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1 **Control of cell shape, neurite outgrowth and**
2 **migration by a novel Nogo-A/HSPG interaction**

3
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30

31 **Summary**

32 Heparan sulfate proteoglycans (HSPGs) critically modulate adhesion-, growth- and migration-
33 related processes. Here we show that the transmembrane protein Nogo-A inhibits neurite
34 outgrowth and cell spreading in neurons and Nogo-A-responsive cell lines via HSPGs. The
35 extracellular, active, 180 aa Nogo-A region called Nogo-A- Δ 20 binds to heparin and brain-
36 derived heparan sulfate glycosaminoglycans (GAGs) but not to the closely related chondroitin
37 sulfate GAGs. HSPGs are required for Nogo-A- Δ 20-induced inhibition of adhesion, cell
38 spreading, neurite outgrowth as well as for RhoA activation. Surprisingly, we show that
39 Nogo-A- Δ 20 can act via HSPGs independently of its receptor Sphingosine-1-Phosphate
40 receptor 2 (S1PR2). We thereby identify a new functional binding receptor for Nogo-A- Δ 20
41 and show that syndecan-3 and syndecan-4 are responsible for Nogo-A- Δ 20-induced effects.
42 Finally, we show in explant cultures *ex vivo* that Nogo-A- Δ 20 promotes the migration of
43 neuroblasts via HSPGs but not S1PR2.

44

45 **Keywords**

46 Nogo-A, HSPG, outgrowth, spreading, adhesion, neuroblast, migration, SVZ, RMS

47 **Introduction**

48 Cell surface heparan sulfate proteoglycans (HSPGs) are highly expressed in the mammalian
49 nervous system (Sarrazin et al., 2011; Yamaguchi, 2001). HSPGs regulate various
50 developmental processes ranging from neuroblast migration, axon growth and guidance to
51 synapse formation and neuronal connectivity (Inatani et al., 2003; Van Vactor et al., 2006;
52 Yamaguchi, 2001). HSPGs transduce signals originating in the extracellular matrix (ECM) or
53 act as obligate co-receptors for several morphogens, growth factors and axon guidance
54 molecules (Bernfield et al., 1999; Sarrazin et al., 2011). Most studies on HSPGs have focused
55 on the regulation of survival-, proliferation- or growth-promoting cues, e.g., fibroblast growth
56 factor (FGF) (Sarrazin et al., 2011), rather than growth-inhibiting and repulsive factors. To
57 our knowledge, only the repulsive activities of EphrinA3, Slit2 and S1P have been reported to
58 critically depend on the presence of cell surface HSPGs so far (Hu, 2001; Irie et al., 2008;
59 Strohlic et al., 2008).

60

61 Nogo-A is a major anti-adhesive and neurite growth-inhibitory protein initially discovered for
62 its role as myelin-associated inhibitor of axonal regeneration in the adult central nervous
63 system (CNS) (Schwab, 2010). In addition to its role in the injured CNS, Nogo-A has been
64 shown to regulate various developmental and plastic processes ranging from synapse
65 formation to neuronal migration (Kempf and Schwab, 2013; Schwab and Strittmatter, 2014).
66 In the adult brain, Nogo-A promotes cell motility and the tangential migration of neuroblasts
67 along the rostral migratory stream (RMS) by triggering cell-cell repulsion (Rolando et al.,
68 2012). At hippocampal and cortical synapses, Nogo-A acts as a negative regulator of long
69 term potentiation and memory stability (Karlsson et al., 2016; Schwab and Strittmatter, 2014).
70 However, it is not known whether Nogo-A-evoked cellular responses are modulated by
71 HSPGs.

72

73 In this study, we identified HSPGs as novel functional receptors for the active Nogo-A
74 domain Nogo-A- Δ 20 (rat amino acid (aa) 544-725 (Oertle et al., 2003)). We found that Nogo-
75 A- Δ 20 activates RhoA and inhibits cell spreading and neurite outgrowth via HSPGs,
76 specifically via the transmembrane HSPGs syndecan-4 and syndecan-3. In addition, we show
77 that Nogo-A- Δ 20 inhibits cell adhesion of neuroblasts in an HSPG-dependent manner and
78 increases neuroblast chain migration *ex vivo*. Our results propose a novel mechanism by
79 which Nogo-A- Δ 20 affects cytoskeletal dynamics by interacting with HSPGs independently
80 of the newly characterized Nogo-A- Δ 20 receptor Sphingosine-1-Phosphate receptor 2
81 (S1PR2) (Kempf et al., 2014).

82

83 **Results**

84 **Cell surface heparan sulfate is required for Nogo-A- Δ 20-induced inhibition of cell** 85 **spreading**

86 Outgrowth of neurites and spreading of cells, e.g. fibroblasts, are strongly inhibited by
87 substrates containing Nogo-A or its active fragment Nogo-A- Δ 20 (Oertle et al., 2003) (Figure
88 1A). To determine a possible role of heparan sulfate (HS), cell spreading inhibition was
89 examined upon enzymatic cleavage of HS. Treatment of Swiss 3T3 cells with heparinase III
90 (HepIII) significantly increased cell spreading by ~45% on the Nogo-A- Δ 20-coated culture
91 dishes when compared to the vehicle (saline) control (Figure 1A,B). Treatment with
92 heparinase I (HepI), which cleaves HS at the level of *O*-sulfated rather than non-sulfated or *N*-
93 sulfated disaccharides (Hovingh and Linker, 1970), resulted in a similar decrease of the Nogo-
94 A- Δ 20 inhibition but required higher enzyme concentrations (Figure S1A).

95 If endogenous HS promotes the Nogo-A- Δ 20 inhibitory effects by directly binding to Nogo-
96 A, excess soluble HS in the culture medium may act as competitive inhibitor and neutralize
97 Nogo-A-mediated cell spreading inhibition. Indeed, acute application of exogenous HS
98 significantly increased cell spreading on a Nogo-A- Δ 20 substrate when compared to control
99 treatment (Figure 1A,C). Similar effects were also observed when HS was added onto Nogo-
100 A- Δ 20-coated plates and washed prior to the plating of the cells, suggesting that Nogo-A-
101 Δ 20-bound HS neutralizes cell spreading inhibition (Figure S1B).

102 To confirm the involvement of HS in Nogo-A- Δ 20 signaling, a HS-deficient mutant CHO cell
103 line, pgsD-677 (Lidholt et al., 1992), was examined. Due to a mutation in the *Ext1* gene
104 encoding for a glycosyltransferase responsible for HS polymerization, pgsD-677 cells do not
105 produce HS (Lidholt et al., 1992). Whereas wild type CHO cells were strongly inhibited in
106 spreading by Nogo-A- Δ 20, spreading inhibition was almost fully abolished in the HSPG-
107 deficient pgsD-677 cells (Figure 1D,E). To confirm that these results are effectively due to the

108 lack of HS, we analysed cell spreading upon re-expression of *Ext1* in pgsD-677 cells. Indeed,
109 *Ext1* re-expression fully restored Nogo-A- Δ 20-mediated cell spreading inhibition (Figure
110 1D,F). The flow cytometry analysis of cell surface HSPGs expression confirmed their absence
111 in pgsD-677 cells, as well as their partial reduction after HepIII treatment and their restoration
112 after *Ext1* re-expression (Figure 1G).

113 **Cell surface heparan sulfate is required for Nogo-A- Δ 20-induced inhibition of neurite** 114 **outgrowth**

115 We examined the functional role of HS in Nogo-A- Δ 20-mediated inhibition of neurite
116 outgrowth using postnatal day (P) 5-8 mouse cerebellar granule neurons (CGNs) as a model
117 system. Notably, CGNs would not adhere if HepIII was applied acutely. Instead, HepIII was
118 applied 12 h after plating for a total duration of 24 h. Delayed treatment of CGNs with HepIII
119 fully abolished the growth-inhibitory effect of Nogo-A- Δ 20: neurite outgrowth was increased
120 by ~92% when compared to the saline control (Figure 2A,B).

121 To extend these findings to other neuronal populations, we analysed the effect of HepIII
122 treatment in postnatal dorsal root ganglion (DRG) neurons and embryonic (E19) cortical
123 neurons. Similar to CGNs, HepIII treatment fully abolished Nogo-A- Δ 20-induced inhibition
124 of neurite outgrowth in DRG (Figure 2C,D) and cortical neurons (Figure 2E,F). Together,
125 these results provide strong evidence for the requirement of HS chains on the surface of
126 Nogo-A responsive cells to promote Nogo-A- Δ 20-mediated inhibition of neurite outgrowth.

127 **Nogo-A- Δ 20 binds heparan sulfate and brain-derived glycosaminoglycans**

128 To investigate a possible direct binding of Nogo-A- Δ 20 to HS, we used an ELISA assay.
129 Biotinylated preparations of HS and heparin, a highly sulfated form of HS (Bernfield et al.,
130 1999), were immobilized and tested for T7-tagged Nogo-A- Δ 20 binding using two different
131 antibodies: an anti-T7 tag and a Nogo-A-specific antibody targeting the Δ 20 domain (11c7
132 (Oertle et al., 2003)). To assess the binding specificity of Nogo-A- Δ 20 to HS, three different

133 variants of chondroitin sulfate (CS), another form of glycosaminoglycans (GAGs), were
134 tested in parallel (CS-A, CS-C and CS-E). In addition, another inhibitory domain of Nogo-A,
135 Nogo-66 (rat aa 1026-1091), which is known to interact with a different receptor complex
136 (Kempf and Schwab, 2013), was tested. Importantly, Nogo-66 inhibits neurite outgrowth but
137 not cell spreading (Kempf and Schwab, 2013). Recombinant Nogo-66-Fc was detected using
138 an Fc-specific antibody. Nogo-A- Δ 20 but not Nogo-66 showed very strong binding to HS and
139 to heparin and significantly less to CS-A, CS-C or CS-E ($p < 0.001$) (Figure 3A). These
140 results were replicated using GAGs extracted from adult rat brains (total GAGs) treated with
141 HepI/III or ChondroitinaseABC (ChABC) to obtain CS-containing GAGs (CS-GAGs) or HS-
142 containing GAGs (HS-GAGs), respectively. Consistent with the above results, Nogo-A- Δ 20
143 bound total GAGs and HS-GAGs very strongly and showed significantly less binding to CS-
144 GAGs ($p < 0.001$) (Figure 3B). No binding of Nogo-66 to total GAGs, HS-GAGs or CS-
145 GAGs was observed (Figure 3B). In order to determine the specificity of the binding of Nogo-
146 A- Δ 20 to CS-GAGs, we tested the binding of the control protein Nogo-A- Δ 21 (rat aa 812-
147 918) (Oertle et al., 2003), which lacks inhibitory activity but is purified under identical
148 conditions, to brain-derived GAGs. No difference in binding was observed between Nogo-A-
149 Δ 21, total GAGs, HS-GAGs or CS-GAGs (Figure S2). Moreover, the absorbance values lie in
150 the same range than those of Nogo-A- Δ 20 binding to CS-GAGs, suggesting than the binding
151 of Nogo-A- Δ 20 to CS-GAGs is likely to be unspecific. Given the fact that the results in
152 Figure 3B, 3D and S2A are standardised against the total GAGs and that the HS:CS ratio in
153 the brain is 1:10 (Deepa et al., 2006), Nogo-A- Δ 20 shows a strong binding preference to HS-
154 GAGs. Together, these results indicate that the key and main binding partner of Nogo-A- Δ 20
155 is HS.

156 Finally, to determine the binding affinity of Nogo-A- Δ 20 to heparin or HS-GAGs, a dose-
157 response binding curve was measured (Figure 3C,D). Binding was saturable and non-linear

158 fitting revealed that Nogo-A- Δ 20 binds to heparin and HS-GAGs with a dissociation constant
159 (K_d) of ~234 nM and ~562 nM, respectively (Figure 3C, D).

160 To assess the ability of Nogo-A- Δ 20 to bind HS under physiological conditions, cell surface
161 binding assays were performed in CHO WT and pgsD-677 cells. Cells were incubated with
162 HA-tagged Nogo-A- Δ 20 for 1 h at 4°C, washed and immunostained for the HA tag (Figure
163 3E). Nogo-A- Δ 20 binding was assessed by measuring the number of Nogo-A- Δ 20 puncta per
164 cell surface area calculated upon 3D reconstruction of the cells. High numbers of Nogo-A-
165 Δ 20 puncta per WT CHO cell were found, whereas no binding of Nogo-A- Δ 20 was detected
166 in CHO pgs-D677 cells (Figure 3F). Similar results were also obtained in 3T3 cells after
167 HepIII treatment (Figure 3G) showing that Nogo-A- Δ 20 binds HSPGs.

168 **Nogo-A- Δ 20 acts via HSPGs independently of S1PR2**

169 Cell surface HSPGs can act as co-receptors by promoting the binding of a ligand to its
170 obligate receptor and thereby altering its activation (Bernfield et al., 1999; Sarrazin et al.,
171 2011). Given the prior identification of the G-protein-coupled receptor (GPCR) Sphingosine-
172 1-Phosphate receptor 2 (S1PR2) as a functional receptor for Nogo-A- Δ 20 (Kempf et al.,
173 2014), HSPGs may enhance or allow the formation of a Nogo-A- Δ 20/S1PR2 complex.
174 Alternatively, HSPGs may transduce Nogo-A- Δ 20 signals independently of S1PR2. In the
175 latter case, we reasoned that HepIII treatment of S1PR2-deficient cells should show a
176 disinhibition effect; in the former case, no effect of HepIII should be observed given the
177 requirement of S1PR2 as obligate receptor. To test this, S1PR2^{-/-} mouse embryonic fibroblasts
178 (MEFs) (Kempf et al., 2014) were treated with HepIII or saline and plated on Nogo-A- Δ 20
179 (Figure 4A, S3A). Strikingly, treatment of S1PR2^{-/-} MEFs with HepIII significantly further
180 increased cell spreading on Nogo-A- Δ 20 when compared to HepIII-treated WT MEFs or
181 S1PR2^{-/-} MEFs alone (Figure 4A, S3A). This suggests that Nogo-A- Δ 20 can act via HSPGs
182 independently of S1PR2.

