts created using IMARIS software. (C-E')
 1062 Representative RCs and PA contacts at PN10-21 in intact and PN5 TX rats. (F-H)
 1063 Quantification of boutons contacting the soma, dendrites and the whole cell for both intact and
 1064 PN5 TX rats at each age. Scale bars: A''& C =10 μ m, B=2 μ m

1065
 1066 **Figure 6. PN development of motor axon collaterals on Renshaw cells.** (A-A') Renshaw
 1067 cells and VACHT⁺ boutons in the ventral horn. (B-D) Calbindin⁺ Renshaw cells contacted by
 1068 VACHT⁺ motor axon collaterals. (E) Graph showing reduction in motor axon collateral density
 1069 on RCs between PN14 and 21. (F-F') Schematics illustrating differences between intact and
 1070 PN5TX rats in development of PA and motor axon inputs to RCs. Scale bars: A=100 μ m, E=10
 1071 μ m

1072
 1073 **Figure 7. Postnatal development of GABApre projections in intact and PN5TX rats.** (A-
 1074 B'') IMARIS software was used to reconstruct 3D images of PA contacting MNs and associated
 1075 P boutons. (C-H'') Representative images of P bouton contacts on PA terminals throughout PN
 1076 development in both groups. (I-K) Quantification of P boutons. (I) Number of P Boutons per 10
 1077 μ m PA terminal surface area. (J) The percentage of PA terminals with greater than 3 P
 1078 boutons. (K) Percentage of PA terminals devoid of P boutons. Scale bars: A-A'=5 μ m, C= 2 μ m.

1079
 1080 **Figure 8. Schematic showing development of GABApre projections (P boutons) and**
 1081 **proprioceptive afferents in normal and neonatally transected rats.** For intact rats, P
 1082 boutons increase as afferents are retracted, but there is a lack of PA retraction in PN5TX rats in
 1083 conjunction with severely attenuated proliferation of P boutons.

1084
 1085 **Figure 9. Monosynaptic reflex excitability of intact and neonatally transected rats.** (A-B)
 1086 H reflex threshold is reduced and Hmax/Mmax ratio is increased in PN14-21 rats following
 1087 neonatal transection. (C) Paired pulse depression of the H reflex in intact and neonatally
 1088 transected rats at PN14-21. Shaded areas highlight the difference in inhibition between intact
 1089 and PN5TX rats. (D-D') Representative traces from intact and PN5TX rats at long (700 ms) and
 1090 short (50 ms) time intervals.

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 1092 **Figure 10. Schematic summarising the PN development of the lumbar spinal**
 1093 **sensorimotor circuitry in intact and neonatally transected rats.** (A) Intact development. (B)
 1094 Development following neonatal transection.

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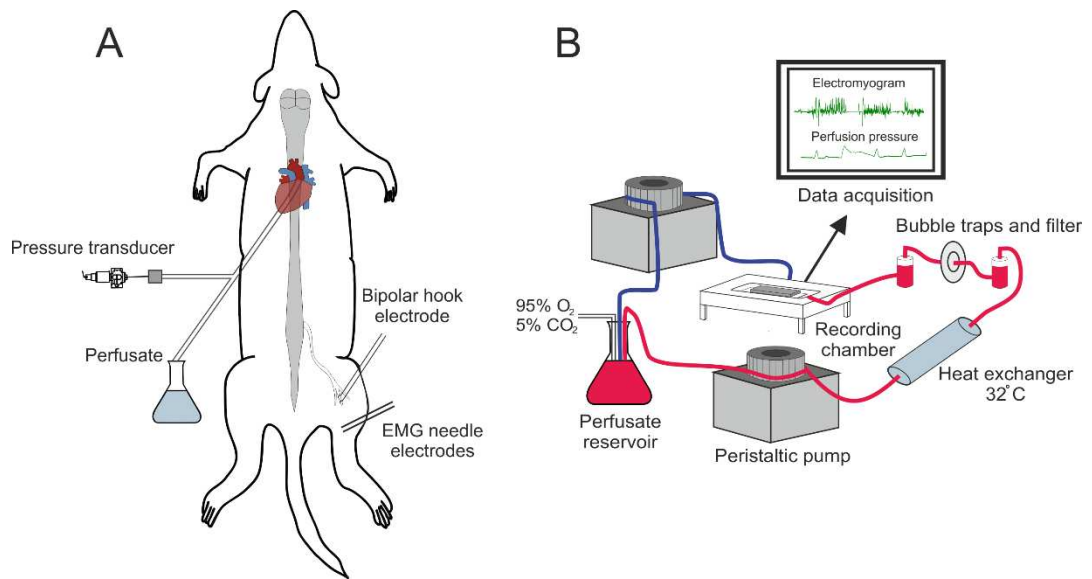
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1102 Figure 1



