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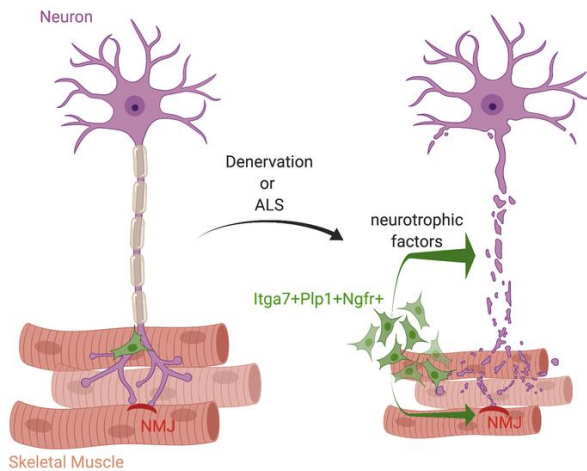
## Activation of skeletal muscle-resident glial cells upon nerve injury

Daisy Proietti, ... , Pier Lorenzo Puri, Luca Madaro

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### Graphical abstract



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1 **TITLE PAGE**

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5

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26 **Conflict of interest statement**

27 The authors have declared that no conflict of interest exists.

28

29 **ABSTRACT**

30 Here, we report on the identification of Itga7-expressing muscle resident glial cells activated by loss  
31 of NMJ integrity. Gene expression analysis at bulk and single cell level revealed that these cells are  
32 distinct from Itga7-expressing muscle satellite cells. We show that a selective activation and  
33 expansion of Itga7-positive glial cells occurs in response to muscle nerve lesion. Upon activation,  
34 muscle glial-derived progenies expressed neurotrophic genes, including Ngfr, which enables their  
35 isolation by FACS. We show that activated muscle glial cells also expressed genes potentially  
36 implicated in ECM remodeling at NMJs. Among them, we observed Tenascin C (Tnc), which was  
37 highly expressed by muscle glial cells activated upon nerve injury, and preferentially localized to  
38 NMJ. Interestingly, we observed that while the activation of muscle glial cells by acute nerve injury  
39 was reversible, upon NMJ repair. By contrast, in a mouse model of Amyotrophic Lateral Sclerosis  
40 (ALS), in which NMJ degeneration is progressive, muscle glial cells steadily increased over the  
41 course of the disease; however, they exhibited an impaired neurotrophic activity, suggesting that  
42 pathogenic activation of glial cells may be implicated in ALS progression.

43

44

45 **INTRODUCTION**

46 Skeletal muscle homeostasis is maintained by a large network of muscle-resident cells, that  
47 coordinate the response to homeostatic perturbations, such as muscle or nerve injury (1, 2).

48 Among these cells, muscle satellite cells (MuSCs) are the bonafide muscle stem cells that  
49 exist in a quiescent state during homeostasis in adult tissues and become activated in response to  
50 acute muscle damage or in chronic degenerative conditions (3–8). In addition to their absolute  
51 requirement for regeneration of injured muscle, recent studies have reported that MuSCs may also  
52 participate in the maintenance of neuro-muscular junction (NMJ) integrity and regeneration upon  
53 nerve injury (9, 10). Nerve injury and repair are events closely associated with the process of muscle  
54 regeneration; however, the precise contribution of MuSCs to NMJ regeneration is poorly understood.  
55 Likewise, whether additional muscle-resident cell types contribute to neurite growth and extension  
56 toward regenerating fibers to restore functional NMJs remains unknown.

57 We and others have recently reported on the activation of muscle-resident mesenchymal  
58 progenitors – the fibro adipogenic progenitors (FAPs), and their expansion following denervation  
59 (11). In addition to FAPs, resident macrophages have also been implicated in the process of NMJ  
60 repair following denervation or injury (9, 10, 12, 13). Interestingly, alterations in function and number  
61 of MuSCs and FAPs have been observed in neuromuscular disorders, such as Amyotrophic Lateral  
62 Sclerosis (ALS), in which loss of NMJ integrity occurs progressively (14, 15)

63 In postnatal life, the disruption of skeletal muscle-nerve cross talk leads to muscle atrophy  
64 and fibrosis (16–18), eventually leading to irreversible paralysis in conditions of complete nerve loss  
65 (i.e. traumatic spinal cord injury) or progressive loss of NMJ (e.g. chronic neuromuscular disorders)  
66 (19). Therefore, identification of the cell types activated by nerve injury and an improved  
67 understanding of their functional interactions is imperative in order to develop novel therapeutic  
68 strategies to counter the effect of muscle denervation.

69 In this study, we performed gene expression analysis in both bulk and single cells isolated from limb  
70 muscle following nerve injury with the aim to identify the cellular players that might contribute to  
71 nerve repair.

72  
73

74 **RESULTS**

75  
76 *Nerve injury activates a neurotrophic program in Itga7+ non-satellite cells*

77 The contribution of Itga7-expressing MuSCs to the maintenance of NMJ integrity, and regeneration  
78 upon nerve injury was previously reported by Liu et al 2015 and Liu et al. 2017 (9, 10). These studies  
79 prompted our interest in the transcriptional profiles of Itga7+ cells isolated from limb muscles of mice  
80 either unperturbed or exposed to nerve injury. To this end, we performed RNAseq analysis on  
81 Itga7+/Sca1-/Ln- cells (from now on, referred to as Itga7+ cells) isolated by FACS from limb muscles  
82 of 3-month-old mice, at 3 days after denervation by sciatic nerve severing, and compared to Itga7+  
83 cells isolated from control mice. Heatmap comparison revealed extensive alterations of the  
84 transcriptional profile in Itga7+ cells isolated post-nerve injury, compared to unperturbed controls,  
85 with a clear bias toward up-regulation of genes which accounted for the vast majority of the  
86 differentially expressed genes (Figure 1a). Notably, most of the upregulated genes were those  
87 associated with neuronal growth and repair pathways, including Nerve Growth Factor Receptor  
88 (Ngfr), Sonic Hedgehog (Shh) Tenascin-C (Tnc), Neuronal Cell Adhesion Molecule (NRCAM), Glial  
89 Cell Derived Neurotrophic Factor (Gdnf), and the glial lineage-specific transcription factors  
90 Oligodendrocyte lineage genes (Olig1) (Figure 1a). Other genes of alternative mesenchymal  
91 lineages, but recently implicated in neurogenesis (e.g. Runx2) (20) were also found upregulated in  
92 Itga7+ cells isolated following nerve injury. Moreover, gene ontology analyses of RNAseq data  
93 predicted the activation of “nervous system development” and “axon guidance” among the main  
94 activated pathways (Figure 1b).

95 The upregulation of neurotrophic genes in Itga7+ cells after denervation suggests that this  
96 population includes cell types endowed with potential nerve repair activities. Thus, in order to identify  
97 this population, we sought to determine whether the activation of the neurotrophic gene program  
98 also occurred in Itga7+ cells isolated from muscles subjected to sciatic nerve crush - a reversible  
99 lesion that is typically followed by repair and restoration of NMJ integrity (21). Indeed, the  
100 upregulation of neurotrophic genes was also detected by qPCR analysis in Itga7+ cells following  
101 sciatic nerve crush (Figure 1c). Interestingly, activation of the same genes was also observed in cells

102 isolated using the Satellite Cells isolation kit (Miltenyi Biotec) magnetic strategy that is based on  
103 lineage marker exclusion of *Itga7*-negative muscle-resident cells (Suppl. Figure 1a).

104         Given the recent studies showing that *Itga7*-expressing cell populations in muscle include  
105 cellular subsets divergent in developmental origin from the actual MuSCs (22), we utilized the  
106 *PAX7<sup>CreER</sup>;tdTomato<sup>ff</sup>* mouse model, which allows MuSCs lineage tracing (23), in order to  
107 unequivocally determine whether the activation of the neurotrophic gene program occurs in MuSCs  
108 or the non-MuSC cell fraction among the *Itga7*<sup>+</sup> cells. In this mouse model, tamoxifen treatment  
109 leads to the permanent expression of tomato fluorescent protein in *Pax7*<sup>+</sup> MuSCs (Suppl. Figure  
110 1b). Seven days after tamoxifen treatment, 3-month-old mice were subjected to nerve injury and  
111 analyzed 3 days later (Figure 1d). As expected, *Itga7*<sup>+</sup>*Tomato*<sup>+</sup> cells isolated from limb muscles of  
112 uninjured mice or following sciatic nerve crush expressed high level of the MuSC-identity genes  
113 *Pax7* and *Vcam1*, whereas the *Itga7*<sup>+</sup>*Tomato*<sup>-</sup> cell population did not express these markers  
114 (Figure 1e). However, upon denervation, only the *Itga7*<sup>+</sup>*Tomato*<sup>-</sup> fraction expressed *Ngfr*, *Shh*, *Tnc*,  
115 *Nrcam* and *Gdnf*, indicating the induction of a neurotrophic signalling pathway in the *Pax7*-negative  
116 fraction of *Itga7*<sup>+</sup> cells (Figure 1e, Suppl. Figure 1c). These observations also indicate that among  
117 the cells isolated with the Satellite Cells isolation kit (Miltenyi Biotec), non-myogenic *Itga7*<sup>+</sup> and *Pax7*  
118 negative cell types might also co-segregate with the myogenic fraction.

119         Overall, these findings show transcriptional activation of a neurotrophic program in the non-  
120 myogenic fraction of *Itga7*<sup>+</sup> cells and suggest their potential involvement in the functional cross-talk  
121 between muscle resident cells and NMJ in response to nerve injury.

122

### 123 *scRNA-seq reveals heterogeneity within the Itga7+ muscle resident cells*

124         Recently, the heterogeneous composition of muscle resident mononuclear cells has been  
125 dissected using high resolution cartography by scRNA-seq. This strategy has revealed the identity  
126 of a new MuSCs-independent myogenic population among the *Itga7*<sup>+</sup> cells, referred to as Smooth  
127 Muscle Mesenchymal Cells (SMMCs) (22). To further determine whether the activation of the  
128 neurotrophic program upon nerve injury occurred in SMMC or in other sub-populations within *Itga7*<sup>+</sup>  
129 cells, we performed single-cell RNA-seq (scRNA-seq) transcriptome profiling in *Itga7*<sup>+</sup> cells isolated

130 from limb muscles of mice 3 days following sciatic nerve crush or control mice. By using the 10x  
131 Genomics' single-cell RNA-seq (scRNA-seq) technology we obtained a total of 3949 cells analyzed.  
132 Clustering analysis identified 11 different groups (Suppl. Figure 2a). Based on markers expression  
133 we could clearly identify three major populations that were composed of multiple sub-clusters (Figure  
134 2a-b). In particular, we detected MuSCs, as Pax7+, Myf5+ and Vcam1+ expressing cells, smooth  
135 muscle mesenchymal cells (SMMCs), as Myh11+ expressing cells and glial cells as Plp1 and Mpz  
136 expressing cells (Figure 2a).