183 In CHO-K1 WT cells, the levels of endogenous S1PRs mRNAs were shown to be below
184 detection limit, and these cells were unresponsive to the S1PR family ligand S1P in a variety
185 of *in vitro* assays (e.g., (Gonda et al., 1999; Okamoto et al., 1998)). Based on these
186 observations, CHO-K1 WT cells are considered as devoid of S1PR expression. To validate
187 this under our experimental conditions, CHO WT cells were treated with the pharmacological
188 S1PR2 antagonist JTE-013 and plated onto Nogo-A- Δ 20 and control substrates (Figure 4B,
189 S3B). As expected, JTE-013 did not antagonize Nogo-A- Δ 20-dependent inhibition of cell
190 spreading (Figure 4B, S3B). The same observation was made in mutant pgsD-677 cells
191 (Figure 4B, S3B). Together, these results suggest that Nogo-A- Δ 20 can exert inhibitory
192 effects via HSPGs in S1PR2-deficient cellular systems.

193 Nogo-A- Δ 20 has been repeatedly shown to activate the RhoA/ROCK pathway and thereby to
194 inhibit cell spreading and neurite outgrowth (Kempf et al., 2014; Niederost et al., 2002). To
195 test whether HSPGs can also mediate Nogo-A- Δ 20-induced downstream signaling, RhoA
196 activation was measured in CHO WT and pgsD-677 cells. In CHO WT cells, a ~250%
197 increase in RhoA activation was observed 20 min after application of Nogo-A- Δ 20, whereas
198 no change was observed in pgsD-677 cells (Figure 4C,D). The inactive Nogo-A fragment
199 Nogo-A- Δ 21 was used as control protein. Further, no change in RhoA activation was
200 observed in the presence of JTE-013 (Figure S4A) suggesting the presence of an S1PR2-
201 independent, HSPG-dependent Nogo-A- Δ 20 signal transduction pathway.

202 To determine whether Nogo-A- Δ 20 inhibition in CHO WT cells could be overcome by
203 blocking RhoA or the downstream Rho-associated kinase (ROCK), CHO WT cells were
204 treated with the RhoA inhibitor C3 transferase or with the ROCK inhibitor Y-27632 and
205 plated onto Nogo-A- Δ 20 (Figure 4E,F). In line with the RhoA activation results, blockade of
206 RhoA or ROCK showed a full rescue of Nogo-A- Δ 20 inhibition (Figure 4E,F).

207 Finally, in order to determine the effect of simultaneous blockade of HSPGs and S1PR2 in
208 cells co-expressing HSPGs and S1PR2, 3T3 cells were treated with HepIII and/or JTE-013
209 and assessed in a cell spreading assay. Strikingly, blockade of HSPGs and S1PR2 showed an
210 additive effect in reducing Nogo-A- Δ 20 induced inhibition of cell spreading (Figure S3C,D).
211 Hence, in cells co-expressing both receptors, Nogo-A- Δ 20 can exert inhibitory effects via
212 both S1PR2 as well as HSPGs. However, as shown by using S1PR2-deficient cells, HSPGs
213 are themselves sufficient to mediate Nogo-A- Δ 20 inhibition and RhoA activation.

214 **Syndecans mediate Nogo-A- Δ 20 inhibition of cell spreading and neurite outgrowth**

215 Membrane-bound cell surface HSPGs consist of two main families: syndecans and glypicans
216 (Bernfield et al., 1999). As opposed to syndecans, glypicans are attached by a
217 glycosylphosphatidylinositol anchor to the membrane and do not exert cytoplasmic signaling
218 roles (Bernfield et al., 1999). The syndecan family consists of four members: syndecan-1 to
219 syndecan-4 (Sdc1-Sdc4) (Bernfield et al., 1999), of which syndecan-4 is the most highly
220 expressed in 3T3 cells (Figure 5A). Interestingly, syndecan-4 has been shown to activate
221 RhoA to promote focal adhesion maturation and stress fibre assembly following engagement
222 with fibronectin (Brooks 2012, Dovas 2006).

223 To test the contribution of syndecan-4 to Nogo-A- Δ 20-induced inhibition of cell spreading
224 and RhoA activation, syndecan-4 was knocked down using lentivirus-delivered ctrl and
225 syndecan-4 shRNA (Figure S5A). Strikingly, knockdown of syndecan-4 fully prevented
226 Nogo-A- Δ 20 inhibition of cell spreading (Figure 5B,C). To test whether Nogo-A- Δ 20
227 activates RhoA via syndecan-4, RhoA activation assays were performed in ctrl vs syndecan-4
228 shRNA cells. The inactive Nogo-A fragment Nogo-A- Δ 21 was used as control protein. No
229 RhoA activation was observed upon syndecan-4 knockdown (Figure 5G). Together, these
230 results suggest that Nogo-A- Δ 20 inhibits cell spreading by activating RhoA via syndecan-4 in
231 fibroblasts.

232 To investigate whether syndecans are also important in Nogo-A- Δ 20-induced inhibition of
233 neurite outgrowth, we first assessed their expression in DIV4 E19 rat cortical neurons and
234 found syndecan-3 to be the most highly expressed (Figure 5D). Remarkably, siRNA-mediated
235 knockdown of syndecan-3 fully prevented outgrowth inhibition on the Nogo-A- Δ 20 substrate
236 (Figure 5E,F; S5B).

237 Further, to test whether syndecan-3 and syndecan-4 directly interact with Nogo-A- Δ 20,
238 microscale thermophoresis binding experiments were performed using recombinant syndecan-
239 3 and syndecan-4 ectodomains. We found that Nogo-A- Δ 20 binds to syndecan-4 and
240 syndecan-3 in a similar affinity range than to brain-derived HS-GAGs with a K_d of \sim 522.1 nM
241 and \sim 865.7 nM, respectively (Figure 5H). Taken together, these data show that Nogo-A- Δ 20
242 binds to and exerts inhibitory effects via syndecan-3 or -4 in a cell type-specific manner.

243 **Nogo-A- Δ 20 promotes neuroblast migration via HSPGs**

244 Nogo-A- Δ 20 was shown to promote the tangential migration of neuroblasts from the
245 subventricular zone (SVZ) to the olfactory bulb along the rostral migratory stream (RMS)
246 through activation of the Rho/ROCK pathway (Rolando et al., 2012). Yet, no molecular basis
247 for this observation was found and we sought to determine the physiological relevance of the
248 Nogo-A- Δ 20/HSPG interaction in this process.

249 To investigate the contribution of HSPGs to SVZ-derived neuroblast migration, postnatal
250 explants of the SVZ and RMS were used as an ex vivo model (Wichterle et al., 1997) and
251 treated with HepIII and/or the Nogo-A- Δ 20 function-blocking antibody 11c7. In this assay,
252 neuroblasts move out of the explant core by chain migration (i.e. associated with each other)
253 as occurs in the RMS in vivo (Wichterle et al., 1997). As previously shown, Nogo-A
254 neutralization by 11c7 induced a significant reduction of the migration area (Figure 6A,B).
255 HepIII treatment induced a similar reduction of the migration (Figure 6A,B). To examine
256 whether HSPGs and Nogo-A- Δ 20 operate through the same pathway, we co-administered

257 HepIII and 11c7. Co-application of HepIII and 11c7 led to a reduction in migration area
258 similar to that obtained upon treatment of HepIII or 11c7 alone (Figure 6A,B) suggesting that
259 Nogo-A- Δ 20 operates through HSPGs in this system.

260 Previous data suggested that Nogo-A sustains neuroblast migration by providing anti-
261 adhesive signals (Rolando et al., 2012). To investigate whether HSPGs participate in Nogo-A-
262 Δ 20-mediated repulsive effects, we asked whether HepIII treatment affected neuroblast
263 adhesion on control vs. Nogo-A- Δ 20-coated substrates in the presence or absence of 11c7.
264 HepIII treatment significantly increased cell adhesion on Nogo-A- Δ 20 to a similar extent than
265 11c7 (Figure 6C). No additive or synergistic effects were observed (Figure 6C), suggesting
266 that Nogo-A- Δ 20 and HSPGs share a common pathway in ex vivo cultures.

267 Finally, to test the role of the previously identified Nogo-A- Δ 20 receptor S1PR2 in neuroblast
268 migration, explants were treated with the S1PR2 blocker JTE-013 or DMSO (vehicle control).
269 No significant effect on the migration area was observed using different concentrations of
270 JTE-013 (Figure 6D,E). Similarly, JTE-013 treatment had no effect on neuroblast adhesion
271 (Figure 6F). Taken together, these data show that Nogo-A- Δ 20 inhibits adhesion and
272 increases migration by providing anti-adhesive signals through HSPGs but not S1PR2.

273

274 **Discussion**

275 Cell-to-cell signaling by ligand receptor interactions as well as interactions with ECM
276 constituents play key roles during developmental processes such as neuronal migration and
277 axon growth. In this study, we identify a novel biochemical interaction between the
278 membrane protein Nogo-A and HSPGs and demonstrate its functional significance in cell
279 spreading, neurite outgrowth, adhesion and neuroblast chain migration.

280

281 Cell surface HSPGs are traditionally viewed as co-receptors that promote the binding of a
282 ligand to its obligate receptor through their large glycosaminoglycan chains (Bernfield et al.,
283 1999; Sarrazin et al., 2011) but do not act as signal-transducing receptors themselves. In the
284 case of FGF and many other morphogens, HS is essential for the ligand/receptor complex to
285 form and to alter its activation (Sarrazin et al., 2011). Surprisingly, our data suggest that this
286 is not the case for Nogo-A- Δ 20 and its S1PR2 receptor (Kempf et al., 2014): Nogo-A- Δ 20 can
287 activate RhoA in S1PR-negative CHO cells and inhibits cell spreading in S1PR2^{-/-} MEFs.
288 Hence, our results strongly suggest that Nogo-A- Δ 20 can signal through S1PR-independent
289 mechanisms. However, when HSPGs and S1PR2 are co-expressed, both pathways can act in
290 parallel, as shown for fibroblasts, or one pathway can gain control of the signaling output, as
291 demonstrated for neuroblasts. Collectively, our experiments reveal that more than one
292 receptor for the active Nogo-A- Δ 20 region exists and that Nogo-A- Δ 20-induced inhibitory
293 effects are regulated in a cell type-specific manner.

294

295 Based on our findings showing the involvement of syndecan-3 and syndecan-4, we may
296 hypothesize that the cytoplasmic tail of syndecans is important for Nogo-A- Δ 20-induced
297 signal transduction upon extracellular binding to the HS chains. A few studies have shown
298 that transmembrane syndecans can act as signaling receptors through their cytoplasmic

299 domains. During cell migration, engagement of syndecan-4 by fibronectin was shown to
300 result in the activation of protein kinase C α (PKC α) upstream of RhoA activation (Bass et
301 al., 2008; Bass et al., 2007; Brooks et al., 2012; Dovas et al., 2006). Even though it is unclear
302 how syndecan-4 signals to RhoA via PKC α , PKC α was shown to activate RhoA via
303 phosphorylation of the Rho guanine exchange factor (RhoGEF) p115 in a different system
304 (Peng et al 2011). It will be interesting to investigate whether Nogo-A- Δ 20 operates via
305 similar mechanisms. In the case of syndecan-3, binding of the heparin-binding growth-
306 associated molecule HB-GAM was shown to result in phosphorylation of the Src kinases c-
307 Src and c-Fyn, and of cortactin, which promotes polymerization and rearrangement of the
308 actin cytoskeleton resulting in neurite outgrowth (Kinnunen et al., 1998). A similar
309 mechanism was proposed for glial cell line-derived neurotrophic factor (GDNF) family
310 ligands and syndecan-3 (Bespalov et al., 2011). However, no link between syndecan-3 and
311 RhoA activation has been reported so far and future studies shall address this point.

312

313 Syndecan-3 is the major HSPG found in neurons of the developing brain and shows abundant
314 expression in major axonal tracts and migratory routes, e.g., in the RMS (Hienola et al., 2006;
315 Nolo et al., 1995; Rauvala et al., 2000). In the adult brain, syndecan-3 is strongly expressed in
316 the hippocampus, cerebellum and cortex and in several axonal tracts (Hsueh and Sheng,
317 1999). Our results show that the anti-adhesive effect of Nogo-A- Δ 20 is accompanied by an
318 HS-dependent increase in neuroblast chain migration. Notably, syndecan-3^{-/-} mice phenocopy
319 the defects in radial and tangential neuronal migration observed in Nogo-A^{-/-} mice (Hienola et
320 al., 2006; Mathis et al., 2010; Mingorance-Le Meur et al., 2007; Rolando et al., 2012).
321 Syndecan-3^{-/-} mice also display a synaptic plasticity phenotype similar to that observed in
322 Nogo-A^{-/-} mice: increased CA1 long-term potentiation (LTP) while baseline transmission and
323 short-term plasticity are not affected (Kaksonen et al., 2002). Given the recent implication of

324 HSPGs in synapse formation and plasticity (Allen et al., 2012; de Wit et al., 2013; Siddiqui et
325 al., 2013), it will be interesting to determine whether Nogo-A also mediates its effects on
326 synapse formation and plasticity via HSPGs (Mironova and Giger, 2013). Overall, the
327 localization of syndecan proteins and their physiological impact in the developing and adult
328 brain are consistent with a functional interaction between Nogo-A and HSPGs *in vivo*.