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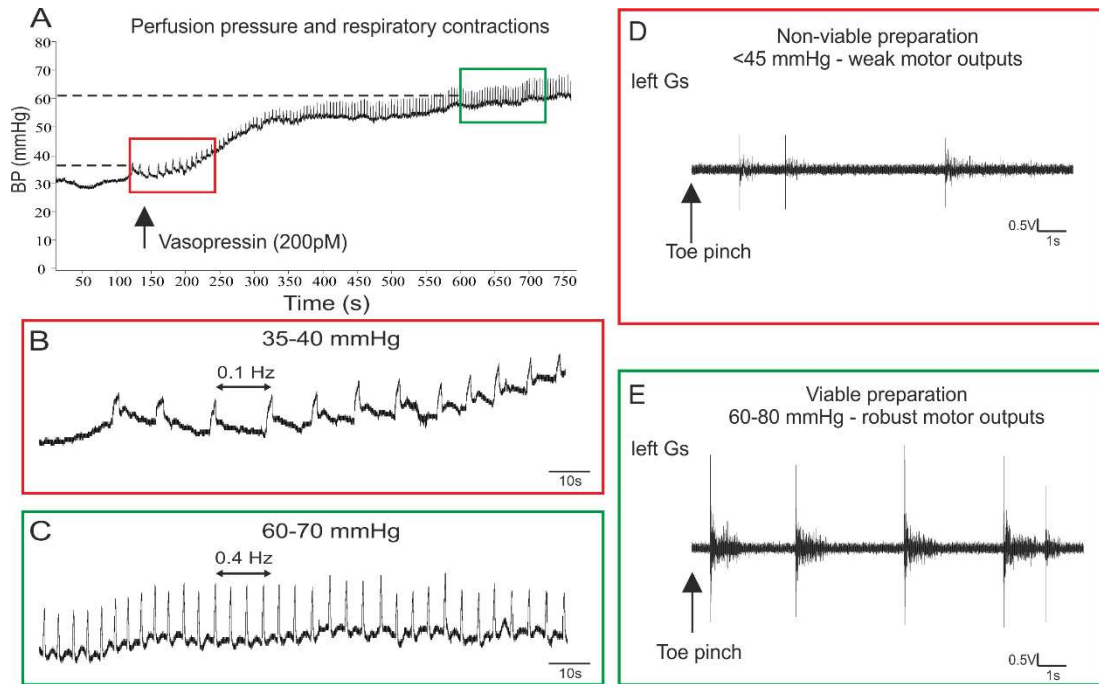
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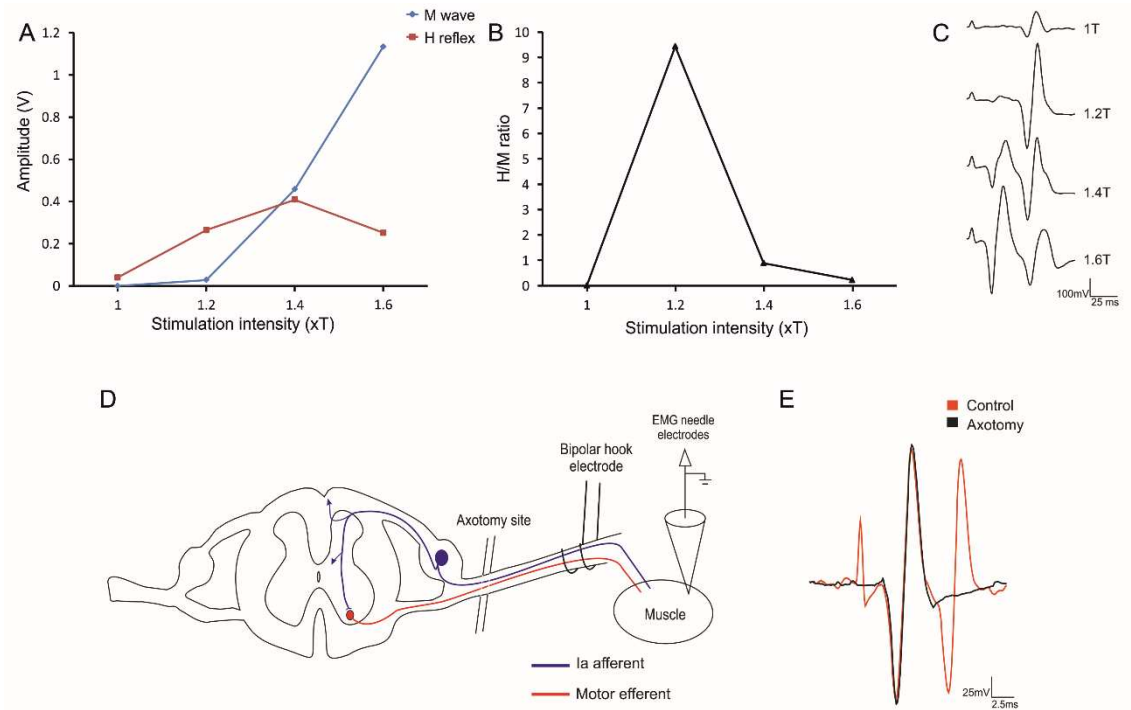
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1123 Figure 3