137 Interestingly, within the SMMCs cell population we could distinguish a Myl9+Rgs5<sup>low</sup> and  
138 Myl9+Rgs5<sup>high</sup> subpopulations (Suppl. Figure 2b). In line with this observation, Rgs5, Myl9 and Plp1  
139 expression was evident in the tomato-negative fraction of tamoxifen treated PAX7.Cre\_tdTomato  
140 mice (Suppl. Figure 2c). The gene expression profile of the smaller clusters (less than 150 cells) was  
141 indicative of myotendinous (Scx<sup>high</sup> expressing cells), endothelial (Pecam1<sup>high</sup>) and mesenchymal (as  
142 Ly6a/Ly6e<sup>pos</sup> and Pdgfra<sup>pos</sup>) lineages (Figure 2b). However, it is likely that the presence of these  
143 clusters is due to contamination from Itga7<sup>low/neg</sup> populations.

144

145 *Plp1+ glial cells are the major Itga7+ population responsive to denervation.*

146 We focused our analysis on the three major cell types identified by scRNAseq analysis  
147 among the Itga7+ cells isolated following nerve injury – i.e. MuSCs, SMMCs and glial cells. We  
148 observed a similar distribution of MuSCs and SMMCs in both healthy and denervated muscle (Figure  
149 3a). By contrast, we observed a specific increase in Plp1+ cells in response to nerve injury. To  
150 validate this finding, we denervated mice by nerve crush injury, and at 12h before harvest we  
151 administered an intraperitoneal injection of EdU. EdU incorporation analysis revealed a significant  
152 proportion of proliferating Plp1+ cells at 3 days following sciatic nerve crush (Suppl. Figure 3a). The  
153 gene expression profile of nerve injury-activated Plp1+ cells largely overlapped with those of glial  
154 cells recently described by both De Micheli et al. and Giordani et al. (22, 24) (Suppl. Figure 3b);  
155 however, there was no significant overlap with other populations (in particular with MuSCs and  
156 SMMCs) (Suppl. Figure 3b). In addition, these cells were enriched in additional markers known to  
157 be expressed by glial/Schwann cells (Suppl. Figure 3c).



158 Furthermore, we noted extensive alterations in gene expression in Plp1+ cells following nerve  
159 injury, compared to control muscle, while only a few genes were significantly altered in MuSCs and  
160 SMMCs (Suppl. Figure 3d). Single cell RNAseq analysis revealed that Plp1+ glial cells are  
161 characterized by a neurotrophic signature (Figure 3b-c and Suppl. Figure 3d), with most of the  
162 upregulated genes coinciding with those identified in the bulk Itga7+ cells shown in Figure 1 – i.e.  
163 Ngfr, Tnc, Gdnf, Runx2 and other genes strictly related to nerve development (Figure 3b-c). Among  
164 these genes, the Ngfr was used to identify these cells, since it was found to be specifically expressed  
165 in glial-cells, and no other muscle resident cells, in response to nerve injury, by an independent  
166 scRNAseq analysis post-denervation (25). It is interesting to note that Hicks et al. have previously  
167 shown that Ngfr is transiently expressed in human MuSC progenitors during the generation of hiPSC-  
168 derived MuSCs (26). Our data indicate that Ngfr expression discriminates glial cells from MuSCs  
169 within a common pool of Itga7+ cells. Indeed, Ngfr+ cells were only detected among the Itga7+  
170 population in the muscle at 3 days after denervation, while only a negligible number of Ngfr+ cells  
171 were present in control muscle (Figure 3d-e, Suppl. Figure 4a-c). There were no significant changes  
172 in the number of Ngfr-Itga7+ cells after nerve injury (Suppl. Figure 4d), thus confirming that the main  
173 cell type responding to denervation are the muscle- resident glial cells. Indeed, the number of Pax7+  
174 cells did not change at 3 days after nerve injury and Pax7 did not co-localize with Ngfr in serial  
175 muscle sections (Suppl. Figure 4e-f). Moreover, the induction of neurotrophic genes, including Ngfr,  
176 but also Plp1, Tnc, Nrcam and Gdnf, was only detected in Ngfr+ cells, but not Ngfr- cells, isolated  
177 from denervated muscle (Suppl. Figure 4g). Finally, the Ngfr+ cells differed in phenotype and  
178 morphology from the Ngfr- population, and unlike the Ngfr- cells, they did not show any myogenic  
179 potential when cultured *in vitro* (Suppl. Figure 5a-b).

180 Interestingly, the presence of Ngfr+ cells was also observed in a mouse model of Spinal Cord  
181 Injury (SCI) where the damage was induced by mechanical lesion of the spinal cord (Suppl. Figure  
182 5c-d). Indeed, at 7 days post spinal lesion, the appearance of Ngfr+ cells was observed in TA muscle.  
183 We found that in unperturbed muscles Plp1 positive cells are close to the neuron structure stained  
184 for Neurofilament (NF-L) that is lost upon nerve injury. However, upon nerve injury, Plp1+ /Ngfr+ cells  
185 were found in a structure reminiscent of the nerve structure and close to bungarotoxin-positive NMJs

186 (Suppl. Figure 4a-c). Moreover, a decline in the number of the Ngfr+ cells and, more importantly, a  
187 decrease in the expression of neurotrophic factors was observed at 30 days after nerve injury, when  
188 the reinnervation process typically occurs (Suppl. Figure 6a-b).

189         Activated glial cells are known to respond to nerve injury and participate in nerve repair and  
190 axon guidance. To exclude the possibility that the presence of activated glial cells within the muscle  
191 was simply caused by co-isolation of adjacent tissues (i.e. nerves), we compared the gene  
192 expression profile of Ngfr+ cells isolated from nerves or muscles at 3 days after nerve injury (Figure  
193 4a). Transcriptome analysis revealed significant differences between the two populations, which  
194 clearly formed two independent clusters with a large subset of differentially regulated genes (Figure  
195 4a-c). Interestingly, among the genes differentially expressed by the two glial cells we found some  
196 genes more represented in muscle derived glial cells (i.e. Tnc, Gdnf), while others are more  
197 expressed in neuron derived cells (i.e. Shh) (Figure 4d). We focused on Tnc since its ablation is  
198 known to delay NMJ recovery in-vivo(27). Tnc protein unequivocally localized close to bungarotoxin  
199 (BTX)-positive NMJ upon denervation (Figure 4e-f and Suppl. Figure 6c). Finally, Tnc protein  
200 expression and localization close to the NMJ was observed in TA muscle at 7 days following spinal  
201 cord injury (Suppl. Figure 6d).

202         These results suggest an involvement of Tnc in the maintenance of NMJ following  
203 denervation.

204

205 *Muscle resident glial cells activated by nerve injury adopt a defective neurotrophic phenotype in a*  
206 *mouse model for ALS.*

207         The symptomatic stage of ALS is characterized by muscle denervation. Motoneuron degeneration  
208 leads to muscle atrophy and muscle weakness, ultimately accelerating disease progression (28). It  
209 is currently unclear whether the disease progression could be influenced by the neurotrophic activity  
210 of specialized cell types. We therefore set to determine whether an increased amount of muscle  
211 resident P1p1+/Ngfr+ glial cells could be observed at sequential stages of disease progression, using  
212 the SOD1<sup>G93A</sup> mouse model of ALS(29).

213 Indeed, Ngfr expression and Ngfr+ cells among Itga7+ cells increased with disease  
214 progression (Figure 5a-d). Ngfr expression displayed a striking increase in muscle derived from  
215 symptomatic SOD1<sup>G93A</sup> at 90- and 140-days of postnatal life with a concomitant reduction of NF-L  
216 positive neurofilament in accordance with the progressive loss of muscle innervation (Figure 5b-d).  
217 Other factors such as Gdnf or Tenascin C also increased significantly in SOD1<sup>G93A</sup> in the late stage  
218 of the disease (140d) compared to age-matched healthy animals (Figure 5d). Overall, these  
219 observations clearly identify muscle glial cells as potential players in the maintenance of nerve to  
220 muscle contact in the context of ALS. Interestingly, the magnitude of the induction of Tnc and Gdnf  
221 was clearly lower compared to acute denervation shown in Figure 1. This may account for the  
222 reduced reinnervation ability of SOD<sup>G93A</sup> leading to muscle paralysis that marks the end-stage of the  
223 disease. In addition to the lower expression of Tnc compared to reversible nerve degeneration, we  
224 also observed a marked difference in Tnc localization in ALS muscle. Indeed, while in reversible  
225 denervation we observed a clear localization of Tnc protein close to the NMJ, in SOD<sup>G93A</sup> muscle  
226 Tnc signal encircled muscle fibers without the NMJ associated pattern (Figure 5e-f). These data  
227 suggest a defect in the response of glial cells during disease progression.

228 To functionally validate the neurotrophic ability of Plp1+Ngfr+ muscle derived glial cells, we  
229 utilized an in-vitro transwell system (Figure 6a-c). Ngfr+ and Ngfr- cells were isolated from limb  
230 muscle of mice subjected to nerve injury or from symptomatic SOD<sup>G93A</sup>, and co-cultured with the  
231 mouse motor neuron-like hybrid cell line (NSC-34), without direct contact through the use of specific  
232 trans-well inserts. Following 72h of co-culture in growth media, Ngfr+ cells from denervated muscle  
233 promoted NSC-34 neuronal differentiation when compared to control cells cultured under standard  
234 neuronal differentiation conditions, as documented by the increase in neurites length and the mean  
235 number of neurites per cell (Figure 6a-c). Conversely, this effect was not observed in NSC-34 co-  
236 cultured with Ngfr- cells. Interestingly, a lower ability to promote NSC-34 differentiation and neurite  
237 elongation was observed in cells co-cultured with Ngfr+ cells derived from SODG93A muscle (Figure  
238 6a-c).

239 These data suggest that an impaired ability of muscle-resident glial cells to adopt a neurotrophic  
240 phenotype in response to nerve injury could contribute to progressive loss of NMJ in ALS muscles.

