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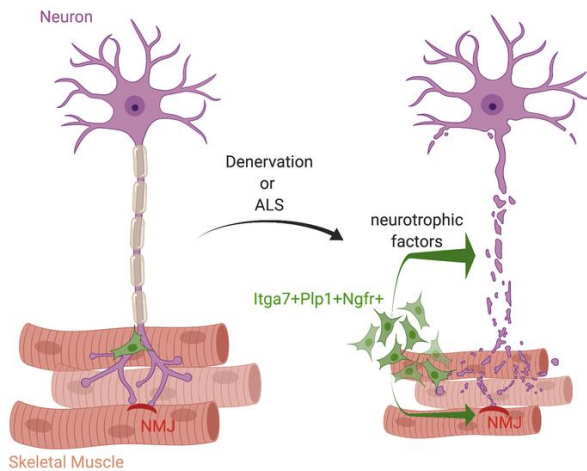
Activation of skeletal muscle-resident glial cells upon nerve injury

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

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1 **TITLE PAGE**

2

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

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^{CreER};tdTomato^{ff}* 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*⁺ cells. In this mouse model, tamoxifen treatment
109 leads to the permanent expression of tomato fluorescent protein in *Pax7*⁺ 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*⁺*Tomato*⁺ 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*⁺*Tomato*⁻ cell population did not express these markers
114 (Figure 1e). However, upon denervation, only the *Itga7*⁺*Tomato*⁻ 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*⁺ 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*⁺ 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*⁺ 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*⁺ 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*⁺
129 cells, we performed single-cell RNA-seq (scRNA-seq) transcriptome profiling in *Itga7*⁺ 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^{low} and
138 Myl9+Rgs5^{high} 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^{high} expressing cells), endothelial (Pecam1^{high}) and mesenchymal (as
142 Ly6a/Ly6e^{pos} and Pdgfra^{pos}) lineages (Figure 2b). However, it is likely that the presence of these
143 clusters is due to contamination from Itga7^{low/neg} 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^{G93A} 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^{G93A} 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^{G93A} 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^{G93A} 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^{G93A} 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^{G93A}, 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^{G93A} 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

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*⁺ 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*⁺ 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^{G93A} mice. However, activated
344 muscle glial cells from symptomatic SOD1^{G93A} 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^{ff} mice were provided by the SBP Animal Facility (La Jolla).
- 359 • Hemizygous transgenic mice carrying the mutant human SOD1^{G93A}(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^{G93A} mice, as indicated in the Figure 5. For
367 the denervation experiments, both male and female C57BL/6J and PAX7CreER/tdTomato^{ff} mice
368 were used. Only female mice were used for the spinal cord injury experiment. As a mouse model of
369 ALS male SOD1^{G93A} mice were used.

370

371 **Cell Lines and Primary Cell Cultures**

372 All cells were cultured in incubators at 37 °C and 5% CO₂. 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 recombinat 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 α -bungarotoxin (Invitrogen, B13422).
395 Myotubes were incubated with the α -bungarotoxin diluted (1:300) in differentiation medium for 1
396 hours at 37°C in 5% CO₂. The number of AchR clusters per field in their longest dimension (≥ 25
397 μm) 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 Figure1a, 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⁻¹; 0.5 ml kg⁻¹) and Zoletil
411 (100 mg ml⁻¹; 0.5 ml kg⁻¹), 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⁻¹;
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 CaCl₂ and 5mM MgCl₂]
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⁺ cells were isolated as TER119⁻/CD45⁻/CD31⁻/Itga7⁺/SCA-1⁻/Ngfr⁺ cells and Pax7-Tomato
431 ⁺ as TER119⁻/CD45⁻/CD31⁻/Itga7⁺/SCA-1⁻/Tomato⁺ (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 μ 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 α -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⁻¹ 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^{-ΔΔCT} 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^{ff} 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/), 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). 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
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606