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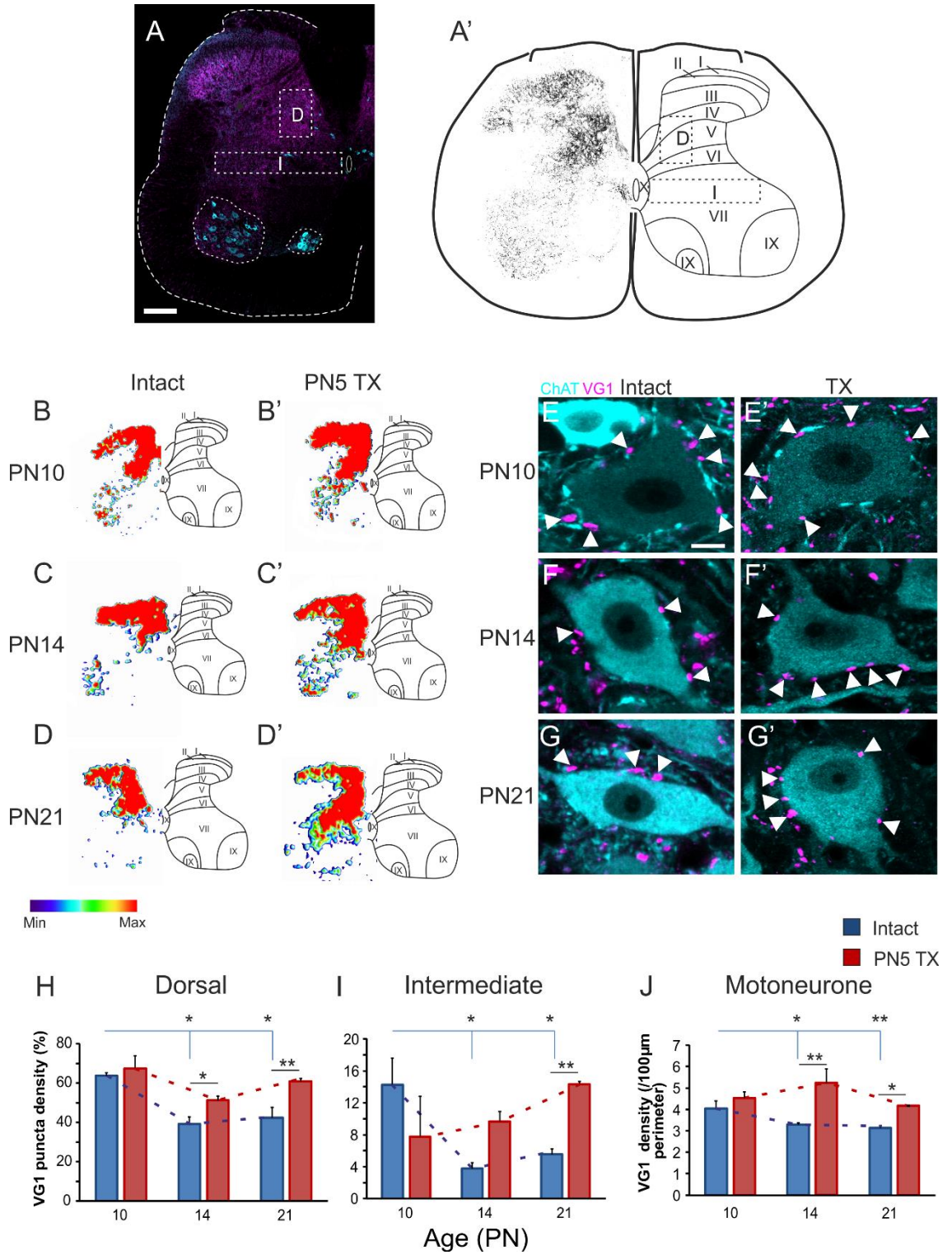
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