241 Next, given the localization of glial cells close to the NMJ, we explored the possibility that they may  
242 play a direct role in the promotion and maintenance of NMJ upon denervation. We tested this  
243 possibility by using a model of AChR clustering in cultured myotubes, previously described by Ngo  
244 and colleagues (30). As shown in Figure 6d-f and in Suppl. Figure7, conditioned media from muscle  
245 derived glial cells promote AChR clustering – as revealed by bungarotoxin staining – in differentiated  
246 C2C12 myotubes. As a positive control, 4h agrin (Agr) treatment was used. Interestingly, while after  
247 a 10' pulse of agrin followed by 4h of release the AChR clustering was reduced, as previously shown  
248 (30), replacement of conditioned media after agrin pulse led to a similar induction of AChR clustering  
249 compared to a full 4h agrin treatment. These observations support a direct role of muscle glial-  
250 released factors in the induction and maintenance of the muscle counterpart of the NMJ. Of note,  
251 we the formation of of AChR clustering was much attenuated when using conditioned media by glial  
252 cells isolated from the SOD<sup>G93A</sup> muscle, further suggesting a functional impairment of muscle glial  
253 cell function during ALS progression.

## 254 **DISCUSSION**

255 scRNAseq-based analysis has been instrumental to unravel the heterogeneity of muscle-  
256 resident cells in unperturbed conditions, while highlighting their dynamic transitions through a  
257 continuum of functional cellular states and trajectories in response to homeostatic perturbations (22,  
258 24, 31–37). These studies have used the typical experimental model of muscle regeneration, by  
259 physical injury, which leads to the sequential activation of multiple cell types, to reveal the identity of  
260 sub-populations endowed with specialized activities and their coordination in response to  
261 regeneration cues. In addition to MuSC activation, scRNAseq analyses has revealed a dramatic  
262 expansion and alteration of gene expression profiles in cells from the inflammatory infiltrate  
263 immediately after acute muscle injury (24, 34, 37). These cells account for the vast majority of the  
264 cell types present in muscles at early time points post injury and establish functional interactions with  
265 other cell types within the regenerative environment, including FAPs and MuSCs, to promote  
266 myofiber regeneration and injury resolution. As part of the regeneration process, the repair of injured  
267 nerve also occurs, although the cellular effectors of this process remain poorly understood. While  
268 earlier studies have suggested the potential contribution of MuSCs in the maintenance of NMJ  
269  
270

271 integrity and regeneration (9, 10), the precise identity of the cell types activated by nerve injury and  
272 their potential neurotrophic activity remain unknown.

273 Unlike muscle injury, nerve injury does not promote muscle regeneration, but leads to  
274 myofiber atrophy and muscle fibrosis (11, 38). These different outcomes are underpinned by  
275 differences in activated cell types. For instance, we have previously observed that muscle  
276 denervation leads to the selective activation of FAPs, which exhibit transcriptional profiles and  
277 biological activities different from FAPs activated in response to acute muscle injury (11, 31).  
278 Importantly, denervation does not trigger the massive infiltration of immune cells observed upon  
279 muscle injury (11). We argue that the lack of inflammatory infiltrate and the consequent reduction in  
280 the amount of inflammatory signals in the milieu of denervated muscles might account for the lack  
281 of activation of multiple cell types in denervated muscles (25). At the same time, the selective  
282 response of muscles to denervation might help to capture specific muscle-resident cells activated by  
283 nerve injury, without the potentially confounding co-existence of other activated cell types.

284 In this study, we describe a population of muscle-resident glial cells that are activated by  
285 nerve injury and might contribute to NMJ repair. These cells express *Itga7* - a cell surface protein  
286 commonly used to prospectively isolate MuSCs (39). Indeed, both previously used FACS strategies  
287 and commercial kits could not distinguish the two populations due to the common antigen surface  
288 marker. A recent study identified a population of smooth muscle mesenchymal cells (SMMCs) within  
289 the FACS-isolated *Itga7*<sup>+</sup> cells that is distinct from MuSCs (22). Interestingly, our results indicate  
290 that muscle-resident glial cells, while sharing *Itga7* expression with MuSCs and SMMCs, exhibit a  
291 distinctive gene expression signature that is enriched in glial cell-specific genes. While some glial  
292 lineage-identity marker was constitutively expressed and could be used for their prospective isolation  
293 in unperturbed muscles (*Plp1*), a subset of neurotrophic genes was selectively expressed in these  
294 cells only in response to nerve injury. Among them, *Ngfr* was instrumental to further isolate the  
295 fraction of *Itga7/Plp1* glial cells activated upon nerve injury (Figure 3). *Ngfr* is a receptor commonly  
296 associated with activated glial cells (40). Since *Ngfr* expression in *Itga7*-positive muscle glial cells is  
297 only observed upon their activation by nerve injury and coincides with the activation of the

298 neurogenic program, it is conceivable that *Ngfr* confers upon glial cells the competence to respond  
299 to neurogenic signals.

300 Although nerve-associated glial-cells (otherwise defined as Schwann cells) are well known,  
301 the biological properties of tissue-resident glial cells have only recently become the object of intense  
302 investigation (41).

303 In the case of skeletal muscle, the specific function of peripheral glial cells and their regulation  
304 in response to homeostatic perturbations, such as in ALS disease, are currently not well known (19,  
305 42). Although several myelinating and non-myelinating cell types (Remak cells and terminal  
306 Schwann cells) have been associated with neuron regeneration, a defined molecular signature able  
307 to discriminate between subpopulations from different anatomical location and different functional  
308 specialization has not been clearly identified. Indeed, the molecular features of terminal Schwann  
309 cells remain mostly unknown, because their scarcity has so far impeded a comprehensive analysis  
310 (43). Our data indicate that single cell RNA-seq based approaches may circumvent this issue due  
311 to their potential to identify transcriptional signatures in a small number of cells within the pool of  
312 cells analysed.

313 Our work identified a population of *Itga7*-expressing cells, distinct from MuSCs and SMMC,  
314 that is selectively activated upon nerve injury and adopt a neurogenic gene expression profile and  
315 functional neurotrophic properties. Interestingly, activated muscle resident glial cells localize in close  
316 proximity to NMJs. While a comparative analysis using the scRNAseq profiles of *Ngfr*<sup>+</sup> cells isolated  
317 either from nerves or from muscles at 3 days post nerve injury revealed clear differences in gene  
318 expression between these two populations, it is possible that they might represent two different  
319 functional states of muscle-resident glial cells. Nevertheless, the differential expression of certain  
320 genes, such as *Tnc*, *Gdnf* and *Shh*, suggests that muscle-resident glial cells adopt different  
321 functional phenotypes in response to nerve injury.

322 The upregulation of *Tnc* in muscle-resident glial cells activated by nerve injury has not been  
323 shown previously and highlights a fundamental difference between the skeletal muscle response to  
324 nerve injury versus myotrauma. In this latter, *Tnc* is typically expressed by other types of resident-  
325 muscle cells (e.g. FAPs) and accumulates within the ECM to regulate MuSC activity (23, 24, 34, 37).

326 Conversely, we show that in response to nerve injury, Tnc accumulates in close proximity to  
327 bungarotoxin (BTX)-positive NMJ. Thus, higher levels of Tnc in muscle-derived glial cells and its  
328 anatomical localization in proximity of NMJ are distinctive features of skeletal muscle response to  
329 NMJ injury. Considering that genetic ablation of Tnc causes delay in NMJ recovery in-vivo (27), we  
330 speculate that muscle glial-cell derived Tnc could contribute to NMJ repair following injury. This  
331 possibility is also supported by the finding that Tnc expression was reduced upon recovery of NMJ  
332 integrity (e.g. up to 30 days after lesion). It is possible that transient deposition of Tnc within the ECM  
333 at NMJ is an important event to promote NMJ repair and is part of a general program by which  
334 muscle glial cells commit to repair injured muscles. Consistently, we found that factors secreted by  
335 muscle glial cells could enhance AChR clustering in cultured myotubes.

336 Further studies will be necessary to investigate the actual contribution of muscle glial cells in  
337 the recovery of NMJ integrity in response to acute lesions or chronic degeneration, and whether  
338 these cells might be amenable to pharmacological manipulation to facilitate nerve repair. In this  
339 regard, pharmacological activation of the neurotrophic potential of muscle glial cells could be  
340 exploited in neurodegenerative disorders, such as ALS. A role for peripheral glial cells in ALS – and  
341 in particular pre-synaptic Schwann cells - has been recently suggested, although the precise  
342 mechanism of their involvement remains unknown (42, 44). We found a progressive increase of  
343 muscle resident glial cells in muscles of the ALS mouse model - SOD1<sup>G93A</sup> mice. However, activated  
344 muscle glial cells from symptomatic SOD1<sup>G93A</sup> mice exhibited reduced activation of neurotrophic  
345 genes, defective Tnc localization, impaired ability to promote neurite outgrowth/differentiation of a  
346 motoneuron cell line and to promote AChR clustering in cultured myotubes, as compared to glial  
347 cells activated in the context of acute reversible denervation. These data suggest that defective  
348 activity of muscle-glial cells could contribute to the pathogenesis of neurodegenerative diseases,  
349 such as ALS.

350

351

352

353 **METHODS**

354 **Mouse Strains**

355 Mouse strains used in this study were:

356

- 357 • C57BL/6J were provided by the Jackson Laboratory (Bar Harbor, USA).
- 358 • PAX7CreER/tdTomato<sup>ff</sup> mice were provided by the SBP Animal Facility (La Jolla).
- 359 • Hemizygous transgenic mice carrying the mutant human SOD1<sup>G93A</sup>(B6.Cg-Tg  
360 (SOD1\*G93A)1Gur/J) gene were originally obtained from Jackson Laboratories (Bar Harbor,  
361 USA).
- 362 • CD1 were provided by Charles River Laboratories, Como, Italy.

363

364 All mice were maintained in a pathogen-free animal facility under standard 12h light/12h dark cycle  
365 at 21 °C with access to red house and to standard chow and water ad libitum. Three-month-old mice  
366 were used for ex-vivo experiments, except for the SOD1<sup>G93A</sup> mice, as indicated in the Figure 5. For  
367 the denervation experiments, both male and female C57BL/6J and PAX7CreER/tdTomato<sup>ff</sup> mice  
368 were used. Only female mice were used for the spinal cord injury experiment. As a mouse model of  
369 ALS male SOD1<sup>G93A</sup> mice were used.