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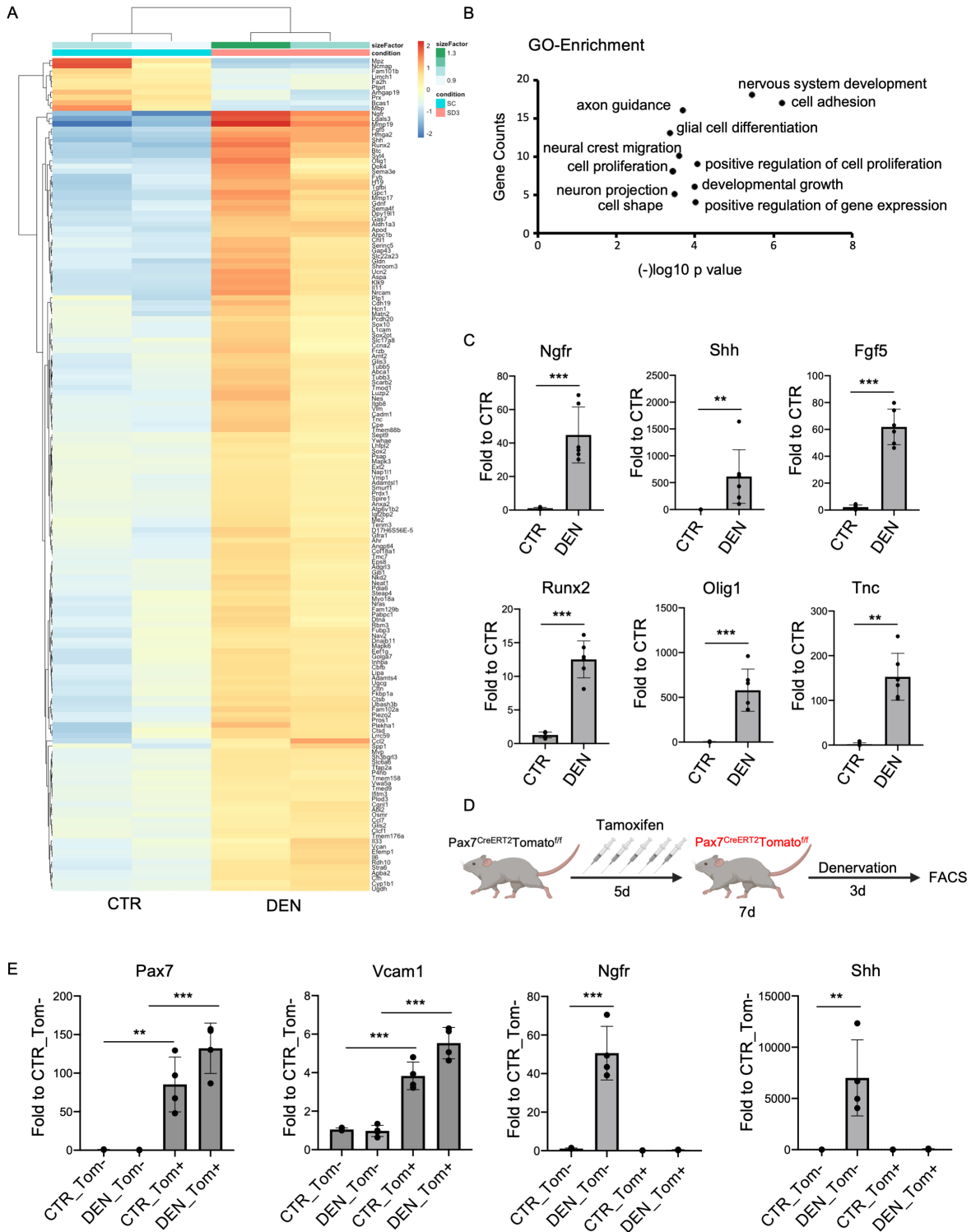
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722