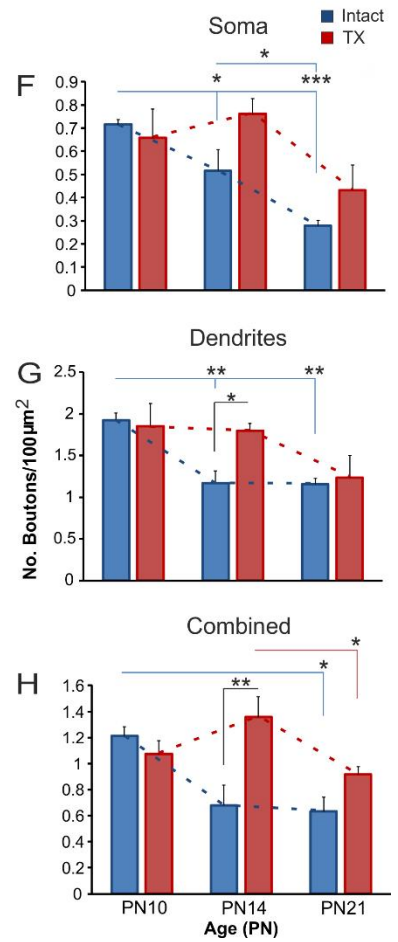
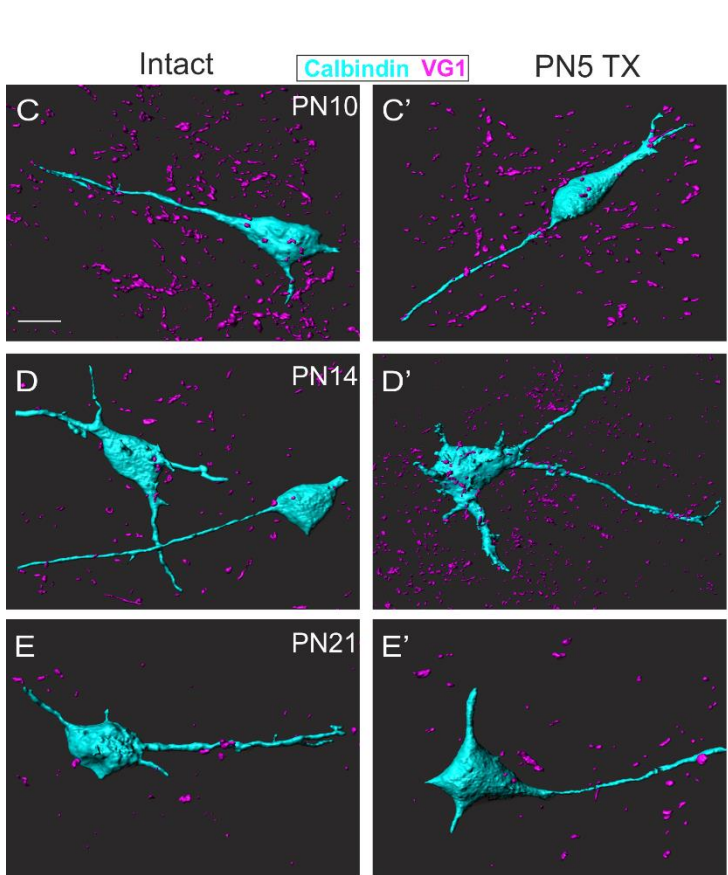
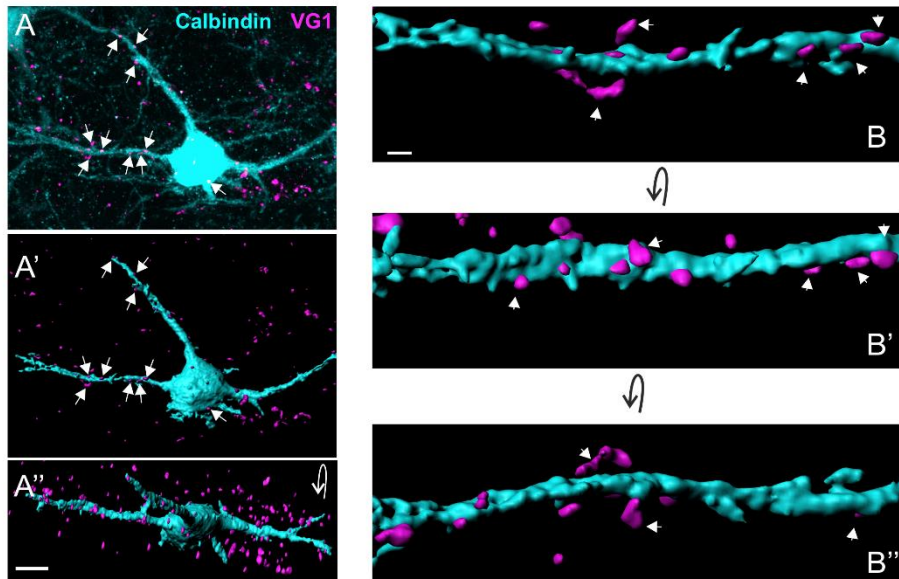
1130 Figure 4