370

371 **Cell Lines and Primary Cell Cultures**

372 All cells were cultured in incubators at 37 °C and 5% CO<sub>2</sub>. We used mouse MN-like NSC-34 cells  
373 (obtained from ATCC) which is a hybrid cell line produced by the fusion of MNs from the spinal cord  
374 embryos with N18TG2 neuroblastoma cells that exhibit properties of MNs after differentiation and  
375 maturation protocols(45). Thus, NSC-34 cells were grown in proliferation media [Dulbecco's Modified  
376 Eagle Medium: Nutrient Mixture *F-12* (DMEM / *F-12*, Sigma-Aldrich, D6421) supplemented with 10%  
377 of fetal bovine serum (FBS, Sigma, F4135) and 1% of Penicillin/Streptomycin (Gibco, 15070-063)].  
378 Differentiation was induced by changing medium for DMEM-F12 plus 0.5% of FBS, 1% of non-  
379 essential amino acids (NEAA, Thermo Fisher Scientific, 11140050), 1% of Penicillin/Streptomycin.



380 Freshly isolated MuSCs and Ngfr+ cells were plated in 24-well plates in GM [DMEM [+Pyruvate]  
381 (Gibco, 41966-029), 20% FBS, 10% Horse Serum, 1% Chick Embryo Extract (CEE)]. Myogenic  
382 differentiation was induced with DMEM and 2% horse serum for 2 or 3 days. NSC-34 and Ngfr+ cells  
383 were also used for co-culture experiments.

384 C2.12 (C2C12) myogenic cells were obtained from ATCC and cultured on 96-well plates in growth  
385 medium [DMEM [-Pyruvate] (Gibco, 61965-026), supplemented with 10% of FBS and 1% of  
386 Penicillin/Streptomycin]. Myogenic differentiation was induced by shifting the cells in differentiation  
387 medium (DMEM [-Pyruvate] complemented with 1% Penicillin/Streptomycin and 2% House Serum).

388

### 389 **Acetylcholine receptor clustering assay**

390 AChRs were considered to be a large AChR cluster when they were  $\geq 25 \mu\text{m}$  in their longest  
391 dimension. C2C12 myotube were treated with 1 nM recombinant Rat Agrin (R&D System, 550-AG)  
392 for 4 hours or 10 minutes in differentiation medium (30). Different C2C12 myotube cultures were  
393 treated with conditioned media of muscle derived glial cells for 4h or after the 10' pulse of agrin.  
394 AChRs were labeled by the binding of Alexa Fluor 488  $\alpha$ -bungarotoxin (Invitrogen, B13422).  
395 Myotubes were incubated with the  $\alpha$ -bungarotoxin diluted (1:300) in differentiation medium for 1  
396 hours at 37°C in 5% CO<sub>2</sub>. The number of AchR clusters per field in their longest dimension ( $\geq 25$   
397  $\mu\text{m}$ ) was measured by ImageJ.

398

### 399 **Denervation**

400 Unilateral hindlimb denervation was performed by clamping the left sciatic nerve under anesthesia  
401 by intraperitoneal injection of 40 mg/kg ketamine (Zoletil®, Virbac) and 10 mg/kg xylazine (Rampum,  
402 BAYER). Upon exposure of the sciatic nerve, the nerve was crushed for three times for 10 seconds.  
403 Alternative, for the bulk RNA-seq in Figure 1a, nerve was cut with a scissor. The lesion was sutured  
404 after the operation.

405

406

407

408 **Spinal cord injury**

409 Three-month-old CD1 mice were used in SCI. To perform SCI, mice were deeply  
410 anaesthetized with a mixture 1:1 of Rompun (Bayer 20 mg ml<sup>-1</sup>; 0.5 ml kg<sup>-1</sup>) and Zoletil  
411 (100 mg ml<sup>-1</sup>; 0.5 ml kg<sup>-1</sup>), the back hairs were shaved, the skin was disinfected with  
412 betadine, and an incision was made to expose the spinal cord. Animals were mounted on a  
413 stereotaxic apparatus with spinal adaptors connected to a cortical PinPoint precision  
414 impactor device (Stoelting) and maintained at 37 °C throughout surgery. To induce a severe  
415 trauma the following parameters were set up: middle, round and flat tip (#4); velocity 3 m s<sup>-1</sup>;  
416 depth 5 mm; dwell time 800 ms. The impact was applied at the thoracic level (vertebrae  
417 T10–T11). Analysis of the graphical impact parameters, operated by the PinPoint software,  
418 was used to identify potential outliers. Behavioural analyses were also used to corroborate  
419 differences in injury severity within groups. Slight lesions were excluded from the study  
420 based on these criteria.

421

422 **Cell preparation and isolation by FACS**

423 Tibialis anterior and gastrocnemius muscles, or nerves, of mice were subjected to enzymatic  
424 dissociation [in PBS with 2 mg/mL Collagenase A (Roche, 10 103 586 001), 2,4 U/mL Dispase I  
425 (Roche, 04 942 078 001), 10 ng/mL DNase (Sigma, 11 284 932 001), 0,4 mM CaCl<sub>2</sub> and 5mM MgCl<sub>2</sub>]  
426 for 60 min at 37° C. The cell suspension was filtered through a 40-µm nylon filter and incubated with  
427 the following antibodies for 30 min: CD45 (Invitrogen, 48-0451-82), CD31 (Invitrogen, 48-0311-82),  
428 TER119 (Invitrogen, 48-5921-82), Sca1 (Invitrogen, 11-5981-82) and Itga7 (AbLab, R2F2), Ngfr  
429 (Miltenyi Biotec, 130-118-793).

430 Ngfr<sup>+</sup> cells were isolated as TER119<sup>-</sup>/CD45<sup>-</sup>/CD31<sup>-</sup>/Itga7<sup>+</sup>/SCA-1<sup>-</sup>/Ngfr<sup>+</sup> cells and Pax7-Tomato  
431 <sup>+</sup> as TER119<sup>-</sup>/CD45<sup>-</sup>/CD31<sup>-</sup>/Itga7<sup>+</sup>/SCA-1<sup>-</sup>/Tomato<sup>+</sup> (Suppl.Figure1b).

432 In Suppl.Figure1a, satellite cell purification was performed by using SC Isolation Kit (Miltenyi Biotec,  
433 Bergisch Gladbach, Germany, 130-104-268).

434

### 435 **Histology Immunofluorescence**

436 For the histological analysis 8  $\mu$ m muscle cryosection were analysed. Both cryosections and cultured  
437 cells were fixed in 4% PFA (Sigma, P6148) for 10 min and permeabilized with 100% acetone for 1  
438 min at RT or with 0.1% Triton for 15 min at RT. Muscle sections and cultured cells were then blocked  
439 for 1h with a solution containing 4% BSA (Sigma, A7030-100G) in PBS. PAX7 staining was performed  
440 by an antigen retrieval protocol. The primary antibodies immunostaining was performed ON at 4°C  
441 and then the antibody binding specificity was revealed using secondary antibodies coupled to Alexa  
442 Fluor 488, 594, or 647 [Invitrogen, Goat anti-Mouse Alexa Flour 647 (1:400, A32728), Goat anti-  
443 Rabbit Alexa Flour 488 (1:400, A32731), Goat anti-Mouse Alexa Flour 488, (1:400, A32723)].  
444 Acetylcholine receptors (AChRs) were revealed with fluorescently labeled Bungarotoxin (BTX)  
445 (1:500 Alexa 594, Invitrogen, B13423). Sections were incubated with DAPI (Thermo Fisher  
446 Scientific, D1306) in PBS for 5 minutes for nuclear staining, washed in PBS, and mounted with  
447 glycerol (3:1 in PBS).

448 The primary antibodies used for immunofluorescences are: rabbit anti-Plp1 (1:100, Cell Signaling,  
449 28702S); mouse anti NFI (1:200, Santa Cruz Biotechnology, SC-20012); rat anti-Ngfr-PE (1:100,  
450 Miltenyi Biotec, 130-118-793); rabbit anti-Tnc (1:100, EMD Millipore Corp, AB19013); mouse anti-  
451 Caveolin-3 (1:1000, BD Transduction Laboratories, 610420); rabbit anti-Laminin (1:400, Sigma,  
452 L9393); anti BIII Tubulin mAb (1:500, PROMEGA, G712A); mouse anti-PAX7 (1:20, Developmental  
453 Studies Hybridoma Bank DSHB, Pax7); mouse anti-Myosin (1:10, Developmental Studies  
454 Hybridoma Bank DSHB, MF20); mouse  $\alpha$ -Tubulin (1:200, Cell Signaling, #2144).

455  
456 The transverse sections and cultured cells were visualized on a Zeiss confocal microscope then  
457 edited using the ImageJ® software. All histological analyses were performed in a blinded fashion.  
458 The figures reported are representative of all the examined fields.

459

### 460 **EdU Proliferation Assay**

461 Cell proliferation was measured by EdU incorporation. 20 mg per kg body weight EdU was  
462 administered intraperitoneally (i.p.) 12h before muscle harvest. Incorporation of EdU was revealed

463 using the “Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 594 dye” (Thermo Fisher  
464 Scientific, C10354) following the manufacture protocol.

465

#### 466 **Co-culture conditions of Ngfr+ cells and NSC34**

467 NSC-34 and Ngfr+ cells were cocultured by using inserts with 1µm porous membrane to avoid direct  
468 contact between populations. NSC-34 were grown independently from Ngfr+ in proliferation media  
469 for 48h in 24-well plates. After 24h, freshly sorted Ngfr+ cells were plated on the upper insert and  
470 transwell co-cultures were maintained for additional 72h in proliferation and differentiation media.

471

#### 472 **RNA analysis by quantitative PCR**

473 RNA was extracted from cells using Qiagen RNeasy mini-kits (Qiagen, 74106) following the  
474 manufacturer’s protocol. Total RNA was quantified with a Nanodrop 8000 spectrophotometer  
475 (Thermo Scientific, Wilmington). First-strand cDNA was synthesized from total RNA using the  
476 Transcriptor First Strand cDNA Synthesis kit (Roche, N808-0234) following the manufacturer’s  
477 protocols. The generated cDNA was used as a template in real-time PCR reactions with 2x Fast Q-  
478 PCR Master Mix (SYBR, ROX) (SMOBIO, TQ1211) and was run on a Roche LC480 machine using  
479 three-step amplification and melt curve analysis. Quantitative real-time PCR reactions consisted of  
480 2x SYBR Green Supermix, 0.25 µmol l<sup>-1</sup> forward and reverse primers and 10 ng cDNA. Relative  
481 gene expression was normalized by dividing the specific expression value by the glyceraldehyde 3-  
482 phosphate dehydrogenase (Gapdh) expression value and calculated using the 2<sup>-ΔΔCT</sup> method.