Proietti et al. Figure 1



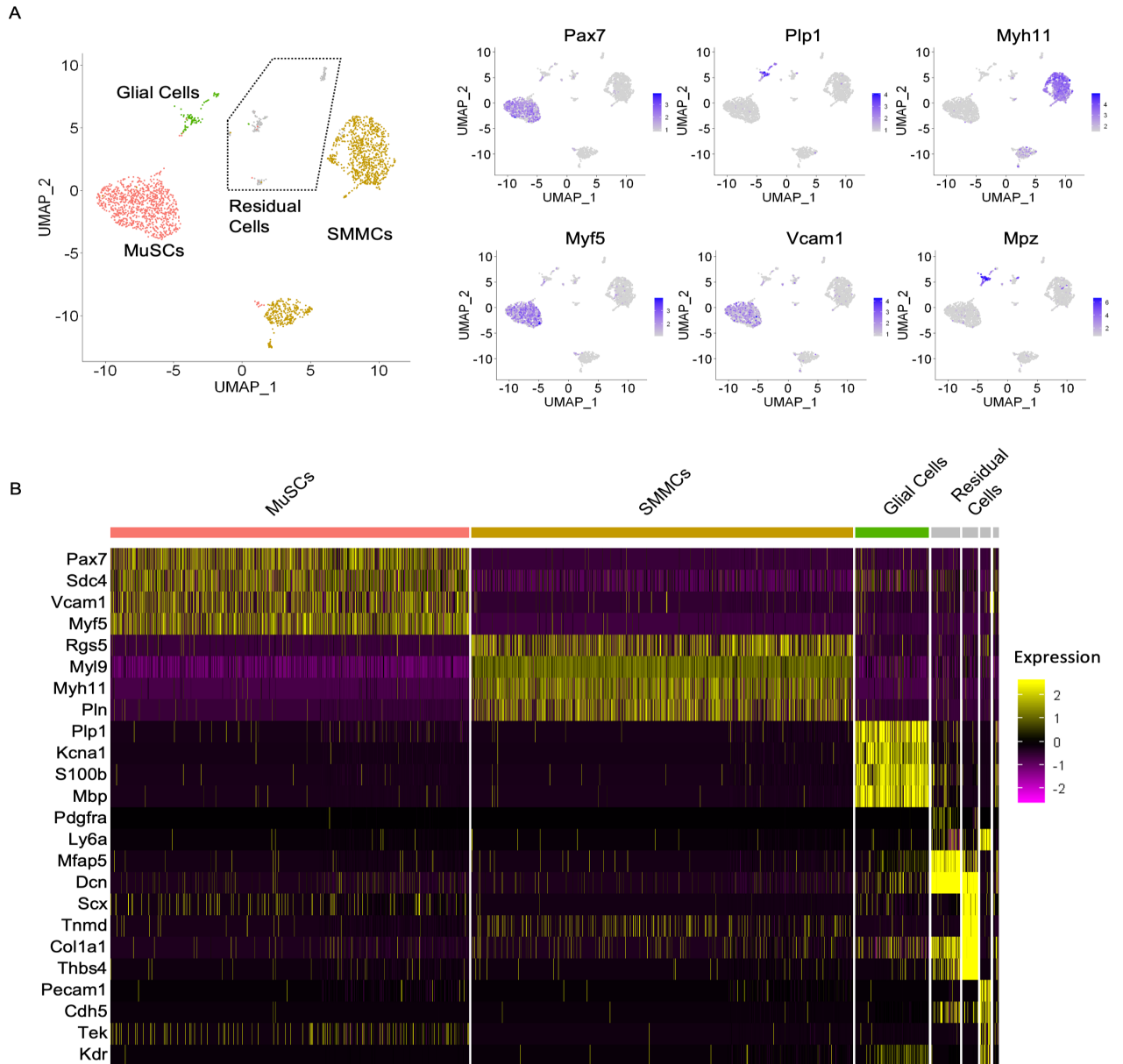
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725 **Figure 1. Activation of a neurotrophic signaling pathway in the Itga7+Sca1-Ln- myogenic cell**
726 **fraction.**