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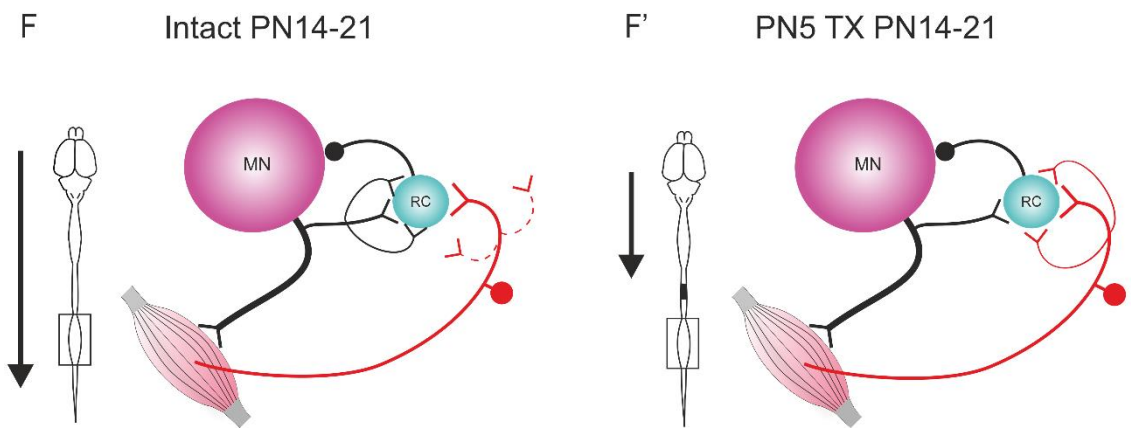
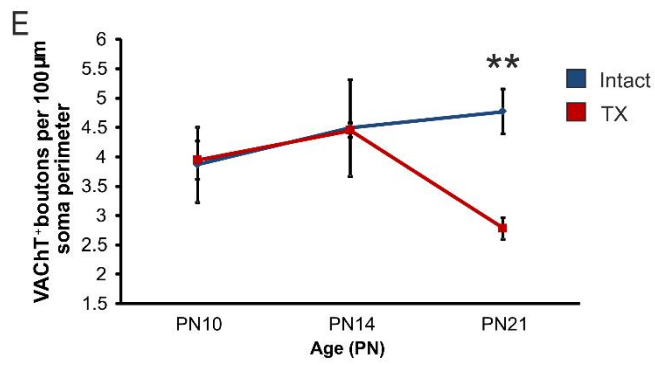
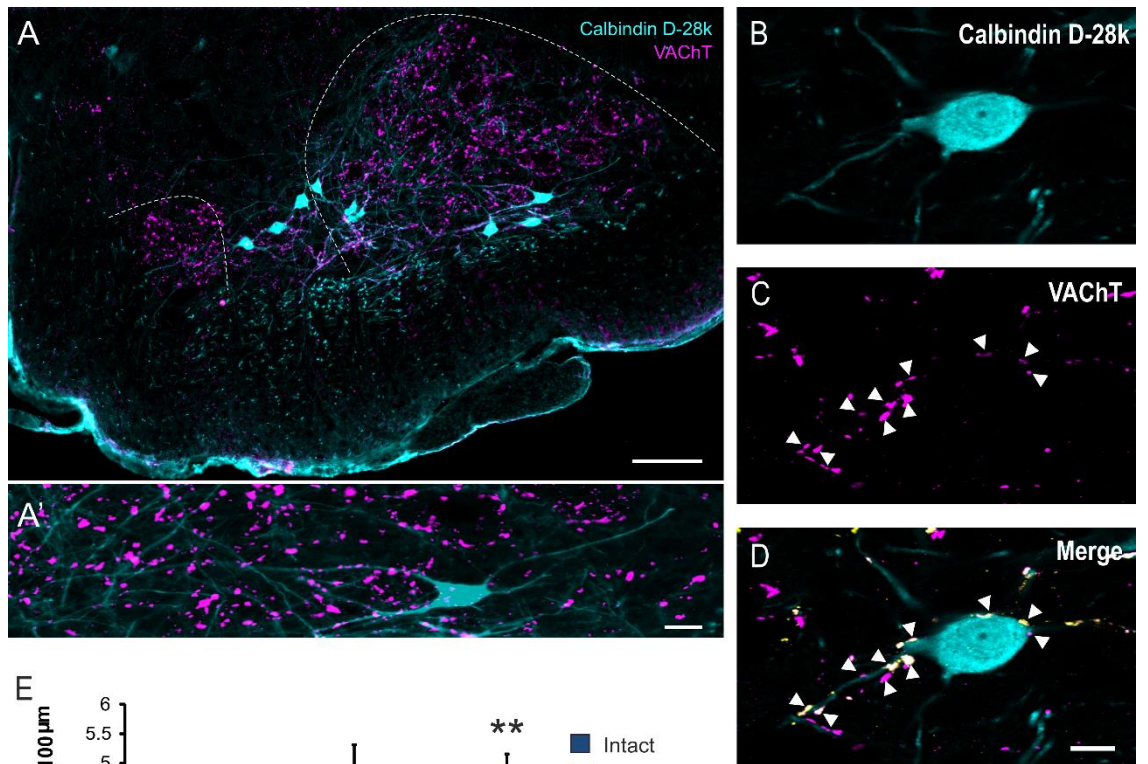
1132 Figure5