483 The following primer sets were used to identify transcripts:

484 Gapdh FW: CACCATCTTCCAGGAGCGAG, Gapdh RV: CCTTCTCCATGGTGGTGAAGAC,

485 Ngfr FW: TGCCTGGACAGTGTTACGTT, Ngfr RV: ACAGGGAGCGGACATACTCT,

486 Shh FW: CACCCCAATTACAACCCCG, Shh RV: CTTGTCTTTGCACCTCTGAGTC,

487 Fgf5 FW: CTGTACTGCAGAGTGGGCAT, Fgf5 RV: AATTTGGCTTAACACACTGGC,

488 Runx2 FW: GCCTTCAAGGTTGTAGCCCT, Runx2 RV: GTTCTCATCATTCCCGGCCA,

489 Olig1 FW: CTCGCCAGGTGTTTTGTTG, Olig1 RV: TAAGTCCGAACACCGATGGC,

490 Tnc FW: CTACCACAGAGGCCTTGCC, Tnc RV: AGCAGCTTCCCAGAATCCAC,

491 Pax7 FW: AGGACGACGAGGAAGGAGACA, Pax7 RV: TCATCCAGACGGTTCCTTT  
492 Vcam1 FW: GCACTCTACTGCGCATCTT, Vcam1 RV: CACCAGACTGTACGATCCT  
493 Nrcam FW: ATGCACAGACATCAGTGGGG, Nrcam RV: GCTTGCCATTGCCTTCTTACC  
494 Gdnf FW: TGGGTCTCCTGGATGGGATT, Gdnf RV: CGGCGGCACCTCGGAT  
495 Rgs5 FW: CGCACTCATGCCTGGAAAG, Rgs5 RV: TGAAGCTGGCAAATCCATAGC  
496 Myl9 FW: GCGCCGAGGACTTTTCTTCT, Myl9 RV: CCTCGTGGATGAAGCCTGAG  
497 Plp1 FW: CCTAGCAAGACCTCTGCCAGTA, Plp1 RV: GGACAGAAGGTTGGAGCCACAA

498

#### 499 **Tamoxifen (Tmx) treatment and denervation**

500 We used PAX7CreER/tdTomato<sup>ff</sup> mice between the ages of 2 and 3 month for Tmx (Sigma, T5648)  
501 injections. Tmx (3 mg) suspended in corn oil was injected intraperitoneally (i.p.) each day for 5 d.  
502 After 7 d from the last injection, we performed the unilateral hindlimb denervation. Tissues were  
503 harvested after 3 d for FACS.

504

#### 505 **RNA-sequencing**

506 MuSC were isolated from mice TA and GA muscle as described. RNA from MuSC was extracted  
507 using RNeasy Mini kit (Qiagen) following the manufacturer's protocol. RNA was shipped to the  
508 sequencing IGA of Udine. The libraries for sequencing were prepared using NuGEN Ovation System  
509 V2 RNA-Seq. For each biological sample two independent experiments were carried out for the  
510 isolation of RNA. All duplicates are pool of three different mice, sorted at different times.

511

#### 512 **RNA-sequencing data processing**

513 For sequencing alignment we used the mouse reference genome assembly GRCm38/mm10  
514 ([http://ftp.ensembl.org/pub/release-76/fasta/mus\\_musculus/dna/](http://ftp.ensembl.org/pub/release-76/fasta/mus_musculus/dna/)), and for transcriptome annotation  
515 we used version 85 of the GRCm38  
516 ([http://ftp.ensembl.org/pub/release85/gtf/mus\\_musculus/Mus\\_musculus.GRCm38.85.gtf.gz](http://ftp.ensembl.org/pub/release85/gtf/mus_musculus/Mus_musculus.GRCm38.85.gtf.gz)). We  
517 used the FASTQC package (v0.11.3) to assess the quality of sequenced libraries. All passed quality  
518 control. Reads were mapped to the reference genome using TopHat2 v.2.1.1. The quality control of

519 the reads distribution along transcripts was performed using infer\_experiment.py from RSeQC  
520 package v2.6.3. All samples had a uniform distribution of reads along transcripts. The sequenced  
521 read counts per annotated gene were derived with the use of htseq-count script distributed with  
522 HTSeq v0.5.4p5. We used the R library package DESeq2 v.1.12.4 for measuring differential gene  
523 expression between two different cell conditions, considering the two RNA-seq experiments as  
524 biological replicates. We picked genes with adjusted P value < 0.001. Gene ontology analysis was  
525 performed using David 6.8 (<https://david.ncifcrf.gov/>). In details Biological Process was predicted on  
526 genes differentially expressed with p adjusted<0.01 with the following setting: threshold counts=2,  
527 threshold EASE=0.1. The most significant 12 functional annotation was illustrated in the figure.

528

### 529 **Single-cell RNA-sequencing**

530 Single-cell RNA-sequencing was performed at IGA facility of Udine - Italy  
531 (<https://igatechnology.com/>). Methanol fixed cells were rehydrated following 10X Genomics  
532 recommendation. In order to remove visible debris, an additional washing in Wash-Resuspension  
533 buffer was introduced. Cell concentration was determined using the Countess II FL Automated Cell  
534 Counter (Thermo Fisher Scientific, Waltham, MA). Trypan Blue staining of the methanol fixed cells  
535 showed that 100% of the cells were dead, indicating that all cells were effectively fixed and  
536 permeabilized.

537 Chromium controller and Chromium NextGEM Single Cell 3' Reagents Kit v3.1 (10X Genomics,  
538 Pleasanton, CA) have been used for partitioning cells into Gel Beads-in-emulsion (GEMs), where all  
539 generated cDNA share a common 10x barcode. Libraries were generated from the cDNA following  
540 manufacturer's instruction and checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA)  
541 and Agilent Bioanalyzer DNA assay (Agilent technologies, Santa Clara, CA). Libraries were then  
542 prepared for sequencing and sequenced on NovaSeq6000 (Illumina, San Diego, CA) with the  
543 following run parameters: Read 1=28 cycles, i7 index=8 cycles, Read 2=91 cycles.

544

545

546

547 **Single-cell RNA-sequencing data processing.**

548 The raw sequencing data were processed by Cell Ranger v 3.1.0 (10X Genomics) with mouse  
549 transcriptome reference mm10 to generate gene-cell expression matrices. Further data analysis was  
550 carried out in R version 3.6.0 using Seurat version 3.1.1(46). The two datasets were set up as  
551 independent Seurat objects. “Cells” that fit any of the following criteria were filtered out: < 200 or >  
552 4,500 expressed genes, or > 10% UMIs mapped to mitochondria. Dataset normalization and  
553 identification of variable features were performed using the NormalizeData() function and the  
554 FindVariableFeatures() with following parameters (selection.method = "vst", nfeatures = 4000).  
555 Integration anchors were computed using the first 20 dimensions, using all the genes present in both  
556 datasets as features to integrate. Finally, we obtained 3949 cells that passed quality control, with an  
557 average of 1,460 genes expressed per cell. For downstream integrated analyses, top 30  
558 components were used for PCA, UMAP and cluster identification (using a resolution of 0.4). Further,  
559 we manually assigned cell population identity based on cell-type-specific markers and merged those  
560 clusters that displayed similar canonical markers. After clustering and cell population identification,  
561 the most highly differentially expressed genes, or putative cluster markers, were identified by a  
562 likelihood-ratio test using the FindAllMarkers() function with the following parameters(only.pos =  
563 TRUE, min.pct =0.5, min.diff.pct=0.25, logfc.threshold = 0.25). Genes differentially expressed in  
564 CTR vs DEN were identified using the FindMarkers() function and subsequently filtered using the  
565 following criteria (pct.1>0.45 or pct.2>0.45; p\_val\_adj<0.01; avg\_logFC< (-0.58) or  
566 avg\_logFC>0.58). Dataset Integration with previously published scRNAseqs (Giordani and De  
567 Micheli) has been performed in Seurat with FindIntegrationAnchors() and IntegrateData() functions  
568 using the first 20 dimensions. From the De Micheli dataset, only the uninjured datapoint ("D0") has  
569 been used for comparison. Both datasets were downloaded from the GEO website.

570

571 **Figure Design**

572 Graphical abstract, Figure 1d, Figure 4a, Figure 6b and Figure 6e were created with BioRender  
573 (<https://biorender.com/>)

574

575 **Statistics**

576 Data are presented as mean with SD. Statistical analysis was performed using Graph Pad Prism 8.0  
577 software (Pad Software). Normality was tested by Shapiro-Wilk test. Unpaired, two-tailed Student's  
578 t test was used to compare the means of two parametric groups, while Mann-Whitney test for two  
579 non-parametric groups. One-way ANOVA with Tukey's post-test was used for comparison among  
580 the different parametric data sets. Significance was defined as  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P <$   
581  $0.001$  (\*\*).

582 The number of biological replicates for each experiment is indicated in the figure legends. RNAseq  
583 data was performed in 2 independent samples derived from different animals. Statistical method was  
584 Deseq2. Right-tailed Fisher's exact test and one-sided Fisher's exact test was used for IPA analyses.  
585 For scRNA-seq, biological sample replicates came also from separate mice. Histological and  
586 Immunofluorescence images are representative of at least 3 different experiment/animals. For cell  
587 culture studies, biological replicates from separate culture wells.

588

589 **Data and code availability**

590 a7int+ mouse bulk RNA-sequencing data, Mouse Single Cells RNA-sequencing data and Ngfr+  
591 Mouse bulk RNA-sequencing data are available at the SRA repository (Accession numbers:  
592 PRJNA623246, PRJNA626530 and PRJNA649152).

593

594 **STUDY APPROVAL**

595 All experiments in this study were performed in accordance with protocols approved by the Italian  
596 Ministry of Health, National Institute of Health (IIS), and Santa Lucia Foundation (Rome) and by  
597 the Sanford Burnham Prebys Medical Discovery Institute Animal Care and Use Committee. The  
598 study is compliant with all relevant ethical regulations regarding animal research and in the respect  
599 of the principles of the 3Rs (Replacement, Reduction and Refinement).



600 **AUTHOR CONTRIBUTIONS**

601 Designing Research Studies, D.P., PL.P. and LM.; conducting experiments, D.P. and L.M.;  
602 investigation, D.P., L.M., M.D., L.G., C.D., S.M.; resources, L.M., A.S., M.A., M.B., C.V., PL.P.;  
603 analyzing data, D.P., L.G., S.A. and L.M.; writing—original draft preparation, L.M.; writing—review  
604 and editing, L.M., L.G., PL.P., B.L.O., C.V., and M.B.; supervision, L.M., PL.P.; project administration,  
605 funding acquisition, L.M. and PL.P.