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



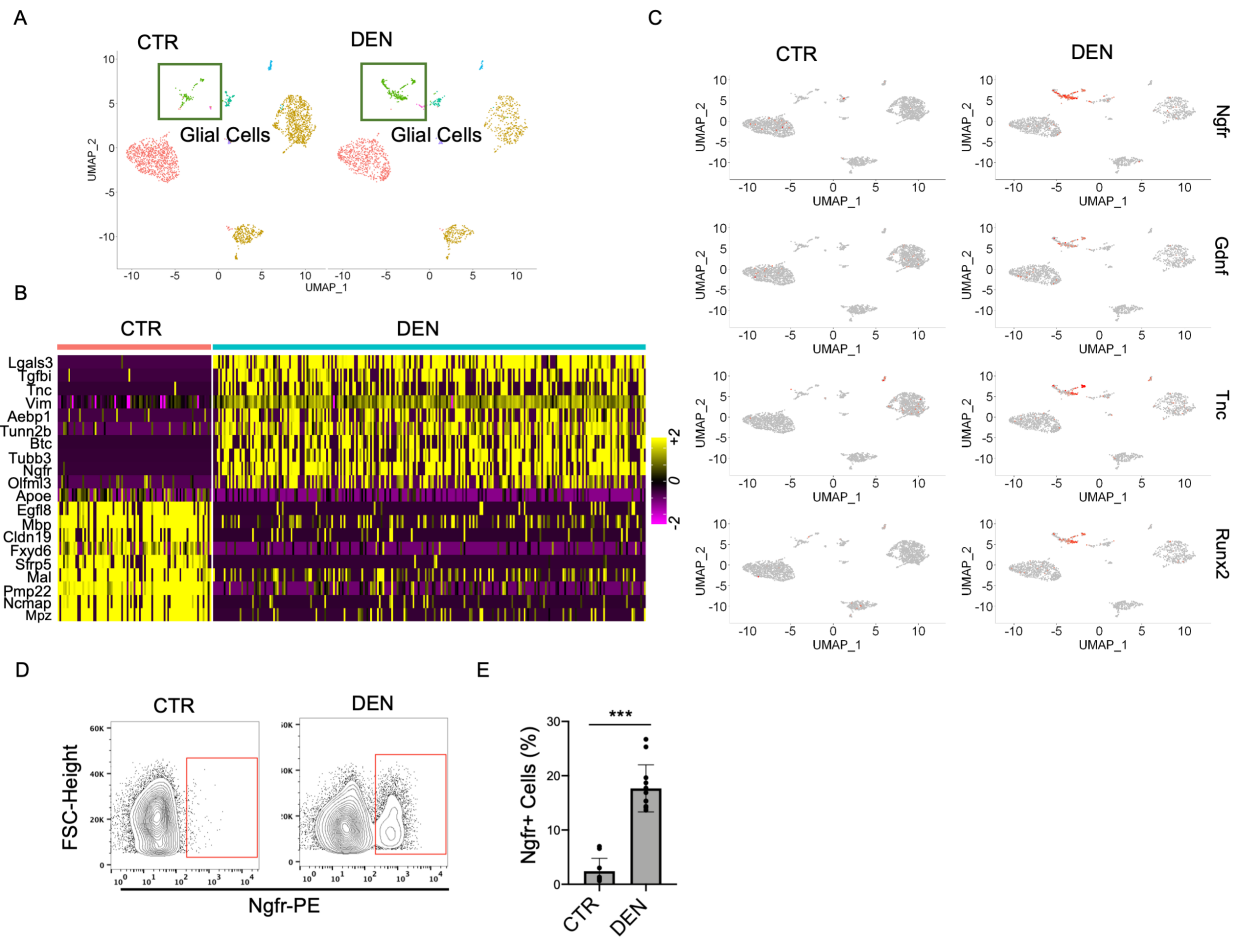
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742 **Figure 2. Itga7+ cell heterogeneity revealed by scRNA-seq analysis.**