IMARIS 3D reconstruction of Renshaw cells

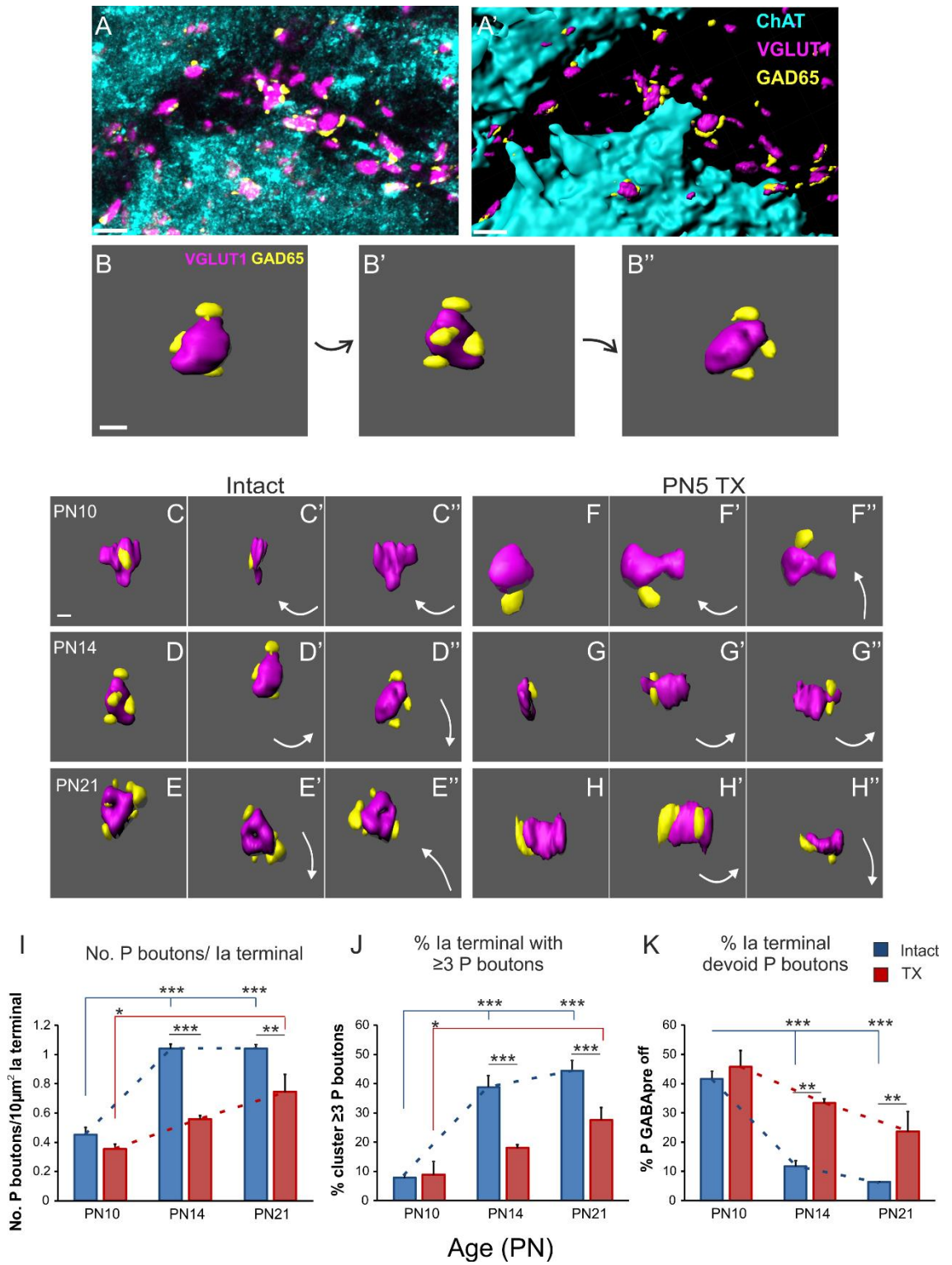


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1134 Figure 6

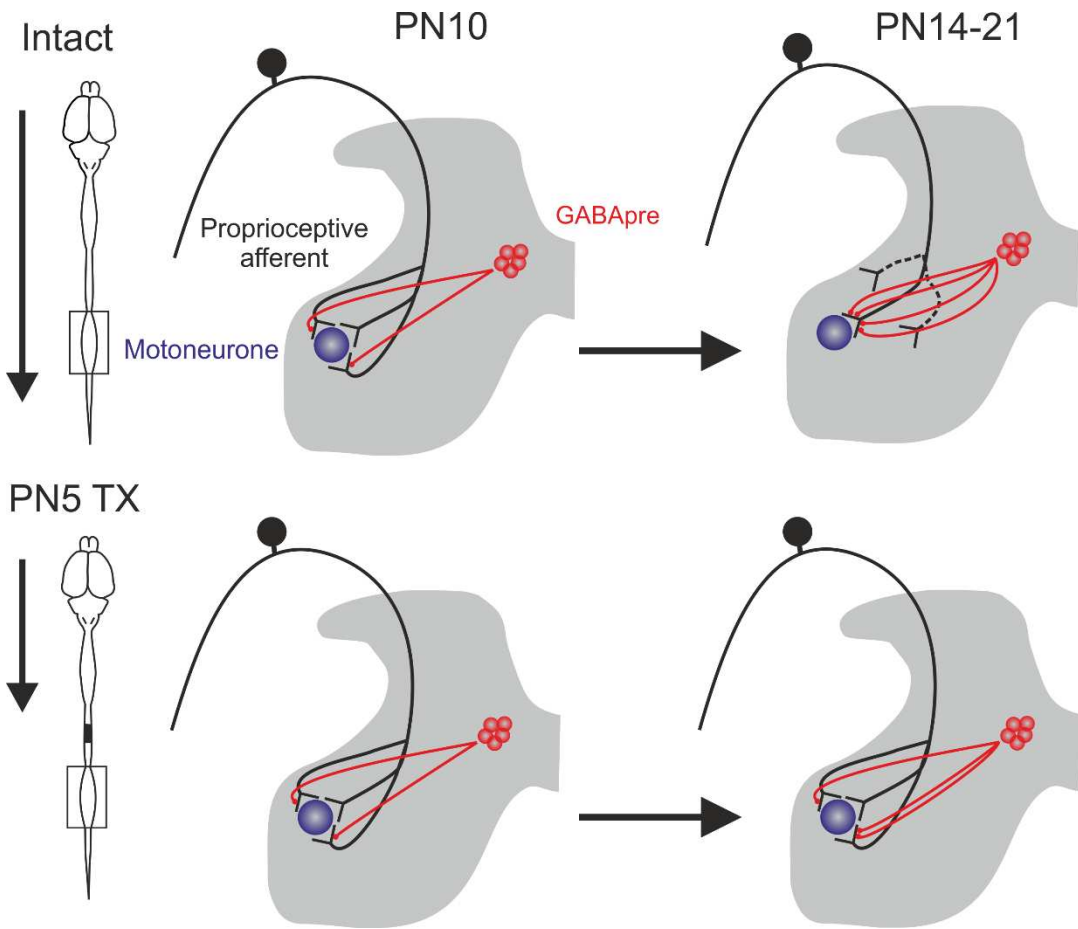