606

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618

619

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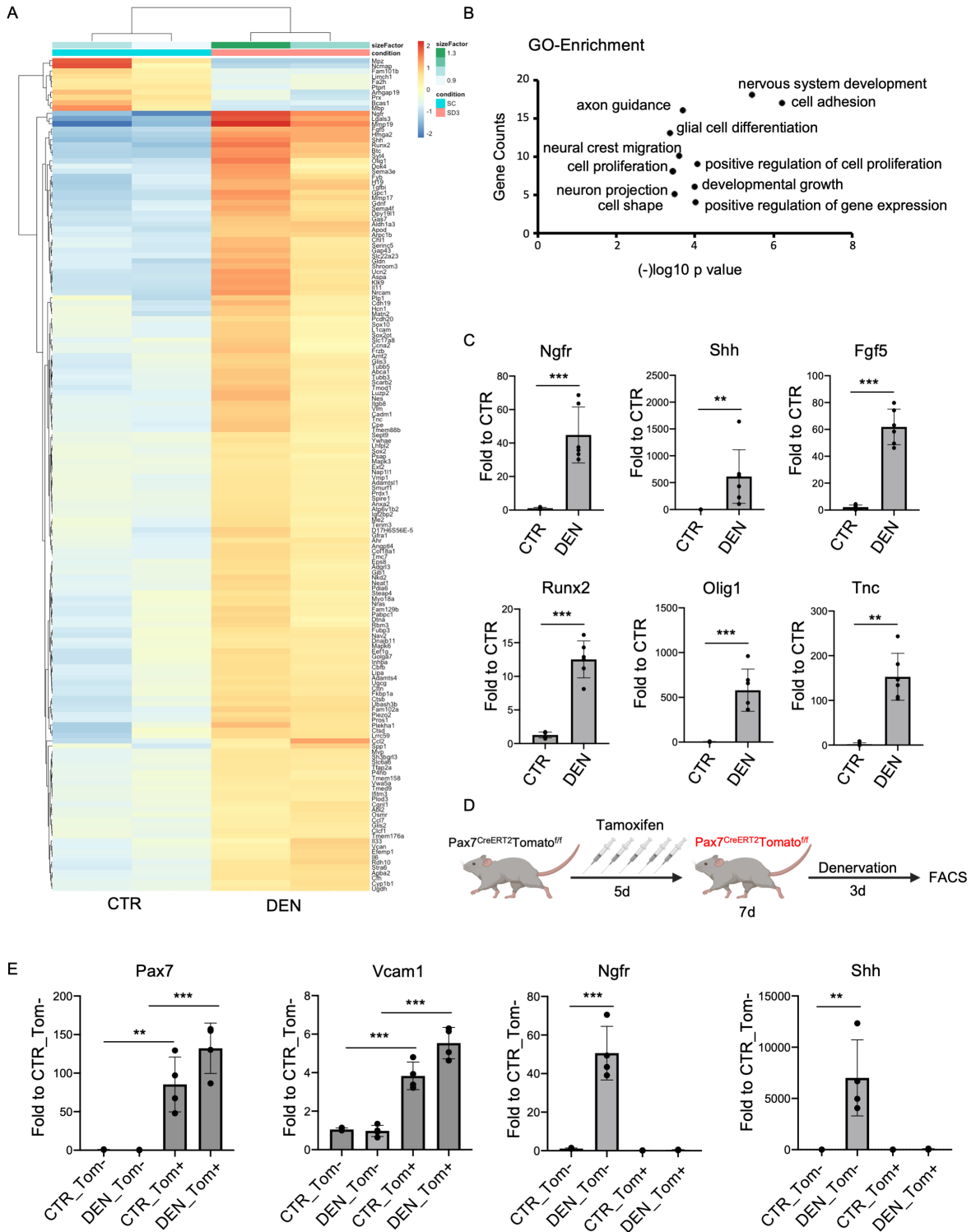
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722

Proietti et al. Figure 1



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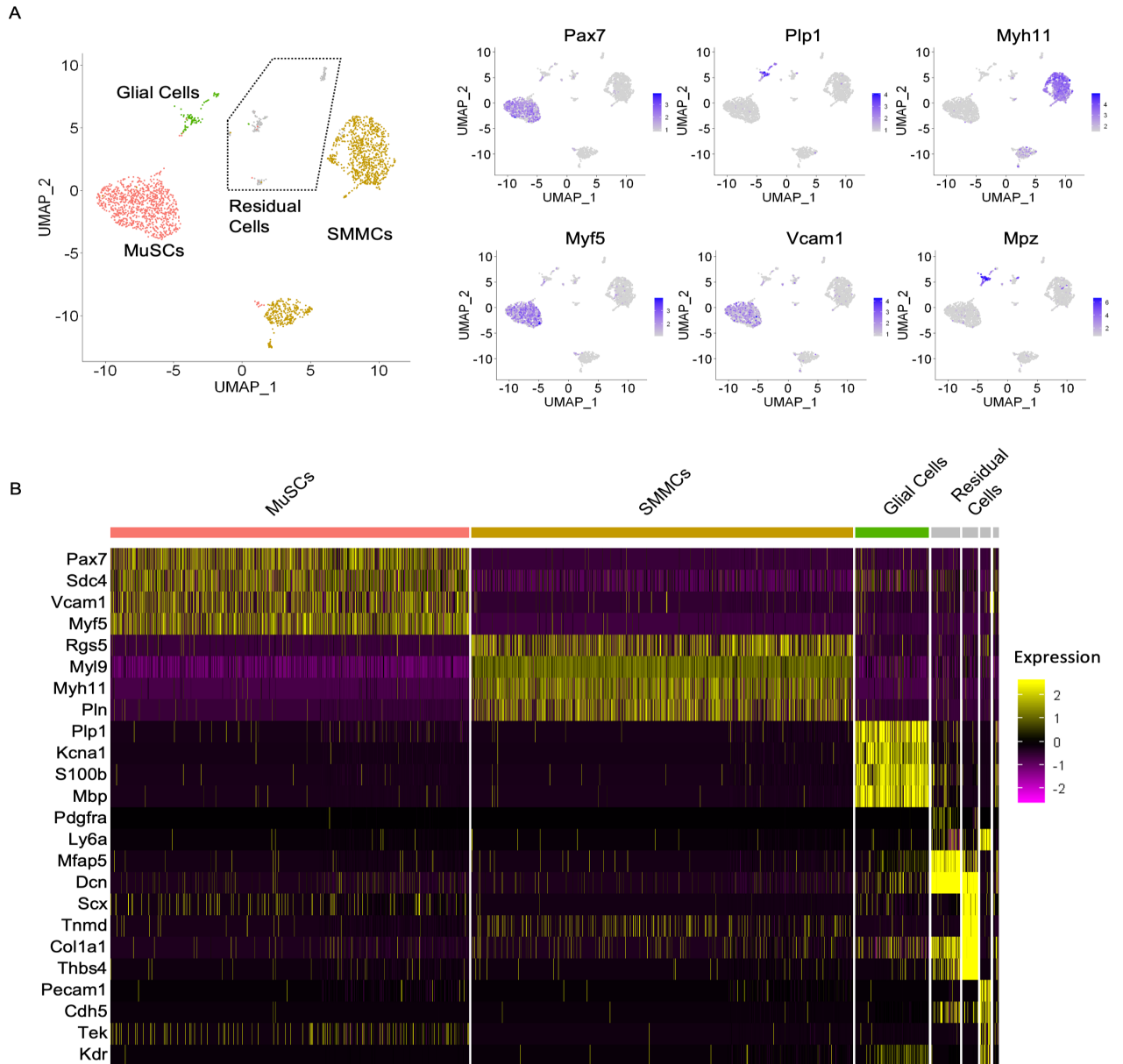
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725 **Figure 1. Activation of a neurotrophic signaling pathway in the Itga7+Sca1-Ln- myogenic cell**  
726 **fraction.**

727 **A)** Heatmap representation of genes significantly deregulated -  $p_{adj} < 0.001$  - in Itga7+Sca1-Ln-  
728 freshly isolated cells derived from denervated (cut) muscle at 3-days post nerve lesion (n=2). **B)** GO-  
729 Enrichment in Biological Function (BF) of genes significantly deregulated -  $p_{adj} < 0.001$  - in  
730 Itga7+Sca1-Ln- cells derived from at 3 days denervated muscle. **C)** qPCR analysis for the expression  
731 of Ngfr, Shh, Fgf5, Runx2, Olig1 and Tnc in freshly isolated Itga7+Sca1-Ln- cells derived from control  
732 and 3 days reversible denervated (crush) muscle. Gapdh was used as housekeeping gene ( $n \geq 5$ ,  
733 Values represent mean  $\pm$  s.d.  $**P < 0.01$  and  $***P < 0.001$ ; by student t-test (Ngfr, Fgf5, Olig1, Runx2,  
734 Shh) or by Mann-Whitney test (Tnc)). **D)** Working model of tamoxifen induced in-vivo treatment. **E)**  
735 qPCR analysis for the expression of Pax7, Vcam1, Ngfr and Shh in freshly isolated Tomato+ and  
736 Tomato- cells derived from control and 3 days denervated muscle of tamoxifen treated  
737 PAX7.Cre\_tdTomato mice. Gapdh was used as housekeeping gene (n=4, Values represent mean  $\pm$   
738 s.d.  $**P < 0.01$  and  $***P < 0.001$ ; by One Way Anova Tukey's Multiple Comparisons test).  
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Proietti et al. Figure 2