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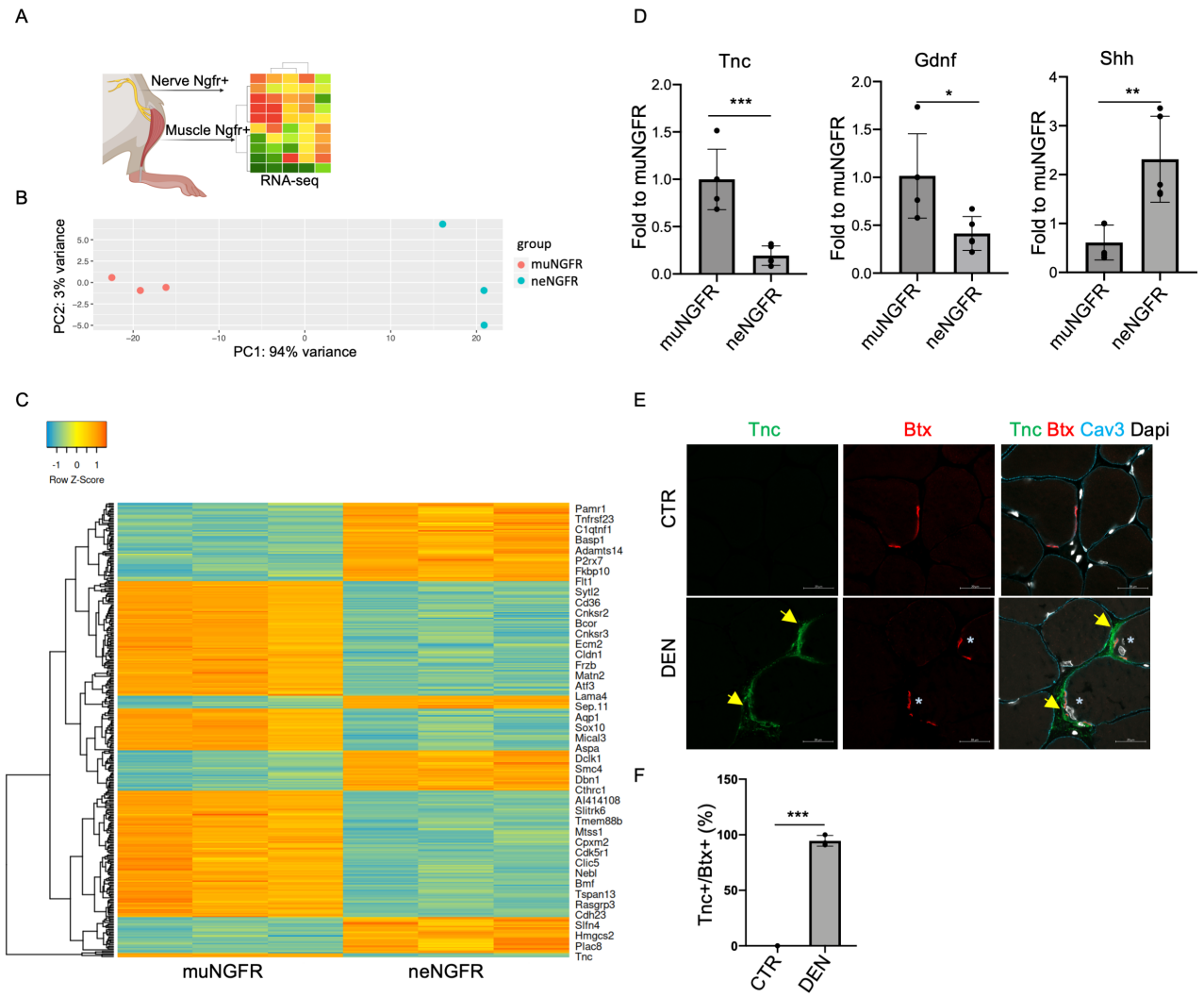
752 **Figure 3. Activation of a neurotrophic signalling pathway in muscle glial cells upon**
753 **denervation.**

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).