329

330 In conclusion, our study shows that Nogo-A- Δ 20 can regulate adhesion, cell spreading,
331 outgrowth and migration of various cell lines, neurons and neuroblasts via a newly identified
332 interaction with transmembrane HSPGs.

333

334 **Experimental procedures**

335 **Plasmids, recombinant fusion proteins, reagents, antibodies and brain-derived**
336 **glycosaminoglycans (GAGs)**

337 A complete description is provided in the Supplemental Experimental Procedures.

338 **Tissue preparation and cell culture**

339 A complete description is provided in the Supplemental Experimental Procedures.

340 **Immunocytochemistry, flow cytometry and RhoA activation assays**

341 Immunocytochemistry, cell surface binding assays, flow cytometry and RhoA
342 pulldown/ELISA experiments were essentially performed as previously described (Kempf et
343 al., 2014). A complete description is provided in the Supplemental Experimental Procedures.

344 ***In vitro* bioassays**

345 3T3 fibroblast spreading assays and neurite outgrowth assays were performed as described
346 previously (Kempf et al., 2014; Oertle et al., 2003). For HepI and HepIII (Sigma) treatment,
347 cells were incubated with 2.5-10 U/ml HepI or HepIII 3 h prior plating and during the
348 spreading assay. For function-blocking experiments, cells were incubated with 1 μ M JTE-
349 013, 5 μ M Y-27632 or 100 μ g/ml C3 30 min prior plating and during the spreading assay.
350 The corresponding solvents were used as controls. For expression of EXT1 in pgsD-677 cells,
351 pgsD-677 cells were transfected with Ext1 cDNA using Lipofectamine 2000 (Invitrogen)
352 according to the manufacturer's instructions. For siRNA experiments, 3T3 cells or E19 rat
353 cortical neurons were transfected with ON-TARGETplus SMARTpool siRNAs using
354 DharmaFECT3 (Dharmacon) according to the manufacturer's instructions. For shRNA
355 experiments, stable 3T3 shRNA cell lines were made using lentiviruses carrying Mission
356 shRNA pLKO lentiviral plasmids (Sigma) containing shRNA against Sdc4 or ctrl shRNA. A
357 complete description is provided in the Supplemental Experimental Procedures.

358 **ELISA**

359 The ELISA was performed according to method described in (Purushothaman et al., 2007)
360 with modifications detailed in Supplemental Experimental Procedures.

361 **Explant assay**

362 P5 explants were prepared from C57/BL6 pups according to (Wichterle et al., 1997) with
363 modifications detailed in Supplemental Experimental Procedures.

364 **Statistical analysis**

365 Statistical analyses were conducted using the statistical software GraphPad Prism 5 or 6
366 (GraphPad Software Inc.). $*p < 0.05$ was considered statistically significant. Calculations
367 were corrected for multiple comparisons as specified.

368 **Author contributions**

369 A.K. and M.E.S. designed the research and wrote the paper; A.K. performed most
370 biochemical and cellular experiments and analyzed data. Z.R. performed flow cytometry
371 experiments. A.M.K., Z.R., A.S., A.S. and B.T. performed some bioassays. J.C.F.K. and
372 J.W.F. performed GAG ELISA experiments and analyzed data. V.G., E.B. and A.B.
373 performed neuroblast migration and adhesion experiments and analyzed data.

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380

381 **References**

- 382 Allen, N.J., Bennett, M.L., Foo, L.C., Wang, G.X., Chakraborty, C., Smith, S.J., and Barres, B.A.
383 (2012). Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA
384 receptors. *Nature* 486, 410-414.
- 385 Bass, M.D., Morgan, M.R., Roach, K.A., Settleman, J., Goryachev, A.B., and Humphries, M.J. (2008).
386 p190RhoGAP is the convergence point of adhesion signals from alpha 5 beta 1 integrin and syndecan-
387 4. *J Cell Biol* 181, 1013-1026.
- 388 Bass, M.D., Roach, K.A., Morgan, M.R., Mostafavi-Pour, Z., Schoen, T., Muramatsu, T., Mayer, U.,
389 Ballestrem, C., Spatz, J.P., and Humphries, M.J. (2007). Syndecan-4-dependent Rac1 regulation
390 determines directional migration in response to the extracellular matrix. *J Cell Biol* 177, 527-538.
- 391 Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., and Zako, M. (1999).
392 Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 68, 729-777.
- 393 Besselov, M.M., Sidorova, Y.A., Tumova, S., Ahonen-Bishopp, A., Magalhaes, A.C., Kuleskiy, E.,
394 Paveliev, M., Rivera, C., Rauvala, H., and Saarma, M. (2011). Heparan sulfate proteoglycan
395 syndecan-3 is a novel receptor for GDNF, neurturin, and artemin. *J Cell Biol* 192, 153-169.
- 396 Brooks, R., Williamson, R., and Bass, M. (2012). Syndecan-4 independently regulates multiple small
397 GTPases to promote fibroblast migration during wound healing. *Small GTPases* 3, 73-79.
- 398 de Wit, J., O'Sullivan, M.L., Savas, J.N., Condomitti, G., Caccese, M.C., Vennekens, K.M., Yates,
399 J.R., 3rd, and Ghosh, A. (2013). Unbiased discovery of glypican as a receptor for LRRTM4 in
400 regulating excitatory synapse development. *Neuron* 79, 696-711.
- 401 Deepa, S.S., Carulli, D., Galtrey, C., Rhodes, K., Fukuda, J., Mikami, T., Sugahara, K., and Fawcett,
402 J.W. (2006). Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide
403 composition for the net-associated proteoglycans. *J Biol Chem* 281, 17789-17800.
- 404 Dovas, A., Yoneda, A., and Couchman, J.R. (2006). PKCbeta-dependent activation of RhoA by
405 syndecan-4 during focal adhesion formation. *J Cell Sci* 119, 2837-2846.
- 406 Gonda, K., Okamoto, H., Takuwa, N., Yatomi, Y., Okazaki, H., Sakurai, T., Kimura, S., Sillard, R.,
407 Harii, K., and Takuwa, Y. (1999). The novel sphingosine 1-phosphate receptor AGR16 is coupled via
408 pertussis toxin-sensitive and -insensitive G-proteins to multiple signaling pathways. *Biochem J* 337 (Pt 1), 67-75.
- 410 Hienola, A., Tumova, S., Kuleskiy, E., and Rauvala, H. (2006). N-syndecan deficiency impairs neural
411 migration in brain. *J Cell Biol* 174, 569-580.
- 412 Hovingh, P., and Linker, A. (1970). The enzymatic degradation of heparin and heparitin sulfate. 3.
413 Purification of a heparitinase and a heparinase from flavobacteria. *J Biol Chem* 245, 6170-6175.
- 414 Hsueh, Y.P., and Sheng, M. (1999). Regulated expression and subcellular localization of syndecan
415 heparan sulfate proteoglycans and the syndecan-binding protein CASK/LIN-2 during rat brain
416 development. *J Neurosci* 19, 7415-7425.
- 417 Hu, H. (2001). Cell-surface heparan sulfate is involved in the repulsive guidance activities of Slit2
418 protein. *Nat Neurosci* 4, 695-701.
- 419 Inatani, M., Irie, F., Plump, A.S., Tessier-Lavigne, M., and Yamaguchi, Y. (2003). Mammalian brain
420 morphogenesis and midline axon guidance require heparan sulfate. *Science* 302, 1044-1046.
- 421 Irie, F., Okuno, M., Matsumoto, K., Pasquale, E.B., and Yamaguchi, Y. (2008). Heparan sulfate
422 regulates ephrin-A3/EphA receptor signaling. *Proc Natl Acad Sci U S A* 105, 12307-12312.
- 423 Kaksonen, M., Pavlov, I., Voikar, V., Lauri, S.E., Hienola, A., Riekkki, R., Lakso, M., Taira, T., and
424 Rauvala, H. (2002). Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-
425 dependent memory. *Mol Cell Neurosci* 21, 158-172.
- 426 Karlsson, T.E., Smedfors, G., Brodin, A.T., Aberg, E., Mattsson, A., Hogbeck, I., Wellfelt, K.,
427 Josephson, A., Brene, S., and Olson, L. (2016). NgR1: A Tunable Sensor Regulating Memory
428 Formation, Synaptic, and Dendritic Plasticity. *Cereb Cortex* 26, 1804-1817.
- 429 Kempf, A., and Schwab, M.E. (2013). Nogo-A represses anatomical and synaptic plasticity in the
430 central nervous system. *Physiology* 28, 151-163.
- 431 Kempf, A., Tews, B., Arzt, M.E., Weinmann, O., Obermair, F.J., Pernet, V., Zagrebelsky, M.,
432 Delekate, A., Iobbi, C., Zemmar, A., et al. (2014). The sphingolipid receptor S1PR2 is a receptor for
433 Nogo-a repressing synaptic plasticity. *PLoS Biol* 12, e1001763.

434 Kinnunen, T., Kaksonen, M., Saarinen, J., Kalkkinen, N., Peng, H.B., and Rauvala, H. (1998).
435 Cortactin-Src kinase signaling pathway is involved in N-syndecan-dependent neurite outgrowth. *J Biol*
436 *Chem* 273, 10702-10708.

437 Lidholt, K., Weinke, J.L., Kiser, C.S., Lagemwa, F.N., Bame, K.J., Cheifetz, S., Massague, J.,
438 Lindahl, U., and Esko, J.D. (1992). A single mutation affects both N-acetylglucosaminyltransferase
439 and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan
440 sulfate biosynthesis. *Proc Natl Acad Sci U S A* 89, 2267-2271.

441 Mathis, C., Schroter, A., Thallmair, M., and Schwab, M.E. (2010). Nogo-a regulates neural precursor
442 migration in the embryonic mouse cortex. *Cereb Cortex* 20, 2380-2390.

443 Mingorance-Le Meur, A., Zheng, B., Soriano, E., and del Rio, J.A. (2007). Involvement of the myelin-
444 associated inhibitor Nogo-A in early cortical development and neuronal maturation. *Cereb Cortex* 17,
445 2375-2386.

446 Mironova, Y.A., and Giger, R.J. (2013). Where no synapses go: gatekeepers of circuit remodeling and
447 synaptic strength. *Trends Neurosci* 36, 363-373.

448 Niederost, B., Oertle, T., Fritsche, J., McKinney, R.A., and Bandtlow, C.E. (2002). Nogo-A and
449 myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA
450 and Rac1. *The Journal of neuroscience* 22, 10368-10376.

451 Nolo, R., Kaksonen, M., Raulo, E., and Rauvala, H. (1995). Co-expression of heparin-binding growth-
452 associated molecule (HB-GAM) and N-syndecan (syndecan-3) in developing rat brain. *Neurosci Lett*
453 191, 39-42.

454 Oertle, T., van der Haar, M.E., Bandtlow, C.E., Robeva, A., Burfeind, P., Buss, A., Huber, A.B.,
455 Simonen, M., Schnell, L., Brosamle, C., *et al.* (2003). Nogo-A inhibits neurite outgrowth and cell
456 spreading with three discrete regions. *The Journal of neuroscience* 23, 5393-5406.

457 Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H., and
458 Takuwa, Y. (1998). EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to
459 multiple signaling pathways, including phospholipase C activation, Ca²⁺ mobilization, Ras-mitogen-
460 activated protein kinase activation, and adenylate cyclase inhibition. *J Biol Chem* 273, 27104-27110.

461 Purushothaman, A., Fukuda, J., Mizumoto, S., ten Dam, G.B., van Kuppevelt, T.H., Kitagawa, H.,
462 Mikami, T., and Sugahara, K. (2007). Functions of chondroitin sulfate/dermatan sulfate chains in brain
463 development. Critical roles of E and iE disaccharide units recognized by a single chain antibody
464 GD3G7. *J Biol Chem* 282, 19442-19452.

465 Rauvala, H., Huttunen, H.J., Fages, C., Kaksonen, M., Kinnunen, T., Imai, S., Raulo, E., and
466 Kilpelainen, I. (2000). Heparin-binding proteins HB-GAM (pleiotrophin) and amphoterin in the
467 regulation of cell motility. *Matrix Biol* 19, 377-387.

468 Rolando, C., Parolisi, R., Boda, E., Schwab, M.E., Rossi, F., and Buffo, A. (2012). Distinct roles of
469 nogo-a and nogo receptor 1 in the homeostatic regulation of adult neural stem cell function and
470 neuroblast migration. *J Neurosci* 32, 17788-17799.

471 Sarrazin, S., Lamanna, W.C., and Esko, J.D. (2011). Heparan sulfate proteoglycans. *Cold Spring Harb*
472 *Perspect Biol* 3.

473 Schwab, M.E. (2010). Functions of Nogo proteins and their receptors in the nervous system. *Nat Rev*
474 *Neurosci* 11, 799-811.