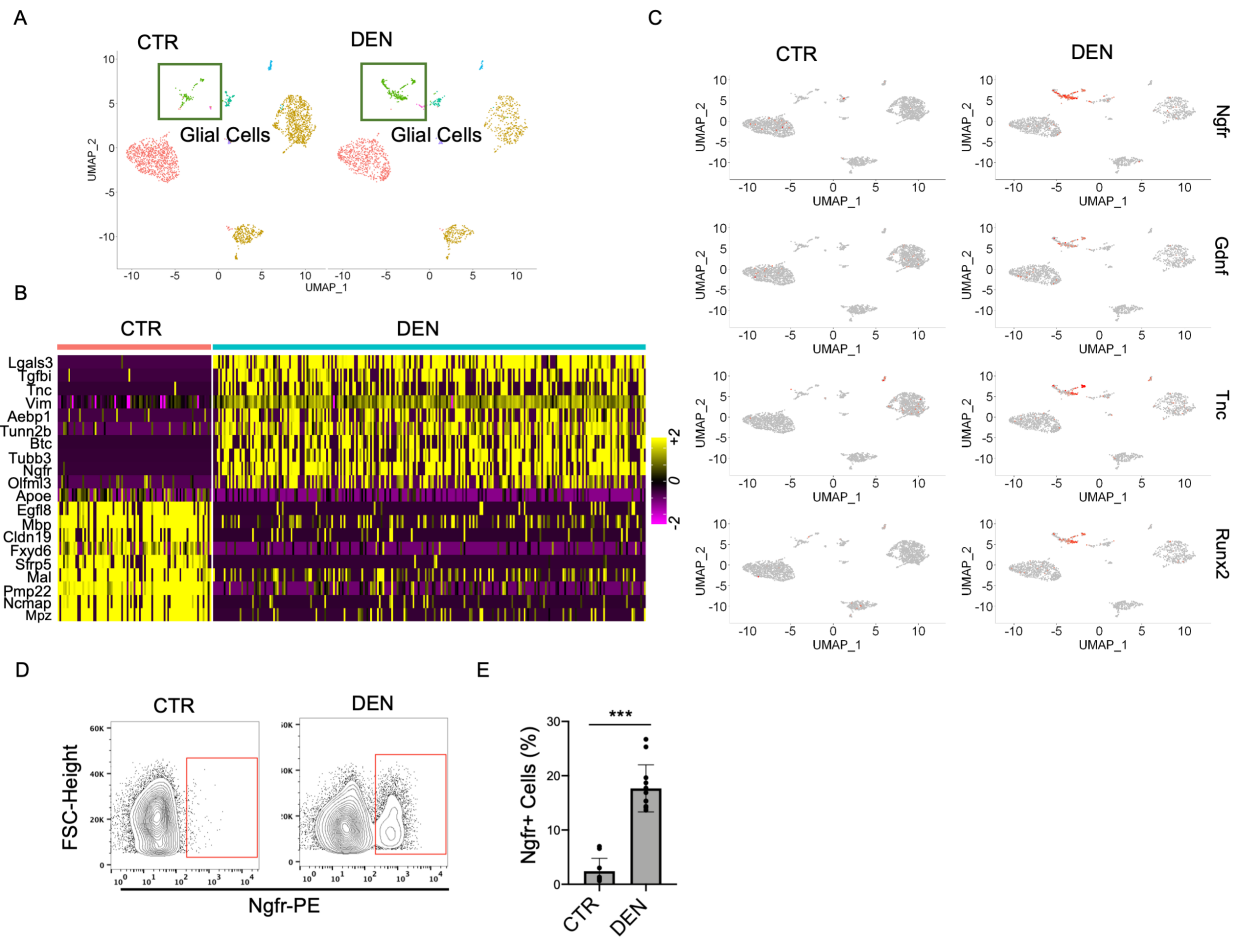
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742 **Figure 2. Itga7+ cell heterogeneity revealed by scRNA-seq analysis.**

743 **A)** Distribution of Pax7, Plp1, Myh11, Myf5, Vcam1 and Mpz transcripts in Uniform Manifold  
744 Approximation and Projection (UMAP)-derived clusters of single cells (Single Cells RNA-seq) of  
745 Itga7+Sca1-Ln- isolated cells from control muscle. **B)** RNA expression heatmap for the given cell  
746 populations (column) and genes (row), sorted by clusters. The canonical markers used to identify  
747 each cluster are plotted (or the most variable genes per cluster in cases where markers were not  
748 already present in the literature).

749

Proietti et al. Figure 3



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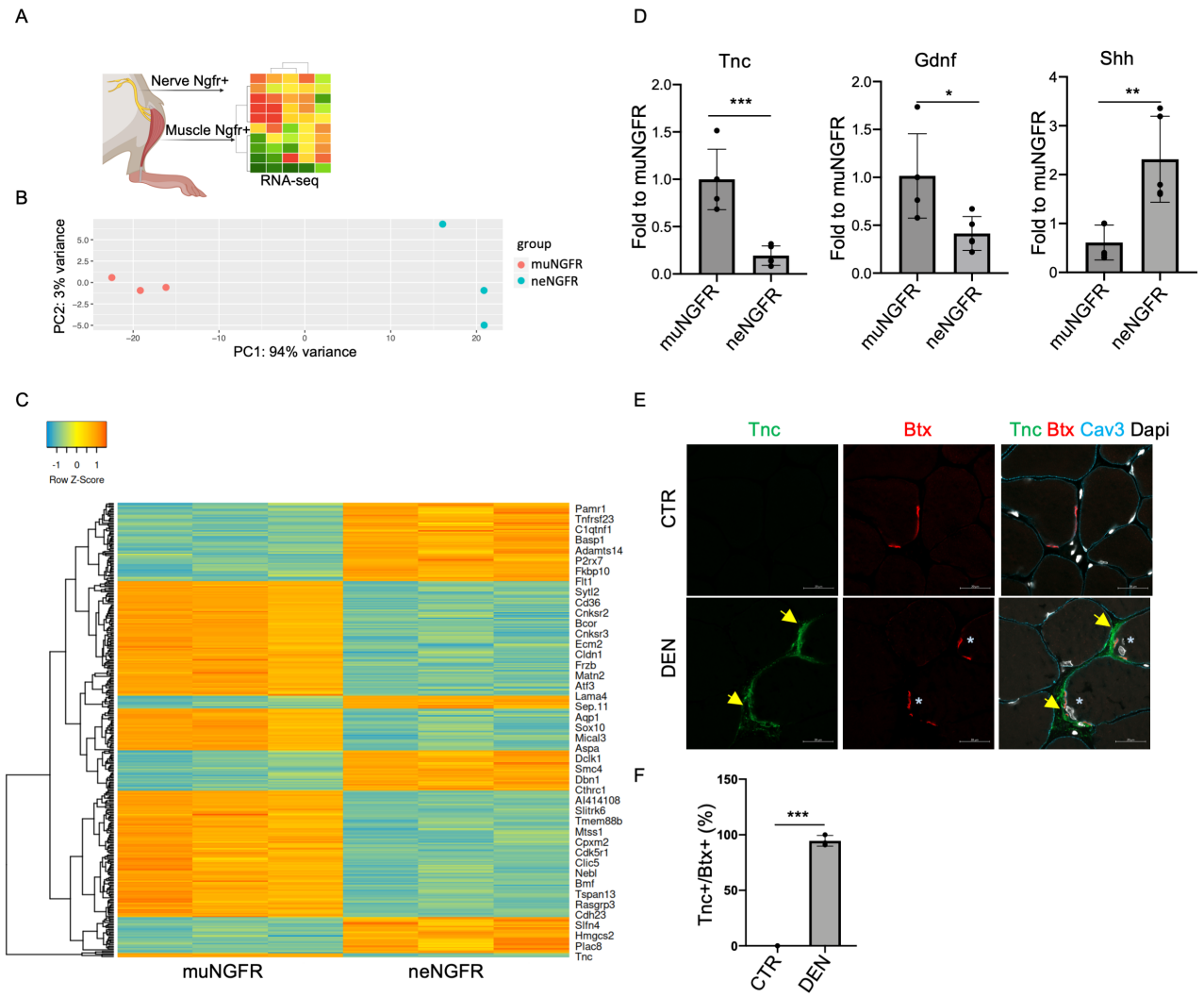
752 **Figure 3. Activation of a neurotrophic signalling pathway in muscle glial cells upon**  
753 **denervation.**

754 **A)** Distribution in Uniform Manifold Approximation and Projection (UMAP)-derived clusters of single  
755 cells (Single Cells RNA-seq) of Itga7+Sca1-Ln- isolated cells from control (CTR-left) and 3-days  
756 denervated muscle (DEN-right). **B)** RNA expression heatmap for Plp1 cell populations isolated from  
757 control and denervated muscle (row) and genes (column), sorted by clusters. **C)** Distribution of Ngfr,  
758 Gdnf, Tnc and Runx2 transcripts in Uniform Manifold Approximation and Projection (UMAP)-derived  
759 clusters of single cells (Single Cells RNA-seq) of Itga7+Sca1-Ln- isolated cells from control (left) and  
760 denervated (right) muscle. **D)** Representative cytofluorimetric plot of Ngfr+ - gated within the  
761 Itga7+Sca1-Ln- population - cells in control (left) and denervated (right) muscle. **E)** Quantification of  
762 Ngfr+ cells was shown in the graphs as a percentage of Itga7+Sca1-Ln- population (n=8 CTR, n=10  
763 DEN, Values represent mean  $\pm$  s.d. \*\*\*P < 0.001; by Mann-Whitney test).

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Proietti et al. Figure 4



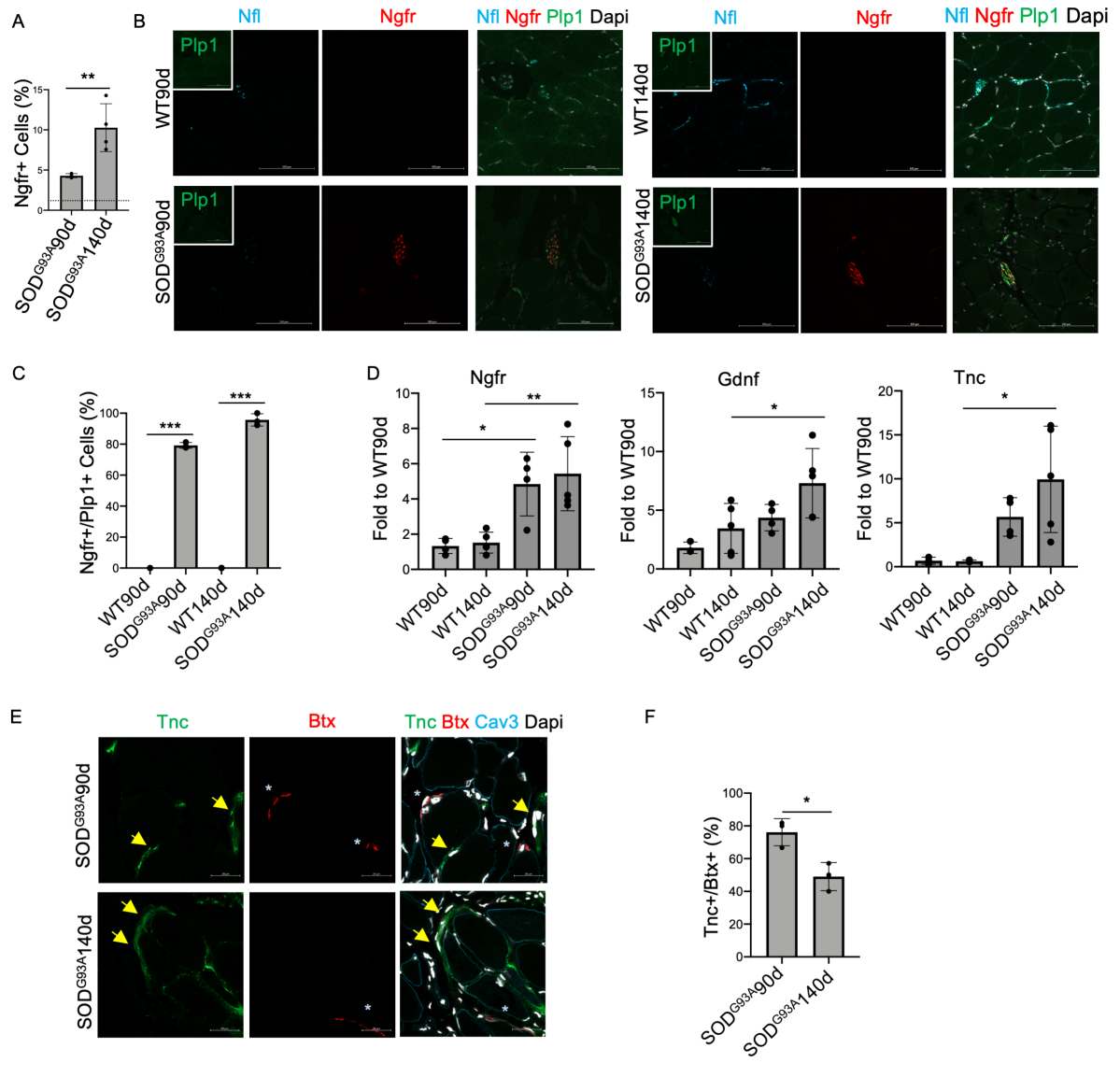
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768 **Figure 4. A specific transcriptional signature distinguishes glial cells in muscle from those**  
769 **residing in the nerve**