764

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Proietti et al. Figure 4



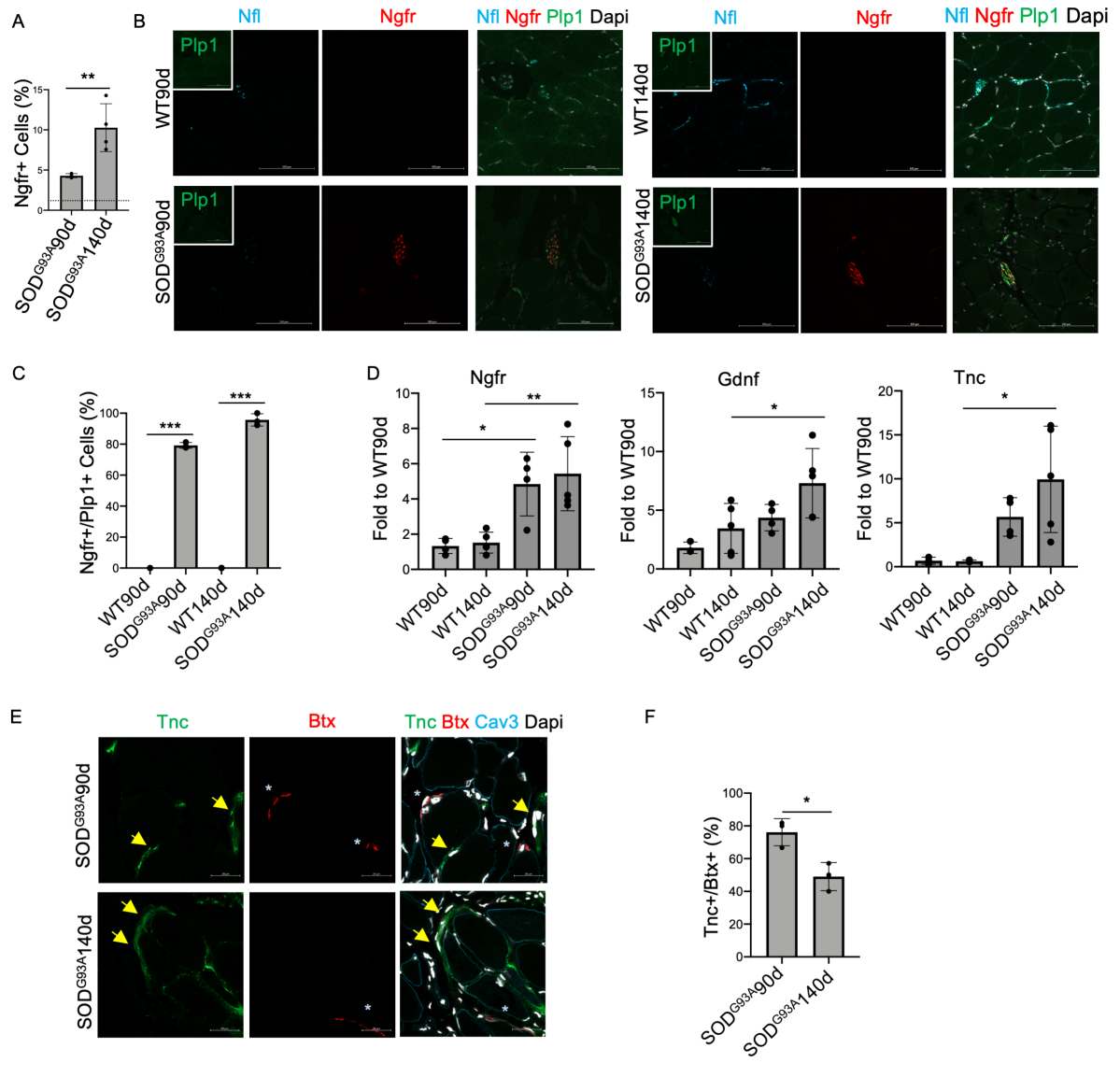
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768 **Figure 4. A specific transcriptional signature distinguishes glial cells in muscle from those**
769 **residing in the nerve**

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 μ 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)