475 Schwab, M.E., and Strittmatter, S.M. (2014). Nogo limits neural plasticity and recovery from injury.
476 *Curr Opin Neurobiol* 27, 53-60.

477 Siddiqui, T.J., Tari, P.K., Connor, S.A., Zhang, P., Dobie, F.A., She, K., Kawabe, H., Wang, Y.T.,
478 Brose, N., and Craig, A.M. (2013). An LRRTM4-HSPG complex mediates excitatory synapse
479 development on dentate gyrus granule cells. *Neuron* 79, 680-695.

480 Strohlic, L., Dwivedy, A., van Horck, F.P., Falk, J., and Holt, C.E. (2008). A role for S1P signaling
481 in axon guidance in the *Xenopus* visual system. *Development* 135, 333-342.

482 Van Vactor, D., Wall, D.P., and Johnson, K.G. (2006). Heparan sulfate proteoglycans and the
483 emergence of neuronal connectivity. *Curr Opin Neurobiol* 16, 40-51.

484 Wichterle, H., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Direct evidence for homotypic,
485 glia-independent neuronal migration. *Neuron* 18, 779-791.

486 Yamaguchi, Y. (2001). Heparan sulfate proteoglycans in the nervous system: their diverse roles in
487 neurogenesis, axon guidance, and synaptogenesis. *Semin Cell Dev Biol* 12, 99-106.

490 **Figure legends**

491

492 **Figure 1. Cell surface HSPGs mediate Nogo-A-Δ20 inhibition of cell spreading. A.**

493 Representative pictures of 3T3 fibroblasts treated with 2.5 U/ml HepIII, 0.1 mg/ml HS or

494 vehicle (saline) and plated on control (ctrl) or Nogo-A-Δ20 substrate. Cells were stained with

495 Phalloidin-Alexa488. **B,C.** Cell spreading quantification of **A.** HepIII (**B**) or HS (**C**) treatment

496 partially reversed Nogo-A-Δ20-mediated cell spreading inhibition. **D.** Representative pictures

497 of CHO WT, CHO pgsD-677 or CHO pgsD-677 expressing *Ext1* cDNA and plated on a

498 control or Nogo-A-Δ20 substrate. **E,F.** Cell spreading quantification of **D.** **E.** The rounding

499 response to Nogo-A-Δ20 is highly impaired in CHO pgsD-677 mutants. **F.** Expression of

500 EXT1 in CHO pgsD-677 cells fully restored Nogo-A-Δ20 inhibition. **G.** Flow cytometry

501 detection of cell surface HSPGs in 3T3 cells (upper panel) or CHO WT and pgsD-677 cells

502 (lower panel) using the 10E4 antibody. HepIII treatment of 3T3 cells reduces HSPG levels.

503 EXT1 expression restores HSPG levels in CHO pgsD-677 cells. WT designates CHO cells.

504 Filled grey curves indicate unstained controls. The fluorescence intensity is displayed on the

505 X-axis (256 bins) and the normalized number of cells per each bin on the Y-axis. Data shown

506 are means ± SEM (n = 8-12 coverslips). **B,C,E,F:** One-way ANOVA with Tuckey's post hoc

507 test; (***) $p < 0.001$. Scale bars: 45 μm. See also Figure S1.

508 **Figure 2. Cell surface HSPGs mediate Nogo-A-Δ20 inhibition of neurite outgrowth. A.**

509 Representative pictures of mouse P7 cerebellar granule neurons (CGNs) treated with

510 500 mU/ml HepIII or vehicle (saline) and plated on a control (ctrl) or Nogo-A-Δ20 substrate.

511 Neurons were stained with βIII-Tubulin. **B.** Total neurite length per cell quantification of **A.**

512 HepIII treatment fully reversed Nogo-A-Δ20-mediated inhibition of neurite outgrowth. **C.**

513 Representative pictures of mouse P7 dorsal root ganglia (DRG) neurons treated with 1 U/ml

514 HepIII or vehicle (saline) and plated on a control or Nogo-A-Δ20 substrate. Neurons were

515 stained with β III-Tubulin. **D.** Total neurite length per cell quantification of **C.** **E.**
516 Representative pictures of DIV5 rat E19 cortical neurons treated with 1 U/ml HepIII or
517 vehicle (saline) at DIV4 and replated on a control (ctrl) or Nogo-A- Δ 20 substrate for 24h.
518 Neurons were stained with Map1b. **F.** Total neurite length per cell quantification of **E.** DIV,
519 days in vitro. Data shown are means \pm SEM (n = 3-9 coverslips). **B,D,F:** One-way ANOVA
520 with Tuckey's post hoc test; (* $p < 0.05$, *** $p < 0.001$; ns: not significant). Scale bars:
521 45 μ m.

522 **Figure 3. Nogo-A- Δ 20 but not Nogo-66 binds Heparin and HS. A-D.** Biotinylated heparin,
523 HS, CS or brain-derived GAGs were coated onto streptavidin-coated wells and analysed for
524 Nogo-A- Δ 20 or Nogo-66 binding by an ELISA-type assay. Average values for the BSA
525 negative control were subtracted from the respective readings. Nogo-A- Δ 20-T7 binding was
526 detected using an anti-T7 or anti-Nogo-A (11c7) antibody and Nogo-66-Fc binding using an
527 anti-Fc antibody. **A.** Binding analysis of Nogo-A- Δ 20 and Nogo-66 to Heparin, HS, CS-A,
528 CS-C or CS-E. **B.** Binding analysis of Nogo-A- Δ 20 and Nogo-66 to brain-derived GAGs
529 treated with heparinase (CS-GAGs) or chondroitinase ABC (HS-GAGs). Total GAGs refer to
530 the untreated GAG fraction. **C.** Saturation curve of Nogo-A- Δ 20 to heparin ($K_d \sim 234$ nM) and
531 brain-derived HS-GAGs ($K_d \sim 562$ nM). Detection was performed using the anti-T7 antibody.
532 **D.** Scatchard plot of **C.** **E.** Representative images of cell surface binding of Nogo-A- Δ 20 to
533 CHO WT and HSPG-deficient CHO pgsD-677 cells. Cells were incubated with 1 μ M HA-
534 tagged Nogo-A- Δ 20 for 30 min on ice and stained using the anti-HA antibody. **F,G.**
535 Quantification of cell surface binding by assessing the number of bound HA-tagged Nogo-A-
536 Δ 20 spots in CHO WT and pgsD-677 cells (**F**) or in HepIII vs. saline-treated 3T3 cells (**G**).
537 Average values for the control were subtracted from the respective measurements. Data
538 shown are means \pm SEM (**A-D:** n = 3 experiments; **F:** n = 10 cells; **G:** n = 30-34 cells). **A,B:**
539 One-way ANOVA with Tuckey's post hoc test; **F,G:** Mann Whitney test (*** $p < 0.001$).

540 **Figure 4. HSPGs mediate Nogo-A- Δ 20 signaling independently of S1PR2.** **A.** Cell
541 spreading quantification of CHO WT and CHO pgsD-677 cells treated with 1 μ M JTE-013 or
542 vehicle (DMSO) and plated on a control (ctrl) or Nogo-A- Δ 20 substrate. Representative
543 pictures are shown in Figure S3A. **B.** Cell spreading quantification of WT and S1PR2^{-/-} MEFs
544 treated with 2.5 U/ml HepIII or vehicle (saline) and plated on a control (ctrl) or Nogo-A- Δ 20
545 substrate. Representative pictures are shown in Figure S3B. **C.** RhoA activation was assessed
546 in CHO WT and pgsD-677 cells 20 min post-incubation with 1 μ M Nogo-A- Δ 20 by western
547 blotting. **D.** Quantification of RhoA-GTP/Total RhoA levels shown in **C.** Nogo-A- Δ 20 does
548 not activate RhoA in pgsD-677 cells. **E.** Representative pictures of CHO WT cells treated
549 with the RhoA inhibitor C3 transferase (0.1 mg/ml), the ROCK blocker Y-27632 (5 μ M) or
550 vehicle (saline). **F.** Cell spreading quantification of **E.** Data shown are means \pm SEM (**B,F**:
551 $n = 6-16$ coverslips; **D**: $n = 3$ experiments. **B,D,F**: One-way ANOVA with Tuckey's post hoc
552 test (** $p < 0.01$; *** $p < 0.001$). Scale bars: 45 μ m. See also Figure S3 and S4.

553 **Figure 5. Syndecans mediate Nogo-A- Δ 20 of cell spreading and neurite outgrowth.** **A.**
554 qRT-PCR expression analysis of syndecans (Sdc) in 3T3 cells. mRNA fold changes are
555 normalized to Sdc1 (100%). **B.** Representative pictures of 3T3 cells treated with a lentivirus
556 expressing Sdc4 or ctrl shRNA for 96 h and replated on a ctrl or Nogo-A- Δ 20 substrate for
557 1 h. Cells were stained with Phalloidin-Alexa488. **C.** Cell spreading quantification of **B.** **D.**
558 qRT-PCR expression analysis of Sdc's in DIV4 rat E19 cortical neurons. mRNA fold changes
559 are normalized to Sdc1 (100%). **E.** Representative pictures of DIV8 rat cortical neurons
560 treated at DIV4 with ctrl or Sdc3 siRNA for 72 h and replated on a ctrl or Nogo-A- Δ 20
561 substrate for 24 h. Cells were stained with MAP1b. **F.** Neurite length quantification of **E.** **G.**
562 RhoA activation was assessed in 3T3 cells expressing Sdc4 or ctrl shRNA 20 min post-
563 incubation with 1 μ M Nogo-A- Δ 20 using a commercially available ELISA kit. Quantification
564 of RhoA-GTP/Total RhoA levels is shown. **H.** Microscale binding analysis of Nogo-A- Δ 20 to

565 recombinant mouse Sdc4 ($K_d \sim 522.1\text{nM}$) or Sdc3 ($K_d \sim 865.7\text{nM}$). Single dots indicate
566 biological replicates in **A** and **D**. Data shown are means \pm SEM (**A,D**: $n = 3$ experiments; **G**:
567 $n = 6$ experiments; **C,F**: $n = 8-16$ coverslips). **C,F,G**: One-way ANOVA with Tuckey's post
568 hoc test (* $p < 0.05$, *** $p < 0.001$; ns: not significant). Scale bars: $45 \mu\text{m}$. See also Figure S5.

569 **Figure 6. Nogo-A- $\Delta 20$ regulates neuroblast adhesion and migration via HSPGs. A.**
570 Representative pictures of neuroblast explants (SVZ+RMS) showing the decrease in
571 migration area of HepIII (500 mU/ml), 11c7 ($1 \mu\text{g}/\mu\text{l}$) and HepIII+11c7-treated explants vs.
572 controls. **B**. Quantification of the migration area. Controls are set to 100% for each
573 experiment. **C**. Adhesion of SVZ-dissociated neuroblasts on a Nogo-A- $\Delta 20$ substrate after
574 treatment with 11c7 and/or HepIII. No synergistic activity is detected by co-treatment of
575 neuroblasts with 11c7 and HepIII in **B** and **C**. **D**. Representative pictures of neuroblast
576 explants (SVZ+RMS) treated with different concentrations of JTE-013 or vehicle (DMSO).
577 **E**. Quantification of the migration in the presence of JTE-013 vs. DMSO. Controls are set to
578 100% for each experiment. **F**. Adhesion of SVZ-dissociated neuroblasts on a Nogo-A- $\Delta 20$
579 substrate after treatment with JTE-013 or DMSO. No significant effect is observed upon
580 treatment with JTE-013 in **E** and **F**. Data shown are means \pm SEM (**B**: $n = 4-5$ experiments;
581 **C**: $n = 3$ coverslips; **E**: $n = 4-5$ experiments, **F**: $n = 5$ coverslips). **B,C,E,F**: One-way ANOVA
582 with Bonferroni's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Scale bars: **A**:
583 $100 \mu\text{m}$.