770 **A)** Experimental setting for RNA-sequencing analysis of Ngfr+ cells derived from denervated muscle  
771 and nerve at 3-days post nerve lesion. **B)** Sample distance - represented as Principal component  
772 analysis (PCA) - of transcriptome of Ngfr+ cells derived from denervated muscle and nerve at 3-days  
773 post nerve lesion (n=3). **C)** Heatmap representation of genes significantly deregulated -  $p_{adj} < 0.001$   
774 – in freshly isolated Ngfr+ cells derived from denervated muscle and nerve at 3-days post nerve  
775 lesion (n=3). **D)** qPCR analysis for the expression of Tnc, Gdnf and Shh in freshly isolated Ngfr+  
776 cells derived from denervated muscle (muNGFR) and nerve (neNGFR) at 3-days post nerve lesion  
777 (n=4, Values represent mean  $\pm$  s.d. . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ; by student t-test (Tnc,  
778 Gdnf) or by Mann-Whitney (Shh)). **E)** Representative immunofluorescence analysis of TA muscle  
779 cryosection derived from control and denervated muscle, stained for Tnc (green), Bungarotoxin (Btx,  
780 red) and Caveolin-3 (Cav3, Cyan). Arrows highlight Tnc and asterisk highlight Btx. Nuclei were  
781 counterstained with dapi. Scale bar = 20 $\mu$ m. **F)** Quantification Bungarotoxin (Btx) and Tnc co-  
782 localization in control and denervated muscle (n=3, Values represent mean  $\pm$  s.d. \*\*\* $P < 0.001$ ; by  
783 student t-test)

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Proietti et al. Figure 5



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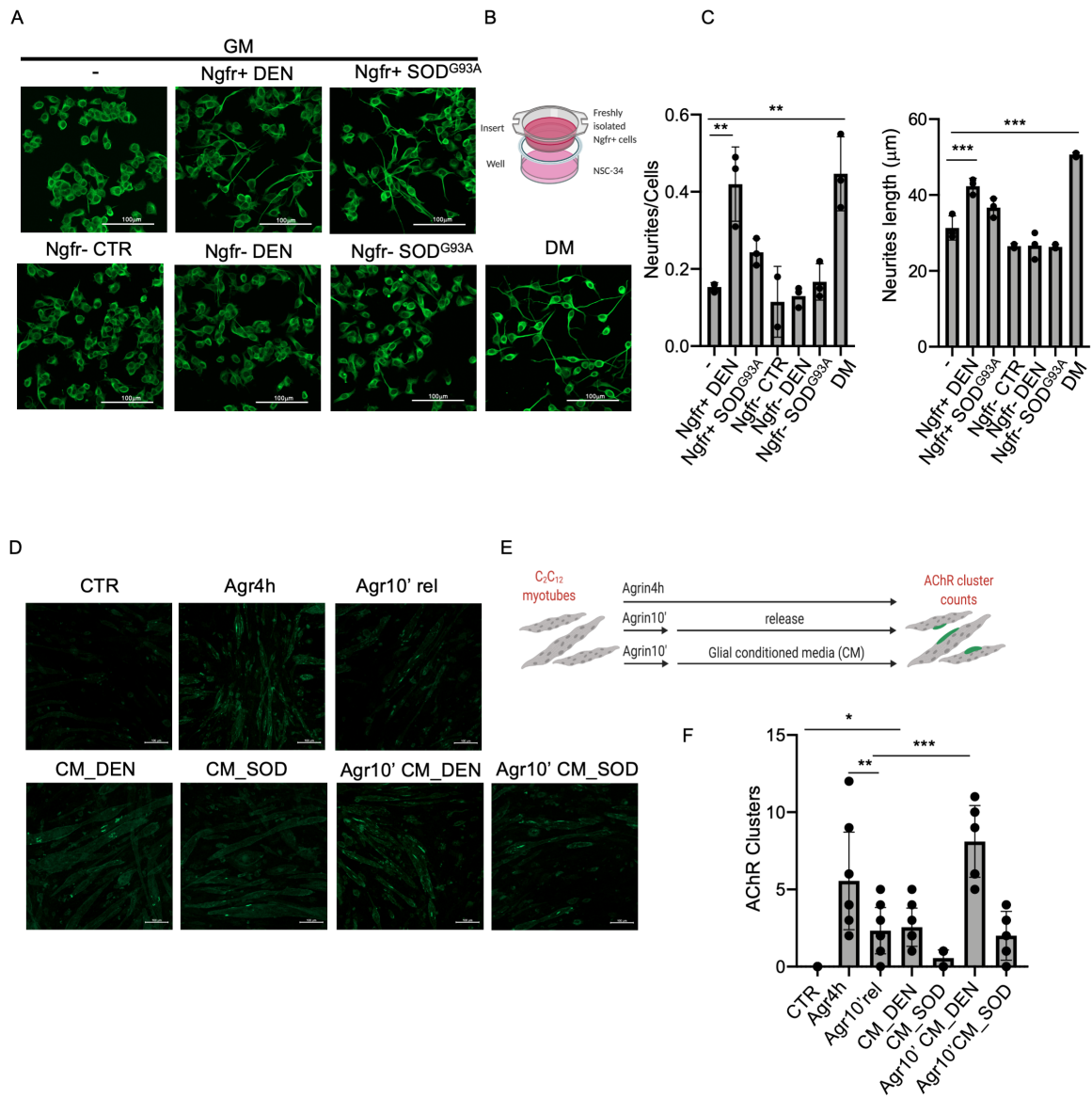
787 **Figure 5. Muscle resident glial cell activation in a mouse model of ALS.**

788 **A)** Ngfr+ cell cytofluorimetric quantification was shown in the graphs as a percentage of Itga7+Sca1-  
789 Ln- population, in 90- and 140-days old SOD<sup>G93A</sup> mice muscle (n=4, Values represent mean ± s.d.  
790 \*\*P < 0.01; by One Way Anova Tukey's Multiple Comparisons test). Dotted line highlight percentage  
791 in WT mice muscle. **B)** Representative immunofluorescence analysis of TA muscle cryosection  
792 derived from 90- and 140-days old SOD<sup>G93A</sup> and WT mice stained for Neurofilament-L (Nfl, Cyan),  
793 Ngfr (red) and Plp1 (green). Nuclei were counterstained with dapi. Scale bar = 100µm. **C)**  
794 Quantification graph of Ngfr+/Plp1+ cells in 90- and 140-days old WT and SOD<sup>G93A</sup> mice muscle  
795 (n=3, Values represent mean ± s.d. \*\*\*P < 0.001; by One Way Anova Tukey's Multiple Comparisons  
796 test) **D)** qPCR analysis for the expression of Ngfr, Gdnf and Tnc in freshly isolated Itga7+Sca1-Ln-  
797 cells derived from WT and SOD<sup>G93A</sup> muscle at 90- and 140-days of post-natal life. Gapdh was used  
798 as housekeeping gene (n=4, Values represent mean ± s.d. \*P < 0.05 and \*\*P < 0.01; by One Way  
799 Anova Tukey's Multiple Comparisons test). **E)** Representative immunofluorescence analysis of TA  
800 muscle cryosection derived from 90- and 140-days old SOD<sup>G93A</sup> and WT mice stained for Tnc  
801 (green), Bungarotoxin (Btx, red) and Caveolin-3 (Cav3, Cyan). Arrows highlight Tnc and asterisk  
802 highlight Btx. Nuclei were counterstained with dapi. Scale bar = 20µm. **F)** Quantification of  
803 Bungarotoxin (Btx) and Tnc co-localization in 90- and 140-days old SOD<sup>G93A</sup> mice muscle (n=3,  
804 Values represent mean ± s.d. \*P < 0.05; by student t-test)

805



Proietti et al. Figure 6



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807

808 **Figure 6. Muscle resident glial cells promote neurite outgrowth and AChR clustering.**  
809 **A)** Representative immunofluorescence analysis of NSC-34 cells in growth media cultured either  
810 alone (-) or in co-culture with Ngfr+ or with Ngfr- cells, both from denervated muscle and SOD<sup>G93A</sup>  
811 muscle at 90-days of post-natal life, and of NSC-34 cells cultured in neurogenic differentiation media  
812 (DM), stained for beta-3-Tubulin (green). Scale bar = 100µm. **B)** Schematic representation of in-vitro  
813 co-culture system. **C)** Quantification of neurites number per cell and length of NSC-34 cultured in  
814 the indicated conditions (n=3, Values represent mean ± s.d. \*\*P < 0.01 and, \*\*P < 0.01; by One Way  
815 Anova Tukey's Multiple Comparisons test). **D)** Representative immunofluorescence analysis of  
816 C2C12 myotubes treated or not with Agrin or conditioned media from glial cells as indicated and  
817 stained with Bungarotoxin (Btx, green). Scale bar = 100µm. **E)** Schematic representation of the  
818 experimental setting. **F)** Quantification of AChR clustering (≥ 25 µm) (n=5, Values represent mean ±  
819 s.d.. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001; by One Way Anova Tukey's Multiple Comparisons test).  
820