784

Proietti et al. Figure 5



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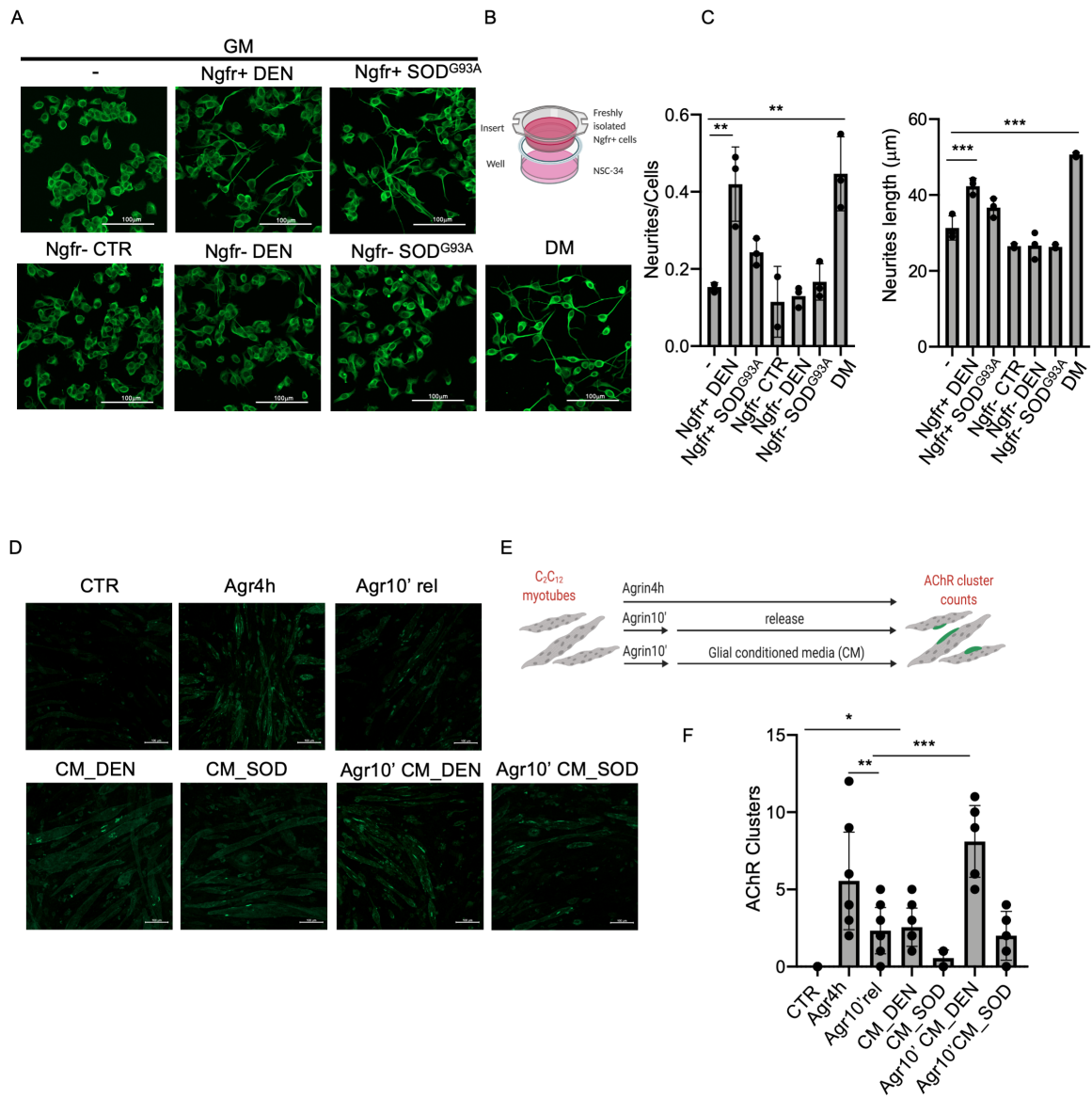
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787 **Figure 5. Muscle resident glial cell activation in a mouse model of ALS.**

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^{G93A} 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^{G93A} 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^{G93A} 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^{G93A} 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^{G93A} 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^{G93A} mice muscle (n=3,
804 Values represent mean ± s.d. *P < 0.05; by student t-test)

805

Proietti et al. Figure 6



806

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^{G93A}
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