Figure 1

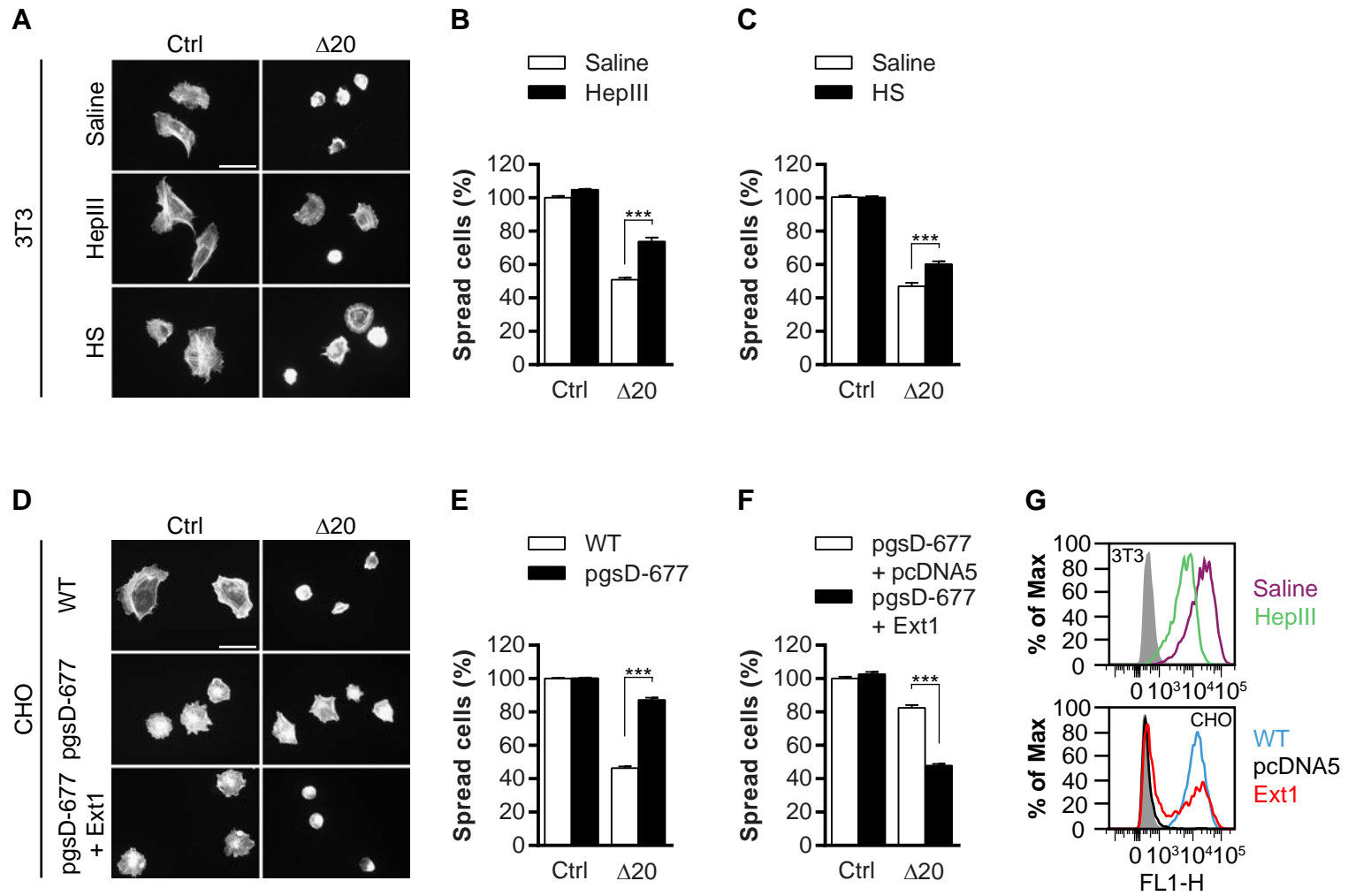


Figure 2

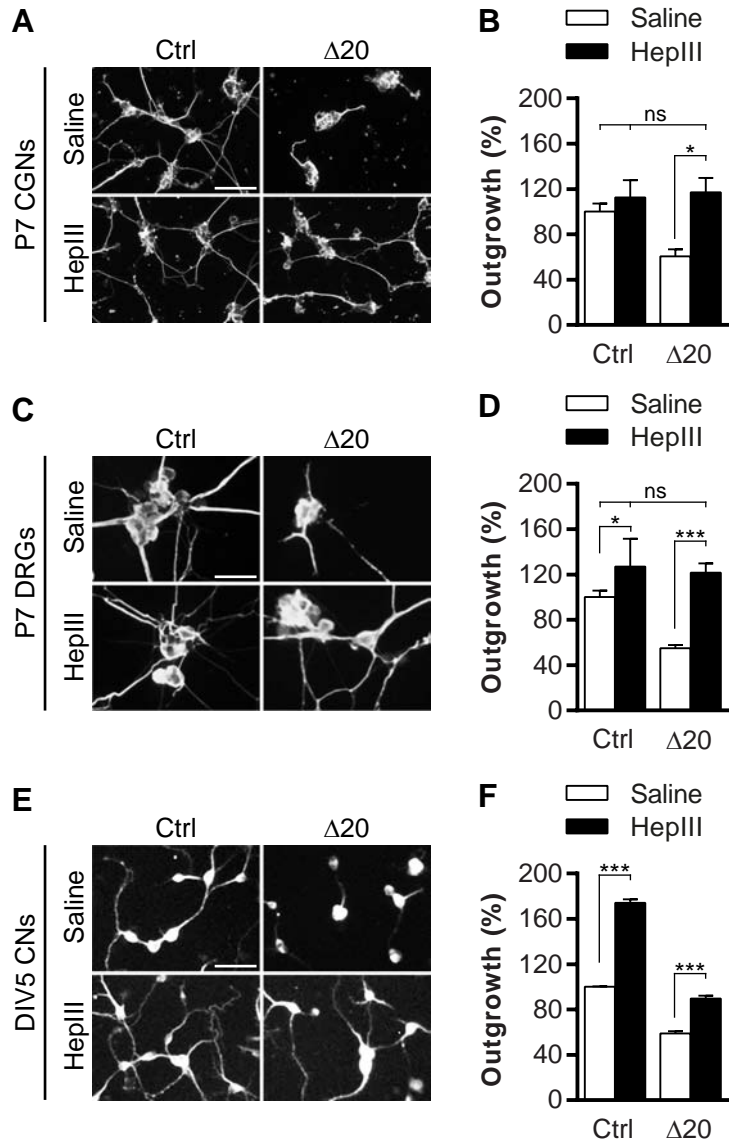


Figure 3

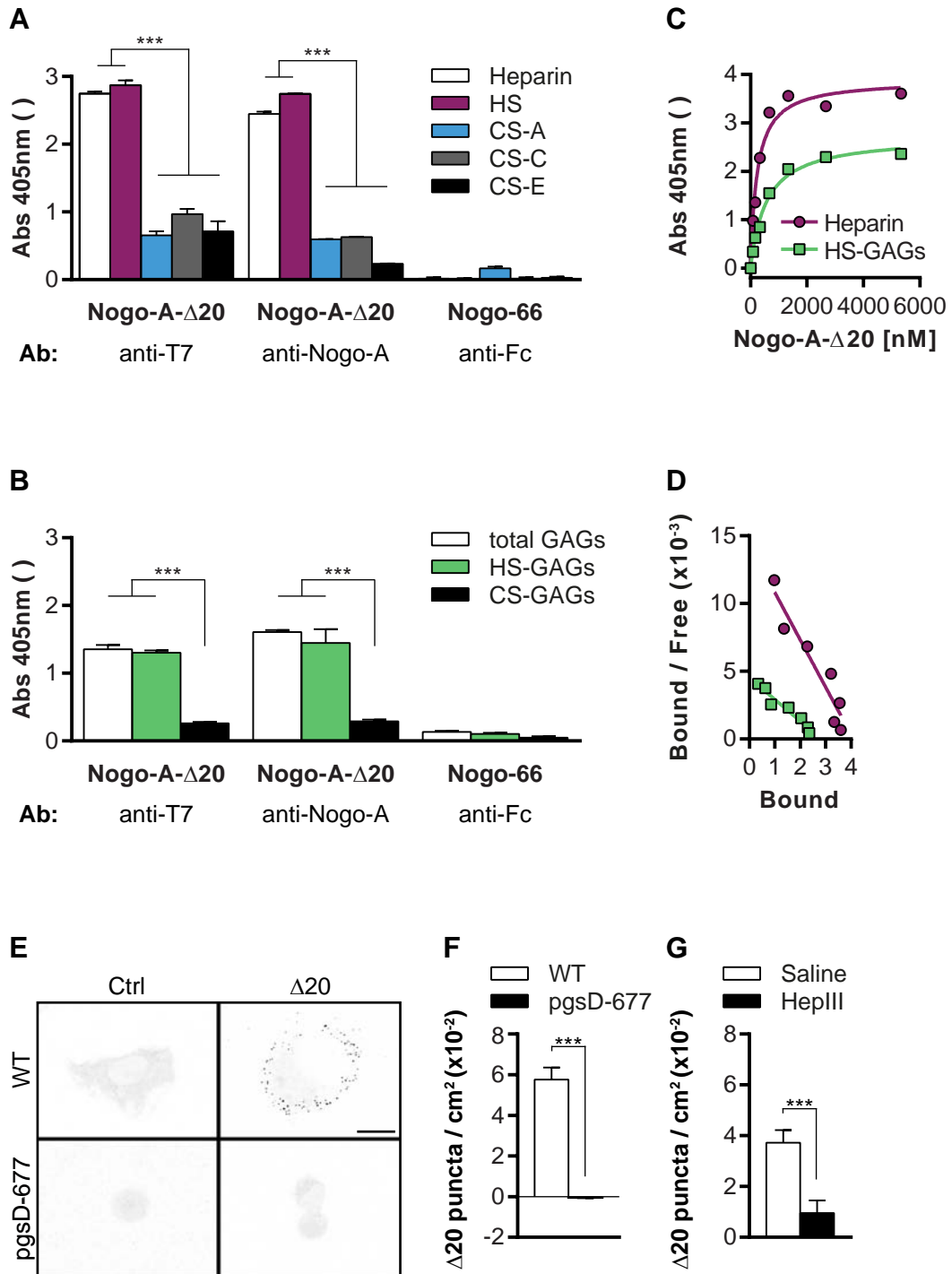
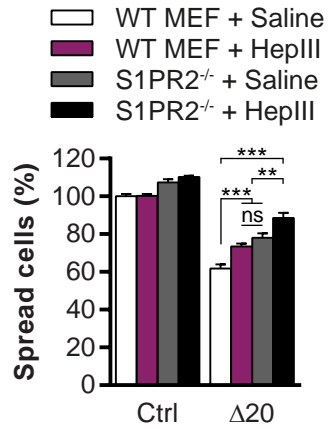
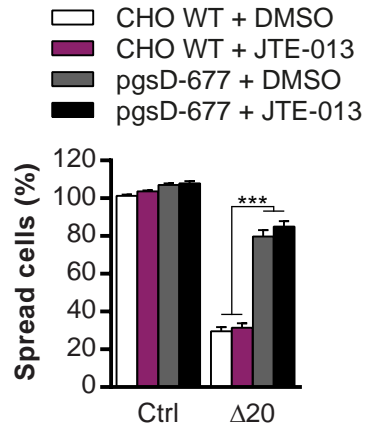


Figure 4

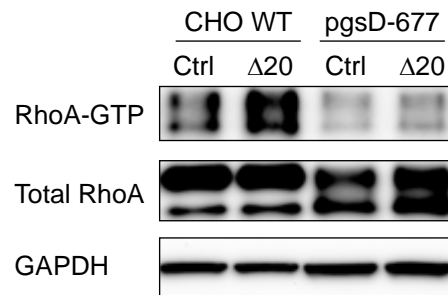
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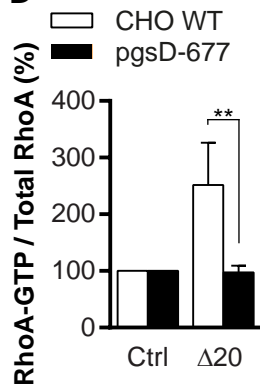
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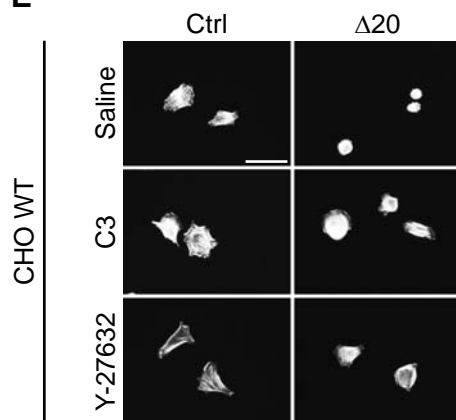
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D