1136 Figure 7



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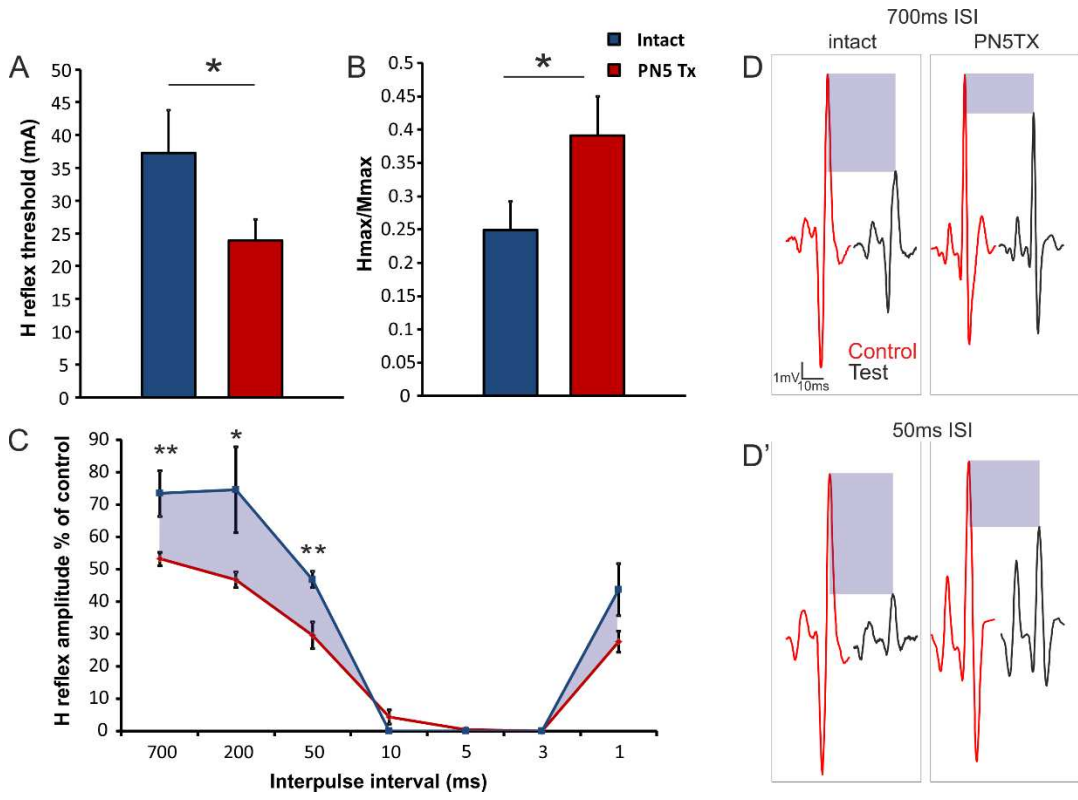
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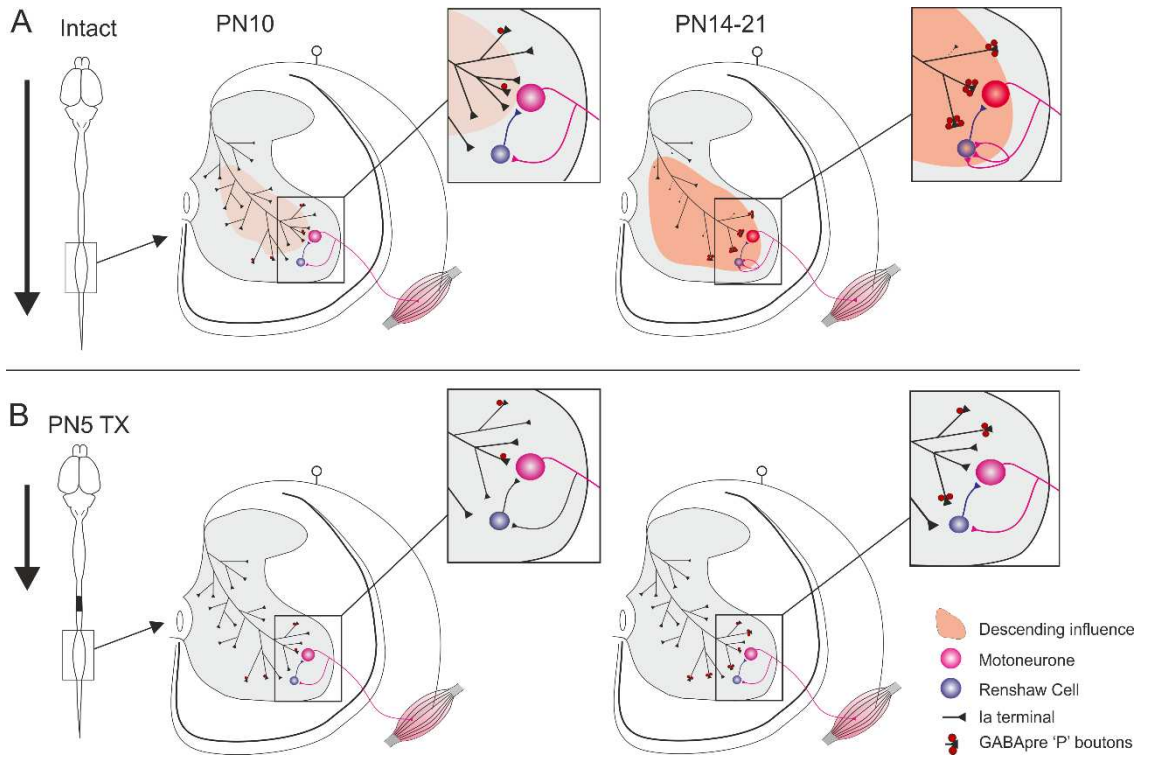
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1145 Figure 9



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1147 Figure 10



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