E



F

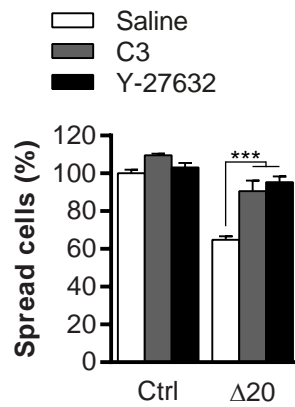


Figure 5

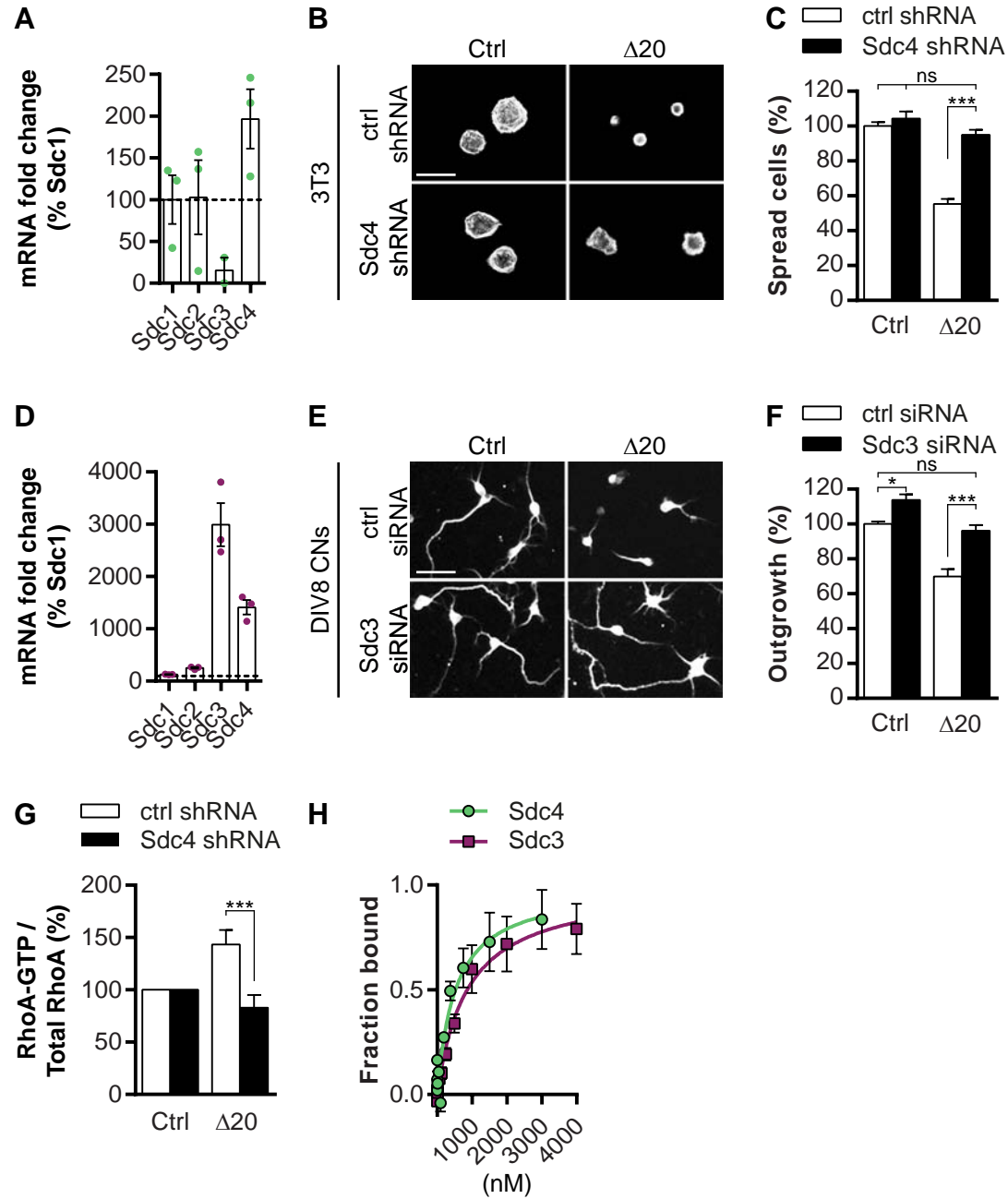


Figure 6

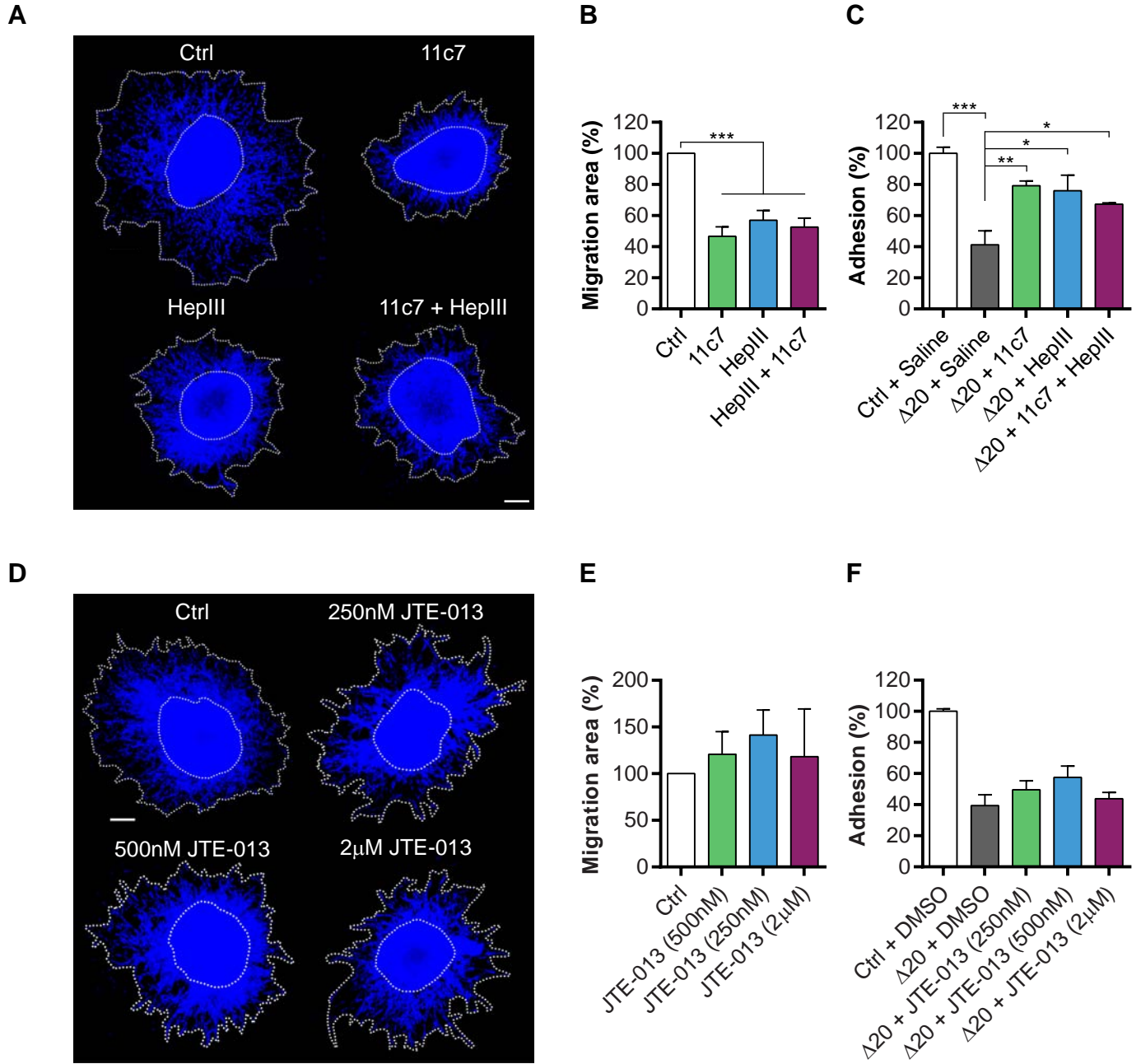


Figure S1

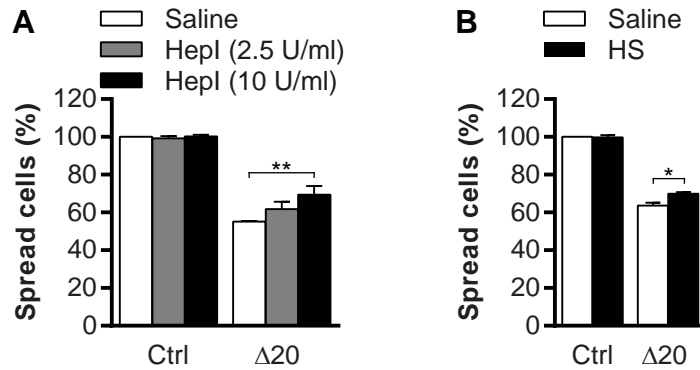


Figure S1. Related to Figure 1. Effect of HepI treatment and pre-incubation of HS and Nogo-A-Δ20 on Nogo-A-Δ20-induced inhibition of cell spreading. **A.** Cell spreading quantification of 3T3 fibroblasts treated with 2.5 U/ml HepI, 10 U/ml HepI or vehicle (saline) and plated on control (ctrl) and Nogo-A-Δ20 substrates. **B.** Cell spreading quantification of 3T3 fibroblasts plated on a substrate consisting of Nogo-A-Δ20 pre-incubated with HS (0.1 mg/ml). Data shown are means ± SEM (**A,B**; n = 3-6 coverslips). **A,B**: One-way ANOVA with Tuckey's post hoc test (* $p < 0.05$, ** $p < 0.01$).

Figure S2

A

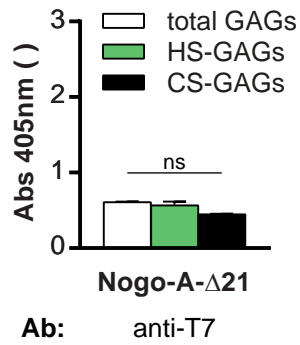


Figure S2. Related to Figure 3. Binding of Nogo-A- Δ 21 to brain-derived GAGs. **A.** Biotinylated brain-derived GAGs treated with heparinase (CS-GAGs) or chondroitinase ABC (HS-GAGs) or untreated (total GAGs) were coated onto streptavidin-coated wells and analysed for Nogo-A- Δ 21 binding by an ELISA-type assay. Average values for the BSA negative control were subtracted from the respective readings. Nogo-A- Δ 21-T7 binding was detected using an anti-T7 antibody. Data shown are means \pm SEM (**A,B**; n = 3 experiments). **A:** One-way ANOVA with Tuckey's post hoc test (ns: not significant).

Figure S3

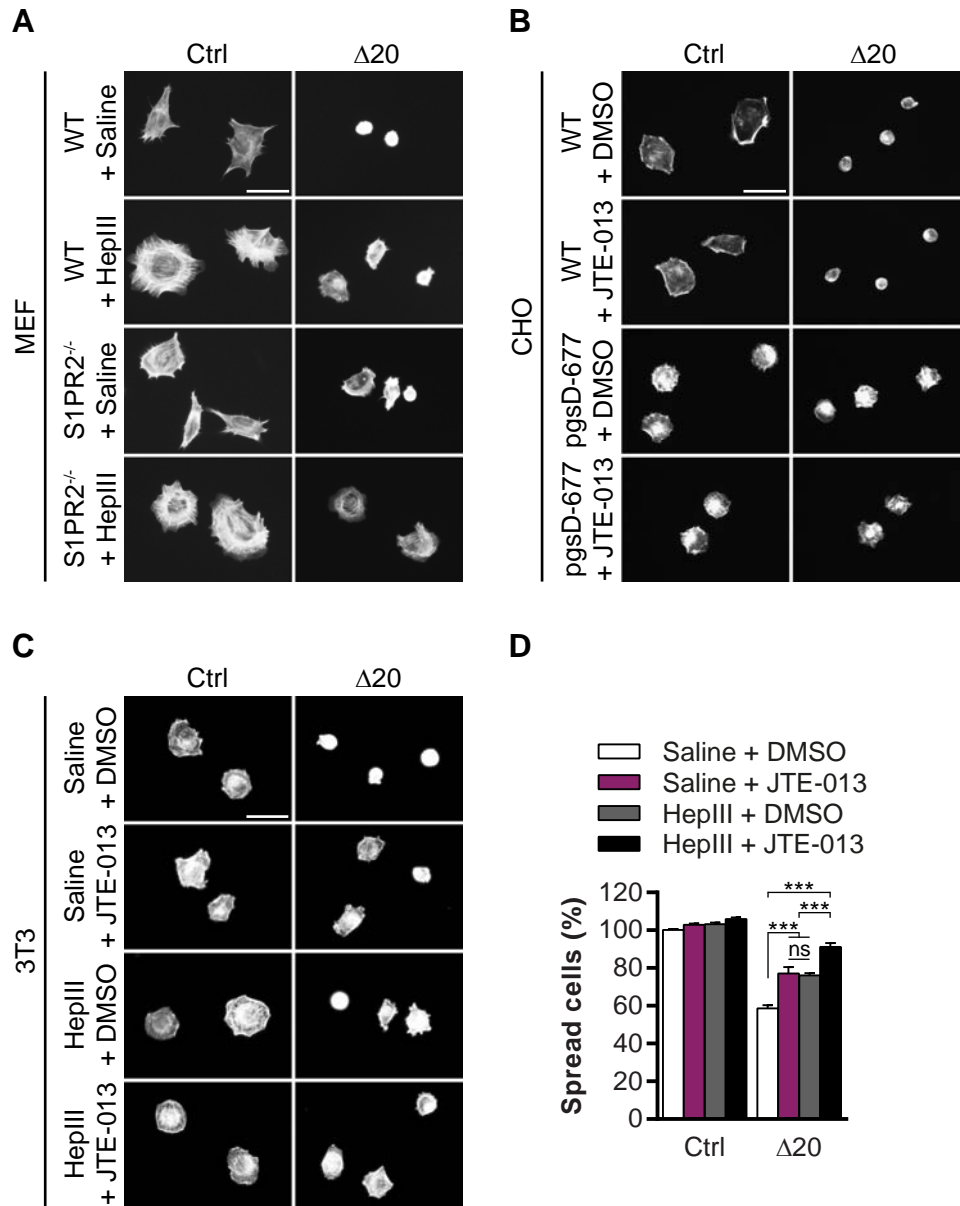


Figure S3. Related to Figure 4. Effects of S1PR2 blockade/knockout and HepIII treatment. **A.** Representative pictures of WT and S1PR2^{-/-} MEFs treated with 2.5 U/ml HepIII or vehicle (saline) and plated on control (ctrl) and Nogo-A- $\Delta 20$ substrates. **B.** Representative pictures of CHO WT and CHO pgsD-677 cells treated with 1 μ M JTE-013 or vehicle (DMSO) and plated on control (ctrl) and Nogo-A- $\Delta 20$ substrates. Quantification of **A** and **B** is shown in Figure 4A and 4B. **C.** Representative pictures of 3T3 cells treated with 2.5 U/ml HepIII or vehicle (saline) and plated on a control (ctrl) or Nogo-A- $\Delta 20$ substrate in the presence of 1 μ M JTE-013 or vehicle (DMSO). **D.** Co-treatment with HepIII and JTE-013 led to an additive effect on Nogo-A- $\Delta 20$ inhibition rescue in 3T3 cells. Data shown are means \pm SEM (**B,D**: n = 5-9 coverslips). **B,D**: One-way ANOVA with Tuckey's post hoc test (** $p < 0.01$; *** $p < 0.001$; ns: not significant). Scale bars: 45 μ m.

Figure S4

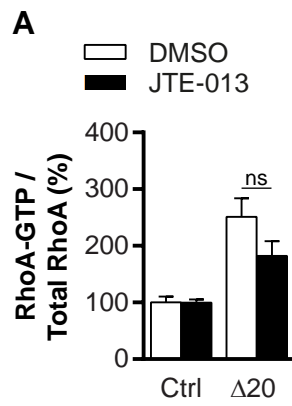


Figure S4. Related to Figure 4. JTE-013 treatment does not affect RhoA activation in S1PR2-negative CHO cells. **A.** RhoA activation was assessed in CHO WT cells 20 min post-incubation with 1 μ M Nogo-A- Δ 20 using an ELISA kit. Data shown are means \pm SEM (n = 3 experiments). Mann Whitney test (ns: not significant).

Figure S5

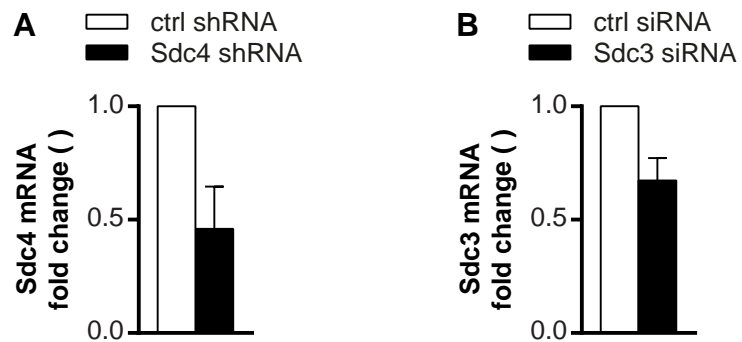


Figure S5. Related to Figure 5. Knockdown efficiency of syndecan shRNA and siRNA constructs. **A.** Quantitative RT-PCR analysis of 3T3 cells expressing lentivirally delivered syndecan-4 (Sdc4) shRNA for 96 h. Scrambled shRNA (ctrl) was used as control and set to 1. Relative quantification of expression levels: Sdc4 (0.460 ± 0.186). **B.** Quantitative RT-PCR analysis of E19 cortical neurons treated at DIV4 with syndecan-3 (Sdc3) or scrambled (ctrl) siRNA for 72 h. Scrambled siRNA was set to 1. Relative quantification of expression levels: Sdc3 (0.673 ± 0.099). Data shown are means \pm SEM (**A,B**: n = 3-4 experiments).

1 **Supplemental Figure Legends**

2

3 **Figure S1. Related to Figure 1. Effect of HepI treatment and pre-incubation of HS and Nogo-A-Δ20 on**
4 **Nogo-A-Δ20-induced inhibition of cell spreading.** **A.** Cell spreading quantification of 3T3 fibroblasts treated
5 with 2.5 U/ml HepI, 10 U/ml HepI or vehicle (saline) and plated on control (ctrl) and Nogo-A- Δ20 substrates. **B.**
6 Cell spreading quantification of 3T3 fibroblasts plated on a substrate consisting of Nogo-A- Δ20 pre-incubated
7 with HS (0.1 mg/ml). Data shown are means ± SEM (**A,B**: n = 3-6 coverslips). **A,B**: One-way ANOVA with
8 Tuckey's post hoc test (* $p < 0.05$, ** $p < 0.01$).

9

10 **Figure S2. Related to Figure 3. Binding of Nogo-A-Δ21 to brain-derived GAGs.** **A.** Biotinylated brain-derived
11 GAGs treated with heparinase (CS-GAGs) or chondroitinase (HS-GAGs) or untreated (total GAGs) were coated
12 onto streptavidin-coated wells and analysed for Nogo-A-Δ21 binding by an ELISA-type assay. Average values
13 for the BSA negative control were subtracted from the respective readings. Nogo-A-Δ21-T7 binding was detected
14 using an anti-T7 antibody. Data shown are means ± SEM (**A,B**: n = 3 experiments). **A**: One-way ANOVA with
15 Tuckey's post hoc test (ns: not significant).

16

17 **Figure S3. Related to Figure 4. Effects of S1PR2 blockade/knockout and HepIII treatment.** **A.**
18 Representative pictures of WT and S1PR2^{-/-} MEFs treated with 2.5 U/ml HepIII or vehicle (saline) and plated on
19 control (ctrl) and Nogo-A-Δ20 substrates. **B.** HepIII treatment of S1PR2^{-/-} MEFs led to an additive effect on Nogo-
20 A-Δ20 inhibition rescue as opposed to S1PR2 knockout or HepIII treatment alone. **C.** Representative pictures of
21 3T3 cells treated with 2.5 U/ml HepIII or vehicle (saline) and plated on a control (ctrl) or Nogo-A-Δ20 substrate
22 in the presence of 1 μM JTE-013 or vehicle (DMSO). **D.** Co-treatment with HepIII and JTE-013 led to an additive
23 effect on Nogo-A-Δ20 inhibition rescue in 3T3 cells. Data shown are means ± SEM (**B,D**: n = 5-9 coverslips).
24 **B,D**: One-way ANOVA with Tuckey's post hoc test (** $p < 0.01$; *** $p < 0.001$; ns: not significant). Scale bars:
25 45 μm.

26

27 **Figure S4. Related to Figure 4. JTE-013 treatment does not affect RhoA activation in S1PR2-negative CHO**
28 **cells.** RhoA activation was assessed in CHO WT cells 20 min post-incubation with 1 μM Nogo-A-Δ20 using an
29 ELISA kit. Data shown are means ± SEM (n = 3 experiments). Mann Whitney test (ns: not significant).

30

31 **Figure S5. Related to Figure 5. Knockdown efficiency of syndecan shRNA and siRNA constructs.** **A.**
32 Quantitative RT-PCR analysis of 3T3 cells expressing lentivirally delivered syndecan-4 (Sdc4) shRNA for 96 h.
33 Scrambled shRNA (ctrl) was used as control and set to 1. Relative quantification of expression levels: Sdc4
34 (0.460 ± 0.186). **B.** Quantitative RT-PCR analysis of E19 cortical neurons treated at DIV4 with syndecan-3 (Sdc3)
35 or scrambled (ctrl) siRNA for 72 h. Scrambled siRNA was set to 1. Relative quantification of expression levels:
36 Sdc3 (0.673 ± 0.099). Data shown are means ± SEM (**A,B**: n = 3-4 experiments).

37

38

39 Supplemental Experimental Procedures

40

41 Recombinant fusion proteins, reagents and antibodies

42 Recombinant proteins Nogo-A- Δ 20 (rat aa544-725) and Nogo-A- Δ 21 (rat aa812-918) were purified as described
43 previously (Oertle et al., 2003). Briefly, BL21/DE3 *E. coli* were transformed with the pET28 expression vector
44 (Novagen) containing His-/T7-tagged Nogo-A- Δ 20, His-/T7-tagged Nogo-A- Δ 21 or His-/HA-tagged Nogo-A-
45 Δ 20 and cultured at 37°C to reach an OD of 0.6 AU. Protein expression was induced by addition of 1 M IPTG for
46 2 h at 30°C. Fusion proteins were purified using Co²⁺-Talon Metal Affinity Resin (Takara Bio Inc.).

47 CS variants and HS were purchased from Seikagaku Corp (Japan) where CS-A is isolated from whale cartilage,
48 CS-C is from shark cartilage, CS-E is from squid cartilage and HS is from bovine kidney. The biotinylated-heparin
49 isolated from porcine intestine was purchased from Sigma.

50 The following primary antibodies were used: mouse anti- β III Tubulin (Promega G712A, clone 5G8; ICC: 1:1000),
51 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen D1306; ICC: 1:1000), mouse anti-GAPDH (Abcam, ab8245;
52 1:20,000), rat anti-HA tag (Roche 11867423001; ICC: 1:200), mouse anti-heparan sulfate (Seikagaku Corp
53 370255-1, clone F58-10E4; FACS: 1 μ g/10⁶ cells), mouse IgM isotype control (BD Pharmingen 557275, clone
54 C48-6; FACS: 1 μ g/10⁶ cells), goat anti-mouse IgG, Fc γ fragment specific (Jackson ImmunoResearch 115-005-
55 071; ELISA: 1 μ g/ml), mouse anti-Map-1b (Santa Cruz sc-58784, clone AA6; ICC 1:2000), mouse anti-Nogo-
56 A (11c7, (Oertle et al., 2003), ELISA: 1 μ g/ml), Phalloidin-Alexa488 (Invitrogen A12379; ICC: 1:500), rabbit
57 anti-RhoA (Cell Signaling 2117; WB: 1:1000), mouse anti-T7 tag (Novagen 69522-3; ELISA: 1 μ g/ml).

58 The following secondary antibodies were used: goat anti-mouse IgG Alexa488-conjugated (Invitrogen A11029;
59 ICC: 1:1000), rat anti-mouse IgM FITC-conjugated (BD Pharmingen 553437, clone II/41; FACS: 1:1000), HRP-
60 conjugated goat anti-rabbit IgG (Jackson ImmunoResearch).

61

62 Brain derived glycosaminoglycans (GAGs)

63 Adult Sprague Dawley rats were sacrificed and decapitated. The brains were cut into smaller pieces before de-
64 lipidation with cold acetone. The tissues were then dried and homogenized in cold pronase buffer. The brain was
65 then treated with pronase overnight and the proteins/peptides were removed by precipitation using trichloroacetic
66 acid, followed by centrifugation. The residual trichloroacetic acid retained in the supernatant (which contains the
67 GAGs) is removed with 5 diethyl ether washes. The GAGs were precipitated with sodium acetate and absolute
68 ethanol overnight at 4°C and recovered after centrifugation. The resulting pellet will be reconstituted in 500 μ l of
69 de-ionized water and stored at -20°C.

70

71 Tissue preparation and cell culture

72 Total myelin protein extracts were prepared from the spinal cords of adult Wistar rats as described previously
73 (Oertle et al., 2003). Swiss 3T3 (ATCC) cells and primary mouse embryonic fibroblasts (MEFs) were maintained
74 in Dulbecco's modified eagle medium (DMEM) (Sigma, Invitrogen) containing 10% neonatal calf serum
75 (Invitrogen). CHO K1 WT (ATCC) and CHO pgsD-677 cells (ATCC) were maintained in DMEM containing
76 10% fetal bovine serum (FBS) (Invitrogen). Primary S1PR2^{-/-} MEFs were described previously (Kempf et al.,
77 2014). P5-8 mouse CGNs, P5-8 mouse DRG neurons and E19 rat cortical neurons were prepared as described
78 previously (Kempf et al., 2014; Oertle et al., 2003). HEK293T (ATCC) cells were maintained in Iscove's modified
79 Dulbecco's medium (IMDM) (Life Technologies) medium supplemented with 4 mM L-Glutamine (Sigma), 1%
80 Penicillin/Streptomycin (Pen/Strep) (Life Technologies) and 10% FBS. Swiss 3T3 ctrl shRNA and Sdc4 shRNA
81 cells were selected with 4 μ g/mL puromycin. All cell lines and primary cells were cultured at 37°C and 5% CO₂.

82

83 Immunocytochemistry

84 Cell lines and primary cells were fixed with 4% paraformaldehyde (PFA) for 15 min, washed and permeabilized
85 with 0.1% Triton X-100. After blocking with 2% goat serum, cells were first incubated with the primary antibodies
86 for 30 min at room temperature and detected using corresponding secondary antibodies in 2% goat serum.

87 For cell surface immunocytochemical detection of Nogo-A- Δ 20, cells were first incubated with 1 μ M HA-tagged
88 Nogo-A- Δ 20 and subsequently with anti-HA antibodies for 1 h each on ice in serum-free medium containing
89 0.02% sodium azide (Sigma). Cells were washed, fixed with 1% PFA and stained with secondary antibodies.
90 Image stacks were acquired using a Leica SP5 confocal microscope equipped with a 63x oil immersion objective
91 (NA 1.4). Stacks were reconstructed in 3D with Imaris (Bitplane) and the cell surface area was measured for each
92 cell. Bound Nogo-A- Δ 20 puncta were counted using the spot function of Imaris and the total number was
93 normalized to the cell surface area for each cell. The average ratio obtained with secondary antibody only controls
94 was baseline-subtracted from each cell.

95

96 Flow cytometry

97 For FACS analysis, non-fixed cells were detached using 0.05% Trypsin/EDTA (Invitrogen), washed 1x in PBS,
98 washed 2x in Tris-Buffer/1%BSA at 4°C and stained with the indicated primary antibodies followed by
99 fluorescently-conjugated secondary antibodies for 30 min each in Tris-Buffer/5%BSA on ice. Cells were
100 immediately analyzed by FACS (BD Canto II). FACS staining was quantitated using the FlowJo (Tree Star Inc)
101 software. The fluorescence intensity is displayed on the X-axis (divided into 256 bins). The % of Max on the Y-
102 axis stands for the number of cells in each bin on the X-axis (FlowJo uses an arbitrary number of 256 bins) divided
103 by the number of cells in the bin that contains the largest number of cells.

104 **RhoA activation assays**

105 3T3 cells were serum-starved overnight and treated for 20 min with 1 μ M Nogo-A- Δ 20 or Nogo-A- Δ 21 control
106 protein. Pulldown of activated RhoA-GTP was subsequently performed using the RhoA Activation Assay
107 Biochem Kit according to the manufacturer's instructions (Cytoskeleton, Inc.). Alternatively, RhoA activation
108 was assessed using the total RhoA ELISA and RhoA G-LISA kit according to the manufacturer's instructions
109 (Cytoskeleton, Inc.). Levels of activated RhoA were normalized to total RhoA levels for each biological replicate.
110

111 ***In vitro* bioassays**

112 3T3 fibroblast spreading assays and neurite outgrowth assays were performed as described previously (Kempf et
113 al., 2014; Oertle et al., 2003). Briefly, 4-well plates (Greiner) were coated with 40-100 pmol/cm² (0.4-1 μ M)
114 Nogo-A- Δ 20 or Nogo-A- Δ 21 (control protein) or 5 μ g/cm² myelin at 4°C overnight. In outgrowth experiments,
115 wells were precoated with 0.3 μ g/ml Poly-L-Lysine (PLL; Sigma) for 1 h at 37°C before the addition of the
116 different substrates. Unbound material was removed by three washes with PBS. Cell lines were detached with 2%
117 (w/v) EDTA in PBS and plated at 7000 cells per cm² for 1 h at 37°C and 5% CO₂, fixed with 4% PFA and stained
118 with Phalloidin-Alexa488. For HepI and HepIII (Sigma) treatment, cells were incubated with 2.5 U/ml HepIII or
119 2.5-10 U/ml HepI 3 h prior plating and during the spreading assay. Higher concentrations of HepIII could not be
120 used under our experimental conditions because of their effects on cell viability. For JTE-013 (Tocris), Y-27632
121 (Sigma) and cell-permeable C3 transferase (CT04, Cytoskeleton), cells were incubated with 1 μ M JTE-013, 5 μ M
122 Y-27632 or 100 μ g/ml C3 30 min prior plating and during the spreading assay. The corresponding solvents or
123 isotype antibodies were used as controls. For expression of EXT1 in pgsD-677 cells, pgsD-677 cells were
124 transfected with Ext1 cDNA using Lipofectamine 2000 (Invitrogen) 48 h prior replating according to the
125 manufacturer's instructions. The percentage of cells that remained round i.e. did not spread was quantified
126 manually in four randomly chosen areas of the well/cover slip and averaged over those areas (n = 1 coverslip).
127 Data were normalized to baseline and plotted as mean \pm SEM from multiple biological replicates.
128 CGNs were plated at 7.5x10⁴ cells per cm², DRGs at 7.5x10³ cells per cm² and cortical neurons at 5x10⁴ cells per
129 cm² onto the various substrates. Neurons were cultured for 24-48 h at 37°C and 5% CO₂, fixed with 4% PFA and
130 stained with anti- β III Tubulin (CGNs and DRGs) or Mab1b (cortical neurons). Treatment of CGNs with
131 500 mU/ml HepIII and DRGs with 1 U/ml HepIII started 12 h post-plating until fixation. Cortical neurons were
132 treated at DIV4 with 1 U/ml HepIII for 3 h and replated for 24 h in the presence of HepIII. The corresponding
133 solvents were used as control. Neurons were imaged with an Axioskop 2 microscope (Zeiss) equipped with a
134 Plan-NEOFLUAR 10X/NA 0.3 objective in a semi-automated way. Mean total neurite length per cell was
135 quantified using the MetaMorph software (Molecular Devices) in four randomly chosen areas of the
136 well/cover slip and averaged over those areas (n = 1 coverslip). Data were normalized to baseline and plotted as
137 mean \pm SEM from multiple biological replicates.
138

139 **siRNA/shRNA**

140 E19 rat cortical neurons were plated at 0.6x10⁶ cells in 6-well plates coated with 0.3 μ g PLL and transfected at
141 DIV4 with 50 nM siRNA using DharmaFECT 3 (Dharmacon) according to the manufacturer's instructions. Three
142 days post-transfection, neurons were detached with 0.25% Trypsin and replated on a Nogo-A- Δ 20 or control
143 substrate for 24 h as described above. Swiss 3T3 cells were plated at 2.9x10⁴ in 24-well plates and transfected
144 with 50 nM siRNA using DharmaFECT 3 (Dharmacon) according to the manufacturer's instructions. 3 days post-
145 transfection, cells were replated on a Nogo-A- Δ 20 or control substrate for 1 h. Following siRNAs were used: rat
146 Syndecan-3 ON-TARGETplus SMARTpool Sdc3 siRNA (L-098896-02-0005), mouse Glypican-1 ON-
147 TARGETplus SMARTpool Gpc1 siRNA (L-049268-01-0005), mouse Glypican-4 ON-TARGETplus
148 SMARTpool Gpc4 siRNA (L-045841-01-0005), mouse Glypican-6 ON-TARGETplus SMARTpool Gpc6 siRNA
149 (L-049420-01-0005) and ON-TARGETplus siRNA non-targeting pool (D-001810-10-0005) (Thermo Scientific,
150 Dharmacon). Quantification of the respective mRNA knockdown was performed by qRT-PCR.
151

152 The following Mission shRNA (Sigma) pLKO lentiviral plasmids containing shRNA against mouse Syndecan-4
153 and non-target shRNA were used for the generation of Swiss 3T3 ctrl and Sdc4 shRNA stable cell lines:
154 TRCN0000331554 and SHC202 (TRC2 vector). Lentiviral plasmids were transfected into HEK293T cells using
155 PEI (polyethyleimine) 25 kDa (Polysciences Inc.). Lentiviruses were concentrated from filtered culture media
156

157 (0.45 μm) by ultracentrifugation at 25000 rpm for 2 h. Quantification of the respective mRNA knockdown was
158 performed by qRT-PCR.

159

160 **Quantitative real-time PCR (qRT-PCR)**

161 Total RNA was isolated with the RNeasy Micro kit (Qiagen) and reverse-transcribed using TayMan Reverse
162 Transcription Reagents (Applied Biosystems). cDNA was amplified using the Light Cycler 480 thermocycler
163 (Roche) with the polymerase ready mix (SYBR Green I Master, Roche). Relative quantification was performed
164 using the comparative CT method. cDNA levels were normalized to the reference genes *Gapdh* and *Rpl19* (mouse)
165 or *Gapdh* and *eF1a1* (rat). Each reaction was done in triplicate. Melting curve analysis of PCR products followed
166 by gel electrophoresis was performed to verify amplicons. Following primers were used:

167

168 mouse *Gapdh*_FWD: 5'- CAGCAATGCATCCTGCACC -3',
169 mouse *Gapdh*_REV: 5'- TGGACTGTGGTCATGAGCCC -3';
170 mouse *Rpl19*_FWD: 5'- TGAGTATGCTCAGGCTACAG -3',
171 mouse *Rpl19*_REV: 5'- GAATGGACAGTCACAGGCTT -3';
172 mouse *Sdc4*_FWD: 5'- TTCTGGAGATCTGGATGACAC -3',
173 mouse *Sdc4*_REV: 5'- CACCAAGGGCTCAATCAC -3';
174 mouse *Gpc1*_FWD: 5'- ACTCCATGGTGCTCATCACTGAC -3',
175 mouse *Gpc1*_REV: 5'- TTCCACAGGCCTGGATGACCTTAG -3';
176 mouse *Gpc4*_FWD: 5'- ACCGACTGGTTACTGATGTCAAGG -3',
177 mouse *Gpc4*_REV: 5'- TTGCAAACGGTGCTTGGGAGAG -3';
178 mouse *Gpc6*_FWD: 5'- : GTCAGCAAAGGTCTTTCAGG -3',
179 mouse *Gpc6*_REV: 5'- GGTCTTTCCTCAGGGTTGTAG -3';
180 rat *Gapdh*_FWD: 5'- CTCTCTGCTCCTCCCTGTTC -3',
181 rat *Gapdh*_REV: 5'- GCCAAATCCGTTACACC -3';
182 rat *eF1 α* _FWD: 5'- GCCACCATACAGTCAGAAGAG -3',
183 rat *eF1 α* _REV: 5'- GAACCACGGCATATTAGCAC -3'.
184 rat *Sdc3*_FWD: 5'- TCCACGACAATGCCATCGACTC -3',
185 rat *Sdc3*_REV: 5'- ACCTACGATCACAGCTACGAGCAC -3';

186

187 **ELISA**

188 The ELISA was modified according to method described in (Purushothaman et al., 2007). Biotinylated GAGs
189 (0.5 μg per well) were immobilized onto a streptavidin-coated 384-well-plate (Pierce/Thermo scientific, IL,
190 USA). Biotinylation of GAGs was performed by EDC and biotin-LC-hydrazide conjugation (Pierce/Thermo
191 Scientific). After biotinylated GAGs were immobilized on the plates, the plates were blocked in 1% BSA and
192 subjected to the binding of recombinant Nogo-66-Fc or Nogo-A- Δ 20. The bound Nogo variants were then
193 recognized by the anti-T7, anti-Fc or 11C7 antibodies. The bound antibodies were detected using anti-mouse-
194 alkaline phosphatase conjugated antibodies followed by a direct measurement of absorbance at 405 nm using p-
195 nitrophenylphosphate (Sigma Aldrich). BSA only controls (no recombinant proteins) measurements were used as
196 baseline in every experiment and subtracted from the other readings. For quantification, the mean \pm SEM of
197 absorbance measurements was determined from three experiments.

198

199 **Explant assay**

200 P5 explants were prepared from C57/BL6 pups according to (Wichterle et al., 1997). Tissues from the SVZ and
201 RMS were embedded in 75% Matrigel growth factor reduced (BD Biosciences) and maintained for 1 day in
202 Neurobasal medium (Invitrogen) supplemented with B27 (1x; Miltenyi), Pen/Strep (20 U/ml; Sigma), and 0.5 mM
203 glutamine (Invitrogen). Antibodies and compounds were mixed with Matrigel: 11C7 (Oertle et al., 2003), 1 $\mu\text{g}/\mu\text{l}$;
204 mouse anti-human IgG, 1 $\mu\text{g}/\mu\text{l}$ (Jackson ImmunoResearch); HepIII, 500 mU/ml (Sigma); JTE-013, 250nM,
205 500nM or 2 μM (Tocris). Only vital explants with cells moving out of the tissue core in chains were analyzed.
206 Explants were fixed in 4% PFA for 40, 1 $\mu\text{g}/\mu\text{l}$ min and stained using 4',6-diamidino-2-phenylindole (DAPI;
207 Fluka) to visualize cell nuclei or labeled for β III Tubulin or Doublecortin (Rolando et al., 2012) to mark
208 neuroblasts.

209 For adhesion experiments, adult SVZs were dissociated and SVZ-cells were either plated on poly-D-lysine (PDL)
210 only or Nogo-A- Δ 20-coated coverslips (12,000 cells/cm² in DMEM/F-12 supplemented with B27) (Rolando et
211 al., 2012). Briefly, glass coverslips (1cm²) were first coated with PDL (5 $\mu\text{g}/\text{ml}$) and then with Nogo-A- Δ 20
212 (100 pmol/cm²). Cells were pre-incubated with HepIII and/or 11c7 or with JTE-013 for 30 min and subsequently
213 plated for 1 h. Cells were fixed, stained with DAPI and for β III Tubulin and scored. The average number of
214 adhered cells was determined by counting in five randomly chosen fields of view of the coverslips.

215

216 **Supplemental References**

217

218 Kempf, A., B. Tews, M.E. Arzt, O. Weinmann, F.J. Obermair, V. Pernet, M. Zagrebelsky, A.
219 Delekate, C. Iobbi, A. Zemmar, Z. Ristic, M. Gullo, P. Spies, D. Dodd, D. Gygax, M. Korte,
220 and M.E. Schwab. 2014. The sphingolipid receptor S1PR2 is a receptor for Nogo-a repressing
221 synaptic plasticity. *PLoS Biol.* 12:e1001763.

222 Oertle, T., M.E. van der Haar, C.E. Bandtlow, A. Robeva, P. Burfeind, A. Buss, A.B. Huber, M.
223 Simonen, L. Schnell, C. Brosamle, K. Kaupmann, R. Vallon, and M.E. Schwab. 2003. Nogo-
224 A inhibits neurite outgrowth and cell spreading with three discrete regions. *J Neurosci.*
225 23:5393-406.

226 Purushothaman, A., J. Fukuda, S. Mizumoto, G.B. ten Dam, T.H. van Kuppevelt, H. Kitagawa, T.
227 Mikami, and K. Sugahara. 2007. Functions of chondroitin sulfate/dermatan sulfate chains in
228 brain development. Critical roles of E and iE disaccharide units recognized by a single chain
229 antibody GD3G7. *J Biol Chem.* 282:19442-52.

230 Rolando, C., R. Parolisi, E. Boda, M.E. Schwab, F. Rossi, and A. Buffo. 2012. Distinct roles of nogo-
231 a and nogo receptor 1 in the homeostatic regulation of adult neural stem cell function and
232 neuroblast migration. *J Neurosci.* 32:17788-99.

233 Wichterle, H., J.M. Garcia-Verdugo, and A. Alvarez-Buylla. 1997. Direct evidence for homotypic,
234 glia-independent neuronal migration. *Neuron.* 18:779-91.

235